In vitro effects of G-Protein Coupled Receptor 120 agonist on osteoblast differentiation and activity in osteosarcoma cells

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In vitro effects of G-Protein Coupled Receptor 120 agonist on osteoblast differentiation and activity in osteosarcoma cells

by

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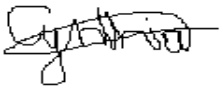
Dedication

I dedicate this dissertation to my mother Sauleta and my late father, my sisters and brother Juvacy, Sikina, Yolanda and Isaac, my Prophet Master Nyangulu together with his family, my Pastor Joseph Tiyesi and my mentors Mrs Dinah Bokaba and Dr Matete Madiba, not forgetting Pastor Landman and his family. Thank you for your endless support, prayers, advice, and encouragement throughout my studies.

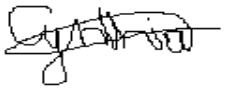
Declaration

I, Cynthia Navela Sithole, declare that this dissertation, which I hereby submit for MSc in Human Physiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:  Date: February 2022

Ethics statement

I, Cynthia Navela Sithole, declare that I have obtained ethical clearance for the research described in this work from the Faculty of Health Sciences Ethics Committee (582/2020). I declare that I have observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the policy guidelines for responsible research.

Signature:  Date: February 2022

Executive summary

Osteoblasts are important bone cells that play a central role in the formation and mineralization of bone. The extracellular-signal-related kinase (ERK) and protein kinase B (AKT) pathways play a role in osteoblast differentiation by regulating the activity of runt-related transcription factor 2 (Runx2). Osterix and Runx2 are major osteoblastic transcriptional factors. Runt-related transcription factor 2 is responsible for the upregulation of osteoblast specific genes including but not limited to: receptor activator of nuclear factor kB ligand (RANKL), osteoprotegerin (OPG), and alkaline phosphatase (ALP).

G-protein coupled receptors (GPCRs) govern many physiological changes in cells. G-protein coupled receptor 120 (GPR120) has gained attention as a target for the development of drugs for several human diseases because of its effects on inflammatory-associated diseases. It has further been shown to affect the formation and function of bone cells; however, the exact mechanisms of the processes are not fully understood.

In this study, various methods were carried out to establish the outcome of a GPR120 agonist (TUG-891) on the formation and function of osteoblasts in human osteosarcoma MG-63 cell line. MG-63 cells were exposed to TUG-891 [0.1-100 µM] in osteogenic medium (OM). Undifferentiated cells were used to investigate TUG-891's impact on cell viability using resazurin assay. Thereafter, TUG-891 was applied to the cells for 7, 14 or 21 days. Later, the following assessments were carried out: Osteoblast differentiation using the ALP activity, calcium mineralisation using alizarin red staining, protein expression (ERK, pERK, AKT, and pAKT) using western blot, and gene expression (*ALP, Osterix, RANKL, OPG, BSP and Runx2*) using quantitative polymerase chain reaction (q-PCR).

TUG-891 had no significant influence on viability of the cells. ALP activity was significantly increased after 7 days ($P<0.01$) and 21 days ($P<0.0001$) of differentiation compared to the OM baseline control. TUG-891 significantly decreased alizarin red S staining in the calcium mineralisation assay after 14 days ($P<0.0001$) and 21 days ($P<0.001$) when contrasted to the OM baseline control. Gene expression was tested for 7 and 14 days. There were no significant effects in gene expression observed for any of the tested genes *(ALP, Osterix, RANKL, OPG, BSP and Runx2*) after day 7 or 14. However, TUG-891 increase gene expression after day 7 and decreased the expression after 14 days compared to OM baseline control.

TUG-891 induced no significant effect on ERK and AKT phosphorylation after 15 minutes, 60 minutes, 4 hours, or 24 hours compared to the baseline control. GPR120 siRNA experiments were carried out for 15 minutes. TUG-891 showed no significant differences in the relative ERK and AKT phosphorylation in the siRNA cells that were used as controls and the GPR120 siRNA cells in comparison to the OM baseline control.

To conclude, TUG-891 reveals negative changes to gene expression and alizarin red staining. This effect may be regulated by the ERK and AKT signalling pathway. However, no significant changes were seen since only preliminary data was presented. These findings may reveal that the GPR120 agonist, TUG-891, may have negative effects on bone health through inhibition of osteoblast activity.

Keywords: Bone remodelling, osteoblasts, MG-63 osteosarcoma cells, G-protein coupled receptor 120, alkaline phosphatase, runt-related transcription factor, mitogen-activated protein kinase.

Research outputs

Poster presentation: Faculty of Health Sciences Research Day, University of Pretoria, August 2021.
Sithole CN, Kasonga AE, van den Bout I. *In vitro* effects of G-Protein Coupled Receptor 120 agonist on osteoblast differentiation and activity in osteosarcoma cells.

A research article titled *In vitro* effects of G-Protein Coupled Receptor 120 agonist on osteoblast differentiation and activity in osteosarcoma cells will be prepared for publication in scientific journals.

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Abbreviations

AC:	Adenylyl cyclase
AKT:	Ak strain transforming
ALP:	Alkaline phosphatase
AMP:	Adenosine monophosphate
ANOVA:	Analysis of Variance
AP-1:	Activator protein 1
APC:	Adenomatous polyposis coli
ATF4:	Activating transcription factor 4
ATPase:	Adenosine triphosphatase
BAT:	Brown adipose tissue
BCA:	Bicinchoninic Acid
BMD:	Bone mineral density
BMMSCs:	Bone marrow mesenchymal stem cells
BMPs:	Bone morphogenetic proteins
BMU:	Bone Multicellular Unit
BRC:	Bone-remodelling Compartment
BSA:	Bovine Serum Albumin
BSP:	Bone sialoprotein
Ca^{2+}:	Calcium ions
CbfA1:	Core-binding factor A1
CBP:	CREB-binding protein
CCK:	Cholecystokinin
CD44:	Cluster of Differentiation 44
CDKi:	Cyclin-dependent kinase inhibitor
Co-Smad:	Common-partner Smads
Col1A1:	Collagen type 1 alpha 1
CREB:	Cyclic-AMP response element binding protein
CSF-1:	Colony-stimulating factor 1
CTGF:	Connective tissue growth factor
DAG:	Diacylglycerol
DC-STAMP:	Dendritic cell-specific transmembrane protein
DEXA:	Dual Energy X-ray Absorptiometry
DHA:	Docosahexaenoic acid
Dkk:	Dickkopfs
Dlx:	Distal-less
DMEM:	Dulbecco's Modified Eagle's Medium
DMP1:	Dentine matrix protein 1
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
Dsh:	Disheveled
EDTA:	Ethylenediaminetetraacetic acid
EGFR:	Epidermal growth factor receptor
ERK:	Extracellular signal-regulated kinase

FBS:	Foetal Bovine Serum
FA:	Fatty acids
FFA:	Free Fatty Acids
FFAR4:	Free fatty acid receptor 4
FGF:	Fibroblast growth factor
FGFR:	Fibroblast growth factor receptor
FOXO:	Forkhead box protein
FRA1:	Fos-related antigen 1
FZD:	Frizzled receptors
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GDP:	Guanosine-5'-diphosphate
GI:	Gastrointestinal
GIP:	Glucose-dependent insulinotropic peptide
GLP-1:	Glucagon- like peptide-1
GLUT4:	Glucose transporter 4
GM:	Gut microbiome
GPCR:	G-protein coupled receptor
GPI:	Glycosylphosphatidylinositol
GPR120:	G-Protein Coupled Receptor 120
GRK:	GPCR kinase
GSK3:	Glycogen synthase kinase 3
GTP:	Guanosine-5'-triphosphate
GTPase:	Guanosine triphosphatases
H^+:	Hydrogen ions
HCl:	Hydrochloric acid
HCO_3^-:	Bicarbonate ion
HPO_4^{2-}:	Hydrogen phosphate ion
IGF:	Insulin-like growth factor
IkB:	Inhibitor of kappa B
IkK:	Inhibitory kappa kinase
IL:	Interleukin
IP_3:	Inositol trisphosphate
JNK:	c-Jun NH2-terminal kinase
LDL:	Low-density lipoprotein
LEF:	Lymphoid-enhancer binding factor
LRP5/6:	LDL receptor-related protein 5 and 6
MAPKs:	Mitogen-Activated Protein Kinases
M-CSF:	Macrophage Colony-stimulating Factor
MEK:	MAPK/ERK Kinase
MKK:	Mitogen-activated protein kinase kinase
MMP-9:	Matrix metalloproteinase 9
MSCs:	Mesenchymal stem cells
mTOR:	Mammalian target of rapamycin
NFAT:	Nuclear factor of activated T-cells
NFATc1:	Nuclear factor of activated T-cells cytoplasmic 1

NF-kB:	Nuclear factor kappa B
NO:	Nitric oxide
NPP1:	Nucleotide pyrophosphatase 1
NTP:	nucleotide triphosphates
OA:	Oleic Acid
OCN:	Osteocalcin
ODF:	Osteoclast differentiation factor
OM:	Osteogenic medium
OPG:	Osteoprotegerin
OPN:	Osteopontin
Osx:	Osterix
PBS:	Phosphate Buffered Saline
PDK1:	Pyruvate dehydrogenase kinase 1
PDZ:	Postsynaptic density 95/discs-large/zona occludens-1
PGE2:	Prostaglandin E_2
PGI2	Prostaglandin I_2
Pi:	Inorganic phosphate
PI3K:	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2:	Phosphatidylinositol-4,5phosphate
PIP3:	Phosphatidylinositol-3,4,5phosphate
PKB:	Protein kinase B
PKC:	Protein kinase C
PKD:	Protein kinase D
PMSF:	Phenylmethyl-sulfonyl fluoride
PLA:	Palmitoleic acid
PLC:	Phospholipase C
PPARγ:	Peroxisome proliferator-activated receptor gamma
PPi:	Inorganic pyrophosphate
p-NPP*:*	*p*-nitrophenyl phosphate
PTH:	Parathyroid hormone
QCT:	Quantitative computed tomography
qPCR:	Quantitative polymerase chain reaction
RANK:	Receptor Activator of Nuclear Factor kB
RANKL:	Receptor Activator of Nuclear Factor kB Ligand
RSK2:	Ribosomal S6 kinases 2
R-Smad:	Receptor regulated Smad
RTKs:	Receptor tyrosine kinases
Runx2:	Runt-related transcription factor 2
SERMs:	Selective estrogen receptor modulators
sFRPs:	Secreted frizzled-related proteins
siRNA:	Small interfering ribonucleic acid
SIBLING:	Small integrin-binding ligand *N*-linked glycoprotein
Smad:	Small mothers against decapentaplegic
SOST:	Sclerostin
SOX2:	SRY-box transcription factor 2

SV:	simvastatin
SVNs:	SV-loaded nano-micelles
TAB1:	TAK1 binding protein
TAK1:	Transforming growth factor-β activated kinase 1
TBS:	Tris-buffered saline
TBS-T:	Tris-buffered saline Tween
TGF-β:	Transforming growth factor-β
TLR4:	Toll-like receptor 4
TNAP:	Tissue-nonspecific alkaline phosphatase
TNF:	Tumour necrosis factor
TNFR:	TNF receptor
TRAF6:	TNF receptor associated factor 6
TRANCE:	TNF-related activation-induced cytokine
TRPM5:	Transient receptor potential cation channel member 5
WIFs:	Wnt inhibitory factors
Wnt:	Wingless-type

Table of Contents

List of Figures

List of Tables

Chapter 1 Introduction

Bone is an active tissue that is constantly repaired throughout life by a process called bone remodelling. This process requires communication between the bone-breaking osteoclasts and the bone-forming osteoblasts.[1] Bone remodelling is crucial for bone homeostasis since an imbalance in bone remodelling favouring resorption may result in bone diseases like osteoporosis.[2] Osteoporosis is characterized by a loss of bone strength, which increases the risk of fracture.[2-3] Osteoporosis is a typical age-related condition that influences one in two women and one in five men.[2,4] Osteoporosis prevalence rises with age, from 2% at 50 to over 25% at 80, especially in post-menopausal women.[4] Therefore, treatments are aimed at decreasing the progression of osteoporosis and preventing bone fractures.

MG-63 cells are osteosarcoma derived cells. They share similar characteristics with primary osteoblasts such as the expression of most integrin subunits.[5] This cell line can be activated using osteogenic medium (OM) containing β-glycerophosphate, ascorbic acid and dexamethasone.[6]

Gene expression varies when the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) signalling pathway is active. Cell growth, differentiation, and survival are all affected by variations in gene expression.[7] For various peptide growth factor receptors and G-protein coupled receptors (GPCRs), the MAPK/ERK signalling cascade is important in mediating cell growth and differentiation.[7] GPCRs regulate many physiological and pathological responses in cells.[8] GPCRs are a large family of receptors with structural motifs in common, such as seven transmembrane helices and being able to activate the heterotrimeric G proteins. Second messenger pathways activate a range of cellular responses after the binding and activation by GPCR ligands.[9-10]

Free fatty acids (FFAs) are able to act as ligands to bind and activate five GPCRs: GPR40, GPR41, GPR43, GPR84 and GPR120.[10] Both medium and long-chain fatty acids can activate G-protein coupled receptor 120 (GPR120), also known as a Free Fatty Acid receptor 4 (FFAR4).[10-12] GPR120 has gained attention as a therapeutic agent due to its modulation of inflammatory-associated diseases.[8,13] TUG-891 is a GPR120 agonist with high potency and selectivity in both human and mice GPR120.[14-15] In cells, TUG-891 is not metabolized and therefore it is beneficial to assist in the understanding of the physiological impacts of GPR120.[16] GPR120 agonists are "functionally selective" and whether GPR120 activation favours the Gα or βarr2 pathway depends on the cell type.[11] Previous studies have also revealed that GPR120 activation via fatty acids (FA) or synthesized ligands like TUG-891 increases calcium levels and the activation of the ERK cascade.[10,17] However, studies on the effects of GPR120 agonists on human osteoblasts cell lines are limited.

1.1 Purpose of research

The aim of this research was to see how the GPR120 agonist TUG-891 affected the differentiation and activity of osteoblasts in MG-63 osteosarcoma cells.

1.2 Method of investigation

The study was conducted *in vitro* on pre-osteoblastic MG-63 osteosarcoma cells. The cells were exposed to OM and TUG-891 for 7, 14 and 21 days to determine their effects on osteoblast formation. The effects of TUG-891 on alkaline phosphatase (ALP) activity, mineralisation, gene, and protein expression were evaluated.

1.3 Objectives

- **Cell viability**

To test the effects of TUG-891 on cell viability in undifferentiated MG-63 osteosarcoma cells using resazurin colorimetric assay.

- **Alkaline phosphatase activity**

To examine the effects of TUG-891 on osteoblast formation in differentiated osteoblasts derived from MG-63 osteosarcoma cells using ALP activity as an early osteoblastogenesis marker.

- **Mineralisation**

To determine the effects of TUG-891 on mineralisation in differentiated osteoblasts derived from MG-63 osteosarcoma cells using Alizarin Red S staining.

- **Gene expression**

To study the effects of TUG-891 on the expression of genes in differentiated MG-63 osteosarcoma cells using quantitative polymerase chain reaction (qPCR) technique for markers (*ALP, Runx2, Osx, BSP, RANKL* and *OPG*) of osteogenesis.

- **Protein expression**

To investigate the effects of TUG-891 on protein (ERK and AKT) expression and activation in differentiated MG-63 osteosarcoma cells using Western blotting.

Chapter 2 Literature review

In this section a short description of bone, its structure and functions will be discussed. Thereafter, different types of cells which make up bone will be explained in more detail. Signalling pathways involved in the differentiation of osteoblasts during osteoblastogenesis will be discussed as well as the osteoblast transcriptional factors including the runt-related transcription factor 2 (Runx2) signalling pathway. The Eph and ephrin signalling pathway, the AKT and mitogen-activated protein kinase (MAPK) pathway in osteoblasts will also be addressed in more detail. Thereafter, bone remodelling and bone modelling will further be addressed together with issues that arise due to imbalances in bone remodelling resulting in osteoporosis. Lastly, G-protein coupled receptors (GPCRs) particularly GPR120, will be discussed together with its signalling pathways and its metabolic health benefits including in insulin resistance, and bone health to give a better understanding of current knowledge and what further studies should focus on.

2.1 Bone

The skeleton is a dynamic and metabolically active organ. It serves a variety of functions such as movement, structural support, calcium and phosphate storage and vital organ protection.[18-19] Metabolically, the skeleton has two main functions: metabolism of minerals by way of phosphate and calcium homeostasis as well as acid-base balance through the buffering of hydrogen ions.[20] The bones that make up the skeleton can be separated into 4 categories: long, short, flat, or irregular bones. The femurs and tibiae are some of the examples of long bones. Short bones include the tarsal and carpal bones. The skull and sacrum are some of the examples of flat and irregular bones, respectively.[21] Long bones are made up of three parts: a tubular shaft termed the diaphysis, which is mostly dense cortical bone; conical metaphyses beneath the growth plates; and spherical epiphyses upwards of the growth plates (Figure 1). Both the metaphyses and

epiphyses are made mainly from the trabecular meshwork bone which is enclosed by a cortical bone shell that is thin and dense.[21] Bones may be formed through endochondral and/or membranous ossification (bone formation). Endochondral bone formation starts by forming skeletal elements as cartilage template which are then substituted by bone. Chondrocytes are responsible for secreting a type X collagen-rich matrix called the osteoid that forms a scaffold which will then be mineralized by osteoblasts.[20] During membranous ossification mesenchymal stem cells will differentiate into mineralizing osteoblasts directly in the area where the bone is formed.[21]

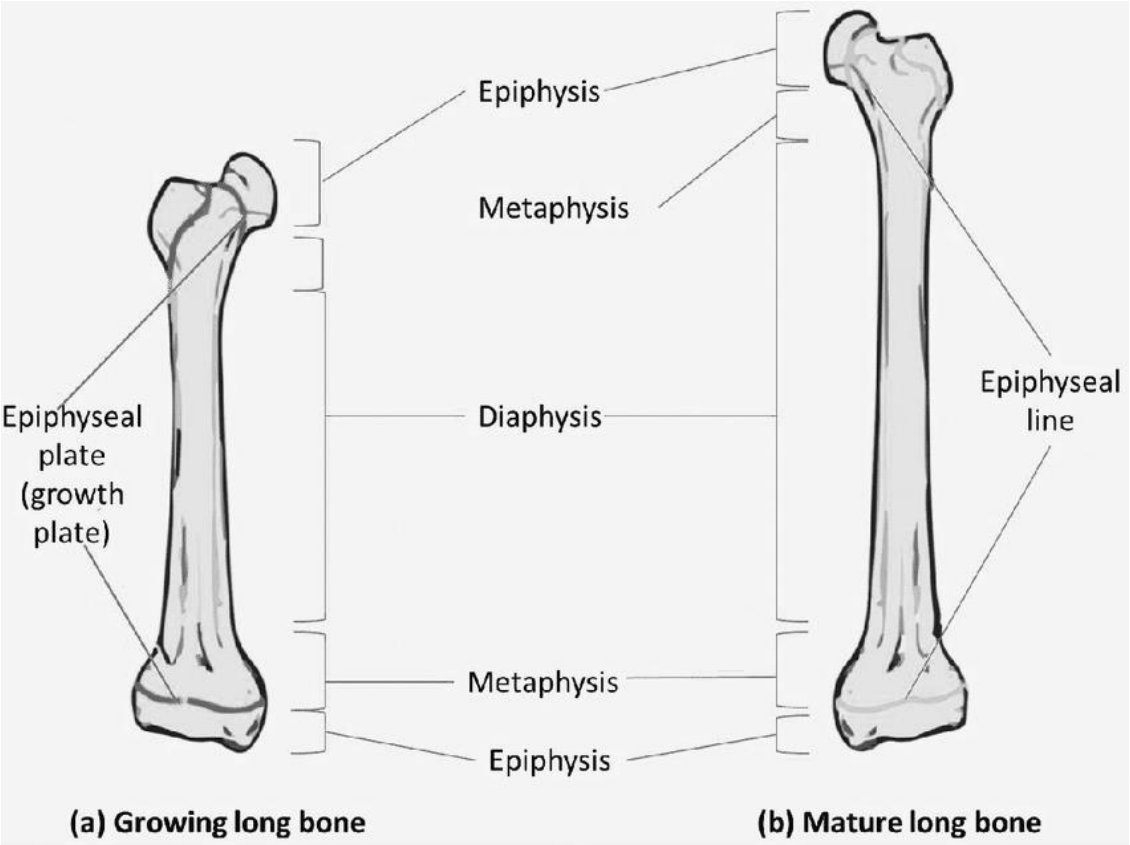


Figure 1: Long bone structure. (a) Epiphyses, epiphyseal plates, metaphysis, and diaphysis of a growing long bone. (b) Epiphyseal plates transforms into epiphyseal lines in a mature long bone.[22]

There are 2 main types of bone: cortical bone, which makes up 80% of the skeleton, and trabecular bone, which makes up the other 20%.[20-21] Cortical bone is stronger, denser and has vascular canals and therefore, forms the outer shell of the bone surrounding the marrow space. Trabecular or spongy bone is less dense and is composed of a honeycomb-like network of trabecular plates and rods distributed throughout the marrow portion; it has low torsional resistance but is more elastic for mechanical support.[20-21] Osteons make up both the trabecular and cortical bone (Figure 2). Cortical bone osteons are called Haversian systems whereas packets are osteons of the trabecular bone.[21] Haversian systems inside the cortical bone, with walls formed from concentric lamellae, are shaped like a cylinder and create a branching network. Packets have a semilunar shape and are made from concentric lamellae.[21] Depending on the species, cortical bone will be less metabolic activity than trabecular bone. An outer periosteal surface and an inner endosteal surface are present in the cortical bone. Fracture repair and appositional growth are achieved through the periosteal activity where bone formation surpasses bone resorption. The endosteal surface, however, has high remodelling activity with resorption exceeding formation due to increased cytokine exposure.[21]

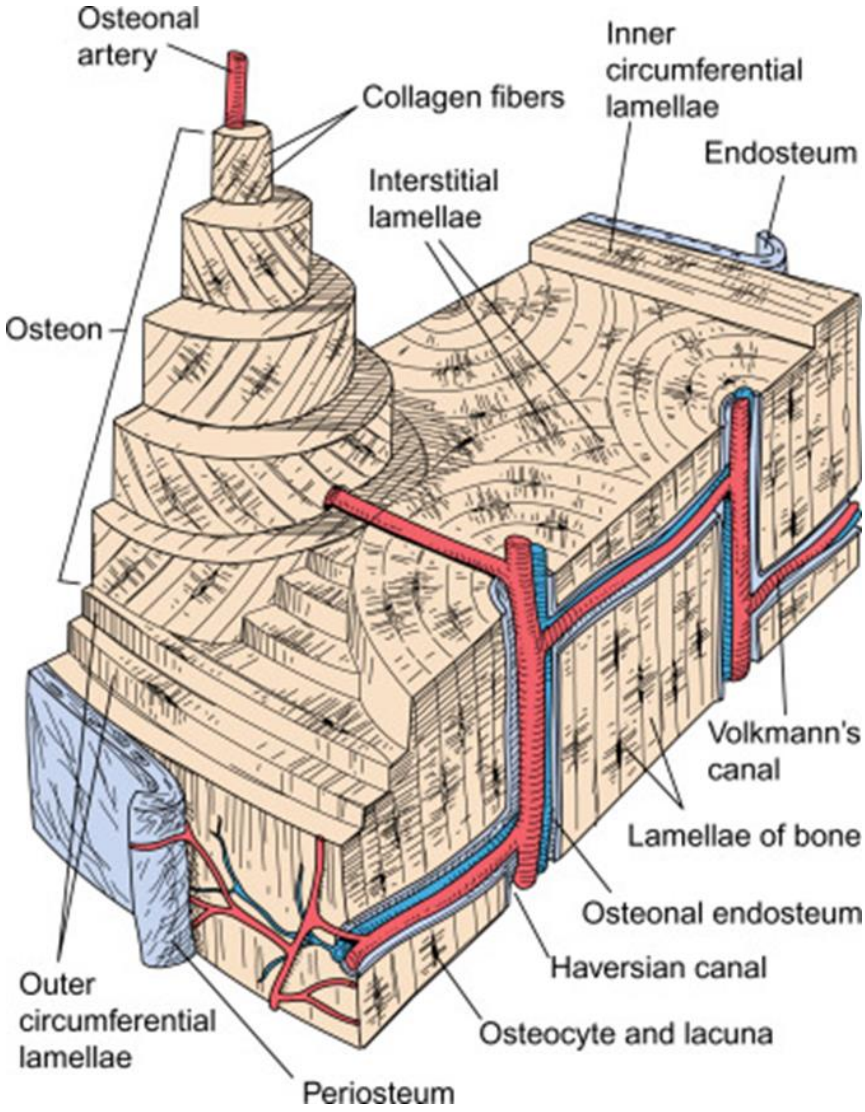


Figure 2: A diagram from a long bone representing a section of cortical bone. An osteon is stretched out above the cross section to aid in the identification of several concentric lamellae and perpendicular collagen fibre orientation in surrounding layers. Image reprinted with permission from Lynnerup N *et al*. [23]

Cortical and trabecular bones are a mixture of osteoid matrix and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] crystal and contain water, specialised bone cells, lipids and non-collagenous proteins.[20] Three helical chains make up collagen fibres, which join to form fibrils. The type I collagen bone matrix is important for bone elasticity

and flexibility. Non-collagenous proteins also make up the matrix and they regulate mineralisation and strengthen the collagen structure. The hydroxyapatite crystal is vital for storing minerals that are needed for homeostasis.[20] Collagen fibrils are established in an cyclic orientation in a lamellar pattern to generate both types of bones.[21] In woven bone, however, the normal lamellar pattern is absent resulting in a weaker bone than lamellar bone. The woven bone can be visible in high turnover conditions like Paget's disease, where bone formation is high.[21]

The cortical surface of a bone is surrounded by a sheath of fibrous connective tissue covering (the periosteum) (Figure 2) via thick collagenous fibres called Sharpey's fibres. Nerve fibres, blood vessels, osteoclasts, and osteoblasts are all present in the periosteum. The endosteum is a membrane that covers the inside of cortical bone, trabecular bone, and the blood vessel canals called the Volkmann's canals which are present in the bone. Additionally, present in the endosteum are the blood vessels and bone cells (osteoclasts, osteoblasts, bone lining cells and osteocytes).[21]

2.2 Bone cells

2.2.1 Osteoclasts

The osteoclast, the only bone-resorbing cell in the body, is a large cell which usually contains around eight nuclei in the cell but may contain as many as 100 in patients with Paget's disease.[21,24-25] The myeloid hematopoietic precursors or macrophage precursor cells of osteoclasts[21,26] are stimulated to fuse and differentiate into mature osteoclasts by the differentiation factors colony-stimulating factor 1 (CSF-1); also known as macrophage colony-stimulating factor (M-CSF);[27] and receptor activator of nuclear factor kappa B (NF-kB)(RANK) ligand (RANKL).[24-25] M-CSF promotes cell proliferation and survival during osteoclast formation, whereas the signalling needed for precursor cells to transition into osteoclasts is stimulated by RANKL.[26] Osteoclast differentiation factor (ODF), osteoprotegerin (OPG) ligand and tumour necrosis factor (TNF)-

related activation-induced cytokine (TRANCE), are other names for RANKL.[28] RANKL is expressed on the surface of osteoblasts, osteocytes, stromal cells, and T cells.[29] RANK is a member of the TNF receptor family and a receptor for RANKL, found on the surface of osteoclasts.[29]

When RANKL binds to RANK, TNF receptor-associated factor 6 (TRAF6) is recruited and the transforming growth factor-β (TGF-β) activated kinase 1 (TAK1)-TAK1 binding protein (TAB1) complex is formed.[24] As a result MAPKs, such as p38 MAP kinase (p38), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK); the NF-κB signalling pathways; ionic calcium/calmodulin-dependent kinase pathways; and AKT pathways, are activated.[24-26,28] This results in the activation of nuclear factor of activated T-cells (NFAT) cytoplasmic 1 (NFATc1), the crucial osteoclast development and function regulator.[24-25]

In the cytoplasm, NF-κB is bound to inhibitor of kappa B (IκB), preventing it from crossing into the nucleus and activating NF-κB nuclear targets. In order for NF-κB to translocate to the nucleus, inhibitory kappa kinase (IkK) is phosphorylated leading to the phosphorylation and destruction of IκB.[24] This frees NF-κB to translocate into the nucleus and activate targets which can lead to the activation of NFATc1. NFATc1 will then activate genes involved in osteoclast formation and function such as dendritic cell-specific transmembrane protein (DC-STAMP), which is involved in the cell-to-cell fusion of osteoclast precursors; and the resorption enzymes cathepsin K and matrix metalloproteinase 9 (MMP-9) which break down the osteoid matrix of bone, and carbonic anhydrase 2 which acidifies the resorption lacuna to break down the hydroxyapatite.[25]

The RANKL/RANK/OPG pathway is key in regulating bone resorption and formation by coupling the activity of osteoclasts and osteoblasts.[30] OPG, is a decoy receptor for RANKL produced by osteoblasts and osteocytes.[20] OPG binds RANKL preventing RANK-RANKL interaction and thereby inhibiting bone

resorption.[20] The RANKL:OPG ratio is crucial for bone resorption. A low ratio decreases osteoclasts and bone resorption whereas a high ratio increases osteoclasts formation and bone resorption.[24] Osteoblasts are also responsible for modulating the differentiation of osteoclasts[25] by also producing RANKL, M-CSF and OPG.[24] Therefore, osteoblasts can maintain the balance between bone resorption and formation by controlling osteoclastic resorption.[24]

2.2.2 Osteoblasts

Osteoblasts are mononuclear cuboidal cells derived from mesenchymal stem cells (MSCs).[19] These same stem cells can give rise to adipocytes, myocytes and chondrocytes in response to specific transcription factors.[19,31] Osteoblasts are the bone forming cells and are therefore responsible for adding a new, mineralized layer of bone after resorption has taken place.[24,26] The osteoblast is a polarized cell and produces osteoid, the organic hydrated protein matrix, towards the bone matrix.[19,32] Osteoid is a very dense type I collagen parallel layer that alternates in orientation and contains small amounts of bone matrix proteins,[32] which are produced in osteoblast formation to cause osteoblastic mineralization.[26] These non-collagenous bone matrix proteins are used as markers of the osteoblast differentiation process and they include but are not limited to osteocalcin (OCN), alkaline phosphatase (ALP), bone sialoprotein (BSP), type I collagen (Col1a1), and osteopontin (OPN). Early indicators of osteoblast differentiation include BSP, ALP, and Col1a1, whereas OCN appears later in the mineralisation process.[31] OPN can be used as a marker of both early and late differentiation as its expression peaks during proliferation and in the later differentiation stages.[31] Bone ALP is a reliable indicator of bone formation, together with other proteins supplies phosphate for bone mineral.[32] OCN is a signalling molecule and also an indicator of osteoblast synthesis activity.[32] OPN is a protein that inhibits the formation of mineral and has been shown to be upregulated by an increase in an inorganic phosphate (Pi).[33] BSP is a small integrin-binding ligand *N*-linked

glycoprotein (SIBLING) that binds calcium to promote biomineralization through mineral nucleation.[34]

Osteoblasts are involved in the formation of the osteoid, which hardens the bone matrix and entraps osteoblasts, which then transforms into osteocytes in intramembranous bone production.[35] Continuous osteoid production leads to the direct formation of bone.[35-36] Although both the intramembranous and endochondral processes start with MSC proliferation and condensation,[36] MSCs initially differentiate into proliferating chondrocytes and generate the earliest cartilage templates that are surrounded by the perichondrium in endochondral bone formation.[36] In cartilage templates, chondrocytes further develop into hypertrophic chondrocytes.[36] MSCs in the perichondrium also differentiate into osteoblasts, which build a bone collar that will eventually form the cortical bone.[36-37] Blood vessels and osteogenic cells infiltrate hypertrophic cartilage, eventually forming the primary ossification centre in the middle of the diaphysis,[37] which leads to the formation of bone and bone marrow.[36] At the proximal and distal epiphyses, secondary ossification centres form, leaving a growth plate between the ossification centres (metaphysis), which supplies the mechanism for further longitudinal growth.[35-36]

2.2.2.1 Biomineralization

A process where minerals are deposited inside or outside of the cells of different organisms is called biomineralization.[38] In bone, osteoblasts are responsible for the formation of hydroxyapatite and there are two steps involved in osteoblastic mineralisation.[38-39] Firstly, a membrane that is rich in phosphatidylserine attracts annexins and allows them to form calcium channels in the matrix vesicles for crystal formation by introducing calcium into the vesicles layer.[33,39] Matrix vesicles are extracellular vesicles created by the polarized budding of chondrocytes and osteoblasts on their surface membranes.[39] Together, annexins and calcium-binding phospholipids and proteins facilitate the accumulation of calcium in the

matrix vesicles.[38-39] Protons will be removed, which will reduce the acidity and allow the hydroxyapatite to condense and form microcrystals needed to produce the strong and dense bone composite.[32] The production of phosphates essential for mineralisation is regulated by the membrane phospholipid C through hydrolysed phosphocholine and phosphor-ethanolamine by the cyto-phosphatase PHOSPHO1 (Figure 3).[32,39] Phosphates are also delivered by Type III Na/Pi co-transporters on both the vesicle matrix and the cell membranes.[38-39] When the solubility limit for the calcium and phosphate accumulation is reached, the hydroxyapatite deposition then takes place within the vesicles of the matrix (Figure 3).[38-39] The mineral is then formed and matures from amorphous calcium phosphate to hydroxyapatite.[39]

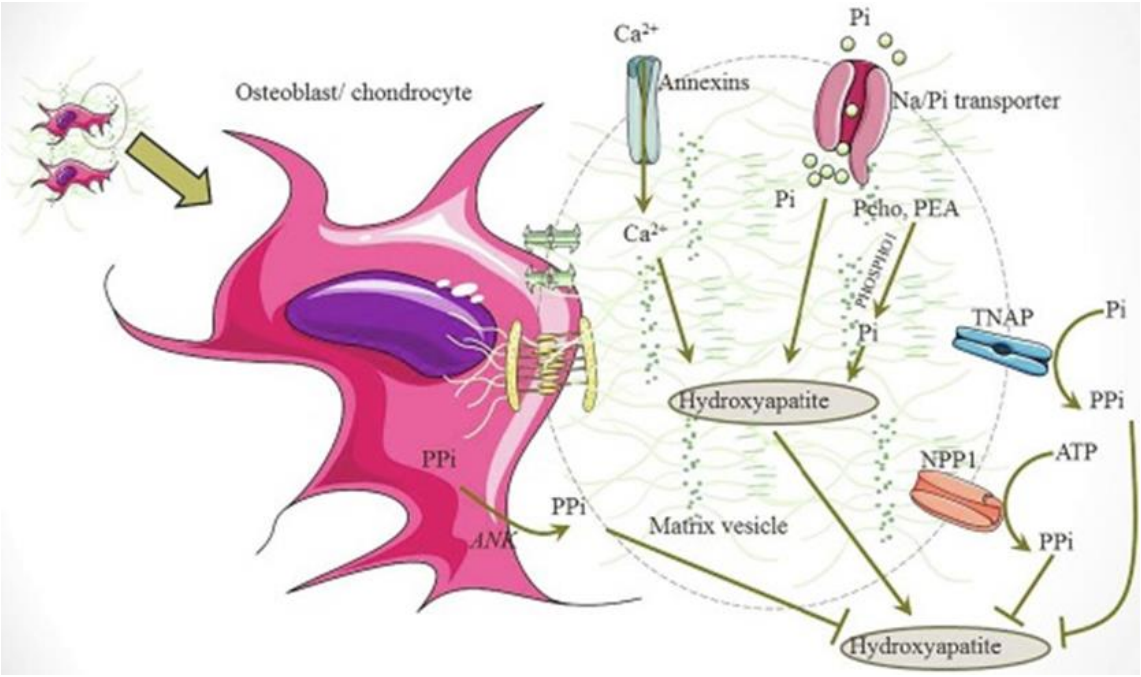


Figure 3: Bone mineralisation and signalling pathways involved. Mineralisation begins when the hydroxyapatite is deposited in the matrix vesicles. The annexin calcium channel provides calcium whilst the sodium phosphate co-transporter together with the help from the actions of the phospho-ethanolamine (PEA) and phosphocholine (PCho) provides the inorganic phosphate for the formation of the crystal hydroxyapatite. The hydroxyapatite then accumulates within the collagen fibrils with the help of the tissue-nonspecific alkaline phosphatase (TNAP) and the

nucleotide pyrophosphatase 1 (NPP1) which forms pyrophosphate (PPi). Ankyrin (ANK) then transports PPi to inhibit further formation of the crystal and leading to the transportation into the extracellular space. Image obtained with permission from Vimalraj S.[39]

Secondly, the hydroxyapatite crystals are distributed into the membrane of the matrix vessel and then drawn into the extracellular space where sufficient calcium and phosphate rates of accumulation are vital.[38] Continuous production of new hydroxyapatite crystals is achieved by the calcium ion (Ca^{2+}) and Pi levels in the extracellular area (Figure 3).[38-39] The hydroxyapatite is then clustered around matrix vesicles and seals bone matrices within the collagen fibrils.[38] The Pi to inorganic pyrophosphate (PPi) ratio is important in this mineralisation step.[38-39] The apical surface of the bone matrix contains ALP which supplies phosphate for bone mineral.[39] ALP plays a role in the formation of Pi from PPi.[33,39] PPi is an inhibitor of hydroxyapatite formation.[33,38] Nucleotide pyrophosphatase 1 (NPP1) from nucleotide triphosphates (NTP) increases the extracellular PPi to repress mineralisation whereas, tissue-nonspecific alkaline phosphatase (TNAP) decreases PPi and increase Pi to promote mineralisation.[33,39] Ankyrin is a transmembrane protein located on the membranes of hypertrophic chondrocytes and osteoblasts and allows for the transportation of PPi from cells (Figure 3).[39] The process is completed at different serum calcium and phosphate concentrations depending on the pH levels.[39] Bone formation is maintained in alkaline environments due to ectoenzyme alkaline phosphatases which remove protons and increase the pH whereas, in bone resorption, the pH decreases due to protons being placed into the resorption pit.[39]

2.2.3 Bone lining cells

Bone lining cells are inactive osteoblasts that line the surface of the bone. These quiescent flat-shaped cells form the endosteum on trabecular bone as well as the endothelial surfaces of mineralized bone, where neither formation nor resorption are taking place.[19,21] The physiological status of bone determines the secretory activity of bone lining cells. In metabolically active bone, the lining cells can

resume their secretory function and expand to appear cuboidal-like. Bone lining cells can produce both OPG and RANKL and therefore, like osteoblasts, can control the differentiation of osteoclasts.[19] Bone lining cells can also control osteoclast resorption by covering the surfaces of bone and preventing osteoclast attachment to the bone matrix when resorption is not needed.[19]

2.2.4 Osteocytes

Osteocytes are another osteoblast-derived cells and are the most numerous and long-lived bone cells in the body,[27] and can survive up to 25 years. Osteocytes form multiple cytoplasmic processes, known as filopodia, which extend from the cell body and gives the cell its characteristic dendritic morphology. Podoplanin is believed to be an important factor in the development of these cytoplasmic processes.[19] Osteocytes are located within lacunae in the bone matrix and the filopodia pass through canals known as the canaliculi. The filopodia play an important role in the transport of nutrients in the bone tissue as well as the cell-to-cell communication between osteocytes.

Once osteoblasts have completed the process of bone formation, some osteoblasts remain embedded in the bone matrix where they will mature into osteocytes.[27] During the maturation of the osteocyte, osteoblast markers such as OCN and ALP, will be downregulated.[19] However, osteocyte markers such as dentine matrix protein 1 (DMP1) and sclerostin (SOST) will be upregulated in order to stimulate osteocyte maturation.[19] The osteocyte plays an important role in translating mechanical load on the bone into biochemical signals which control the bone remodelling cycle.[25] These biochemical signals include nitric oxide (NO), ATP, calcium (Ca2+), and prostaglandins (PGE_2 and PGI_2).[19] Osteocytes also express bone matrix proteins (e.g. OCN, galectin 3 and cluster of differentiation 44 (CD44)) which are important for the adhesion of the cell to the bone and for regulating the mineral exchange within the lacunae.[21]

2.3 Osteoblast transcriptional factors

A variety of homeodomain proteins, including the Smads, lymphoid-enhancing factor (LEF) and members of the activator protein (AP) family govern osteoblast differentiation. Runt-related transcription factor 2 (Runx2), osterix (Osx) and activating transcription factor 4 (ATF4) are considered to be the master genes that govern osteoblast differentiation.[31]

The expression of Runx2, also known as the core-binding factor A1 (CbfA1) is the earliest noticeable event which takes place during osteoblast formation.[23-24] Runx2 belongs to the Runt family of transcription factors, which also includes Runx1 and Runx3, and is expressed in multipotent MSCs, osteoblasts, and chondrocytes. Runx2 also plays an important role in the development of the mammary glands.[40] The two promoters, P1 and P2, that control the transcription of the Runx2 gene are expressed in osteoblasts as well as chondrocytes.[40] Runx2 forms a heterodimer with Cbfb, allowing it to bind to deoxyribonucleic acid (DNA) more effectively.[40] This will allow for the upregulation of genes involved in the osteoblast differentiation and function such as *ALP*, *Col1A1*, *OCN* and *BSP*.[24] Vascular invasion of the cartilage moulds does not occur in Runx2 knockout, and bone formation cannot occur demonstrating the crucial role of Runx2 in osteoblast formation.[18]

Osx is another crucial transcriptional regulator of osteoblasts that controls the later stages of the formation of bone.[31] Osx is also crucial for the upregulation of osteoblast specific genes.[24] Although Runx2 and Osx are both essential for osteoblast differentiation at the early and late stage, Runx2 serves as the master regulator that is upstream of Osx.[31] The importance of Osx is highlighted by the fact that Osx-knockout mice cannot form bone even though the MSCs of these Osx-knockout mice express normal amounts of Runx2.[31,41] This further demonstrates that Osx is downstream of Runx2. AKT, also known as Protein kinase B, is important for increasing the protein stability, and osteogenic and

transcriptional activity of Osx during bone formation.[26] Runx2 activity can control AKT-mediated signalling pathways allowing for Runx2 to control the activity of Osx.[26]

ATF4 along with its substrate, ribosomal 6 kinase 2 (RSK2) are important in the terminal differentiation of osteoblasts as well as the expression of BSP and OCN.[31] During the final stages of osteoblast development, RSK2 phosphorylates and activates ATF4. RSK2 and ATF4 will then regulate the formation of type I collagen, which is the major component of bone matrix.[31] Runx2 has been shown to interact with ATF4 to stimulate OCN expression in osteoblasts.[42] This suggests that ATF4 may regulate the activity of Runx2 and therefore play a critical role in osteoblast differentiation.

2.4 Osteoblast signalling

There are various signalling pathways that contribute to osteoblast differentiation,[26] these pathways include but are not limited to the fibroblast growth factor (FGF), MAPK, AKT, bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF- β), and wingless-type MMTV integration site family (Wnt)/β-catenin proteins.[26]

2.4.1 The wingless-type MMTV integration site family (Wnt)/β-catenin pathway

Wnts are a family of 19 glycoproteins that are secreted to mediate biological processes such as embryogenesis.[43] The family of seven-transmembrane frizzled receptors (FZD) are stimulated by Wnt ligands.[31] The Wnt ligands interacts with one of two low-density lipoprotein (LDL) receptor-related proteins (LRPs) and one of ten FZD G-protein coupled receptors to stimulate any of four intracellular signalling pathways.[43] Wnt proteins can be classified into two distinct classes: the canonical stimulating proteins and the non-canonical stimulating proteins.[31] The canonical Wnt signalling pathway requires the binding of FZD and

LRP to Wnt ligands to stabilise β-catenin in the cytoplasm.[31] In the non-canonical pathway, Wnt5a interacts with FZD receptors to activate the protein kinase C (PKC) pathway to increase intracellular calcium levels or to stimulate changes in the cytoskeleton through Rho/JNK signalling pathways.[31] The canonical Wnt signalling pathway was identified as a major transcriptional regulator of osteoblasts by studying rare bone diseases.[20,31] In the absence of Wnt, adenomatous polyposis coli (APC), axin, and glycogen synthase kinase 3 (GSK3) form a multi-subunit β-catenin degradation complex where β-catenin is ubiquitinated.[20] In the canonical pathway, the interaction of Wnt ligands to FZD and LRP receptors causes the binding of dishevelled (Dsh) to Axin proteins which dissociates the β-catenin degradation complex. As a result, β-catenin is phosphorylated and translocated into the nucleus.[31,43] The soluble β-catenin then binds and stimulates several transcriptional factors such as LEF,[20,31] leading to osteoblast proliferation and differentiation.[20] The non-canonical signalling pathway may also contribute to the osteoblast differentiation,[44] although its significance in bone formation is poorly understood.[43] Various intracellular and extracellular proteins influence the canonical and non-canonical pathways.[43] These proteins include the secreted frizzled-related proteins (sFRPs), Wnt inhibitory factors (WIFs), dickkopfs (Dkks), Wise, SOST and connective tissue growth factor (CTGF). WIFs and sFRPs proteins interact with Wnts to prevent Wnts binding to FZD receptors. Similarly, Dkks, SOST and CTGF interacts with LRPs to prevent Wnt signalling. Dkks inhibits the canonical pathway only but sFRPs and WIFs can inhibit both pathways.[31,43] The Wnt signalling pathways and associated proteins are crucial for the differentiation of MSCs to mineralizing osteoblasts.[24] The activation of the Wnt signalling pathways leads to the activation of Runx2 transcriptional factor which controls osteoblast formation.[45]

2.4.2 Transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP)

TGF-β proteins play a role in the proliferation, differentiation, and growth of cells.[46] Skeletal development and maintenance are regulated by both TGF-β and BMPs.[47] The activation of the MAPK pathway or the receptor regulated mothers against decapentaplegic (Smad) (R-Smads) and common-partner Smads (Co-Smads) transduce both TGF-β and BMP signals.[47] Three known isoforms of TGF-β exist: TGF-β1, TGF-β2 and TGF-β3; with only TGF-β1 being the most prevalent in bone tissue.[47] In the TGF superfamily, ligand-receptor binding is initiated by the heterodimeric serine-threonine kinase receptor complex.[46] Bone resorption can lead to the release and activation of inactive TGF-β proteins from the bone matrix. Activated TGF-β proteins can then stimulate osteoblastic bone formation.[47] In this way, TGF-β1 can regulate bone remodelling by linking bone formation to bone resorption. Previously, studies on the mechanisms of TGF-β for osteoblast regulation showed that TGF-β1 triggers bone matrix thickness and osteoblast growth *in vitro* while also suppressing the late stage of osteoblast differentiation.[47] TGF-β increases parathyroid hormone (PTH) functional receptors while PTH causes the formation of TGF-β1 and TGF-β2 in osteoblasts to regulate osteoblastogenesis.[47] TGF-β activates inhibitory Smads to inhibit BMP signalling and the late stages of osteoblast formation.[47]

BMPs and TGF-β share structural similarities but have distinct ways of interacting to the receptors.[46] BMPs are found in skeletal tissue and can stimulate bone formation by upregulating Runx2 and Osx expression through the Smad and MAPK pathways.[47] BMPs 1 to 7 have all been indicated to play a crucial in skeletal development by enhancing osteoblastogenesis.[31,47] In osteoblast cultures, BMP-2, -4 and -6 have been found to be expressed highly, further indicating their crucial role in osteoblast formation.[31] BMP signalling takes place when BMP binds to type I or II receptor kinase to form the heterodimeric BMP receptor-ligand complex. In the complex, the active type II receptor trans-

phosphorylates type I receptor which then activates the canonical Smad-mediated and the non-canonical p38 MAPK pathway.[46] In the canonical pathway, type I receptors can phosphorylate R-Smads leading to the nuclear translocation of Smad4 which will upregulate Runx2 expression. In the non-canonical pathway, the type I receptor kinase subunit phosphorylates and activates TAK1, triggering a kinase cascade including MAPK kinase (MKK) 3 and MKK6, which phosphorylates and activates p38 MAPK resulting in the transcription of Runx2.[46] TGF-β and BMP signalling pathways both have the key osteoblast regulator, Runx2, as a final gene target.[46] These pathways are therefore crucial for osteoblast formation. Inhibition of BMP signals can be achieved through secreted polypeptides such as noggin, chordin, gremlin, and twisted gastrulation which act as antagonists to modulate BMP signals.[47] BMP and Wnt signalling might also co-operate and co-regulate each other as BMP-2 has been shown to activate β-catenin signalling, possibly by inducing the expression of canonical Wnts, to promote osteoblast differentiation.[48]

2.4.3 Fibroblast growth factors (FGF) signalling

The FGFs are a family of roughly 22 signalling proteins that can bind to five FGF receptors (Fgfrs).[49] These signalling proteins regulate bone formation by binding to four tyrosine kinase receptors (Fgfr1-Fgfr4).[31] These receptors are found in the immature and adult skeleton and have been shown to be expressed in tissues that produce osteoblasts and in differentiated osteoblasts.[31] Fgfr1 signalling has been shown to stimulate differentiation of osteoblast progenitor cells, while suppressing differentiation in mature osteoblasts.[31] In osteoblasts, there are three likely FGF ligands (FGFs 2, 9 and 18) that signal Fgfr1. During embryonic development, FGFs 9 and 18 signalling are crucial, while FGF2 seems to have a significant role in differentiated osteoblasts.[31] FGF2 has been shown to phosphorylate and activate Runx2 via the MAPK pathway, indicating that FGF2 is critical in the control of Runx2 activity and the formation of bone.[31] The MAPK and PKC are two FGF signalling pathways that have been associated to

osteoblast differentiation. FGF signalling, however, seems to inhibit canonical Wnt signalling indirectly through upregulation of the transcription factor SRY-box transcription factor 2 (Sox2).[48]

2.4.4 Ak strain transforming (AKT) signalling

The Ak strain transforming (AKT) (also known as protein kinase B, PKB) is a family of three serine/threonine protein kinases (AKT1–3) which regulates many essential physiological activities such as cell growth, proliferation, and survival.[49-51] AKT is the principal target of phosphatidylinositol-3,4,5 trisphosphate (PI3K) signalling.[51] Phosphatidylinositol-3,4,5phosphate (PIP3) is produced by PI3K, which recruits and activates pyruvate dehydrogenase kinase 1 (PDK1) and AKT in this signalling pathway.[52] PI3K can result in the translocation of AKT to the cell membrane where it is phosphorylated and subsequently fully activated by the mammalian target of rapamycin (mTOR) C2/Rictor complex and PDK1.[49] Once activated AKT will phosphorylate several different substrates such as GSK3β, mTOR, endothelial NO synthase, and BCL2 Associated Agonist Of Cell Death (Bad).[52] Growth factors and other extracellular signals can activate the PI3K/AKT signalling pathway.[50-51] AKT can modulate cellular growth by activation of mTOR to promote protein synthesis. The suppression of the forkhead box protein (FOXO)-mediated cyclin-dependent kinase inhibitor (CDKi) and p27 transcription by AKT can enhance proliferation. The FOXO transcription factor family is phosphorylated by AKT, which regulates cell survival by lowering the expression *Bim* gene, a pro-apoptotic target of FOXO, or by phosphorylation of Bad for direct deactivation of FOXO.[49]

In osteoblasts, the PI3K/AKT signalling pathway is critical for normal growth and maintenance of the skeletal.[49] By preventing nuclear translocation of FOXOs, AKT has been demonstrated to promote bone formation and osteoblast survival.[52] Suppression of the PI3K/AKT signalling pathway was found to enhance osteoblast damage.[50] Since Runx2 does not contain an AKT consensus

phosphorylation site, direct phosphorylation interactions between Runx2 and PI3K/AKT is unlikely. However, *AKT1* knockout mice have shown a reduced Runx2 expression.[53] AKT has further been shown to inactivate GSK3β and cause nuclear exclusion of *FOXO1* to enhance Runx2 activity.[54] Inhibition of GSK3β by AKT, activates β-catenin and increases activate LEF target genes.[49] As mentioned previously, β-catenin and LEF are important regulators of the canonical Wnt pathway and are crucial for osteoblast formation. In addition, AKT together with BMP2 regulates the differentiation of osteoblasts from MSCs.[52]

2.4.5 Eph and Ephrin signalling

The ephrin ligands (efnA and efnB) bind to ephrin receptors (EphA and EphB), the largest group of receptor tyrosine kinases (RTKs) known.[55] The transmembrane efnB and glycosylphosphatidylinositol (GPI) anchored efnA families comprises the two subfamilies of the ephrin ligands. There are 14 known Eph receptors and 8 ephrin ligands.[56] The efnA ligand interacts with its receptor in a "lock-and-key" manner, whereas the efnB ligand interacts with its receptors by changing its conformation that causes a "induced fit" conformation.[55] In combination with the activation of their respective receptors, ephrin ligands can induce a "reverse" signal that is unique from the "forward" signal.[55] When the binding of ephrin ligands and receptors occurs, a bidirectional signal is activated, whereby both the receptor (forward) and the ligand (reverse) signalling can activate downstream signalling pathways to create diverse consequences.

In bone, osteoclasts express the ephrin ligand, ephrinB2, and while osteoblasts express the receptor, EphB4.[57] The ephrinB2/EphB4 form a bidirectional signalling pathway[58] to regulate the differentiation of these bone cells to reduce resorption and improve bone formation.[57] The ephrinB2 signalling inhibits the c-fos-NFATc1 pathway to decrease osteoclast formation whereas, EphB4 signalling improves osteoblast formation by inducing osteogenic markers including Runx2[57-58] possibly through the ERK pathway.[57] Zhao *et al*. revealed an

increase in osteoclast formation with a loss of ephrinB2 reverse signalling.[57] Wang *et al.* proved that Insulin-like growth factor 1 (IGF-1) signalling is vital in bone cell differentiation through the ephrin B2/EphB4 pathway.[58]

2.4.6 MAPK pathway in osteoblasts

ERK, p38 and JNK pathways are the main MAPK pathways that regulate bone formation in osteoblasts.[59] In osteoblastic cells, BMP-2 can activate MAPK indicating that these pathways have unique roles in regulating ALP and OCN expression. BMP-2 stimulates JNK and p38 via protein kinase D (PKD) function during osteoblast differentiation, independent of PKC activity.[31]

2.4.6.1 ERK MAPK pathway

ERK is a key regulator of Runx2 activity in the skeletal system.[59] Both ERK1 and ERK 2 are expressed in osteoblasts.[59] The ERK1/2 pathway is crucial in the early proliferation and differentiation of osteoblasts.[60] The ERK pathway mediated signal transduction is initiated when extracellular stimuli bind to RTKs. This binding promotes the stimulation of Ras-guanosine-5'-diphosphate (GDP) by the exchange of guanyl nucleotide.[60] Ras- guanosine-5'-triphosphate (GTP) attaches and activates Ras-binding domain of rapidly accelerated fibrosarcoma (Raf) which stimulates MAPK/ERK kinase (MEK)1/2 to activate ERK1/2.[60] ERK1/2 acts on downstream transcription factors to promote the expression of target genes that regulate the biological properties of cells.[60] When MEK1/2 activates ERK, the active ERK binds to OCN and BSP promoters in osteoblasts through ERK and Runx2 interaction (Figure 4a).[59] *Erk1* knockout and *Erk2* conditional knockout in osteoblast mice show reduced bone mineralisation.[59] In osteoblast-like cells, activation of the ERK signalling pathway can prevent apoptosis, improve cell survival, and boost cell proliferation.[61] Besides Runx2, ERK has a number of additional recognised targets that help in osteogenesis such RSK2 and Fos-related antigen 1 (FRA1) (Figure 4a). RSK2 phosphorylates ATF4 and is required for osteoblast differentiation to regulate the formation of col1a1.[62]

FRA1 is a Fos family of subunits of activator protein 1 (AP-1) transcriptional complex that plays an important role in mineralization in mature osteoblasts. FRA1-deficent osteoblasts show an increased ALP activity but reduced mineralization capacity.[59]

2.4.6.2 p38 MAPK pathway

There are four isoforms of p38 MAPK, p38α, p38β, p38γ and p38δ.[63] p38 MAPK may convey signals through phosphorylation of transcription factors directly or indirectly by stimulating other kinases that later phosphorylate its downstream substrates.[63] Inhibition of p38α and p38β by the p38 MAPK inhibitor SB203580 has been demonstrated to inhibit the early stages of osteoblastogenesis *in vitro*.[63] MKK3 and MKK6 are the MAPK kinases that primarily activate p38, where MKK3 activates every isoform, except for p38β (Figure 4b).[59] An analysis of mice without either MKK3 or MKK6 found that both contribute to long bone mineralisation in dose-dependent ways although MKK6 has a significant contribution to calvaria mineralisation.[59] Although p38α and p38β have unique functions, some functions of both isoforms and deletion of both isoforms results is developmental defects which have not been observed with deletion of either of the isoforms.[59] The p38 MAPK pathway, like ERK MAPK, promotes osteoblast development in part by phosphorylating Runx2.[59] Runx2 activity promotes interaction with the transcriptional co-activator cyclic-adenosine monophosphate (AMP) response element binding protein (CREB)-binding protein (CBP) (Figure 4b). As a result, MAPK activity is required for the construction of a complex that includes Runx2, CREB, and CBP.[59] A study showed that p38 MAPK inhibition interferes with the differentiation process of osteoblasts.[63] Furthermore, all markers of differentiation including ALP and OCN were decreased in the presence of the inhibitor.[63]

Osteoblast differentiation can also be regulated by the distal-less (*Dlx)* genes (*Dlx1-6*) which represents the homeobox-containing transcription factors.[64] The *Dlx* genes have also been demonstrated to mediate the stimulation of Runx2 and

Osx through BMP-2 signalling.[64-65] *Dlx5* is only detected in osteogenic MSCs[65] and acts as a p38 substrate (Figure 4b).[59] The overexpression of *Dlx5* has been linked to increased osteoblast differentiation whilst *Dlx5* deletion impairs osteogenesis.[64-65] The effects of p38 in osteoblasts are also modulated by Osx, which is activated downstream of Runx2.[59]

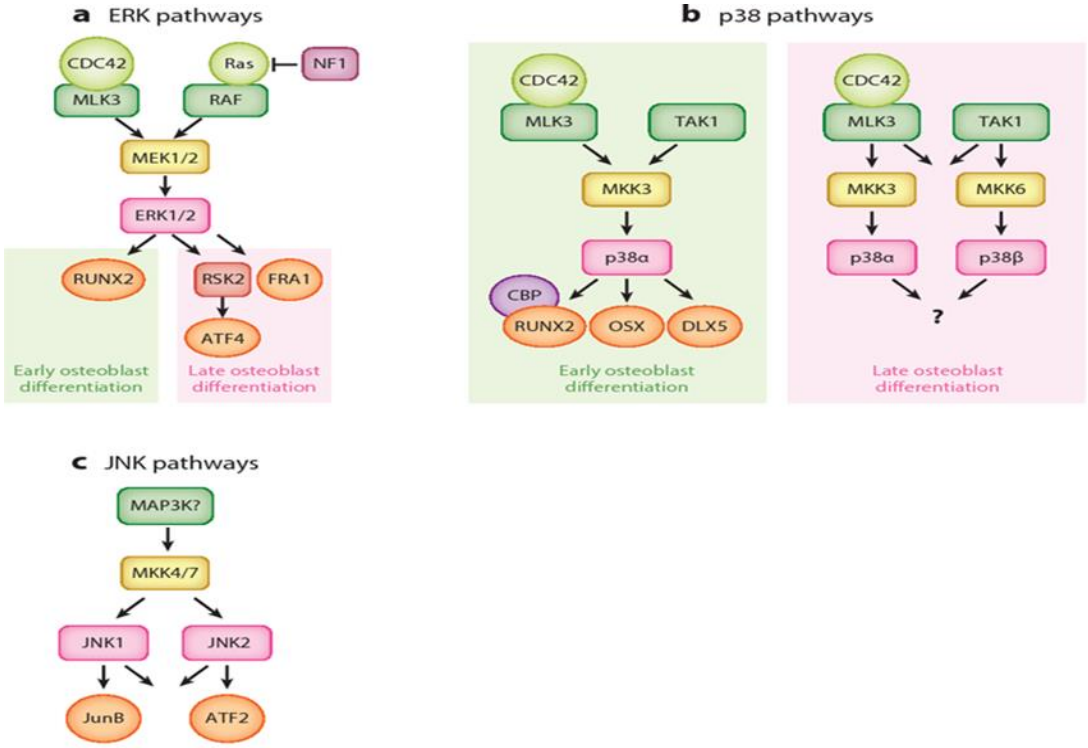


Figure 4: ERK, p38 and JNK pathways in osteoblasts. Activated cell division control 42 (CDC42) activates the mixed lineage kinase 3 (MLK3) by phosphorylation which further activates MAPK/ERK kinase (MEK1/2) or MAPK kinase (MKK)3/6. a) MEK1/2 may also be activated by small-GTPases such as Ras interactions with the rapidly accelerated fibrosarcoma (Raf). MEK1/2 activates ERK1/2 which phosphorylates Runx2 for the differentiation of osteoblasts early and FRA1 and ribosomal 6 kinase 2 (RSK2) which later activates the activating transcription factor (ATF) 4 to promote the differentiation of osteoblast in the later stages. b) During the early stages of osteoblast differentiation, MKK3 may be activated by transforming growth factor-β (TGF-β) activated kinase 1(TAK1) to activate p38α which acts on Runx2, Osterix, Dlx5 or CBP. On the later stages, TAK1 together with MLK3 may also activate MKK6 to activate p38β and together with p38α affect osteoblast genes. c) Certain MAPK kinase kinases activates MKK4/7 which then

phosphorylates and activates JNK1/2 which then targets ATF2 and JunB. Image obtained with permission from Greenblatt MB *et al*.[59]

2.4.6.3 JNK MAPK pathway

The JNK MAPK has three isoforms, JNK1, JNK2 and JNK3. Although it is the least understood pathway in osteoblasts, the JNK pathway seems to be an important osteoblast differentiation promoter.[59] Unlike the p38 pathway, which requires only MKK3 or MKK6 for fully stimulation, JNK needs both MKK4 and MKK7 (Figure 4c).[59] McGonnell *et al.* that JNK1 phosphorylation of Runx2 is suppressive, preventing the early stages of differentiation, however, because the function of JNK is related to later stages of differentiation, this inhibition of Runx2 could be linked to diminished Runx2 function in mature osteoblasts.[49] JNK1 activated c-Jun signalling together with NFAT were demonstrated to be vital in osteoclast differentiation regulated by RANKL.[28] JNK has the ability to bind and phosphorylate RSK2 directly even when ERK is known to act upstream of RSK2.[59] JNK regulates AP-1 activity by activating JUN, JUNB, or JUND (Figure 4c).[59] JUN and JUNB knockout mice show defects in osteoblast proliferation and blockage in osteoblast differentiation whereas bone mass is increased in JUND knockout mice.[66] JUN is dimerised and regulated by ATF2 transcription factor, which is also a JNK substrate. Mineralization at the growth plate is reduced in ATF2-deficient mice, indicating a deficit in osteoblast activity.[59]

2.5 Bone modelling and remodelling

The skeleton is constantly built and rebuilt by two processes known as bone modelling and remodelling.[20-21] Bone modelling refers to bone turnover that occurs during growth to modify bone structure, whereas bone remodelling refers to bone turnover that helps maintain calcium balance by recycling bone while preserving its shape.[67]

2.5.1 Bone modelling

The shape and size of bone is modified due to physiologic effects or mechanical forces on the skeleton causing the skeleton to react to the stresses it encounters.[21] This process is known as bone modelling. Bone modelling mostly occurs in growing children as is important for growth. According to Wolff's law, long bones change shape in response to the forces they encounter.[21] In the metaphysis, the periosteal surface experiences osteoclastic resorption, whereas the inner endosteal surface experiences new bone production, transforming the epiphysis into a diaphysis.[20] Bone resorption and production should be uncoupled during bone modelling to retain skeletal structure ensure growth of the bones.[20-21] In adults, bone modelling is less frequent than remodelling and at skeletal maturity, bone modelling is mostly completed.[20]

2.5.2 Bone remodelling

Bone remodelling, is a process that continues throughout life, whereby formation and resorption occurs in such a way as to keep the structure of the bone balanced.[21] In bone remodelling, old bone is resorbed, or in other words the trabecular osteons are removed, and new bone is formed. Old packets are replaced with newly synthesised bone matrix.[21] Thereafter, the mineralization of the matrix takes place to prevent micro-damage of bone so as to preserve bone strength and mineral balance.[21] The bone remodelling cycle is also important for maintaining an optimum amount of blood calcium, bone remodelling is also necessary as the resorption of bone increases serum calcium while formation decreases serum calcium.[68] During bone remodelling, resorption and formation of bone must be tightly coupled.[21,69] Osteoclasts and osteoblasts function antagonistically to maintain this balance.[20] Several processes take place in the bone remodelling cycle (Figure 5).[20] The cycle takes place within the basic multicellular unit (BMU) or bone remodelling compartment (BRC) which contains, osteoblasts, osteoclasts, osteocytes and capillary blood supply.[20-21] Remodelling

sites may appear at random, but they are sometimes tailored to regions in need of repair.[21]

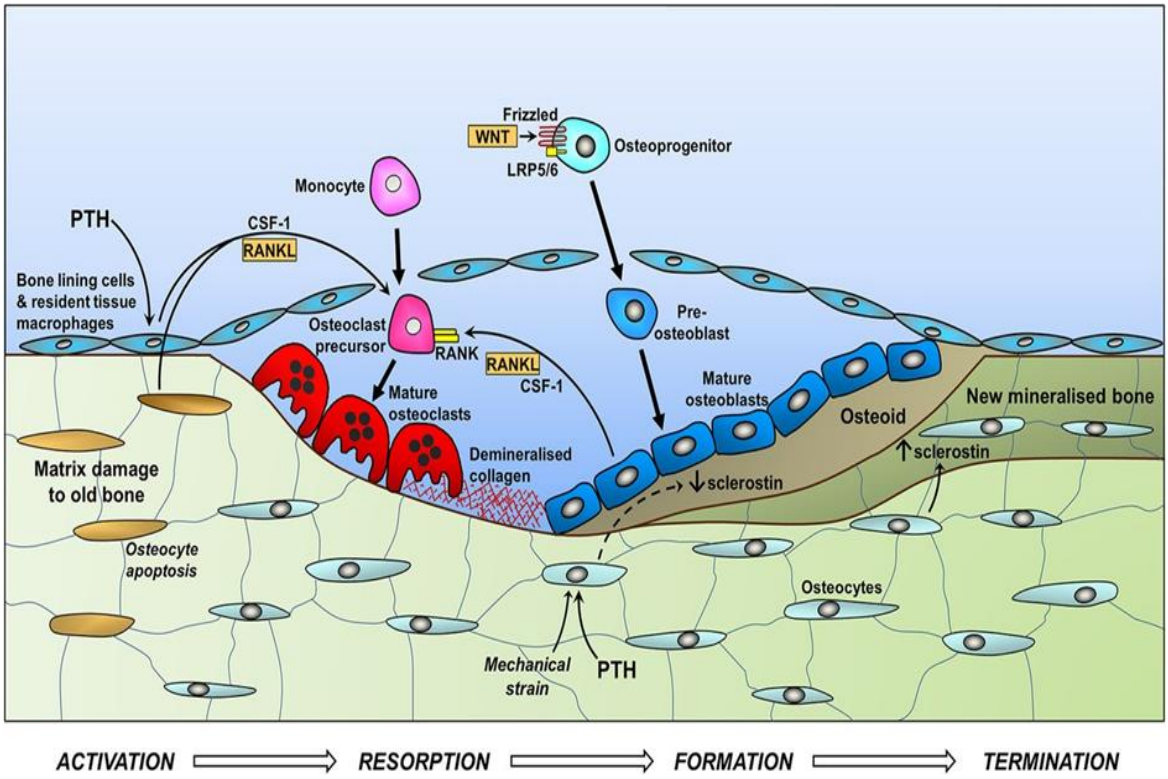


Figure 5: Schematic representation of the bone remodelling processes in the endosteal surface of trabecular bone. The process takes place within the bone remodelling unit containing bone cells. Different types of bone cells are activated in specific stages of the cycle. Osteocyte apoptosis causes changes to parathyroid hormone (PTH) to increase the activation of osteoclast precursors which then forms pre-osteoclast. The receptor activator of nuclear factor kappa B ligand (RANKL) and colony-stimulating factor 1 (CSF-1) causes osteoclast formation. The Wnt pathway play a role in bone formation causing a decrease in sclerostin (SOST). SOST binds to low-density lipoprotein receptor-related protein 5/6 (LRP5/6) to block the Wnt pathway. Image republished with permission from Freudenthal B *et al*.[30]

During *activation*, bone lining cells separate to expose the bone surface after micro-damage which causes apoptosis of osteocytes.[20,24] Apoptosis osteocytes causes the release of paracrine factors or PTH changes, which increases the recruitment of monocyte lineage osteoclast precursors (Figure 5).[20] These precursor cells are drawn from the circulation and are activated together fuse and form large, multinucleated pre-osteoclasts.[20-21] Osteocytes also control the differentiation and activation of osteoclasts by secreting RANKL.[20] Pre-

osteoclasts attach to the bone and form a sealing zone where they can secrete factors to resorb the bone.[21]

Resorption starts once the osteoclasts have attached to the bone surface and formed a ruffled border (Figure 5).[20] The RANKL:OPG ratio; interleukin (IL) 1 and 6, CSF, PTH, vitamin D, and calcitonin regulates osteoclast formation, activation as well as resorption.[21] The osteoclasts use the H^+-ATPase proton pumps together with chloride channels to pump hydrogen ions into the resorption pit to dissolve it.[20-21] These hydrogen ions acidify the compartment to a pH of about 4.5.[21] To maintain electroneutrality, acidic vesicles are merged with the ruffled border and electrogenic proton pump are connected to chloride channels on the pit.[21] The hydrochloric acid (HCl) dissolves the solid hydroxyapatite to calcium (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}) ions as well as water.[68] Carbonic anhydrase II catalyses the reaction between water and carbon dioxide to synthesize protons and bicarbonate ion (HCO_3^-). This activity allows HCl to be continuously released into the resorption region.[68] The Cathepsin K enzyme is released into the resorption pit to digest the collagen-rich bone matrix.[20] PTH signalling causes bone matrix break-down collagen matrix digestion by RANKL and CSF-1-mediated osteoclast recruitment, differentiation and activity (Figure 5).[30] Programmed cell death terminates the process to avoid excessive resorption.[20]

Reversal takes place when resorption switches to formation.[21] There are two essential processes during this phase: the resorbed area is prepared to allow osteoblasts to lay down the new bone matrix; and additional signalling occurs to ensure the coupling of osteoclasts and osteoblasts to ensure the all the resorbed bone is replaced.[20] The resorbed bone surface is prepared by the osteoblasts which remove collagen matrix which is not mineralised and deposit mineralised collagen.[20] Although the exact signals that couple resorption to formation are not fully understood, reversal phase cells are likely to be the ones sending and reserving such signals.[20] Osteoclasts may couple bone resorption to formation

since they emit cytokines and express Ephrin receptors on their surface.[20] Other proposed coupling signals include TGF-β, IGF-1/2, and BMP-2 which are derived from the bone matrix.[20-21] The reversal phase is also controlled by the changes in strain gradient when osteoclasts resorb cortical bone. The base of the lacunae contains more strain compared to the margins of the Howship's lacunae. Osteoclasts and osteoblasts become activated due to changes in the strain gradient.[21]

Bone *formation* signals can be exerted by mechanical activation and the endocrine signal PTH through osteocytes.[27] Under resting conditions, osteocytes produce SOST, which binds to LRP5/6 and blocks Wnt signalling, thereby inhibiting osteoblast formation. Mechanical loading or PTH signalling in osteocytes can inhibit SOST production and therefore activate bone formation by activating Wnt signalling (Figure 5).[27] To make new bone and control mineralisation, osteoblast progenitors differentiate and release matrix vesicles to concentrate calcium and phosphate while PPi and proteoglycans are enzymatically destroyed.[20-21] Proteoglycans immobilises calcium ions within the matrix vesicles[19] whereas PPi inhibits the formation of bone matrix,[70] therefore, degradation of these proteins causes the release of calcium ions from within the matrix vesicles leading to bone formation.[19]

In the resting or *termination phase*, osteoblasts undergo either apoptosis,[27] form bone lining cells or are buried within the matrix to become osteocytes once mineralisation is completed.[20] The signalling of termination is influenced osteocytes which secretes Wnt antagonists such as SOST (Figure 5).[20]

2.6 Osteoporosis

The resorption and formation of bone are tightly coupled in the bone remodelling cycle.[20] Paget's disease, hyperparathyroidism, osteopetrosis and osteoporosis, can all be caused by a loss of the coupling of osteoclast and osteoblast activity.[20,26] The most prevalent metabolic bone pathology is osteoporosis, which

occurs due to increased osteoclast and decreased osteoblast activity leading to fragile bones. The accompanying fragility fractures that occur as a result of the disease are linked to increased morbidity and mortality.[20] Osteoporosis causes bone fragility and fractures and is characterised by a decreased bone mineral density (BMD).[71]

2.6.1 Diagnosis

The imaging technology that evaluates the BMD called dual energy X-ray absorptiometry (DEXA) is used to detected osteoporosis.[20] Should the DEXA measurement give a T-score of less than or equal to -2.5, the patient can be considered to have osteoporosis.[20] The results of plain radiography or quantitative computed tomography (QCT) scans may also indicate osteoporosis.[20] Bone loss occurs in both men and women as part of the aging process and it is associated with increased fracture risks.

2.6.2 Pathophysiological mechanisms

Osteoporosis can be divided into primary osteoporosis, which is more common and includes postmenopausal and age-related osteoporosis,[20] and secondary osteoporosis that is mainly due to pharmacological interventions or systemic or endocrine disorders such as vitamin D deficiency and hyperparathyroidism, connective tissue diseases, genetic diseases, certain medications, and metabolic disorders.[20,72] Vitamin D stimulates calcium and phosphate absorption from the gut for bone mineralisation, therefore, its deficiency may lead to hyperparathyroidism and bone resorption.[73] Glucocorticoid therapy and immobilization are the most prevalent causes of secondary osteoporosis.[20] Although useful in the treatment of a range of diseases, glucocorticoids negatively affects skeletal development leading to osteoporosis.[74] Glucocorticoid therapy supresses bone formation and causes bone resorption by directly upregulating peroxisome proliferator-activated receptor gamma (PPARγ), SOST and RANKL

activity.[74] These conditions can cause increased bone fragility and therefore increase the risk of fracture.

Bone loss in primary osteoporosis, will typically occurs around the third decade in both men and women. At this age the sex steroid concentrations (oestrogen in women and testosterone in men) are still within the normal range.[3] In women, oestrogen concentrations decrease rapidly with the onset of menopause. In men, the sex hormone-binding globulin concentrations doubles and the amount of bioavailable testosterone decreases during a man's life. Furthermore, because testosterone may be converted to oestrogen, bioavailable oestrogen concentrations in men fall by roughly 50% with aging.[3] This decrease in oestrogen concentrations leads to an increase in bone resorption that exceeds bone formation resulting in an uncoupling of the bone remodelling process.[3] Oestrogen supresses RANKL and increases OPG and thus decreases the RANKL:OPG ratio to inhibit osteoclast formation and activity.[3] In osteoblasts, oestrogen promotes MSCs differentiation to osteoblasts and inhibits apoptosis.[3]

Traditionally, vitamin D deficiency, oestrogen deficiency and secondary hyperparathyroidism are the prominent pathophysiological models in postmenopausal osteoporosis, however, recent studies have shown that processes contributing to osteoporosis are extensive.[2]

An increase in the incidence of fractures is linked to an increase in the demand for osteoporosis management.[75] Low bone mass is an important component of risk fracture in osteoporosis, which is defined by BMD.[75-76] Low bone mass is not the only factor that raises the risk of bone fractures; age, gender, and non-skeletal factors all have a role.[75] Salari *et al*. reported the prevalence of osteoporosis has been estimated to be 18.3% worldwide, with ages ranging from 15 to 105.[76] With 23.1% and 11.7%, respectively, women were observed to be more prevalent than men.[76] Africa had the highest prevalence, with 39.5% of those aged 18 to 95 being affected.[76] In Sub-Saharan Africa alone, the prevalence of osteoporosis

was reported to range from 18.2 to 65.8% excluding South Africa.[77] Sub-Saharan Africa was estimated to have the lowest health resource inputs in comparison to the rest of the world.[77] In South Africa, the rates of hip fracture were reported by ethnic groups.[78] When compared to the Coloured men and women, the Indian group had a greater rate of fractures. However, both of these groups had greater rates than the Black South African group.[78] From about 80 years of age, the hip fracture rates for the Indian group were greater than for the White South African group.[78] These statistics highlight the need to prioritise the prevention and treatment of osteoporosis.

2.6.3 Treatments

Oestrogen levels and bone remodelling are the main targets for pharmacological interventions.[71] Mechanisms of treatment involve stimulating PTH synthesis and OPG production and inhibiting IL-1, 4, 6 and M-CSF, as well as enhancing osteoblast proliferation.[71] Pharmacological drugs used for the treatment of osteoporosis include substances which suppress bone resorption such as bisphosphonates, which inhibits osteoclast activity, selective oestrogen receptor modulators (SERMs), oestrogen replacement therapy, calcitonin (an osteoclast inhibitor), denosumab which binds RANKL, and calcium and vitamin D supplements. Anabolic substances which enhance bone formation through osteoblast function stimulation include teriparatide (PTH), strontium ranelate, and romosozumab which inhibits SOST.[2,71] Although numerous clinical trials proved the importance these pharmacological treatments for osteoporosis, long-term usage of these interventions have negative side effects.[71]

Many countries have employed natural substances derived from plants to treat osteoporosis, and they have demonstrated to have less adverse effects and to be better for long-term use.[71] However, further studies of specific cellular and molecular pathways of these natural compounds are required before they may be used to treat osteoporosis.

2.7 G-protein coupled receptors (GPCRs)

Bone remodelling and growth, as well as inflammation, homeostasis maintenance, and metastasis are some examples of biological functions in which G-protein coupled receptors (GPCRs) are vital.[79] GPCRs are cell-surface receptors that share a α-helical seven transmembrane structure. Activation of GPCRs triggers intracellular signalling cascades and regulates second messengers by coupling to heterotrimeric G-proteins and their downstream effectors.[11,80]

There are three families of proteins that act as primary signalling effectors for most GPCRs: GPCR kinases (GRKs), arrestins and the key transducers, heterotrimeric G proteins containing α, β, γ subunits.[79] The β and γ subunits form a dimer (Gβγ dimer) and remain connected throughout the signalling cycle. The Gα proteins further subdivided into four main classes: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$.[79]

2.7.1 GPCR signalling

Binding of an agonist molecules leads to the activation of the GPCR receptor in the G protein mediated signalling.[81] When a GPCR is activated, the G-protein binds the receptor and the Gα subunit conformationally shifts to allow for GDP to dissociate and GTP to bind in place of the GDP.[79,81] Thereafter, the binding of GTP leads to the separation the Gα subunit from the Gβγ dimer.[81] The unbound Gα and Gβγ proteins can trigger various effector substrates and pass down the signal to secondary messengers.[81] The Gα protein leads to the regulation of molecules such as calcium, potassium channels, adenylyl cyclase (AC) etc.[81] For downstream signalling, effectors like phospholipase and potassium channels can be activated by Gβγ protein.[79,81] The AC and intracellular cAMP levels are regulated by the Gs and Gi pathways respectively, whereas phospholipase C (PLC) activates Gα which stimulates an increase in the intracellular calcium levels.[79] The rapid turnoff of the signal is through the receptor phosphorylation by

GRKs followed by arrestin binding.[82] When the GRKs phosphorylate the GPCR, the phosphorylated GPCR then attaches to arrestins, causing the G protein connection to dissociate and the receptor–arrestin complex to enter into the cell and results in the signal being turned off.[79] Therefore, GRK protein stability regulation could be a possible feedback mechanism for controlling GPCR signalling and basic cell functions.[79] By triggering arrestin-mediated GPCR downstream signalling and halting G protein signalling, the arrestin family proteins control GPCR signal transduction.[79] The arrestin binds clathrin-coated pits through the adaptor protein (AP2) on specific sites in their C-termini for receptor internalisation.[82] GPCRs have been used as targets for about 50-60% of modern therapeutics.[80-81]

2.7.2 G-protein coupled receptor 120 (GPR120)

G-protein coupled receptor 120 (GPR120), also known as free fatty acid (FFA) receptor 4 (FFAR4),[24,83] belongs to the rhodopsin GPCR family and is highly conserved throughout many species. GPR120 together with GPR40, GPR41, GPR43, GPR84, and GPR119 receptors are stimulated by FFA which can act as signalling molecules through these GPCRs.[11,84] GPR120 is thought to function as a lipid sensor in the body and, together with GPR40, can be stimulated by medium- to long-chain FFA. [11,83] Long-chain FFA can also stimulate GR119, while short-chain FFA stimulate GPR43 and GPR41.[11] In macrophage cells and adipocyte cells, GPR120 has shown insulin sensitising and anti-inflammatory effects.[11] Activating the pro-survival AKT signal and blocking the pro-death pathway induced by the glucocorticoid, could cause osteoblast survival in omega-3 FA activation of GPR120.[85] Omega-3 FA may stimulate osteoblastogenesis and inhibit bone resorption by modifying membrane function, regulating calcium balance, and boosting osteoblast activity through GPR120.[85] The activation of GPR120 can stimulate Gα or β-arrestin 2 pathways in bone cells to cause various effects within the cells.[24,83]

2.7.3 The GPR120 Gα and β-arrestin 2 signalling

In the Gα signalling, activated GPR120 couples with Gα[84,86] to stimulate the PKC pathway and increase intracellular calcium concentrations (Figure 6).[84] The coupled Gα causes PLC activation which leads to the cleavage of phosphatidylinositol 4,5 bisphosphate (PIP_2), forming inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of calcium, whereas DAG, together with calcium, activates PKC.[86] GPR120 activation induces ERK1/2 activation,[86] and stimulate PI3K/AKT pathways in cell lines expressing GPR120 (Figure 6).[84] Numerous signalling pathways can activate ERK1/2 including those triggered by GPCR.[86] Hudson *et al.* revealed that TUG-891 activates GPR120 and causes rapid and sustained phosphorylation of ERK1/2 through Gα signalling.[15,69] Epidermal growth factor receptor (EGFR) transactivation is also been shown to play a role in the sustained activation of ERK1/2.[15,87]

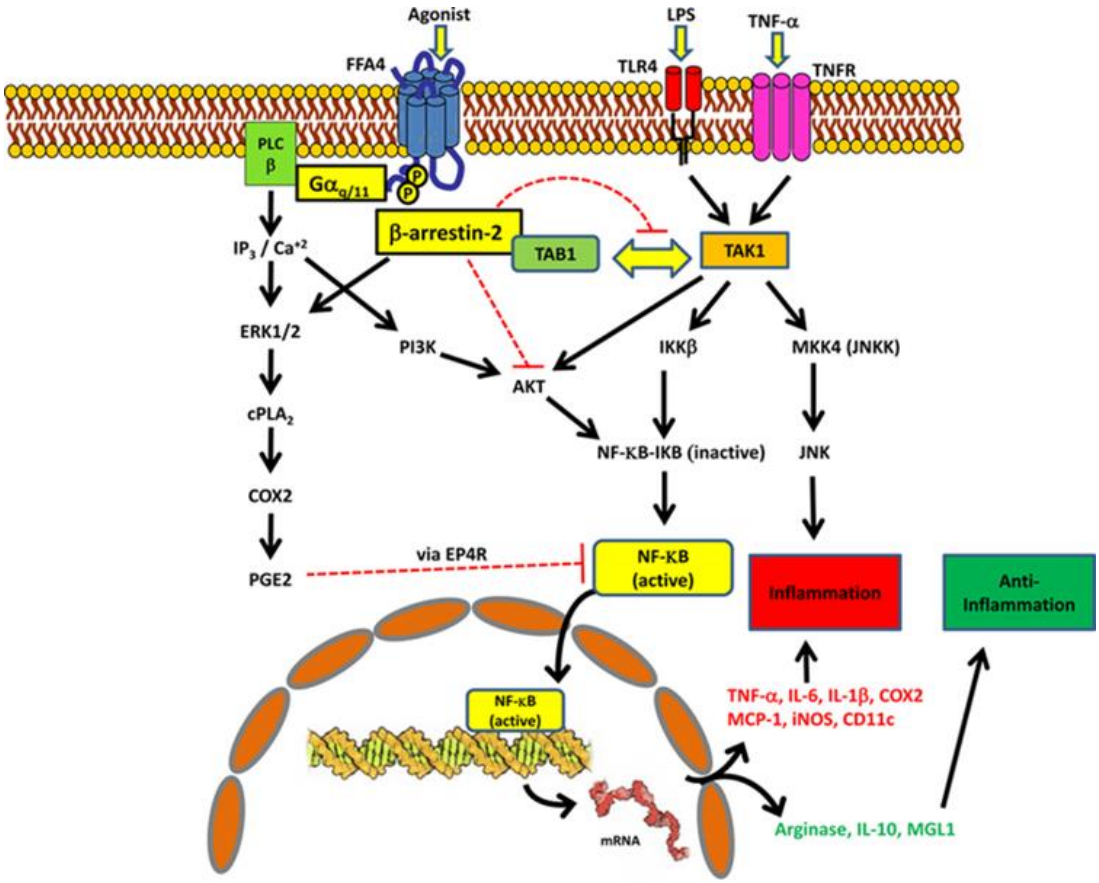


Figure 6: GPR120 anti-inflammatory signalling pathways in macrophages. GPR120/FFA4 activation stimulates either the Gα pathway or the β-arrestin pathway. The Gα pathway signals through the Phospholipase C (PLC)/ Inositol trisphosphate (IP3) to increase calcium levels leading to PI3K/AKT/NF-kB activation. The ERK pathway may activate cytosolic phospholipase A2 ($cPLA_2$)/ cyclooxygenase-2 (COX2)/prostaglandin E_2 (PGE2) which may inhibit NF-KB through the prostaglandin E2 receptor 4 (EP4R). Toll-like receptor 4 (TLR4) and tumour necrosis factor (TNF) receptor (TNFR) activation by Lipopolysaccharide (LPS) and TNF-α respectively, activates the TAB1-TAK1 complex which activates the NF-kB via IKKβ or JNK through MKK4 to increase inflammation. GPR120/β-arrestin inhibits the activation of NF-kB and JNK and enhances anti-inflammatory effects by preventing the binding TAK1 to TAB1 and the activation of the AKT pathway. Image obtained from Moniri N.[87]

TNF-α and toll-like receptor 4 (TLR4) pathways mediate many pro-inflammatory responses, and these pathways overlap at TAK1 activating TAB1 to regulate downstream inflammatory effects by activating NF-kB and JNK pathways (Figure 6).[11] As RANKL is a member of the TNF family of receptors, RANKL can activate the TAK1-TAB1 complex. The development of the TAK1-TAB1 complex is prevented by β-arrestin 2 signalling (Figure 6), and this may indicate that unsaturated FAs illicit anti-osteoclastogenic through β arrestin 2 mediated inhibition of TAK1-TAB1.[24] Activated GPR120 can recruit cytosolic β-arrestin 2 to form a complex and cause the receptor to desensitise and the complex to internalise.[11,86] The internalised GPR120- β-arrestin 2 complex binds to TAB1 to prevent the binding of the TAB1 with TAK1, ultimately inhibiting downstream pro-inflammatory pathways.[11]

2.7.4 GPR120 and metabolic health benefits

GPR120 maintains metabolic balance in a variety of tissues by sensing long-chain FAs from dietary fat.[88] GPR120 has been shown to be involved in the sensitivity of insulin and anti-inflammation.[17] In macrophages, docosahexaenoic acid (DHA) anti-inflammation effects have been demonstrated to be mediated by GPR120.[85]

GPR120 agonist, TUG-891, has been demonstrated to decrease blood glucose by increasing the production of the hormone incretin, stimulate the anti-inflammatory effects of macrophages,[84] improve the absorption of glucose in the liver, decrease hepatic steatosis, and improve insulin resistance and sensitivity.[87] These results demonstrate the potential of GPR120 in the treatment of metabolic disorders.

2.7.4.1 GPR120 and insulin resistance

In metabolic disorders such as, obesity and type 2 diabetes high plasma FFA levels have been detected.[89] This can lead to steatosis and insulin resistance in the liver, muscle and adipose tissues.[11] It has been shown that insulin secretion is stimulated by acute FFA exposure in while it is impaired by chronic FFA exposure.[11] FFAs could be crucial in both hyper- and hypo-insulinemia during type 2 diabetes development due to their multiple and opposing effects on insulin secretion.[11] Due to the various roles of insulin in the body, insulin resistance is linked to a dysfunctional energy metabolism.[14] Insulin plays a role in transporting glucose to tissues such as the liver for FA synthesis; in muscles for protein synthesis and ATP production; and adipose tissues for triglyceride formation.[90] The use of GPR120 in the treatment of type 2 diabetes sparked interest because it recognise FFAs.[11] Studies have shown that activation of GPR120 increases the secretion of intestinal incretins that enhance pancreatic insulin secretion such as glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK).[87] GPR120/Gα signalling increases intracellular calcium levels leading to the activation of the transient receptor potential cation channel subfamily M member 5 (TRPM5), which will stimulate GLP-1 and CCK secretion.[14,84] Thereafter, GLP-1 and CCK will increase the proliferation and survival of pancreatic β-cells which secrete insulin.[14]

Furthermore, due to its anti-inflammatory effects on macrophages, GPR120 activation can help maintain insulin sensitivity by inhibiting inflammation.[14] Oh *et*

al. used a GPR120 agonist (cpdA) which is selectively potent for GPR120 and showed similar anti-inflammatory effects as omega-3 FAs in macrophages *in vitro* and *in vivo.*[91] The cpdA agonist together with DHA were reported to positively influence insulin sensitivity and glucose tolerance by preventing the activation of the pro-inflammatory pathways such as NF-κB, JNK and TAK1 pathways.[91] Paschoal *et al.* reported that both GPR120 and PPARγ activation *in vivo* may positively impact glucose tolerance and insulin sensitivity.[92]

In rodent models of diabetes associated with insulin resistance and impaired insulin secretion, GPR40 and GPR120 coactivation were shown to demonstrate long-lasting glycaemic improvement.[93] In contrary, a study conducted by Simone *et al.* found that the reported anti-inflammatory and insulin-sensitizing effects of omega-3 FAs may not require GPR120 signalling.[94] Therefore, further investigations on the effects of GPR120 in insulin resistance and its mechanisms are required.

2.7.4.2 GPR120 and inflammation

Studies have demonstrated that omega-3 FA supplementation could reduce the severity of several inflammatory diseases through GPR120.[11] GPR120 agonism has been shown to decrease the expression of pro-inflammatory cytokines, IL-6 and TNF-α, while increasing anti-inflammatory cytokines like IL-10.[87] This could be due to the GPR120 β-arrestin pathway that suppresses inflammation through TLR2, TLR4 and TNF receptor (TNFR).[11] As previously mentioned, the GPR120 β-arrestin2 pathway can inhibit the pro-inflammatory MAPK and NF-κB signalling pathways through inhibition of TAK1-TAB1 complex whereby GPR120 physically interacts with β-arrestin2 and prevents the signalling through NF-kB and JNK.[11] Therefore, GPR120 agonists may be used to treat a wide range of disorders mediated by one or more of these pathways.[11] However, Moran *et al.* showed that in diabetic mice FA regulated both anti-hyperglycaemic and anti-inflammatory effects, whereas GPR120 receptor knockout mice had no impact.[84]

In fact, GPR120 knockout mice revealed sensitivity to a high-fat diet resulting in the enhancement of inflammation and downstream effects of inflammation.[84] A new anti-inflammatory mechanism that could be useful in the treatment of inflammatory bowel disease mediated by GPR120 in specific intestinal epithelial cell types was discovered.[95] GPR120 had similar effects in intestinal epithelial cells as it did in monocytes and macrophages, inhibiting the NF-kB pathway and promoting anti-inflammation via the β-arrestin2 pathway.[95] This new mechanism could improve the understanding of the effects that GPR120 have on the intestinal inflammation disorders such as the Crohn's disease. However, contrasting studies reveals that there is still much to be understood about the role of GPR120 in mediating anti-inflammatory effects of FAs.

2.7.4.3 GPR120 and adipogenesis

Adipose tissue highly express GPR120 rather than GPR40 suggesting that GPR120 is involved in adipocyte differentiation.[96] Furthermore, GPR120 knockout using small interfering ribonucleic acid (siRNA) causes the proportion of lipid droplets in cells to decrease as well as the expression of PPARγ to further decline during adipogenesis.[96] GPR120 activation reduces insulin resistance by promoting adipogenesis to maintain a balance in lipid metabolism.[14] In primary adipocyte cells, GPR120 activation increases glucose transporter 4 (GLUT4) activity using the PI3K/AKT pathway, and this effect is not seen when GPR120 is knocked out.[11] Schilperoort *et al.* proposed that GPR120 activation stimulates mitochondrial fission in a calcium-dependent manner to possibly enhance metabolic activity in brown adipocytes.[97] Again, GPR120 directly stimulates brown adipose tissue (BAT) activity using mechanisms that are dependent and independent of GPR120.[97] GPR120 knockout by siRNA supresses adipocyte differentiation and inhibits the expression of key adipocyte regulators.[87] Song *et al.* proposed that GPR120 might promote adipogenesis via the PPARγ.[17] Another study showed that TUG-891 enhanced the uptake of triglyceride-derived FA by BAT to reduce the weight of the body.[97]

2.7.5 GPR120 and bone health

GPCRs are important regulators of bone development and remodelling with their mutations leading to bone diseases.[79] To regulate osteoblast function, GPCRs communicate via a variety of canonical pathways.[79] In GPCRs, the phosphorylation of GRK and the signalling of β-arrestin regulates osteoblast function.[79] GPR40 and GPR120 have been shown to be expressed in osteoclasts and osteoblasts.[12,85,98] GPR120 influences skeletal physiology indirectly by affecting the formation of intestinal and pancreatic beta cell hormones that affect bone and have a direct impact on skeletal function.[98] Numerous studies on the impact of polyunsaturated long-chain FA on osteoclasts and osteoblasts have been conducted.[98] Some studies have further looked at the role of GPR120 on bone health and will be discussed in further detail.

2.7.5.1 GPR120 studies *in vitro*

A study by Ahn *et al.* has shown that in mature bone cells omega-3 FAs activates GPR120.[13] GPR120 was further shown to stimulate bone formation by increasing β-catenin activity in osteoblasts and suppress bone resorption by suppressing the NF-κB activation in osteoclasts.[13] Using DHA, Kishikawa *et al.* has shown that GPR120 inhibits osteoclast formation by directly inhibiting osteoclast precursor differentiation *in vitro*.[99] Previously, studies have revealed that osteoclast formation could be inhibited by GPR120 signalling by targeting NF-κB and MAPK signalling pathways.[24] *In vitro* studies performed by Kasonga *et al.* has revealed that in the presence of high levels of omega-3 FA, GPR120 stimulates osteoblast activity while suppressing the activity of osteoclasts.[24] Interestingly, this study has shown that the omega-9 FFA, oleic Acid (OA), stimulated the expression of osteoblast genes through GPR120 in MC3T3-E1 pre-osteoblasts, however inhibited osteoclastogenesis in RAW264.7 murine macrophages independently of GPR120.[24] This may suggest that activation of GPR120 by a ligand is cell dependent. Recently in our laboratories, using RAW264.7 murine macrophages,

we reported that GPR120 activation via TUG-891 inhibits osteoclast formation and resorption through the inhibition of reactive oxygen species.[100]

In an *in vitro* study, similar to the canonical Wnt signalling, the PTH receptor interacted with Dsh protein to activate the β-catenin pathway in bone cells.[13] Natural and synthetic GPR120 agonists such as palmitoleic acid (PLA) and TUG-891 increase the expression of osteoblast genes in MC3T3-E1 pre-osteoblasts through the GPR120 receptor.[24] Furthermore, GPR120 stimulation by FA or synthetic agonists has been shown to also increase the intracellular calcium levels and cause an increase in the activation of the ERK cascade.[17] GPR120 has been reported to increase osteoblast differentiation and proliferation.[12] Gao *et al*, showed that GPR120 activation by low concentrations of TUG-891 in bone marrow mesenchymal stem cells (BMMSCs) promoted adipogenesis via the p38 pathway while high concentrations of TUG-891 stimulated osteoblast formation through the ERK pathway.[12-13] On top of the cell-dependent effects previously mentioned, this study reveals that GPR120 activation may also have varying dose-dependent effects.

2.7.5.2 GPR120 *in vivo* studies

It has been reported that the activation of GPR120 *in vivo* can be achieved by both omega-3 and omega-6 dietary poly-unsaturated long-chain FAs at levels that are safe for human consumption.[101] An *in vivo* study by Kishikawa *et al.* revealed that DHA inhibited LPS-induced production of osteoclasts and bone resorption in mice through GPR120 signalling.[99] Furthermore, high availability of omega-3 FAs inhibited bone resorption and stimulated bone formation in fat-1 transgenic mice that over-expressed both omega-3 FAs and GPR120.[13]

In conclusion, the benefits of GPR120 are not limited only to the above-mentioned but go further beyond. For instance, Milligan *et al.* has shared some therapeutic opportunities for GPR120 in cancer.[102] Some studies have shown that GPR120 knockout in mice with both GPR120 and GPR40 activation by the platinum

induced FA 16:4(n-3) in GPR120 knockout mice improved cisplatin-induced DNA damage in tumour cells to inhibit tumour growth.[102] Although there were some contrasting results by other studies, there is potential in GPR120 antagonists to decrease the development of resistance to platinum containing chemo-therapeutics.[102] Furthermore, research by Wang *et al.* reported that in breast cancer GPR120 activation promotes chemo-resistance by increasing the expression of the ABC transporter and FA synthesis.[103]

Therefore, GPR120 shows promise as a therapeutic target for a range of disorders. However, to fully understand the role of GPR120 in the body, and in the bone in particular. More studies are necessary. There are few studies on the effects of GPR120 in osteoblast function and activity using osteosarcoma cells. Furthermore, the mechanisms of action of GPR120 in osteoblasts has not been fully studied. Therefore, more research is needed to unlock the potential of GPR120 signalling in the treatment of bone diseases.

Chapter 3 Materials and methods

3.1 Logistics

All experiments were carried out in the laboratories of Department of Physiology and the Centre for Neuroendocrinology at the University of Pretoria. Photomicrographs were taken at the Department of Physiology at the University of Pretoria.

3.2 Ethical consent

Ethical consent was obtained from the Faculty of Health Sciences Research Ethics Committee at the University of Pretoria. (Reference number 582/2020, Appendix A)

3.3 General laboratory procedures

3.3.1 Materials and reagents

Resazurin, trypan blue, Triton X-100, and all other chemicals of research grade were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagles Medium (DMEM) (Cat #11594486) and 0.5% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Cat #10779413) were purchased from GIBCO (Invitrogen Corp, Carlsbad, CA, USA) and foetal bovine serum (FBS) was bought from Capricorn Scientific (Cat #FBS-GI-12A) (Ebsdorfergrund, Germany). Consumable plasticware such as culture flasks, culture plates, pipette tips, syringes, etc. were purchased from Lasec (Midrand, GP, SA). TUG-891 (GPR120 agonist) was supplied from Research and Diagnostic Systems (Minneapolis, USA). PD184352 (MEK inhibitor) was supplied by Sigma Aldrich (St. Louis, MO, USA). Clarity Western ECL Substrate was bought from Bio RAD (Hercules, CA, USA). M-MuLV reverse transcriptase was purchased from New England Biolabs (Ipswich, MA, USA). The bicinchonic acid (BCA) protein assay kit was purchased

from Thermo-Fischer Scientific (Rockford, IL, USA) and the primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat #G9545) was supplied by Sigma Aldrich (St. Louis, MO, USA); antibodies against AKT (Cat #4691) and pAKT (Cat #4060) were purchased from Cell Signalling Technology (Danvers, Massachusetts, US); the antibody against ERK (Cat #29162) was supplied by Signalway Antibody (College Park, MD, USA) and the antibody against pERK (Cat #AF1018) were supplied from Research and Diagnostic systems (R & D systems, Minneapolis, MN, USA). Primers for ALP, RUNX, Osx, OPG, GAPDH and RANKL were synthesized and purchased from Inqaba Biotec (Pretoria, GP, SA).

3.3.2 Preparation of buffers and solutions

Table 1. Preparation and storage of buffers and solutions used in this study.

Compound	Preparation and storage
Buffers: 1. Phosphate buffered saline (PBS)	For 10X stock solution: 80 g NaCl, 2 g KCl, 2 g KH_2PO_4 and 10,5 g Na_2HPO_4 in 1 L double distilled water (ddH_2O).pH adjusted to 7.4. Working solution: stock solution diluted 10 times in ddH_2O and autoclaved. Aliquots stored at 4˚C in the dark.
2. Trypsin-EDTA working solution	50 mL Trypsin-EDTA prepared in 450 mL autoclaved sterile PBS. Aliquots stored at -20˚C in the dark.
3. Freeze medium	40% FBS, 50% serum free DMEM and 10% dimethyl sulfoxide (DMSO).
4. Trypan blue dye, 0.5%	0.5 g prepared in 100 mL PBS.
5. Resazurin solution, 0.02% (w/v)	0.02 g diluted in 100 mL ddH_2O.

6. Osteogenic media (OM):β-glycerophosphate	For 10 mM solution: 0.017 g β-glycerophosphate prepared in 8 mL complete DMEM*.
Ascorbic acid	50 mM prepared in ddH_2O. Aliquots stored at -80˚C in the dark, diluted to 50 µM in complete DMEM*.
Dexamethasone	1 mM prepared in 100% ethanol. Aliquots stored at -80˚C in the dark, diluted to 1µM in complete DMEM*.
7. ALP assay buffer	5 mM 4-nitrophenyl phosphate disodium, 0.5 mM $MgCl_2$, 0.1% Triton X-100 in 50 mM Tris-HCl with the pH adjusted to 9.5.
8. Alizarin Red S staining solution, 2% (w/v)	0.2 g Alizarin Red S prepared in 10 mL ddH_2O.
9. Lysis buffer: RIPA buffer	50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1%Triton X-100, 2 mM EDTA and 50 mM NaF with the pH adjusted to 7.4.
10. Loading buffer (6X)	0.5 M Tris [pH 6.8], 10% SDS, 30% glycerol,0.03% bromophenol blue and 6% β-mercaptoethanol.
11. Tris-glycine transfer buffer	For 10X stock solution: 144 g glycine and 30.3 g Tris filled to 1 L withddH_2O. Stored at room temperature. For 1X solution: 100 mL 10X Tris-glycine transfer buffer, 200 mL methanol and 700 mL ddH_2O.

*Complete DMEM – DMEM supplemented with 10% FBS.

Table 2. Preparation and storage of primers and antibodies used in this study.

Compound	Preparation and storage
1. Primers: Beta-actin GAPDH GPR120 ALP RUNX2 Osx OPG RANKL	All primers were reconstituted to 100 µM in nuclease free water. For 10 µM working solution: 10 µL of forward and 10 µL reverse primer prepared in 80 µL nuclease free water. Aliquots stored at -20˚C in the dark. (Primer sequences can be found in Table 3 and Table 5)
2. Antibodies: GAPDH ERK pERK AKT pAKT	1 mg/mL in PBS. Aliquots stored at -20˚C in the dark.

3.3.3 Cell culturing and maintenance

This project made use of cell lines and *in vitro* assays. All *in vitro* assays were performed at least three times independently and where appropriate three technical repeats were included in each assay. Descriptive statistics were performed on each assay to determine the mean, standard deviation, and standard error of the mean as appropriate.

The MG-63 osteosarcoma cell line (MG63-C) was purchased from Cellonex (South Africa). MG-63 cells were maintained in complete DMEM supplemented with 10% FBS. This cell line has previously shown to express high levels of alkaline phosphatase and parathyroid hormone (PTH)-unresponsive receptors coupled to adenylate cyclase as well as 1,25(OH)2D3 receptor sites.[104] 1,25(OH)2D3 increases the production of bone specific protein, osteocalcin.[104] Therefore, due to these characteristics of MG-63 osteosarcoma cells, this cell line offered a useful experimental model for studying osteoblastic properties and osteoblast-produced molecules in an established human cell line.

MG-63 cells were cultured under sterile conditions at 5% CO_2 and 37˚C in a humidified incubator.[60] Cells were sub-cultured at 80% confluency in T-75 flasks by removing the medium, rinsing the cells twice with PBS (Table 1 #1) and adding 5 mL Trypsin-EDTA (Table 1 #2) (1X) for 7 minutes at 37˚C in a humidified incubator until cells detach.[5] Fresh medium was then added, and cells were aspirated and placedx into a 15 mL tube to be centrifuged at 4 000 xg for 30 seconds (Centrifuge 8x15, Nedtex Co., Taipei, Taiwan). Thereafter, cells were removed and re-suspended in 4 mL of fresh media. The cells were aspirated, and 500 000 cells were placed into a new culture flask. Medium was replaced 1-2 times per week.[105] To freeze cell stock away, cells were frozen in cryovials at a density of $1x10^6$ cells per mL in freeze medium (Table 1 #3) and placed into long-term storage in liquid nitrogen when not in use, or in -80˚C freezer for short-term storage. The cell count, passage and dates were recorded. Cells were only used in experiments up until a passage of 30.[5] All cell culture work was performed in a sterile laminar flow cabinet to prevent contamination.

3.3.4 GRP120 agonist (TUG-891), vehicle preparation and PD184352 (MEK inhibitor) (Stock solution preparation and storage)

Stock concentrations of 100 mM TUG-891 and 10 mM PD184352 were prepared in DMSO. Aliquots were stored at -80°C freezer in the dark until needed. The

stock solutions for TUG-891 were freshly diluted to the required concentrations ranging from 0.01-100 µM in complete DMEM. Stock solutions for PD184352 were diluted to 10 µM in complete DMEM. The highest concentration of DMSO did not exceed 0.1% during the experiments and was used as the vehicle control (VC).

3.3.5 Trypan blue exclusion and cell counting

Trypan Blue dye (0.5%) exclusion method and a haemocytometer were used to obtain cell counts. Cells were trypsinized, centrifuged at 4 000 xg for 30 seconds (Centrifuge 8x15, Nedtex Co., Taipei, Taiwan) and re-suspended in DMEM. A preparation was made up of 20 µL re-suspended cells and 80 µL phosphate buffered saline (PBS). A 1:1 dilution further working solution was made using 20 µL of Trypan Blue dye (Table 1 #4) and 20 µL of the cell solution. Thereafter, 20 µL was pipetted onto the haemocytometer under a coverslip. The viable cells were counted in four quadrants using an Olympus BH2 microscope (Olympus, Tokyo, Japan). Non-viable cells stained blue and were not counted. The total number of viable cells over the four quadrants were averaged and multiplied by 10^5 to obtain the number of viable cells per mL.[106]

3.4 Methods for experiments

3.4.1 GPR120 expression in MG-63 osteosarcoma cells

To determine the expression of GPR120 in MG-63 osteosarcoma cells, reverse transcriptase PCR (RT-PCR) was carried out. PxE 0.2 Thermal Cycler (Thermo Fischer Scientific, Waltham, MA, USA) was used for PCR.

RNA was isolated from undifferentiated MG-63 cells using ISOLATE II RNA Mini Kit (Bioline Meridian Bioscience, UK). The cells were lysed by adding the lysis buffer together with the 2-mercaptoethanol and vortexed vigorously. The lysate was then filtered and centrifuged at 11 000x g for 1 minute at 4ºC (Sigma 1-14

microcentrifuge, Germany). The RNA binding conditions were adjusted by adding 70% ethanol and homogenising the lysate. The binding of RNA was done by loading the lysate into the ISOLATE II RNA Mini Column and then centrifuged at 11 000x g for 30 seconds (Sigma 1-14 microcentrifuge, Germany). The silica membrane was desalted by adding the Membrane Desalting Buffer and centrifuging at 11 000x g for 1 minute to dry the membrane (Sigma 1-14 microcentrifuge, Germany). The DNA was digested by applying DNase I reaction mixture directly to the centre of the silica membrane and incubated at room temperature for 15 minutes. The DNase I reaction mixture was prepared by diluting the Reaction Buffer DNase in 10% of the reconstituted DNase I. The silica membrane was washed and dried using the Wash Buffer. The eluted total RNA was placed immediately on ice. RNA (1 µg) was reverse transcribed into cDNA using M-MuLV reverse transcriptase incubated in the oven at 42˚C for 1 hour. Thereafter, the enzyme was inactivated at 90˚C and the DNA was stored at -20˚C until needed.

The reaction mixture contained 0.5 µM of forward and reverse primer, 1 µl of the prepared template DNA, 1X KAPA2G Robust HotStart Ready Mix (KAPA biosystems, Cape Town, South Africa) and nuclease-free water (up to 10 µl). GAPDH was used as a loading control. PCR primers were produced by Inqaba Biotec (Pretoria, South Africa) (Table 3) and are osteoblast-specific genes. The samples were centrifuged at 2 000 xg for 10 seconds (MyFuge, Benchmark Scientific Inc., Edison, NJ.USA), placed in a PxE 0.2 Thermal Cycler, and amplified on a 3-step cycling using the cycles given in Table 4. The details for GAPDH and GPR120 primer can be found in appendix F. PCR products were resolved in 1% agarose gel and 5 µl of the 5 ng/µl Kapa express DNA ladder was used (KAPA biosystems, Cape Town, South Africa). The PCR products were visualized with 0.005% ethidium bromide using a gel documentation system attached to a monochrome scientific grade camera (E- Box 1000/26M, Vilber Lourmat, Cedex, France).

Table 3: RT-PCR primers which were used. (Abcam, Cambridge, MA, USA)

Gene	Forward Primer sequence (5' – 3')	Reverse Primer sequence (5' – 3')
GAPDH	GAAAGCCTGCCGGTGACTAA	GCCCAATACGACCAAATCAGAG
GPR120	TCTTCTCCGACGTCAAGGGC	GGCCAGCACCAGAGGGATAG

Table 4: The cycles and their duration used in the PxE 0.2 Thermo cycler for RT-PCR.

Cycles	Temperature	Duration	Step
1	95˚C	3 minutes	Initial denaturation
35	95˚C	15 seconds	Denaturation
	60˚C	15 seconds	Annealing
	72˚C	15 seconds	Extension
1	72˚C	1 minute	Final extension

3.4.2 Resazurin assay for the detection of cell viability

Resazurin is a blue water-soluble dye that has been used previously for determining *in vitro* cell viability in numerous cells including MG-63 cells.[107-108] Furthermore, it is very stable and non-toxic to cells.[107] Resazurin becomes reduced to a highly fluorescent resorufin, pink dye by dehydrogenase enzymes in viable cells.[109] The reduction of resazurin is equivalent to the number of live cells.[108] This method was carried out to determine the amount of resorufin present after exposing the cells to different concentrations of TUG-891. MG-63 osteosarcoma cells were trypsinized and cultured into a sterile 96-well plate in complete DMEM at a density of 16 000 cells per cm^2 and incubated for 24 hours to allow for attachment. A positive and negative control was used where cells were exposed to 0.1% DMSO (negative control) or complete DMEM. Triton X-100 (positive control) was used as an inducer of cell death. Triton X-100 is a non-

ionic detergent that permeabilizes the cell membrane causing cell death and lowering cell viability while preserving the protein structure and the protein-protein interactions.[110] It was expected that the positive control would contain a higher amount of resorufin compared to the negative control. After attachment, different concentrations of TUG-891 [0.01, 0.1, 1.0, 10 and 100 µM] were added to the wells and incubated for 48 hours. At the end of the culture period, 10% resazurin solution made up of 0.02% (w/v) resazurin (Table 1 #5) and DMEM media were pipetted into each well including the control wells to assess the metabolic activity of the cells.[109] The plate was incubated for 1-4 hours at 37˚C in the dark to allow the colour,[109] to change from dark blue to become reduced to a fluorescent light purple/violet colour known as resorufin. The absorbance values were measured using an Epoch Micro-plate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at a wavelength of 570 nm and using 600 nm as the reference wavelength. Results were analysed using Graph-Pad to obtain graphs that were shown as percentage of the negative control. This experiment comprised of three biological repeats each seeded in three experimental repeats.

3.4.3 Osteoblast differentiation

For osteogenic differentiation to occur, complete DMEM supplemented with OM containing β-glycerophosphate, ascorbic acid and dexamethasone (Table 1 #6) is required for at least three weeks.[6] Normally, to support cells *in vitro*, OM offers nutrients, extracellular matrix proteins, and growth factors.[111] Furthermore, there is evidence that serum may act as an antioxidant for cells.[111] β-glycerophosphate serves as a derivation point of phosphate for bone mineral formation as well as induction of gene expression via the phosphorylation of ERK1/2. Ascorbic acid mediates osteogenic differentiation by increasing the secretion of Col1a1.[6,112] Dexamethasone has been reported to prevent apoptosis of bone marrow derived MSCs in confluent cultures and further promotes MSC proliferation. Dexamethasone may further enhance osteoblast differentiation by modulating Runx2 phosphorylation.[6] After 14 and 21 days of treatment, the level of GPR120

protein was substantially higher than after 7 days of induction.[12] By day 7, MG-63 cell proliferation and cellular ALP activity had both increased significantly, according to research.[60] Early osteogenic markers like Runx2, ALP and Osx can be detected as early as day 7, however late markers like OCN can only be detected from 14 and 21 days.[12,60] Therefore, based on these previous studies, osteoblast differentiation was performed for 7, 14, and 21 days in this current study.

3.4.4 Alkaline phosphatase (ALP) activity assay as an early marker for the detection of osteoblast differentiation

Alkaline phosphatases (ALPs) are recognised as the biochemical markers for osteoblast formation and evidenced to be involved in skeletal mineralisation.[33,110] At an alkaline pH, ALPs play a role in the conversion of the colourless *p*-nitrophenyl phosphate (*p*-NPP) to a yellow end-product *p*-nitrophenol.[110] In foetal rat calvaria, the ALP activity has been shown to be relative to the rate of collagen production *in vitro*.[110] An ALP activity assay was used to measure the enzymatic activity of MG-63 cells exposed to TUG-891 for 7, 14 and 21 days of osteoblast differentiation. The negative control did not contain the osteogenic media whilst the baseline control had the osteogenic media for each of the time intervals. It was expected that the baseline control would have a higher ALP activity than the negative control. MG-63 cells were seeded at 16 000 cells per cm^2 into a sterile 48-well plate in complete DMEM and incubated for 24 hours to allow attachment. Cells were differentiated in the presence of OM and TUG-891 [0.01, 0.1, 1, 10 and 100 µM] which was replaced every 2-3 days to keep the experimental contents fresh during the differentiation period.[113] Cells were fixed with 4% formaldehyde for 5 minutes after 7, 14 and 21 days of differentiation. ALP was assayed using a colorimetric assay where the rate of *p*-nitrophenol formation was measured. The cells were then incubated with ALP assay buffer (Table 1 #7) for 60 minutes. Thereafter, 100µL of the ALP assay buffer was transferred to a new 96-well plate and an increase in absorbance was read at 405 nm with 650 nm as

a reference wavelength using an Epoch Micro-plate Spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA). Results were analysed using Graph-Pad to obtain graphs. All results were expressed as a percentage of the negative control. Three biological repeats and three experimental repeats were done for this experiment.

3.4.5 Alizarin Red S staining for the detection of calcium in the mineral matrix

The effects of GPR120 agonists on osteoblast function were determined using an Alizarin Red S stain. Alizarin red S staining allows for the measurement of extracellular matrix deposited calcium.[114] Alizarin Red S staining was performed after 7, 14 and 21 days of differentiation to determine the effects of TUG-891 [100 µM] on mineralisation. The negative control cells had no osteogenic media. The baseline control cells had osteogenic media for each time point and therefore, was expected to have more mineral deposits than the negative control. The cells were fixed with 3.7% formaldehyde for 1 hour. The cells were then washed using PBS at a pH of 4.2 for 5 minutes and then stained in a 2% (w/v) solution of Alizarin Red S (Table 1 #8) (Merck, Germany) for 10 minutes. The stained cells were rinsed with double distilled water (ddH2O) and allowed to air dry overnight. The cells were then visualised using a light microscopy where photomicrographs were taken using an Olympus BH2 microscope equipped with an SC30 camera (Olympus, Tokyo, Japan). Thereafter, alizarin red was eluted using 10% acetic acid and neutralized with 10% ammonium hydroxide. The absorbency was read at 405 nm using an Epoch Micro-plate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Results were analysed using Graph-Pad and the results were expressed as a percentage of the baseline control.[115] Three biological repeats and three experimental repeats were done for this experiment.

3.4.6 Quantitative-Polymerase Chain Reaction (q-PCR) for the detection of osteoblast specific genes

Polymerase Chain Reaction is an technique used to amplify specific DNA from small source DNA.[116] In this study, PCR was used to analyse the expression of early and late markers of osteoblast specific genes for 7 and 14 days. The negative control did not have osteogenic media and baseline control had osteogenic media for each time point. It was expected that the expression of osteoblast genes would be higher in the baseline control than the negative control. MG-63 osteosarcoma cells were trypsinized and seeded into a sterile 48-well plate in DMEM at a density of 16 000 cells per cm^2 and incubated for 24 hours to allow attachment. Cells were differentiated in the presence of osteogenic media and TUG-891 [100 µM] which were replaced every 2-3 days. Osteoblast cells were analysed for early and late bone specific gene expression markers for up to 14 days of differentiation. Genes for early bone biomarkers were analysed after 7 days of differentiation. These markers included *ALP, RUNX2, BSP, OPG, Osx,* and *RANKL*. The primers for each gene were selected using the Primer-BLAST tool.[117] The details for the primers can be found in appendix F.

The isolation of RNA was done using the ISOLATE II RNA Mini Kit (Bioline Meridian Bioscience, UK) as mentioned above. RNA (1 µg) was reverse transcribed into cDNA using M-MuLV reverse transcriptase to obtain DNA which was stored at -20˚C until when it was needed. The primers that were used in this study (Table 5) are osteoblast-specific genes and were synthesized by Inqaba Biotec (Pretoria, South Africa). The cDNA was added to forward and reverse primers for the osteoblast-specific genes mentioned below. SensiFAST SYBR No-ROX Mix (Bioline Reagents Ltd, United Kingdom) and PCR primers synthesized by Inqaba Biotec (Pretoria, South Africa) were used for q-PCR. The samples were centrifuged at 2000 xg for 10 seconds (MyFuge, Benchmark Scientific Inc., Edison, NJ.USA), placed in a Light cycler 96 (Roche Diagnostics, Basel, Switzerland) and amplified on a 3-step cycling using the cycles given in

Table 6.[109] Relative gene expression was analysed using the -$2^{\Delta\Delta CT}$ method using β-actin as the loading control.[109] Data was expressed relative to the negative control. Graph-Pad was used to obtain graphs. Three biological repeats and two experimental repeats were done for this experiment.

Table 5: qPCR primers which were used. (Abcam, Cambridge, MA, USA)

Gene	**Forward Primer sequence (5' – 3')**	**Reverse Primer sequence (5' – 3')**
β-actin	CAGAGCCTCGCCTTTGC	CTCGTCGCCCACATAGGA
ALP	ACGTGGCTAAGAATGTCATC	CTGGTAGGCGATGTCCTTA
Runx2	GCTGTTATGAAAAACCAAGT	GGGAGGATTTGTGAAGAC
Osterix	GCAGCTAGAAGGGAGTGGTG	GCAGGCAGGTGAACTTCTTC
RANKL	GCGTCGCCCTGTTCTTCTAT	GGAACCAGATGGGATGTCGG
OPG	GCCCTGACCACTACTACACA	TCTGCTCCCACTTTCTTTCC
BSP	CTATGGAGAGGACGCCACGCCTGG	CATAGCCATCGTAGCCTTGTCCT

Table 6: The cycles and their duration used in the Light cycler 96 for q-PCR.

Cycles	**Temperature**	**Duration**	**Step**
1	95˚C	60 seconds	Initial denaturation
45	95˚C	15 seconds	Denaturation
	60˚C	30 seconds and plate read	Extension
1	60-95˚C	Various	Melt curve

3.4.7 Western blotting for the detection of mitogen-activated protein kinase (MAPK) protein expression

Western blotting is used to separate proteins based on their molecular weight and assess their expression level.[118] This method was performed to determine the level of ERK phosphorylation and AKT phosphorylation, in determining whether the MAPK pathway and the AKT pathway are activated by GPCRs.

Therefore, different pathways which might have been activated by GPCRs in osteoblasts were examined. MG-63 osteosarcoma cells were trypsinized and seeded into a sterile 6-well plate in DMEM at a density of 16 000 cells per cm^2 and incubated for 24 hours to allow for attachment. Cells were cultured for 15 minutes, 60 minutes, 4 hours, and 24 hours in the presence of osteogenic media containing TUG-891 [100 µM]. The negative control did not contain osteogenic media and the baseline control had osteogenic media for each time interval. When GPR120 was silenced using siRNA, MG-63 cells were seeded for 24 hours for attachment, thereafter, X-tremeGENE™ siRNA Transfection Reagent (Sigma-Aldrich) and siRNA were diluted in serum-free media according to the manufacturer's instructions.[24] The cells were exposed to control and GPR120 siRNA in serum free medium for 24 hours. Thereafter, the cells were then exposed to OM, 10 µM PD184352 (PD) and TUG-891 [100 µM] for 15 minutes before protein extraction was performed.

Extraction of proteins was done as follows: the cells were washed in ice-cold tris-buffered saline (TBS) before being lysed in 100 µl radioimmunoprecipitation assay (RIPA) (Table 1 #9) lysis buffer supplemented with 5% protease inhibitor cocktail (Cat #P2714) (Sigma Aldrich, St. Louis, MO, USA), 5% phosphatase inhibitor cocktail (Cat #P2850) (Sigma Aldrich, St. Louis, MO, USA) and 0.3M phenylmethyl-sulfonyl fluoride (PMSF) (Cat #P7626) (Sigma Aldrich, St. Louis, MO, USA) for 30 minutes on ice.[60,109] The samples were vortexed every 10 minutes. The lysate was centrifuged at 13 000 xg for 30 min using a Jouan Br4i centrifuge (DJB Labcare, Buckinghamshire, UK) at 4°C for the removal of non-lysed fragments.[109] The supernatant was stabilised using a 6X loading buffer containing bromophenol blue (Table 1 #10). The supernatant was stored at -80ºC in a dark place until when needed. The purified proteins were quantified using a micro bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, UK).[60,109]

Protein separation involved equal amounts of protein (15 µg/µl) that were loaded in sample buffer containing 1% β-mercaptoethanol and loaded onto a 12% polyacrylamide gel at 120 to 150 mV.[109] Proteins were electro-transferred using the Bio-Rad transfer system (Bio-Rad, Hercules, CA, USA) onto nitrocellulose membranes using a Tris-glycine transfer buffer (Table 1 #11) for an hour and 15 minutes.[109] The membranes were then blocked with TBS-tween (TBS-T) solution containing 5% bovine serum albumin (BSA) powder for an hour.[109] The membranes were then washed for 30 minutes then probed with specific rabbit polyclonal primary antibodies against ERK(1:1000), pERK (0.1 µg/ml), AKT(1:1000), GAPDH (1:1000), and pAKT (1:2000) at 4ºC overnight. GAPDH was used as the housekeeping gene. The membranes were incubated with Goat anti-rabbit IgG Antibody HRP Conjugate (Cat #170-5046) (0.8 mg/ml) for one hour.[109] The blots were, thereafter, developed using a Clarity ECL Western Blotting Substrate (Bio-Rad, Hercules, CA, USA) and visualised using Chemidoc MP (Bio-Rad, Hercules, CA, USA).[109] The images were analysed on Image J software and densitometric data for band intensities was generated.[25] Three biological repeats and two experimental repeats were done for this experiment.

3.5 Sample size and data analysis

Outcomes of interest derive from a single factor experimental design considering TUG-891 at six concentrations [0, 0.01, 0.1, 1, 10, 100 µM]. In each experiment (biological repeat) three technical repeats were made. In these well controlled laboratory experiments very little variation between technical repeats were expected and so also between biological repeats. In view of the latter a minimum of 14 degrees of freedom (df) for residual mean squares estimates were required for sample size to be adequate. At least four biological repeats were planned to ensure at least 18 df for residual mean squares estimate, however due to time constraints a minimum of two biological repeats were conducted. Data was analysed using one-way analysis of variance (ANOVA) to compare the exposure of MG-63 cells to TUG-891 concentrations to MG-63 cells in the positive control.

To avoid false positives the Bonferroni correction was applied when doing multiple comparisons, pairwise or in contrasts. The standard deviation used to derive confidence intervals follow as the square root of the residual mean square. At least three biological repeats were done at each TUG-891 concentration, each following from triplicate (technical) repeats for resazurin and Alizarin red S staining. Western blot and qPCR were done in duplicates. All western blot images were quantified using Image J software. Testing was done at the 0.05 level of significance, i.e., a p-value < 0.05 denotes a statistically significant result. Graph-Pad Prism version 5.00 software (GraphPad Software Inc., California, USA) was used to obtain graphical results.

Chapter 4 Results

4.1 GPR120 is expressed in MG-63 osteosarcoma cells

To test whether GPR120 was expressed in MG-63 osteosarcoma cells, RT-PCR was used to visualise GPR120 transcription. RNA (1 µg) was isolated from MG-63 cells using the ISOLATE II RNA Mini Kit and reverse transcribed to cDNA using M-MuLV reverse transcriptase. Specific primers for GAPDH and GPR120 were used for RT-PCR.

MG-63 osteosarcoma cells were shown to express GPR120. The expected length of the GPR120 DNA fragment is 212 base pairs while that of GAPDH is 150 base pairs. A cell line that does not express GPR120 which could be used as a negative control could not be obtained. In lane 3 (Figure 7), except for a band at ~600bp we also observe bands at ~300bp and ~200bp. The multiple bands observed may be due to issues relating to primer specificity or genomic DNA contamination. The most intense band at ~200bp in lane 3 is close to the expected GPR120 length of 212bp, confirming the expression of GPR120 in MG-63 osteosarcoma cells. Therefore, further experiments were carried out.

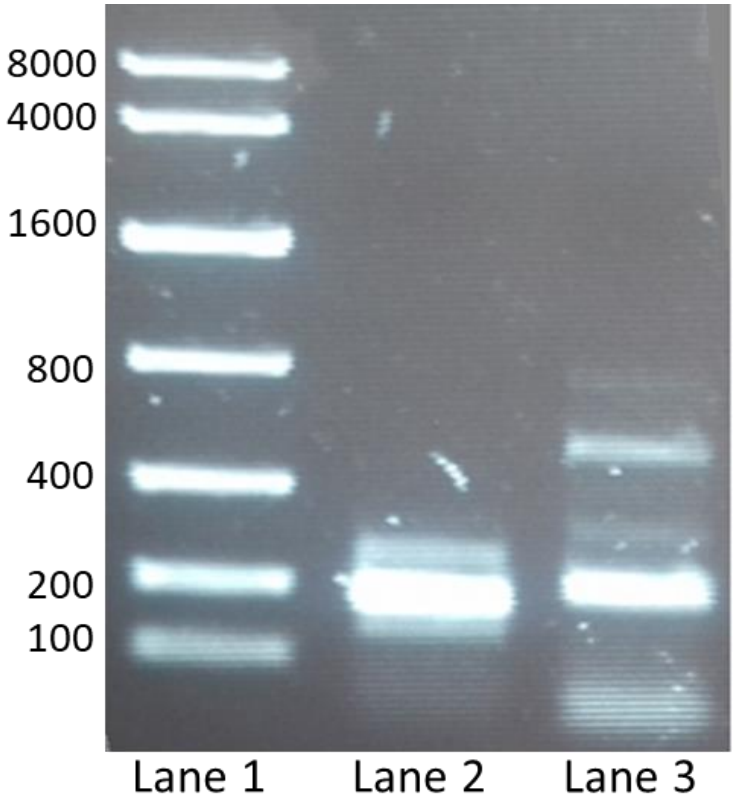


Figure 7: The expression of GAPDH and GPR120 in MG-63 osteosarcoma cells.

Visualisation of the DNA fragments generated by RT-PCR with primers specific for GAPDH as loading control and for GPR120. Lane 1= Molecular ladder, lane 2= GAPDH, lane 3= GPR120.

4.2 GPR120 agonist (TUG-891) did not affect the cell viability of undifferentiated MG-63 osteosarcoma cells

To assess whether GPR120 activation affects cell viability of MG-63 osteosarcoma cells, a resazurin assay was conducted. Resazurin measures the metabolic capacity of cells. Viable cells reduce resazurin into resorufin resulting in a colour change which can be measured using a spectrophotometer. The spectrophotometric data correlates directly to cell numbers and can indicate differences in cell viability.[119]

MG-63 cells were treated with a range of concentrations of TUG-891 [0.01-100 µM] for 48 hours after which cell metabolism was assessed. The data represents the average of three independent experiments each with three technical repeats. There was a significant increase ($P<0.05$) in cell viability of the vehicle control cells (V+) compared to the negative control cells (V-). None of the TUG-891 concentrations induced a statistically significant change in metabolism with all p values above 0.05 when compared to V+ (Figure 8). Triton X-100 exposure was included as an inducer of cell death and as expected, reduced cell viability by almost 70% ($P<0.0001$) when compared to the untreated or DMSO treated controls.

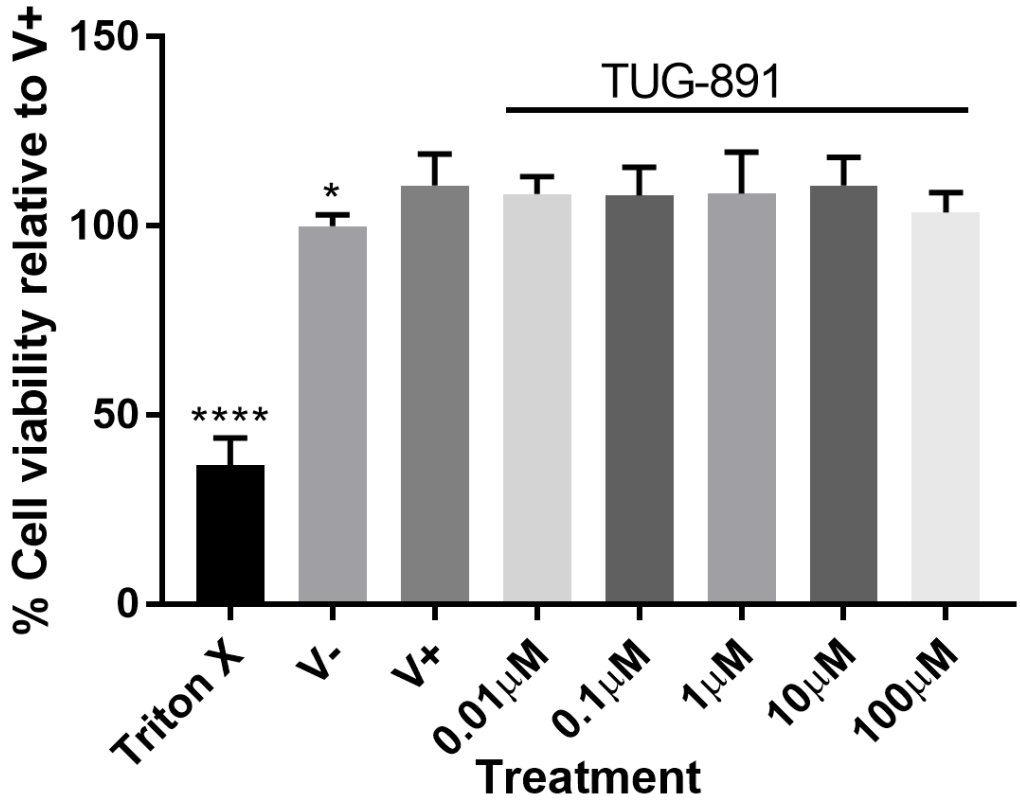


Figure 8: The effects of TUG-891 on MG-63 osteosarcoma cell viability

Averages of three experiments with S.E.M. of MG-63 osteosarcoma cell viability after 48-hour exposure to 0.1% Triton X-100, media only (V-), medium containing DMSO (V+),

or indicated concentrations of TUG-891 dissolved in 20 mg/ml DMSO. *P < 0.05, ****P < 0.0001. ANOVA analysis was done with Bonferroni correction to compare TUG-891 concentrations, V- and Triton X to V+.

4.3 TUG-891 induced an increase in ALP activity in MG-63 cells

To determine whether TUG-891 can activate GPR120 to induce MG-63 osteoblast differentiation, the level of osteoblast differentiation was assessed by alkaline phosphatase activity assay. Alkaline phosphatase enzymes are widely used as biochemical markers of osteoblast phenotypes,[110] because phosphatase activity increases during osteoblast differentiation. The level of osteoblast differentiation was indirectly measured by determining the relative quantity of ALP.

MG-63 cells were treated with osteogenic medium (OM) and a range of concentrations of TUG-891 [0.01-100 µM] for 7, 14 and 21 days. The negative control cells (OM-) did not contain osteogenic medium. The baseline control (OM+) cells contained OM only for each time point. In three independent repeats of day 7, 14 and 21, there was no significant increase in ALP activity in OM+ cells when compared to the control OM- cells. Even so, TUG-891 exposure induced a significant increase in ALP activity when compared to OM+ cells at day 7 ($P<0.01$) and day 21 ($P<0.0001$) for 100 µM concentration (Figure 9). At day 14, there was no significant changes in ALP activity in all the tested TUG-891 concentrations compared to the OM+ cells. Thus, while OM medium did not induce a significant increase in ALP activity, high concentrations of TUG-891 did increase ALP activity significantly.

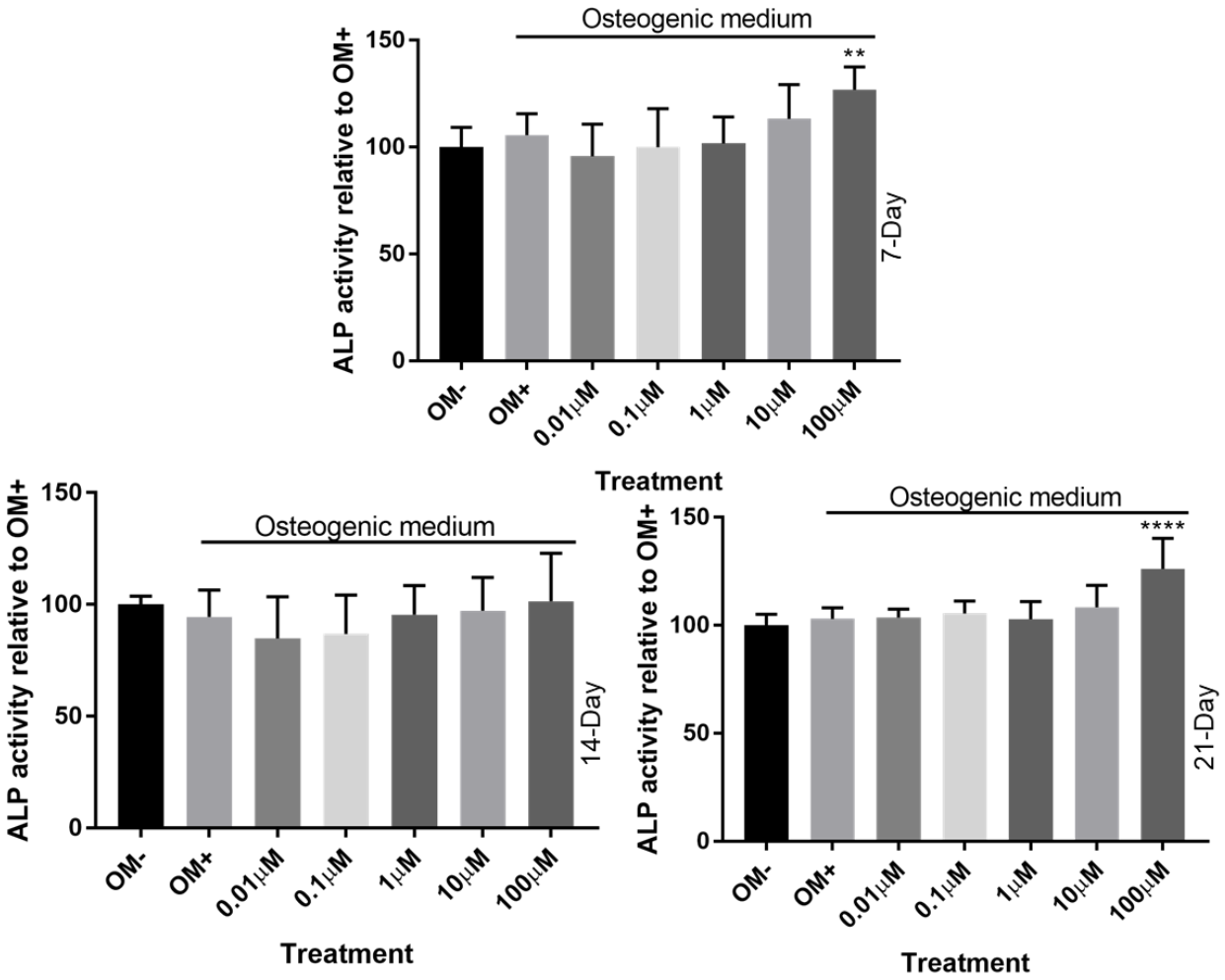


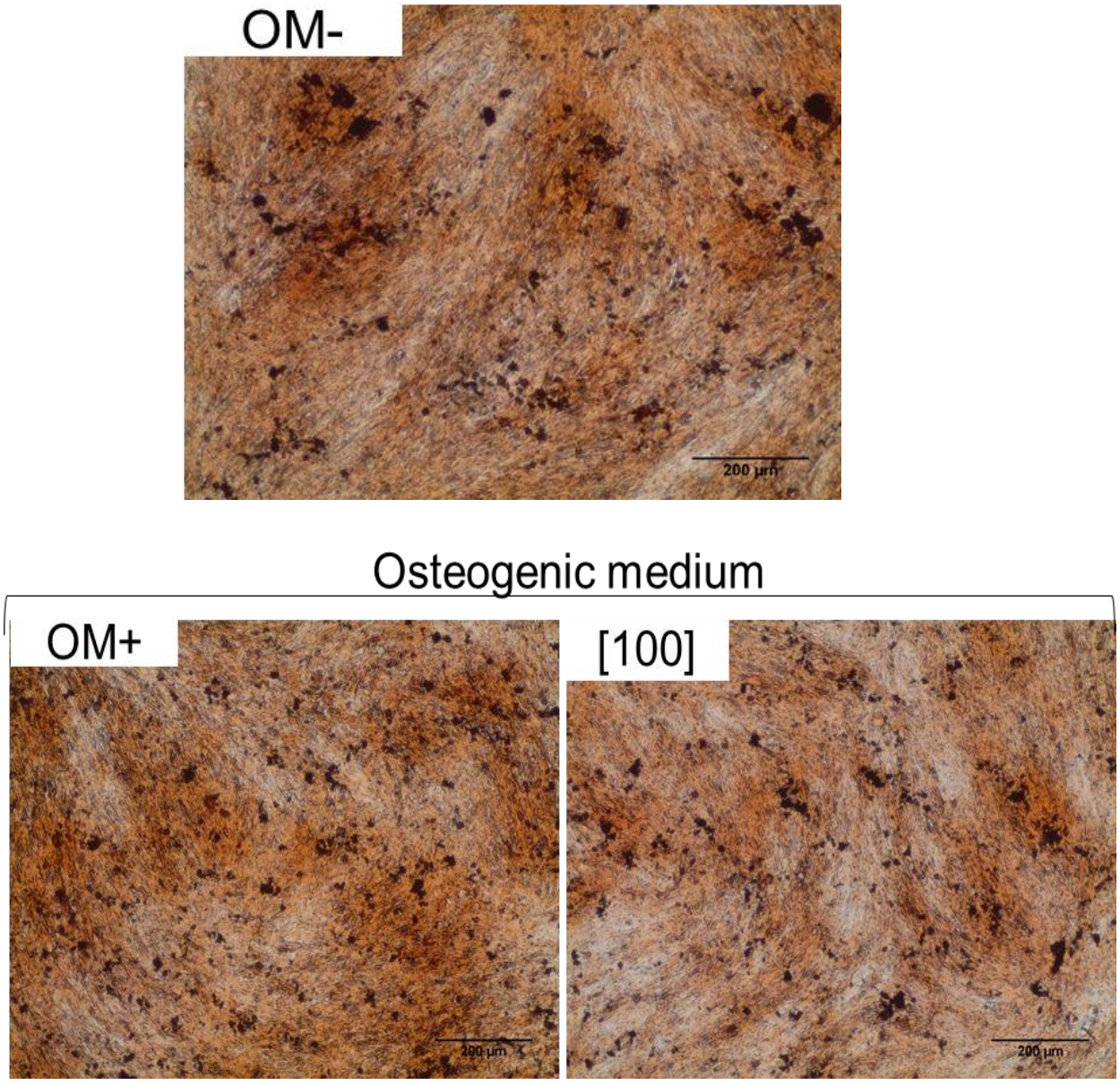
Figure 9: The effects of TUG-891 on ALP activity in MG-63 cells after 7, 14 and 21 days of exposure.

Osteoblast differentiation represented as ALP activity relative to OM+ in MG-63 cells. ALP activity was measured relative to OM+ at 405 nm and 650 nm wavelength where 650 nm was used a reference wavelength. OM- = negative control cells without osteogenic media. OM+= baseline control cells with osteogenic media, [0.01-100 µM] = TUG-891 concentrations. Data represents the average of three independent experiments conducted in triplicate. **p<0.01, ****p<0.0001. ANOVA analysis was done with Bonferroni correction to compare TUG-891 concentrations, and OM- to OM+.

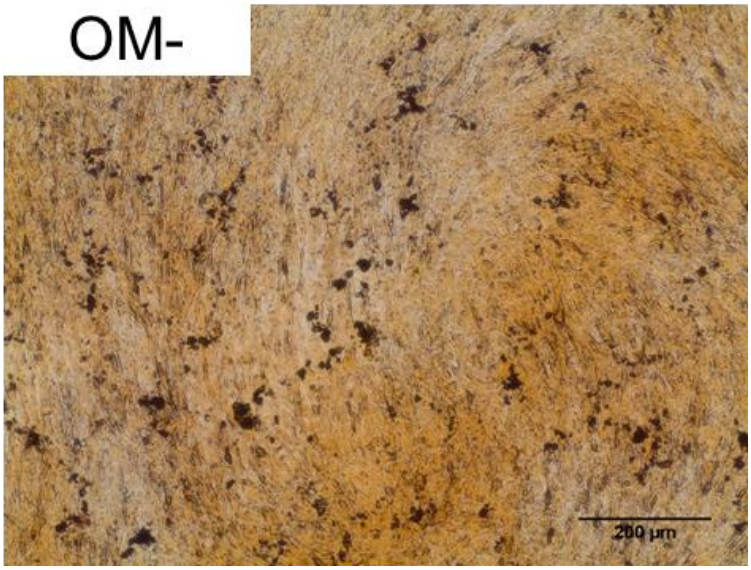
4.4 TUG-891 decreased Alizarin red S staining in MG-63 cells

To evaluate whether TUG-891 influences calcium mineralisation in MG-63 cells, osteoblast mineralisation was visualised using alizarin red s staining. Alizarin Red is a stain that binds to calcium deposits. After washing, the alizarin red stain was eluted from cells and its concentration determined by absorbance to quantify mineralisation.

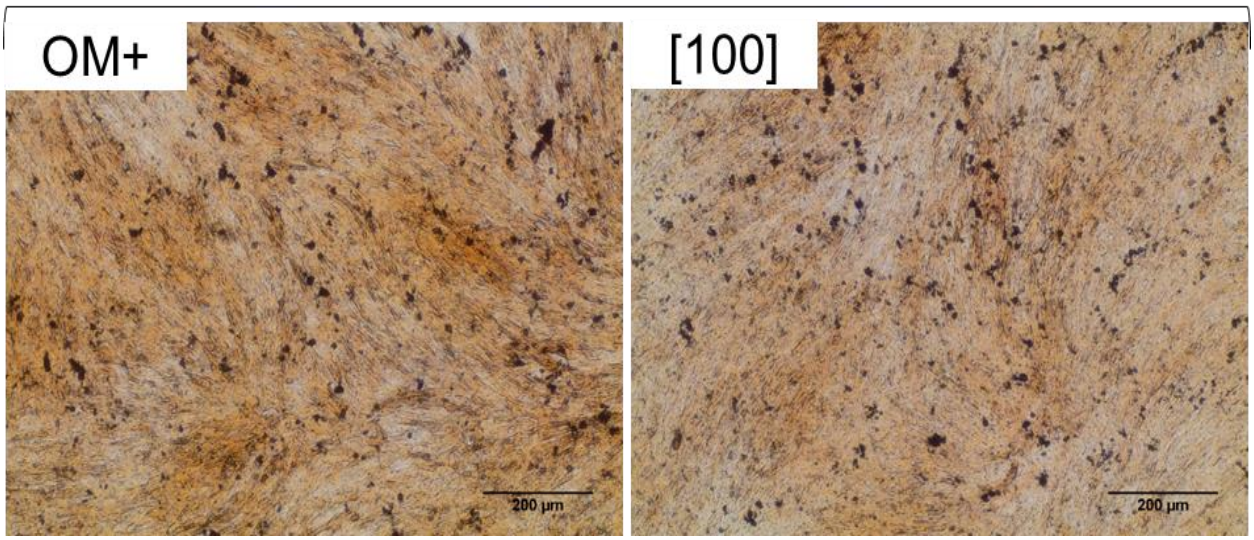
MG-63 cells were treated with OM containing 100 µM TUG-891 for 7, 14 and 21 days. Control cells were either treated with non-OM medium as negative control or OM medium only as a baseline control for each time point. The cells were fixed, washed, and stained with 2% (w/v) of alizarin red S stain for 10 minutes. The stained mineralised granules were allowed to dry overnight, and photographs were taken (scale bar: 200µm) (Figure 10). On the photographs, there were not many discrepancies between the baseline (OM+) and negative control (OM-) on days 7-21. In the cells exposed to TUG-891 however, a smaller number of stained granules were visible compared to the OM+ cells. Staining appeared to be more intense on day 7 (Figure 10a) with the stain appearing to diminish on days 14 and 21, (Figure 10b and 10c) respectively. Quantification of the amount of dye bound in cells indicated no significant differences in the staining between OM- and OM+ cells at day 7-21 (Figure 11). Furthermore, no significant differences in the amount of staining between TUG-891 and OM+ cells at day 7 of exposure. However, after 14 and 21 days, there was a significant decrease ($P<0.0001$) and ($P<0.001$) respectively, in staining for the cells exposed to 100 µM concentration of TUG-891 compared to the OM+ cells (Figure 11).



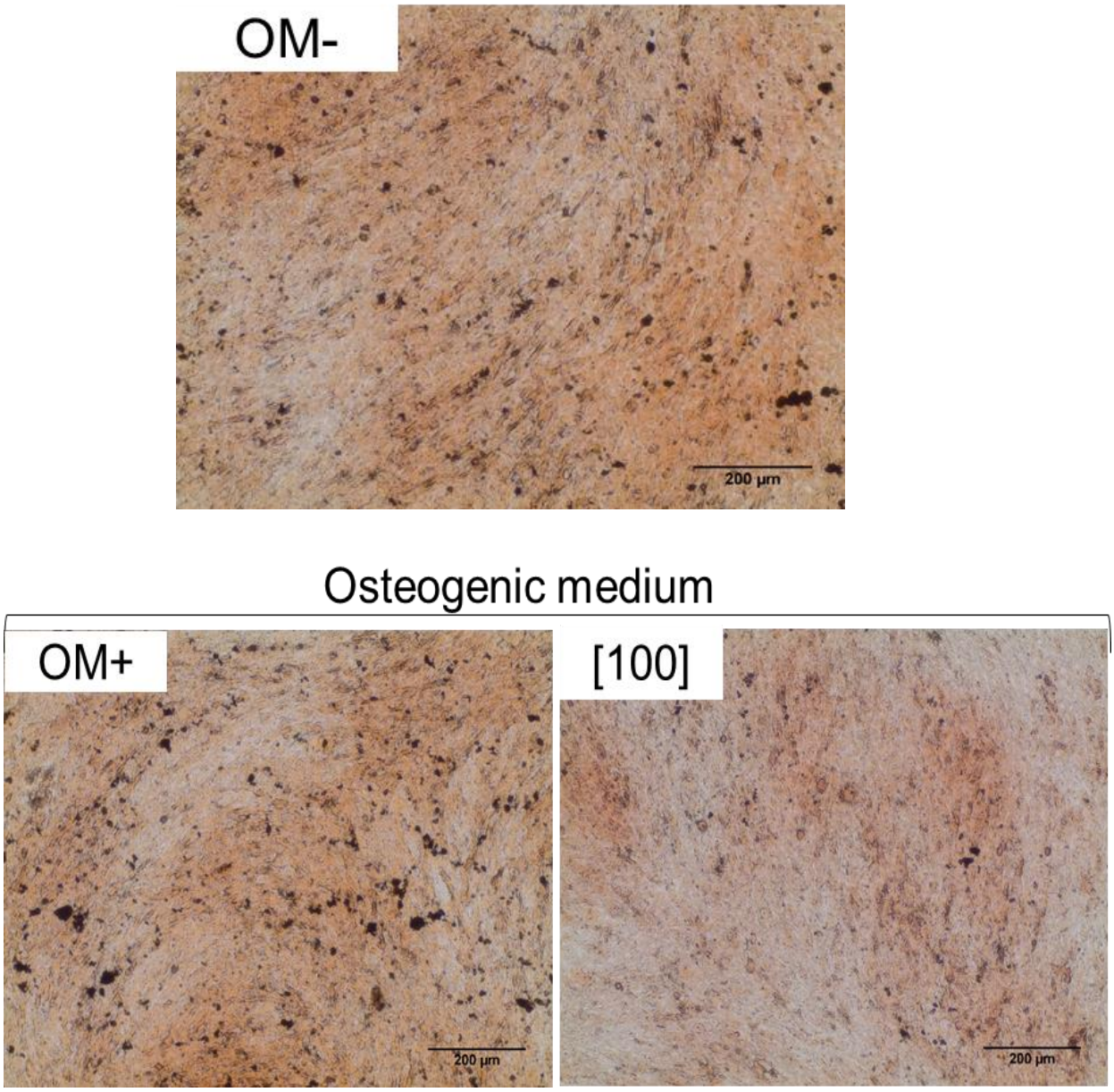
a) 7-day differentiation



Osteogenic medium



b) 14-day differentiation



c) 21-day differentiation

Figure 10: The effects TUG-891 on stained granules in differentiated MG-63 osteosarcoma cells after a) 7, b) 14 and c) 21 days of exposure.

Photomicrographs of alizarin red stained MG-63 cells. The cells were stained with 2% (w/v) of alizarin red S stain and left to dry overnight, and photographs were taken (scale bar: 200µm). Images are representative of three independent experiments conducted in

triplicate. OM- = negative control cells without osteogenic media. OM+= baseline control cells with osteogenic media and [100 µM] = TUG-891 concentration.

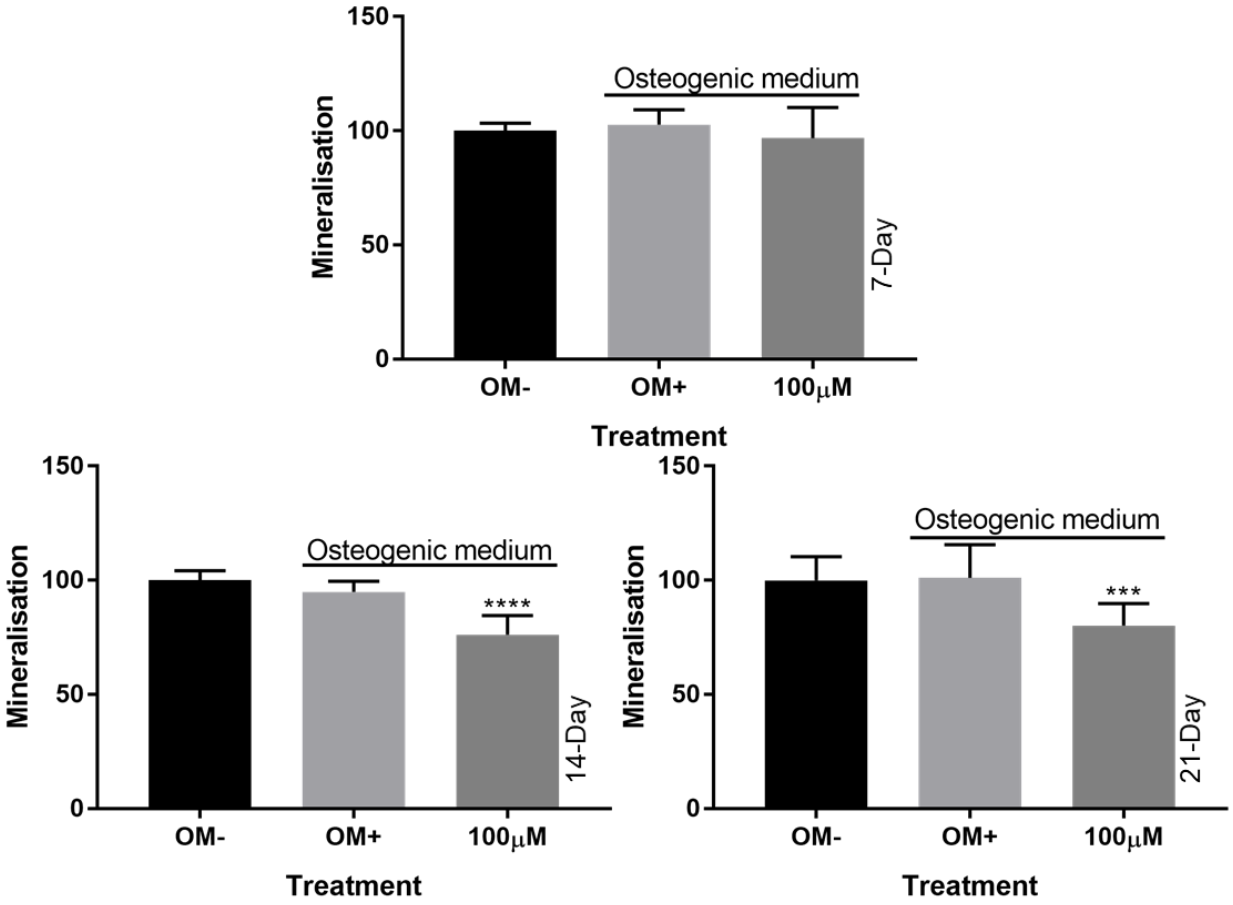


Figure 11: The effects TUG-891 on Alizarin red S staining in MG-63 osteosarcoma cells after 7, 14 and 21 days of exposure.

Graphical representation of Alizarin red S staining as mineralisation in MG-63 cells. Alizarin red S stain was eluted with 10% acetic acid and neutralised with 10% ammonium hydroxide. The amount of staining was measured relative to OM+ at 405 nm wavelength. OM- =negative control cells without osteogenic media. OM+= baseline vehicle control cells with osteogenic media and 100 µM = TUG-891 concentration. Data is an average of three independent experiments conducted in triplicate. ****P < 0.0001, ***P < 0.001.

ANOVA analysis was performed with Bonferroni correction to compare the TUG-891 concentration, and OM- to OM+.

4.5 TUG-891 did not induce any significant changes to osteoblastic gene expression in MG-63 cells after 7 or 14 days

To assess whether TUG-891 induces osteoblastic gene expression in OM stimulated MG-63 osteosarcoma cells, Quantitative-Polymerase Chain Reaction (q-PCR) was performed. The biomarkers for osteoblast differentiation were analysed after 7 and 14 days for markers of differentiation. Early markers analysed are *ALP, RUNX2, BSP, OPG,* and *RANKL* while the late marker *Osx* was analysed.

MG-63 osteosarcoma cells were grown in the presence of OM and TUG-891 [100 µM] for 7 and 14 days. The baseline control contained OM (OM+) whereas, the negative control contained media only (OM-) for each time point. Two independent experiments were performed for each gene. At the end of the culture period, RNA was isolated from the cells and reverse transcribed to cDNA. The cDNA was added to the primers for the osteoblast-specific genes. The samples were amplified, and gene expression was analysed using Actin as the loading control. The data was then represented graphically. After 7 days, no significant differences were seen between the expressions of *Runx2, Osx, ALP, BSP, OPG* and *RANKL* in OM+ cells compared to the OM- cells (Figure 12). Furthermore, TUG-891 did not show any significant differences in gene expression in any of the genes tested when compared to the OM+ cells at 7 days (Figure 12). However, TUG-891 showed an increasing trend in gene expression of *Runx2, Osx, ALP, BSP* and *RANKL* when compared to OM- except for *OPG* expression.

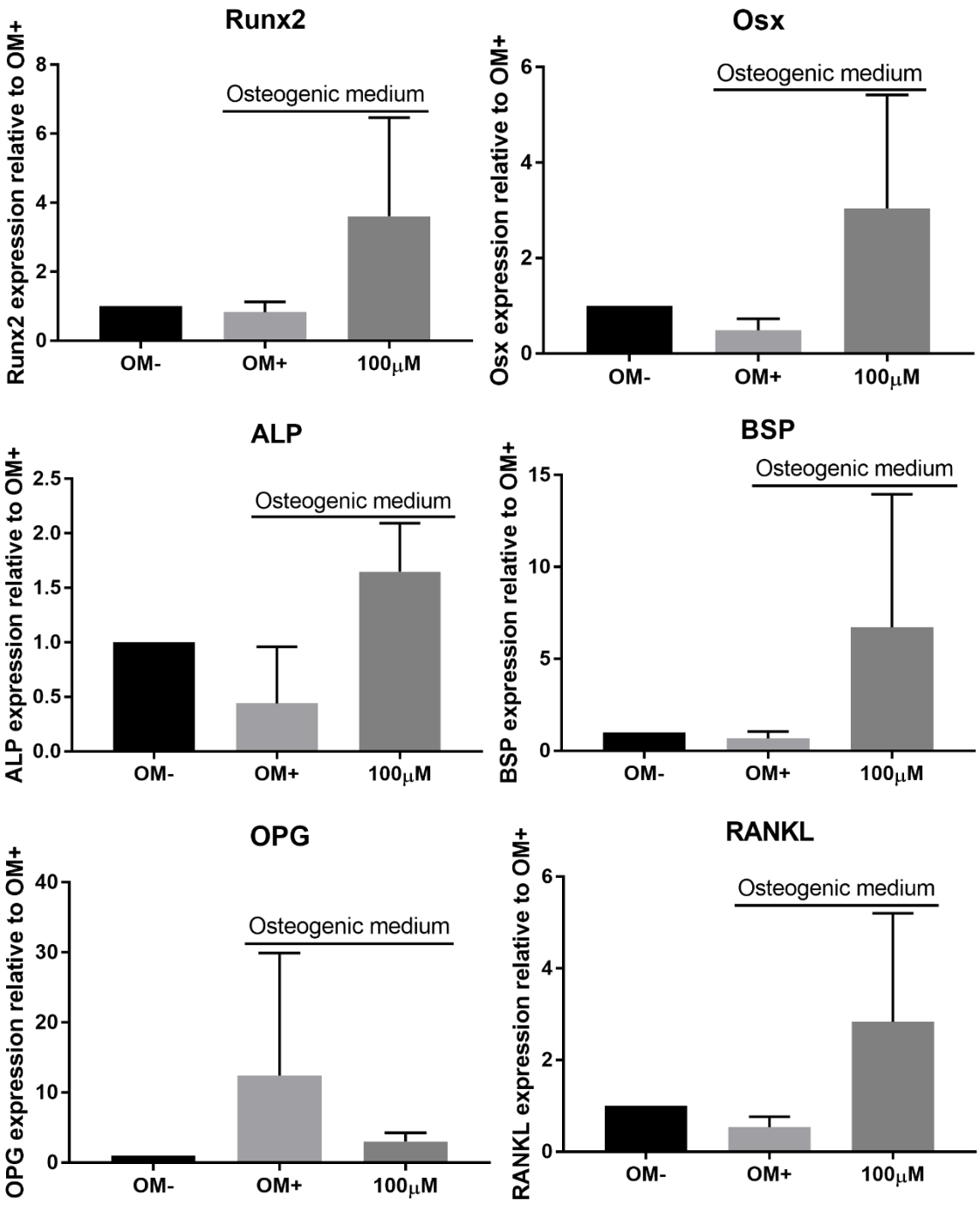


Figure 12: The effects of TUG-891 on gene expression in MG-63 cells at 7 days of osteoblast differentiation.

Graphs representing gene expression for *RUNX2*, *Osx*, *ALP*, *BSP*, *OPG* and *RANKL*. The graphs represent the average of two independent experiments. Data was analysed

using $2^{-\Delta\Delta CT}$ method with β-actin used as a housekeeping gene. Graphs were obtained using Graph-Pad. OM-=negative control. OM+= baseline control containing OM. 100 µM = TUG-891 concentration. The TUG-891 concentration and OM- was compared to OM+.

After 14 days, OM+ cells did not induce any significant differences in gene expression for all the tested genes when compared to the OM- cells (Figure 13). Again, TUG-891 did not show significant changes in gene expression when compared to the OM+ cells (Figure 13). However, TUG-891 revealed a decreasing trend in gene expression for all the tested genes compared to OM+ cells.

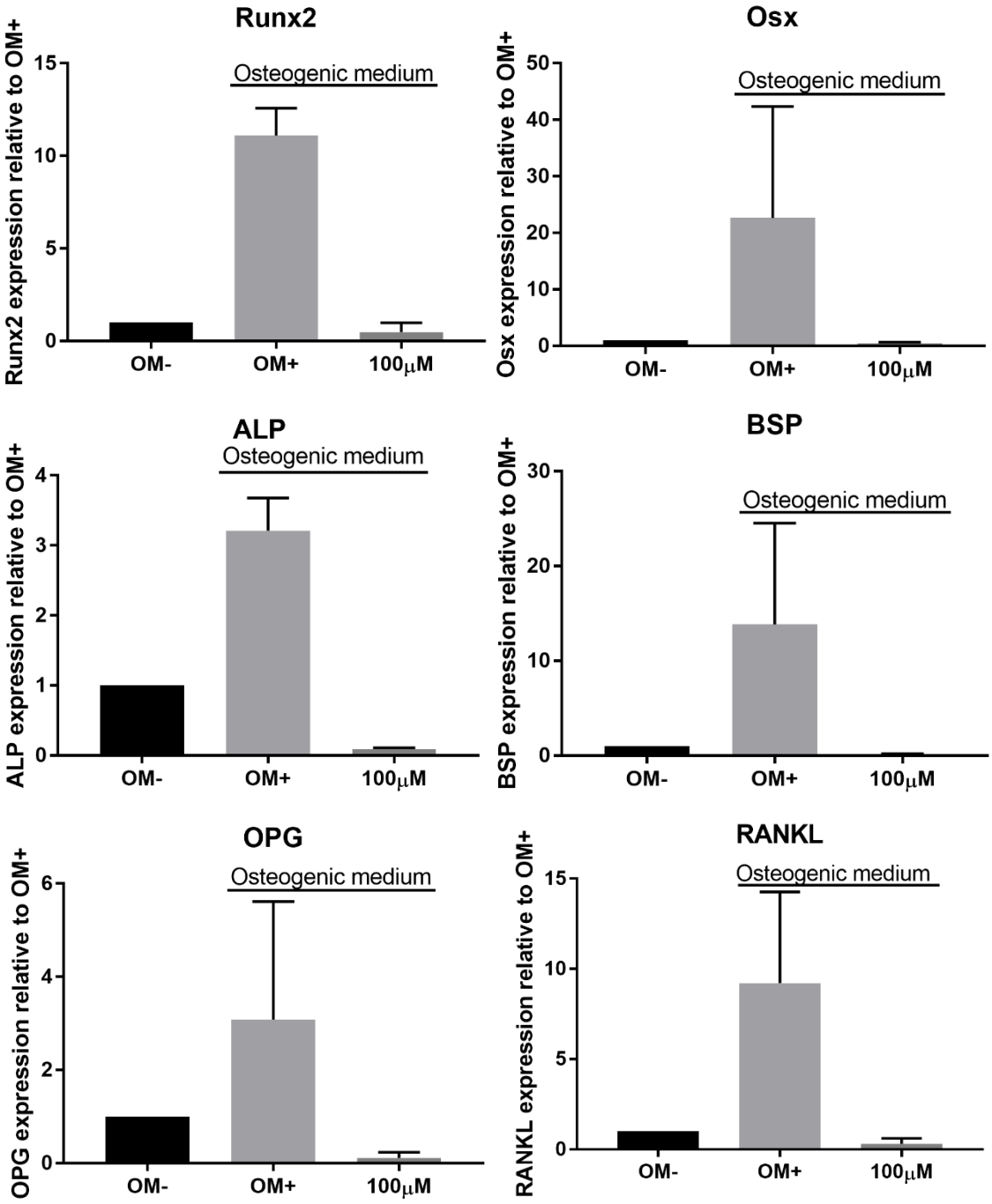


Figure 13: The effects of TUG-891 on gene expression in MG-63 cells at 14 days of osteoblast differentiation.

Graphs representing gene expression for *RUNX2, Osx, ALP, BSP, OPG* and *RANKL*. Two independent technical repeats are represented by the graphs. The data was

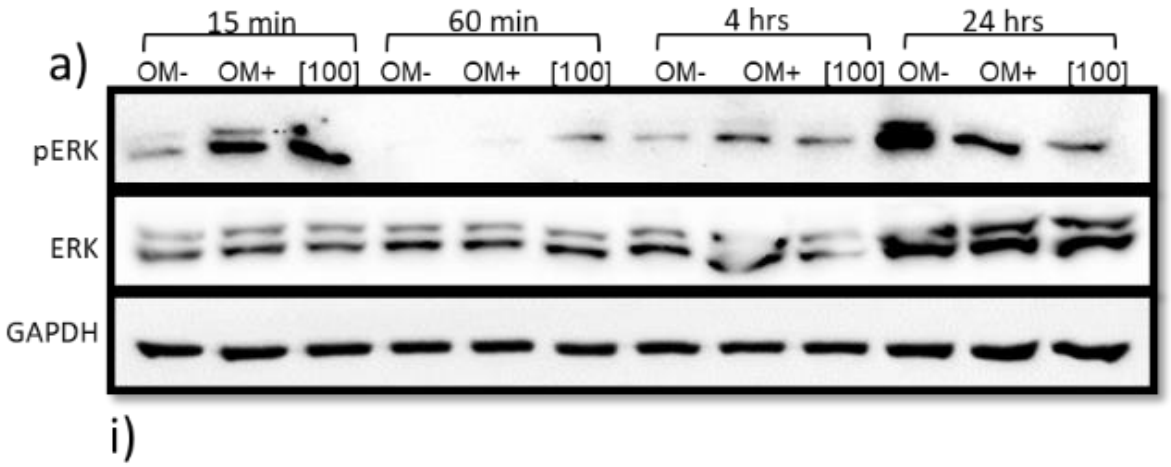
analysed using $2^{-\Delta\Delta CT}$ method with β-actin used as a housekeeping gene. Graphs were obtained using Graph-Pad. OM-=negative control OM+= baseline control containing OM 100 µM = TUG-891 concentration. TUG-891 concentration, and OM- was compared to OM+.

4.6 TUG-891 induced no significant effect on ERK phosphorylation in MG-63 cells

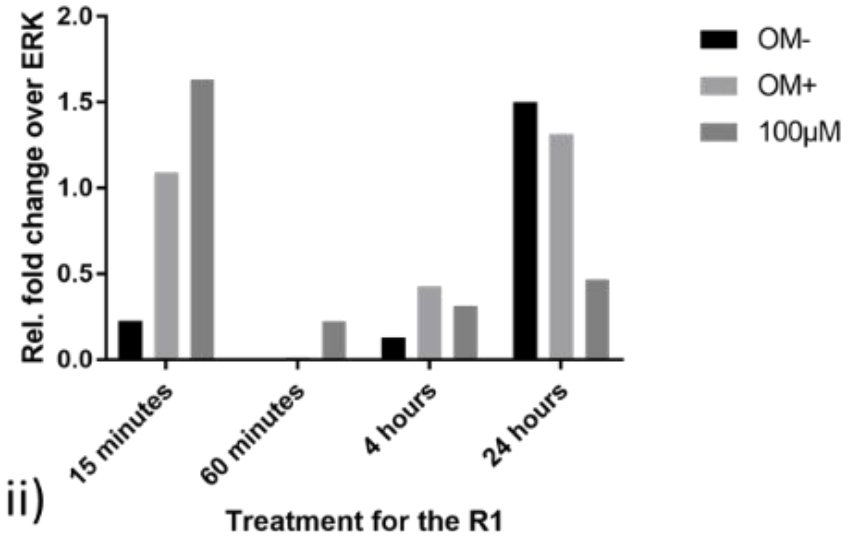
To determine whether GPR120-mediated osteogenesis is dependent on ERK signalling pathway in early differentiating MG-63 osteosarcoma cells, the relative fold change over total ERK signal was determined.

MG-63 cells were exposed to 100 µM TUG-891 for 15 minutes, 60 minutes, 4 hours, and 24 hours in the presence of OM. OM- is a negative control that contained no osteogenic medium. OM+ is a baseline control with osteogenic medium for each time point. Only two biological repeats were conducted due to time limitations and preliminary data was presented (Figure 14). In both repeats, the results revealed a phasic expression of the proteins for the tested time intervals and ERK phosphorylation (pERK) was observed at 15 minutes already. However, the kinetics of pERK differed between the repeats. For instance, OM- seemed different at different time points within each repeat although OM- did not contain any treatment. In the first repeat, the bands intensities for ERK blots remained quite consistent from 15 minutes to 4 hours (Figure 14ai) and the intensity increased after 24 hours in both the controls and TUG-891 cells. The intensity of pERK blots decreased drastically after 60 minutes and remained low after 4 hours, but after 24 hours the blot for OM- intensified than that of OM+ and TUG-891. In the second repeat, the intensity of ERK blots remained similar that of the first repeat with an increase after 24 hours (Figure 14bi). Blot intensities for pERK also showed a similar trend to the first repeat, however blots intensified after 4 hours compared to 24 hours. These intensities were increased in the controls compared to TUG-891.

After the obtained bands were quantified using Image J software, the data was normalised using GAPDH band intensity and the data was represented as fold change over total ERK signal. Graphs were then acquired using Graph-Pad. Statistical significances could not be reported due to time constraints and only two biological repeats were conducted. The relative fold change over total ERK signal remained similar after 15 minutes and 4 hours for both the repeats (Figure 14ii). In the OM+ cells, the relative fold change visibly increased after 15 minutes and 4 hours compared to the OM- cells. However, TUG-891 decreased the relative fold change after 4 hours in the first repeat but increased the change in the second repeat. In the first repeat, only TUG-891 revealed the relative fold change over the total ERK signal after 60 minutes. At 24 hours, OM- showed higher fold change compared to OM+, and OM+ compared to TUG-891 (Figure 14aii). In the second repeat, the relative fold change over the ERK signal could not be observed after 60 minutes or 24 hours except for OM+ at 24 hours (Figure 14bii).



i)



ii)

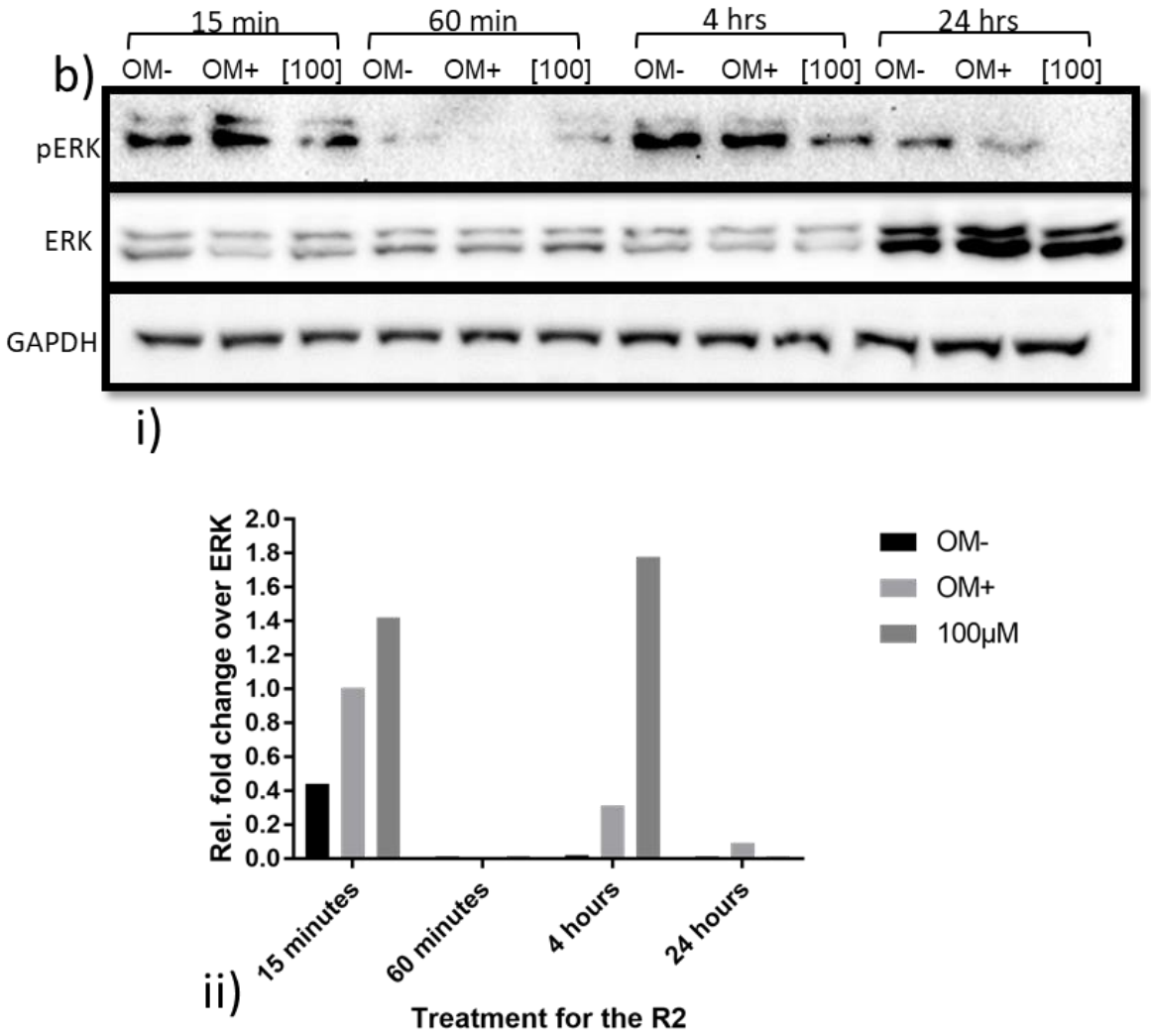


Figure 14: The effects TUG-891 on the expression of pERK, ERK and GAPDH and the relative fold change over the total ERK in MG-63 osteosarcoma cells after 15 minutes, 60 minutes, 4 hours, and 24 hours.

The first repeat is shown in figure a) and the second repeat in figure b). The pERK, ERK, and GAPDH blots are depicted in figure i) of the two independent experiments. These blots were developed using a Chemidoc. The relative fold change over the total ERK signal are shown in figure ii) of the two repeats at different time intervals in MG-63 cells. The blots were quantified using Image J software and graphs were obtained using Graph-Pad. OM-=negative control cells without the osteogenic media. OM+=baseline control cells with osteogenic media.TUG-891 (100 µM). R1= repeat 1, R2= repeat 2.

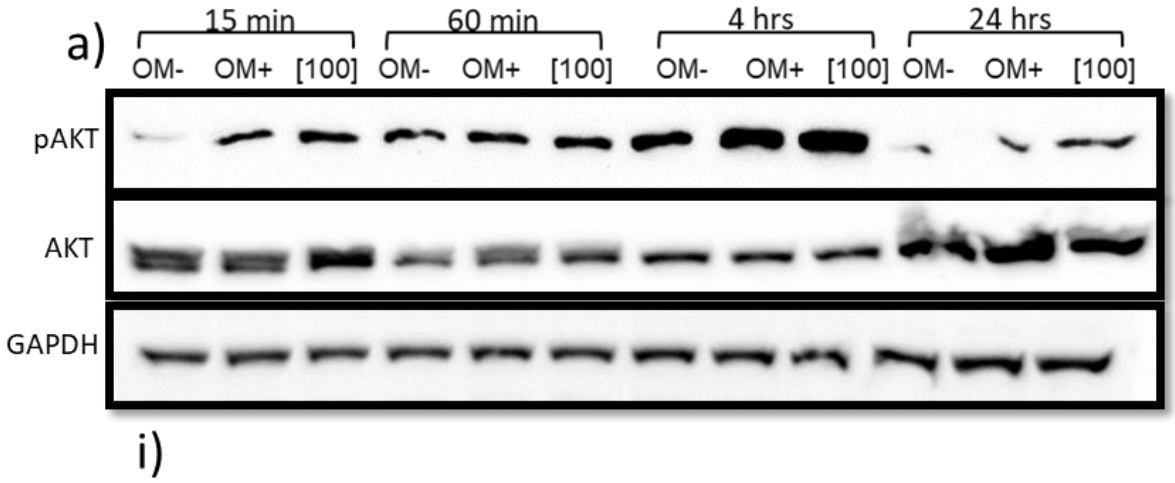
4.7 TUG-891 induced no significant effects of AKT phosphorylation in MG-63 cells

To determine whether AKT signalling pathway is affected by GPR120-mediated osteogenesis in early differentiating MG-63 osteosarcoma cells, the relative fold change over total AKT signal was determined.

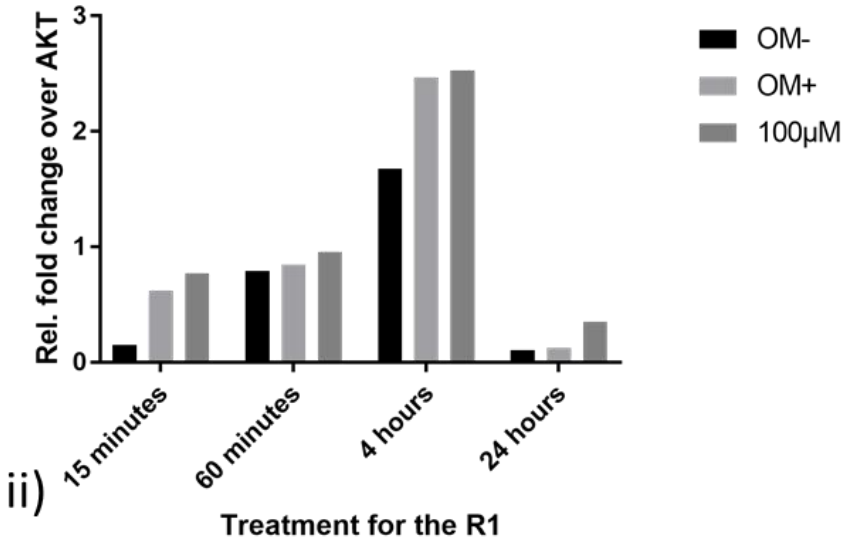
MG-63 cells were exposed to 100 µM TUG-891 for 15 minutes, 60 minutes, 4 hours, and 24 hours in the presence of OM. At each time point, the baseline control (OM+) contained osteogenic media whilst the negative control OM- did not. Only two biological repeats were conducted due to time restrictions and therefore, the presented data is preliminary. In both repeats, the phosphorylation of AKT (pAKT) was seen already after 15 minutes, however the band intensities were different when comparing both repeats (Figure 15i). Despite the fact that OM- did not contain any osteogenic media, it appeared to be different at different time points for each repeat. Although pAKT revealed different kinetics in the two repeats, there was a peak in band intensities observed at 4 hours. However, after 24 hours the intensity decreased drastically (Figure 15). In the first repeat, the OM- blot intensity was less intense and similar to the intensities of all the treatments at 24 hours (Figure 15ai). In the second repeat, the OM- and OM+ blots at 15 minutes, 60 minutes and 24 hours were less intense compared to the TUG-891 blots. The total AKT bands remained slightly consistent from 15 minutes to 4 hours, but the intensities increased after 24 hours in all the treatments of both repeats (Figure 15bi).

The bands were quantified using Image J software and the results were normalised using GAPDH and the data was represented as fold change over total AKT signal. Graphs were then acquired using Graph-Pad. There were no statistically significant changes reported as only two independent repeats were performed due to time restrictions. The first and second repeat revealed similar trends in the relative fold change over total AKT signal across the different time

points. A peak was seen after 4 hours (Figure 15ii). OM+ showed higher relative fold change compared to the OM- cells in both repeats across the different time intervals except after 60 minutes in the second repeat. TUG-891 also increased the fold change across the different time points in both repeats compared to OM+ except after 4 hours in the second repeat (Figure 15ii).



i)



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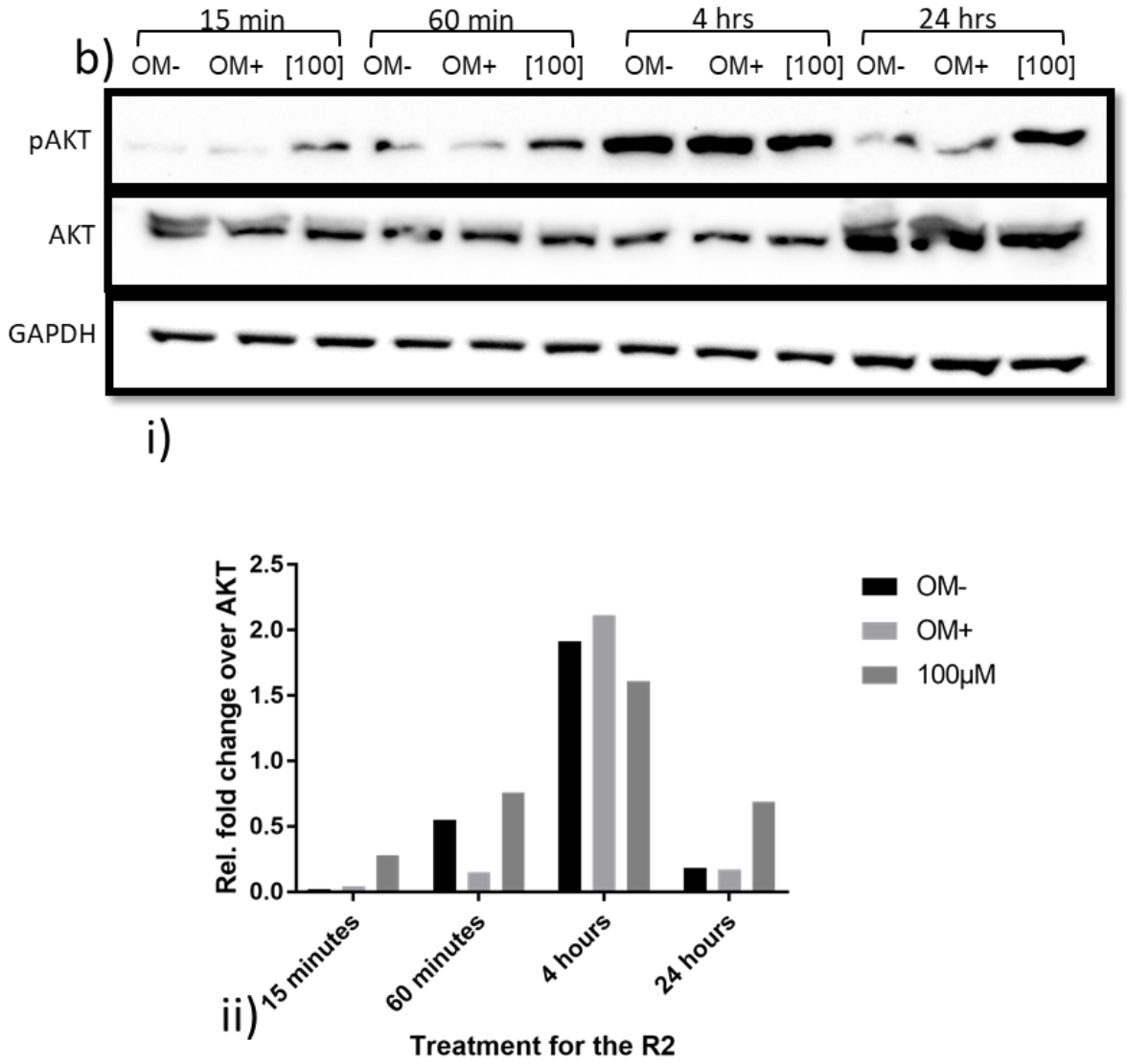


Figure 15: The effects TUG-891 on the expression of pAKT, AKT and GAPDH and the relative fold change over the total AKT in MG-63 osteosarcoma cells after 15 minutes, 60 minutes, 4 hours, and 24 hours.

The first repeat is shown in figure a) and the second repeat in figure b). The pERK, ERK, and GAPDH blots are depicted in figure i) of the two independent experiments. These blots were developed using a Chemidoc. The relative fold change over the total AKT signal are shown in figure ii) of the two repeats at different time intervals in MG-63 cells. Relative band densities were obtained after the blots were analysed using Image J software and graphs were obtained using Graph-Pad. OM-=negative control cells without

the osteogenic media. OM+=baseline control cells with osteogenic media.TUG-891 (100 µM). R1= repeat 1, R2= repeat 2.

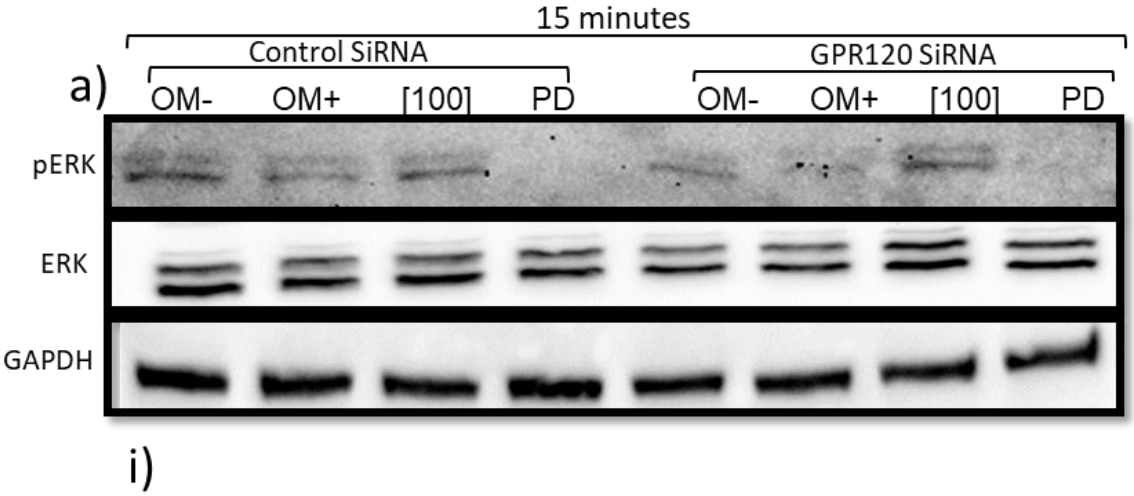
4.8 GPR120 siRNA does not significantly alter TUG-891 dependent ERK phosphorylation

A further study which included the silencing of GPR120 was conducted. A highly selective and non-competitive MEK inhibitor (PD184352) was used. MG-63 cells were transfected with the control (1:100) or GPR120 (1:100) siRNA using the transfection reagent (1:20) overnight. Thereafter, the cells were exposed to 100 µM TUG-891 or 10 µM of PD184352 (PD) for 15 minutes in the presence of OM. The baseline control indicated by OM+ consisted of osteogenic medium. The negative control OM- consisted of medium only. Only two biological repeats were performed therefore, preliminary data was presented.

The band intensities for total ERK remained relatively constant in both the control and GPR120 siRNA cells of the different treatments for both repeats (Figure 16i). In both repeats, the pERK bands were not easily detected for PD184352 exposure for the control or GPR120 siRNA cells. The OM- bands for pERK appeared less intense than the OM+ and TUG-891 cells in both the control and GPR120 siRNA cells in the second repeat (Figure 16 bi). However, OM- appeared more intense than the OM+ blots in the first repeat. Furthermore, pERK blots showed higher TUG-891 intensity in the control siRNA cells than the GPR120 siRNA cells in the second repeat (Figure 16bi). However, the two biological repeats were different.

The bands were quantified using Image J software to obtain the densities and represented graphically. The results were normalised using GAPDH and the data was represented as fold change over the total ERK signal. Significant changes could not be reported due to time limitations. Two biological repeats were

conducted. The two repeats did not show similar trends in the relative fold change over the total ERK signal. The MEK inhibitor PD inhibited the relative fold change over total ERK drastically compared to the OM+ cells (Figure 16ii). The relative fold change over the total ERK in the OM+ cells decreased compared to OM- cells in the first repeat for the control and GPR120 siRNA cells whereas it increased in the second repeat. The GPR120 siRNA did not appear to inhibit TUG-891 in the GPR120 siRNA cells compared to the control siRNA cells in both repeats.



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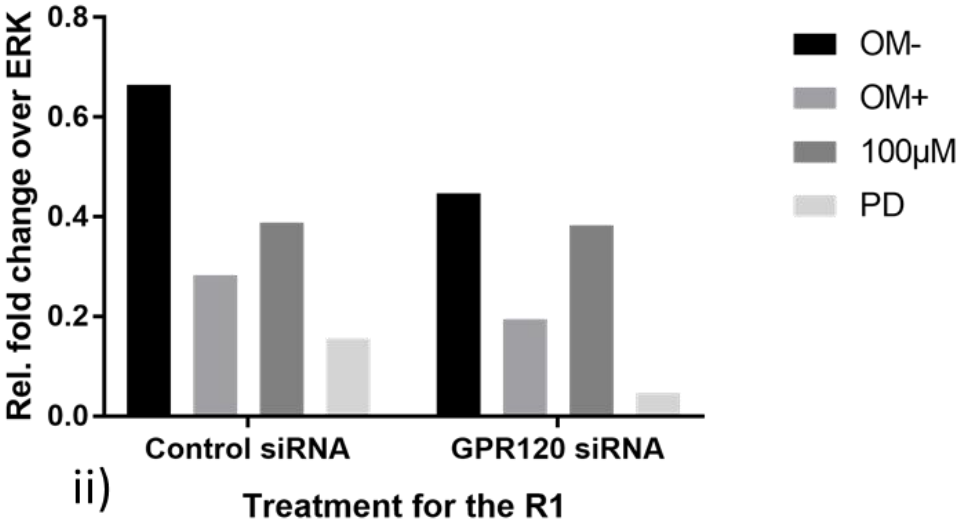

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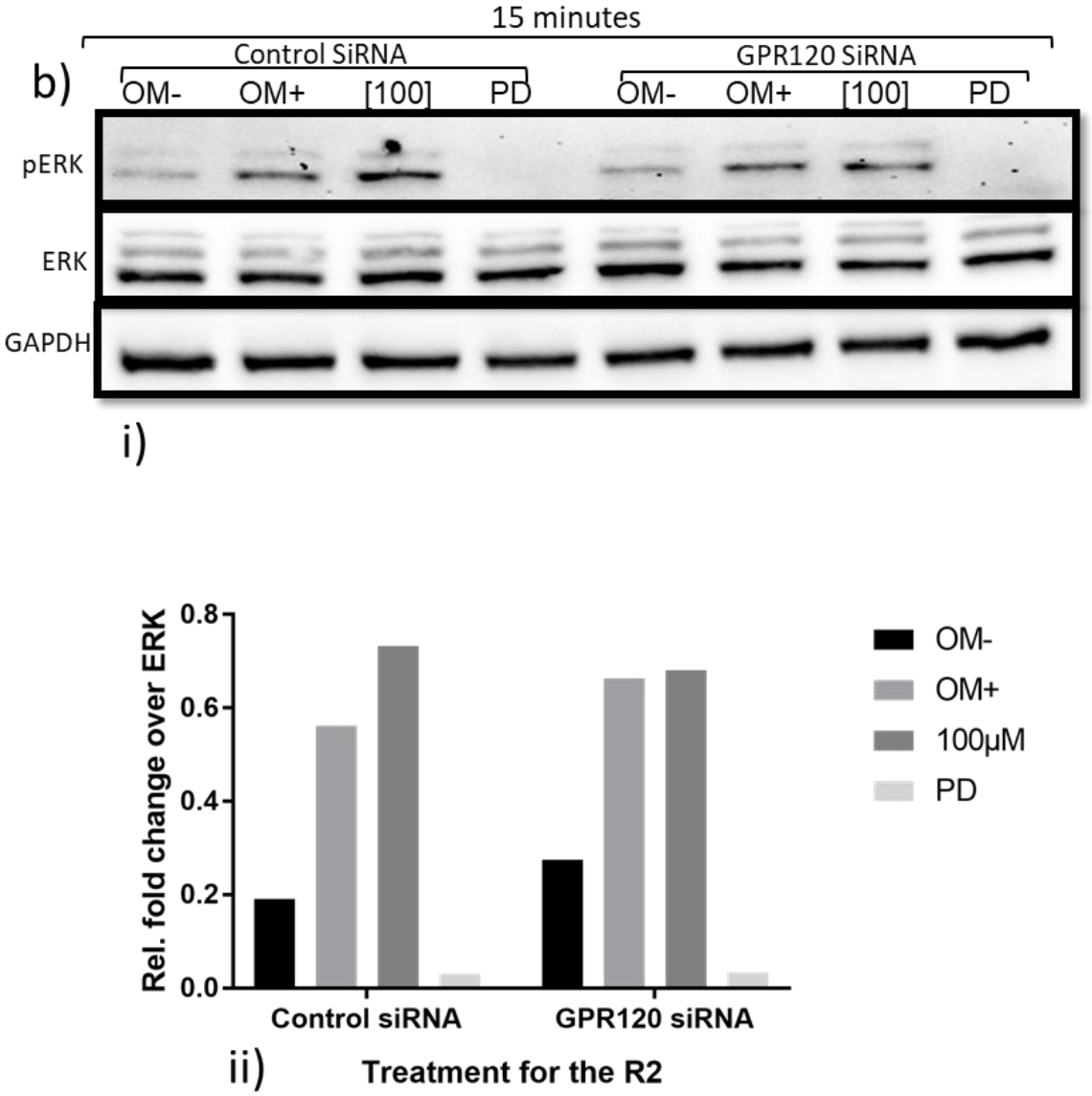


Figure 16: The effects of TUG-891 on pERK, ERK and GAPDH expression and the relative fold change over the total ERK signal in MG-63 cells at 15 minutes for control and GPR120 siRNA.

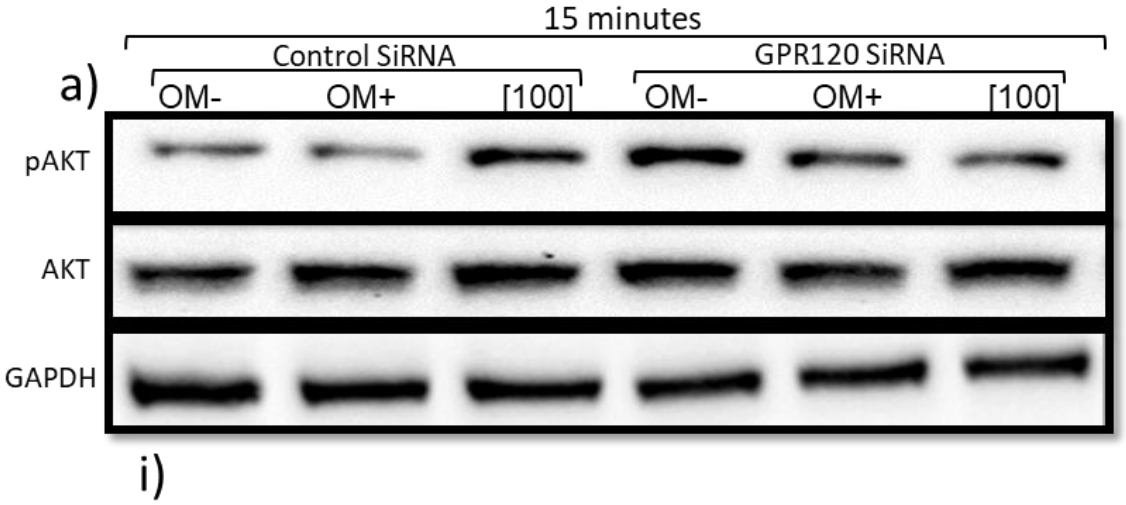
The first repeat is shown in figure a) and the second repeat in figure b). The pERK, ERK, and GAPDH blots are depicted in figure i) of the two independent experiments. These blots were developed using a Chemidoc. The relative fold change over the total ERK signal are shown in figure ii) of the two repeats in the control and GPR120 siRNA in MG-63 cells after 15 minutes. Relative band densities were obtained after the blots were

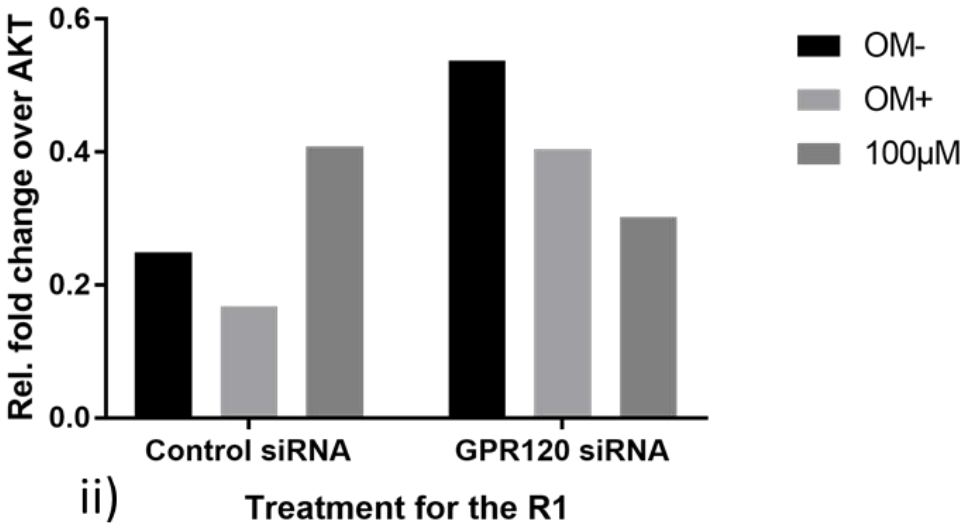
analysed using Image J software and graphs were obtained using Graph-Pad. GAPDH was used to normalise the data. OM-=negative control cells without the osteogenic media. OM+=baseline control cells with osteogenic media. PD= PD184352 an ERK inhibitor. TUG-891 (100 µM). R1= repeat 1, R2= repeat 2.

4.9 TUG-891 does not induce significant AKT phosphorylation in GPR120 siRNA MG-63 cells

MG-63 cells were transfected with control or GPR120 siRNA overnight. The cells were then exposed to 100 µM TUG-891 for 15 minutes in the presence of OM. OM+ is a baseline control whilst OM- is a negative control. Two biological repeats were presented. Similar to pERK, repeat 1 appears different from repeat 2. No clear differences can be seen in the pAKT and AKT band intensities for the control and GPR120 siRNA cells for all the different experimental groups in both repeats (Figure 17i). The pAKT blots in the first repeat revealed higher band intensity for TUG-891 cells compared to the controls in the control siRNA cells (Figure 17ai). However, OM+ in the control siRNA cells appears different from the OM+ in the GPR120 siRNA cells. The total AKT in the second repeat appears to have double bands whereas repeat one only shows single bands (Figure 17bi).

The bands were quantified using Image J software and the results were normalised using GAPDH and the data was graphically represented as fold change over total AKT signal. There were no significant differences recorded as only two repeats were conducted. Again, similar to ERK, the relative fold change over the total AKT showed different trends for the two repeats (Figure 17ii). In both repeats, the relative fold change over AKT in OM+ cells appeared lower than the OM- cells. TUG-891 showed an increased trend in the relative fold change over the total AKT in the control siRNA cells compared to GPR120 siRNA cells in both repeats but the GPR120 siRNA appeared not to be effective (Figure 17ii).





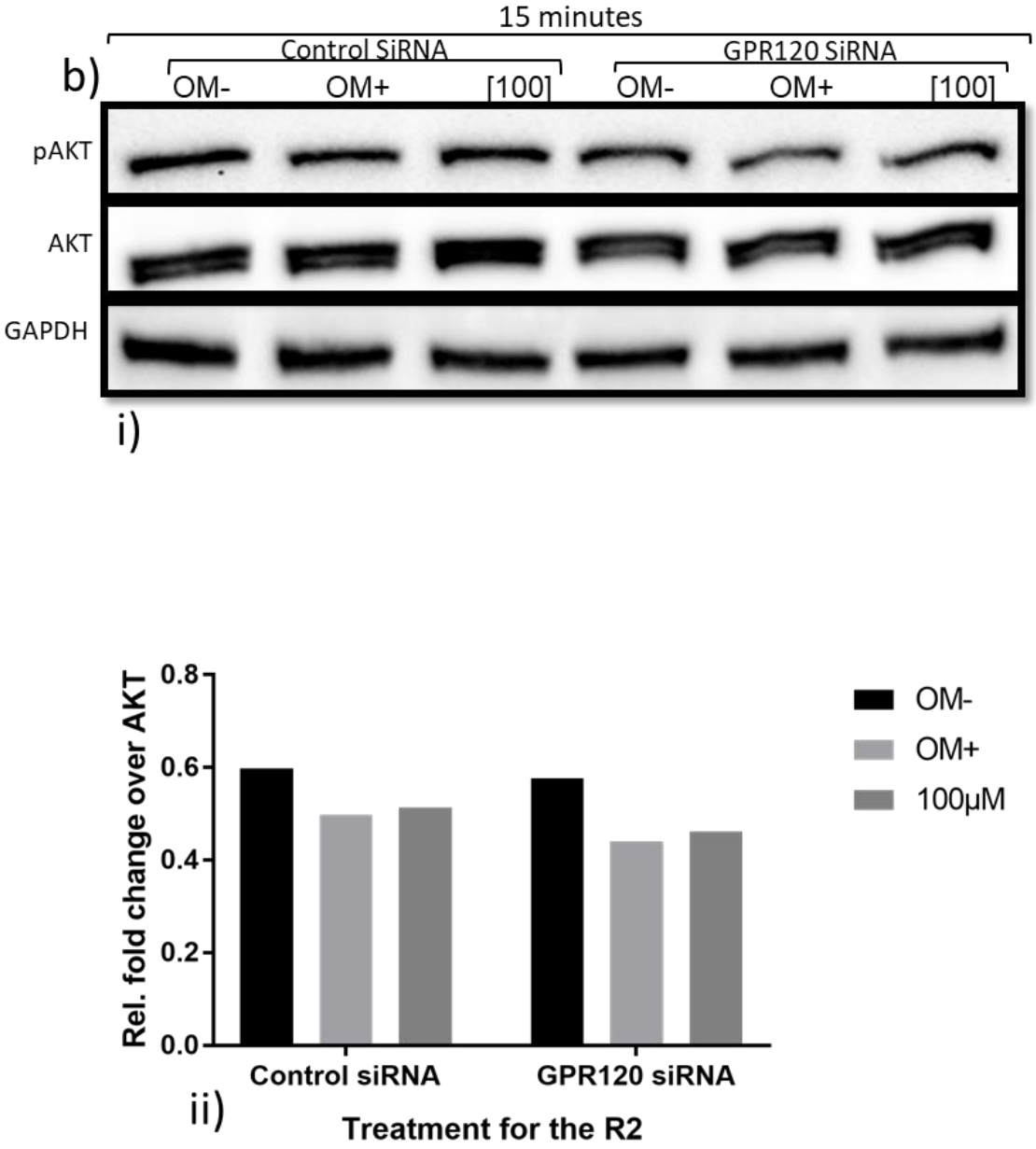


Figure 17: The effects of TUG-891 on pAKT, AKT and GAPDH expression and the relative fold change over the total ERK signal in MG-63 cells at 15 minutes for control and GPR120 siRNA.

The first repeat is shown in figure a) and the second repeat in figure b). The pAKT, AKT, and GAPDH blots are depicted in figure i) of the two independent experiments. These blots were developed using a Chemidoc. The relative fold change over the total AKT signal are shown in figure ii) of the two repeats in the control and GPR120 siRNA in MG-

63 cells after 15 minutes. Relative band densities were obtained after the blots were analysed using Image J software and graphs were obtained using Graph-Pad. GAPDH was used to normalise the data. OM-=negative control cells without the osteogenic media. OM+=baseline control cells with osteogenic media. TUG-891 (100 µM). R1= repeat 1, R2= repeat 2

Chapter 5 Discussion

Osteoporosis is characterised by reduced bone mass and strength.[120] It is a common systemic bone metabolism disease that leads to an increase in bone fragility.[120] GPR120 is involved in a number of activities, including adipogenesis, inflammation and metabolic regulation.[83] GRP120 has been acknowledged in protecting against bone diseases like osteoporosis and osteoarthritis.[85] This is due to the fact that GPR120 can increase omega-3 osteoblastic bone production by stimulating β-catenin activation and decrease osteoclastic bone resorption by decreasing NF-κB signalling.[85] Furthermore, GPR120 has the capacity to control BMMSC bi-potential differentiation in a dose-dependent manner.[85]

This *in vitro* study involved MG-63 osteosarcoma cells which were differentiated into osteoblast-like cells using OM and exposed to TUG-891, a GPR120 agonist, at varying concentrations. The effects were determined using osteoblast specific assessment methods such as ALP activity, bone mineralisation, gene expression and activation of ERK and AKT pathways. This study aimed at determining the effects that TUG-891 had on early differentiating osteoblasts and the effects on the ERK and AKT pathways. The results will be discussed and compared to previous studies.

5.1 Overview of the research findings

The expression of GPR120 was tested using RT-PCR on MG-63 osteosarcoma cells. Besides the band at the expected length of GPR120 DNA fragment, other bands were observed which could have been due to DNA contamination or due to primer specificity issues. Since the primers were not known to be intron-spanning, during the preparation of the cDNA the DNase enzyme was used to digest the DNA to avoid DNA contamination. Therefore, the MG-63 cells were confirmed to express the GPR120 gene (Figure 7) and therefore, further experiments were carried out. However, additional experiments such as western

blotting or another PCR experiment using a different primer set were required to confirm the expression of GPR120 in MG-63 cells. However, a different primer set for an additional PCR experiment was not available. Nonetheless, in this study, GPR120 expression using western blotting was tried but could not be presented due to non-specific binding of the antibody.

5.1.1 Resazurin assay for cell viability

Cell viability assessments using resazurin assay does not cause destruction of the cells.[121] The resazurin dye enters the cells and becomes reduced to resorufin, and the extent of the conversion is the measure of cellular viability.[121]

Comparing to previous studies

GPR120 may play a vital role in the process of proliferation of bone-related cells. Gao *et al*. demonstrated that at the early stages, GPR120 mediated omega-3 FA induced bone growth by increasing the proliferation of osteocytes.[12] A subsequent study also indicated that TUG-891 significantly promoted cell viability and increased proliferation of BMMSCs.[12] In a study that was previously conducted in our laboratories, we reported that TUG-891 had no effect on cell viability of RAW264.7 murine macrophages.[100] Philippe *et al.* showed that GW9508, a dual GPR40 and GPR120 agonist, had no significant impact on cell viability of MC3T3-E1 pre-osteoblast cells at 0-50 µM concentrations , and in fact for the highest dose of 100 µM, there was a slight significant increase of cell proliferation.[122] These findings demonstrate the varying effects that GPR120 agonists may have on bone cell proliferation in bone cells. There are currently no studies that have shown the effects of GPR120 agonists in MG-63 osteosarcoma cells.

In our current study, the cell viability of undifferentiated MG-63 osteosarcoma cells was assessed using resazurin assay after exposure to TUG-891 for 48 hours at various concentrations. TUG-891 did not cause any significant changes

in cell viability at the tested concentrations compared to the vehicle control (Figure 8). Therefore, TUG-891 was shown to have no effect on cell viability in MG-63 osteosarcoma cells. The differences between the findings of this current study and previous studies could be due to the different bone cell types used which may be at different proliferative stages.

5.1.2 TUG-891 exposure induces alkaline phosphatase activity

One of the major characteristics of differentiated osteoblasts is an increase in ALP activity as well as the expressions of bone markers to help in bone mineral deposition and ultimately improving mineralisation.[114] ALP is expressed at an early phase of differentiation and involved in the initiation of mineralization.[123]

Comparing to previous studies

Philippe *et al.* have previously shown that OM upregulated ALP activity in a time-dependent manner in MC3T3-E1 cells.[122] Interestingly, GW9508, a GPR40 and GPR120 agonist, was shown to significantly increase ALP activity in MC3T3-E1 pre-osteoblast cells in the absence of OM.[122] This may indicate that the increase in ALP activity can be induced independently of osteoblast differentiation. Kasonga *et al.* reported that ALP activity was significantly increased in OM+ MC3T3-E1 murine pre-osteoblast cells when compared to OM- cells.[24] In contrast this current study showed that there was no significant difference in ALP activity between OM- and OM+ cells (Figure 9), suggesting that exposure to OM+ did not lead to osteoblast differentiation.

Reasons that OM+ may not have induced osteoblast differentiation

According to Czekanska *et al.*, ALP levels in MG-63 cells reached a peak after 28 days of differentiation, which may explain why there were no significant changes observed between OM- and OM+ at any of the experimental days of differentiation in this current study.[124] Kasonga *et al.* further reported that TUG-891 cells caused a significant increase in ALP activity relative to baseline control

in control MC3T3-E1 cells, but did not induce ALP activity when GPR120 expression was silenced.[24] Similarly, in this present study, TUG-891 (100 µM) significantly increased ALP activity when compared to the baseline control at day 7 and day 21 but not day 14 (Figure 9). However, as there was no difference in ALP activity seen between OM- and OM+, this data suggests that TUG-891 may induce ALP activity independently of osteoblast differentiation. These findings are similar to that of Philippe *et al*. who reported GPR40 to have induced ALP activity independent of pre-osteoblast differentiation.[122]

5.1.3 TUG-891 decreased alizarin red staining

Alizarin red S staining and Von Kossa staining are some of the widely used assessments for calcium mineralisation targeting anionic phosphates and calcium cations respectively.[125] In this current study, the ability of MG-63 cells to produce calcified extracellular matrix was tested using alizarin red staining. There were no significant differences in mineralisation between the OM- and OM+ cells through 7 to 21 experimental days. This may again indicate that osteoblast differentiation did not take place and that extending the differentiation days could have provided with meaningful results. After 7 days of exposure, there was no significant differences in the staining between TUG-891 and the baseline control. However, after 14 and 21 days, there was a significant decrease in the staining (Figure 11). Gao *et al.* has reported that GPR120 activation by TUG-891 (50 µM) could promote Alizarin red S staining and mineralization in bone marrow MSCs.[12] In contrast, this present study showed that TUG-891 (100 µM) decreased alizarin red S staining in MG-63 osteosarcoma cells. The use of MG-63 cells and a higher concentration of TUG-891 in this present study may explain the differences seen between this study and that of Gao *et al.* This present study made use of an immortal osteosarcoma cell line whereas by Gao *et al*. made use of primary BMMSCs which are normal somatic cells.[126] The MG-63 cell line shows an immature osteoblast phenotype that develops in long term culture. The BMMSCs on the other hand, are primary stem cells which tend to retain their differentiated

phenotype *in vitro*.[113] The differences in the cell stages between the BMMSCs and the MG-63 cells may affect the way TUG-891 affects osteogenesis in these two cell lines. Similar to the MG-63 cells, the MC3T3-E1 cells are also an immature osteoblast cell line. Interestingly, the findings of this present study are similar to those of Philippe *et al.* who reported that GW9508 significantly stimulated ALP activity but inhibited the mineralisation process in MC3T3-E1 cells.[122] These results could suggest that TUG-891 may exert differential effects on osteogenesis depending on the stage at which the osteoblast cells are exposed.

Comparing to previous studies

Vater *et al.* have previously suggested that MSCs expressing high ALP levels in standard medium do not mineralise in OM.[127] However, contrary to this, some studies show that mineralisation is greater in osteogenic cultures compared to standard medium in MSCs expressing high levels of ALP.[111] This present study showed that there were no differences in the number of stained granules in the OM and in the cells without osteogenic medium, and furthermore that the stain diminished over time in the different experimental groups (Figure 10). There were no changes observed in the OM- cells compared to OM+ cells for each of the time points (Figure 10), this could be due to technical issues. A previous study has shown that even with no calcium deposits in the extracellular matrix, MG-63 cells form a cell layer after 14 days of differentiation.[124] This finding suggests that MG-63 cells may be unable to mineralise,[124] which may explain why no differences were observed between OM- and OM+ cells in this present study.

Reasons that OM+ may not have induced osteoblast differentiation

Czekanska *et al.* have suggested that the MG-63 osteosarcoma cells may possibly be blocked in a pre-osteoblast state.[113] Nevertheless, some studies have shown that MG-63 can mineralise,[128] while others have shown they cannot.[124] These differences may be due to a variety of factors including, the supplier of the

cells, the passage of cells, the type of OM used, etc. This may suggest that the MG-63 cells are not ideal for mineralisation experiments as their ability to mineralise remains controversial. However, the MG-63 cells have been shown to express high levels of osteoblast specific genes when treated with OM.[124]

5.1.4 TUG-891 regulates the expression of osteoblast specific genes

The changes of the osteoblast specific genes, *Runx2, ALP, Osx, OPG, RANKL*, and *BSP* in MG-63 cells treated with 100 µM TUG-891 for 7 and 14 days were assessed. We report in this study that there were no significant changes in gene expression in OM+ MG-63 cells when compared to OM- cells at day 7 (Figure 12) or day 14 (Figure 13) in all the tested genes. This is due to time limitations and only two biological repeats were conducted. However, trends in both day 7 and day 14 were observed in gene expression. TUG-891 appeared to increase the expression of *Runx2, ALP, Osx, RANKL*, and *BSP* compared to OM+ cells after day 7 except for *OPG* with no statistical significances (Figure 12). After 14 days, OM+ increased gene expression of all the tested genes compared to OM- cells. TUG-891 decreased the expression of all the tested genes compared to OM+ (Figure 13). Again, no statistical changes were observed. BSP functions in the late stages of osteoblast differentiation to promote bio-mineralization.[34] Osx regulates transcription in the final stages of bone tissue formation, therefore it is expressed in differentiating and differentiated osteoblasts,[31] but no statistical changes were observed for both *BSP* and *Osx* genes in the OM+ cells compared to the OM- cells after 14 days. These results may indicate that the MG-63 cells may not be able to reach the later stages of osteoblast differentiation where we expect to see the expression of these genes. Czekanska *et al.*, have previously reported that ALP levels reached a peak after 28 days of differentiation in MG-63 cells.[124] Therefore, this shows that previous studies have successfully differentiated MG-63 cells. However, it should be noted that qPCR experiments were conducted for 14 days due to time constraints. Extending the length of the

qPCR experiments to 21 days or even 28 days may result in a better expression of the osteoblast marker genes tested in this study.

Comparing to previous studies

Kasonga *et al.* reported that *Runx2* and *BSP* gene expression were all significantly higher in MC3T3-E1 cells containing osteogenic medium compared to OM- cells after 7 days.[24] Similarly, Chen *et al.* showed that *Runx2* and *ALP* gene expression was not detected in MC3T3- E1 cells cultured in α-MEM alone but when the cells were treated with the bone resorption supernatant for 7 and 14 days, *Runx2* and *ALP* gene expression was detected.[129] *Runx2* and *ALP* have further been shown to be highly expressed in differentiating MG-63 osteosarcoma cells.[124] These results indicate that Runx2 and ALP may serve as good indicators of osteoblast differentiation. As previously mentioned, the MG-63 cells may be blocked in a pre-osteoblastic state which may explain why, in this present study, stimulation of MG-63 cells with OM did not induce mineralization or ALP activity. Therefore, no significant changes were seen in the expression of the *Runx2* and *ALP* genes in OM exposed MG-63 cells indicating that the blockage in MG-63 cells occurs upstream of *Runx2* and *ALP* gene expression. However, it was previously reported that MG-63 cells were able to express the necessary genes for osteoblast differentiation but were unable to progress into mineralizing osteoblasts.[124] These results could indicate that, in the MG-63 cell line, similar to the results obtained in alizarin red staining, gene expression could be inhibited.

The GPR120 agonist, TUG-891, showed no significant difference in the gene expression compared to OM+ cells at day 7 and day 14. However, after day 14 of exposure, TUG-891 showed a decrease in gene expression compared to OM+ cells (Figure 13). However, this decrease was not significant. Nevertheless, these results may indicate that TUG-891 decreases osteoblast differentiation in MG-63 osteosarcoma cells. This decrease in osteoblast differentiation is further supported by the significant decrease in alizarin red staining after TUG-891

exposure however, the significant increase in ALP activity after TUG-891 exposure contradicts the above-mentioned results. This suggests that the decrease in *ALP* gene expression by TUG-891 at day 14 did not result in a decrease in ALP activity possibly due to a lack of translation of *ALP* genes. The contradictory *ALP* gene expression and ALP activity assay data may be explained by many contributing factors such as: only two biological repeats were conducted due to time limitations; the MG-63 cells may be blocked in pre-osteoblastic stages; gene expression was not tested at day 21 which may have provided with better findings to enable appropriate comparisons. Once again, this indicates the importance of gene expression assays to determine osteogenesis in the MG-63 cells.

Comparing to the above findings

Although ALP has been shown to play an important role in early bone mineralization, high levels of serum ALP are seen in bone degenerative diseases such as osteoporosis.[38,130] As previously mentioned, Phillipe *et al*. reported that GW9508 can increase ALP activity in MC3T3-E1 cells without OM,[122] suggesting that an increase in ALP activity may not equate to osteoblast formation. Phillipe *et al*. further reported that GW9508 decreased alizarin red staining and Runx2 gene expression at day 13 in MC3T3-E1 cells stimulated with OM. Similarly, this present study reported an increase in ALP activity at day 7 and 21 and a decrease in Runx2 expression at day 14 and a decrease in alizarin red staining at day 14 and 21 by TUG-891 in MG-63 cells. However, gene expression did not show any significant differences. Similar to GPR40, these results might suggest that GPR120 agonists can stimulate ALP activity in pre-osteoblast cells, independent of osteoblast formation,[122] while inhibiting mineralization and osteoblast gene expression.

Discussing TUG-891 findings in comparison to previous studies

Kasonga *et al.* reported that TUG-891 increased the expression of *Runx2, OPG* and *BSP* gene expression in control shRNA expressing MC3T3-E1 murine pre-osteoblast cells after 7 days, and this effect was inhibited when GPR120 expression was silenced.[24] However, Kasonga *et al.* did not measure the expression of genes after 14 days.[24] Gao *et al.*, did evaluate the expression of osteoblast specific genes after 14 days in BMMSCs exposed to TUG-891.[12] This study found that TUG-891 significantly increased the expression of *Runx2, ALP*, and *OCN* when compared to the OM+ cells after 14 days.[12] In contrast, Phillipe *et al.* have reported that GW9508 decreased the expression of *Runx2* in primary bone marrow cells after 13 days.[122] These conflicting results indicate that there is still much to be understood about the mechanisms of action of GPR120 agonists in osteoblasts. It is suggested that the agonists may have stimulatory effects early in osteoblast differentiation while having inhibitory effects in the later stages.[122] Gao *et al.* made use of 50 µM TUG-891 while this present study used 100 µM. This may also indicate that TUG-891 may have differing effects depending on the concentration used. However, these concentrations are both supraphysiological. According to Hudson *et al*., TUG-891 was reported to show effects at pEC_{50} of approximately 7 which is equivalent to the EC_{50} of 100 nM concentration.[15] Furthermore, Lui *et al*., reported TUG-891 to have inhibited DU145 cell proliferation after lysophosphatidic acid exposure at an IC_{50} of 73 nm which was consistent with 100 nM EC_{50} values.[131] However, in this current study, 100 µM concentration of TUG-891 was used in correspondence to the effects reported by Gao *et al*. concerning TUG-891. Gao *et al*. reported that low concentrations of TUG-891 (0.1-1 µM) induced adipogenesis while high concentrations (30-100 µM) induced osteogenesis.[12] Furthermore, we previously reported in our laboratories that TUG-891 (100 µM) significantly inhibited osteoclast formation in RAW 264.7 murine macrophages.[100] Nevertheless, Gao *et al.* made use of BMMSCs while in this present study, we used MG-63 cells. The use of different cell lines and concentrations of TUG-891 may have further

contributed to the conflicting findings. GPR120 agonists are known to stimulate different signalling pathways depending on the specific cell type used.[11]

5.1.5 TUG-891 showed no effect to the relative fold change over the total ERK and AKT signals

Motivation for the time points chosen to perform western blotting

MAPKs, as revealed by several studies, are key factors in the regulation of osteoblast cell line commitment and differentiation by enhancing the activity of Runx2.[132] In this study, western blotting was used to analyse the relative fold change over the total ERK and AKT signals. These relative fold changes were determined after 15 minutes, 60 minutes, 4 hours, and 24 hours after TUG-891 exposure in the presence of OM to measure the activation of ERK and AKT signalling pathways. Agonist activation of GPCRs has been shown to activate ERK phosphorylation at approximately 2 to 5 minutes.[133] However, in osteoblasts, using MG-63 cells, ERK activation peaked at 15 minutes, according to Niu *et al*.[60] Lee *et al*. reported a 30-minute increase in ERK, P38, and JNK protein levels, which peaked at 60 minutes and then gradually dropped.[134] After MG-63 cells were treated with melatonin, which is known to limit cell proliferation in human MG-63 cells, phosphorylation of ERK was reduced after 4, 24, and 48 hours.[135] Therefore, 15 minutes, 60 minutes, 4 hours, and 24 hours were the timepoints used in western blotting based on these previous studies. There are studies which showed that ERK phosphorylation peaked at day 12 of osteogenic culture in mature primary calvarial osteoblasts.[136] Furthermore, in the study, the phosphorylation levels of osteoblast master regulators were further shown to peak at day 6.[136] Ge *et al.* reported that the earliest effects of transgenes were seen at day 10, just after initial transgenic expression was detected at day 7.[137] Therefore, further studies can investigate the effects of TUG-891 on ERK activation at earlier or later time points than the tested points in this current study.

Discussion of the ERK results

In this current study, only two biological repeats were conducted for both ERK and AKT and preliminary data was presented. Therefore, statistical significances could not be reported. The band intensities for pERK and pAKT in OM- cells appeared different over the different time points. This occurrence may be due to technical issues and emphasises the need for at least three biological repeats to make better conclusions. However, multiple biological repeats could not be conducted as time was limited. The relative fold change over total ERK was increased in OM+ cells when compared to the OM- cells after 15 minutes in both repeats. This showed that ERK phosphorylation was induced in early differentiating MG-63 cells after exposure to OM. However, more biological repeats are required to confirm this. The total ERK bands together with the total AKT bands intensified after 24 hours, possibly because the amount of protein collected after 24 hours was almost double the amount measured for the previous time periods during protein quantification. It is possible that the amount of protein doubled as a result of the longer exposure duration. However, this reason does not completely justify our findings. Other issues could have contributed to these findings, such as technical issues. TUG-891 appeared to increase the relative fold change over ERK signal after 15 minutes (Figure 14). Using the siRNA to silence GPR120 revealed no conclusive findings in this study (Figure 16). More biological repeats could have resulted in more accurate results because ERK showed different kinetics in the two repeats. Nevertheless, MAP kinases have distinct roles in regulating ALP and OCN where Runx2 has been proven to be induced by BMP.[31] BMP can activate ERK, JNK and p38 in osteoblastic cells.[31] Activated ERK signalling pathway acts on the downstream transcription factors and promotes the expression of target genes to regulate the biological properties of the cells.[60] While some studies have shown that the inhibition of the ERK1/2 pathway causes the inhibition of the proliferative capacity of the osteoblastic cells,[12,60] other studies however, have shown that ERK inhibition enhances cell proliferation in osteoblasts.[138-139] Furthermore, ERK1/2 activation has been

shown to downregulate osteogenesis by negatively regulating osteoblastic differentiation factors such as BMP-2, Col1a1, and OCN.[60,140]

Comparison to previous studies

A study by Lin *et al.* used murine calvaria bone cells and pre-treated the cells for 1 hour with ERK inhibitors (PD98059 and U0126) after which differentiation was determined. Osteoblast differentiation was significantly increased compared to the vehicle (DMSO)-treated control in a time and dose-dependent manner.[138] Furthermore, Lin *et al.* showed that PD98059 appeared to stimulate ALP activity to promote cell differentiation,[138] which may indicate that ERK has a negative role on osteoblast differentiation. Consistent with the study by Lin *et al.*, Niu *et al.* used human osteoblast-like MG-63 cells to test the effects that simvastatin (SV) and SV-loaded nano-micelles (SVNs) had on the ERK1/2 pathway.[60] When MG-63 cells were pre-treated with ERK1/2 pathway inhibitor using PD98059, the phosphorylated ERK1/2 protein levels were significantly inhibited in the experimental groups compared with the group of MG-63 cells without the inhibitor.[60] While the ERK1/2 pathway was inhibited, changes in the ALP activity and protein levels of the osteogenic factors such as *Osx* and *OCN* were tested to clarify the role of ERK in SV and SVNs mediated osteoblastic differentiation. ALP activity was significantly increased at 7 days in all the tested groups compared to control regardless of whether the cells were pre-treated with an inhibitor.[60] Niu *et al.* further showed that the cellular Osx and OCN protein expressions were significantly upregulated compared to the control and osteogenic differentiation was enhanced,[60] whereas, in this present study, *Osx* gene expression was decreased at day 14 with no statistical changes compared to the control and with no prior treatment with an ERK inhibitor. Contrary to the above findings, Yang *et al.* have shown that ERK phosphorylation can promote osteogenic differentiation in MG-63 cells.[141] In this study, insulin was shown to increase osteoblast differentiation however this effect was inhibited when ERK inhibitors were used, suggesting that ERK played a role in inducing osteoblast differentiation. These

findings further reveal that the role of ERK signalling in the osteogenic differentiation of MG-63 cells remains controversial and further experiments are needed to confirm these findings.

Although no statistical significances could be reported for gene expression and protein expression, the increase in the relative fold change over total ERK and the increase in gene expression after day 14 in OM+ cells compared to OM- cells suggests that ERK may be involved in osteogenesis in the MG-63 cells. However, further studies are needed to confirm this. The role of ERK signalling pathway in osteoblast differentiation is poorly understood due to controversial findings seen in different studies. This present study showed that TUG-891 decreased osteoblast markers although the effect on ERK phosphorylation was not shown to be significant. This may indicate that TUG-891 decreased osteogenesis independent of ERK signalling pathways. However, only two repeats were conducted and preliminary data was presented for qPCR and western blotting. Therefore, further repeats are required to confirm this indication.

Discussion of the AKT results

GPR120 activation can cause pAKT stimulation.[10,142] This present study showed no significant differences in AKT phosphorylation as only two biological repeats were performed and the data presented was preliminary. Similar trends were observed in the first and second biological repeats. The relative fold change over the total AKT reached a peak after 4 hours but decreased drastically after 24 hours. The relative fold change over the total AKT in OM+ cells appeared to increase compared to OM- cells in both repeats. This suggests that AKT phosphorylation was stimulated in MG-63 osteosarcoma cells and that TUG-891 may require the AKT pathway to exert its effects on osteogenesis. However, further research is needed to confirm this. TUG-891 revealed opposing trends to the relative fold change over the total AKT in the two repeats but seemed to increase the relative fold (Figure 15) therefore, conclusions could not be made

unless further experiments are to be conducted. Similar to ERK, using the siRNA to silence GPR120 revealed no conclusive findings in this study as the two repeats showed different trends regarding the relative fold change over the total AKT (Figure 17). Further biological repeats could have provided with better results and meaningful interpretation of the findings. Also, important to note is that there were differences seen in OM- cells over the different time points. This could be due to technical issues as OM- cells did not contain any treatment at any time interval.

Significance of AKT pathway

Runx2 and PI3K/AKT signalling have been linked to osteogenesis in a positive feedback loop, with PI3K/AKT signalling inducing *Runx2* expression and activity.[143] Baker *et al.* have shown that *in vitro* osteogenic development of human bone marrow derived MSCs requires PI3K/AKT signalling.[143] GPR120 agonists are known to stimulate the PI3K/Akt pathway through Gα signalling.[84] This may suggest that, in the MG-63 cells, TUG-891 may have activated the Gα signalling pathway. Previous studies have shown TUG-891 can upregulate *Runx2* expression in differentiating MC3T3-E1 cells at 7 days, however these effects where suppressed when β-arrestin 2 was silenced.[24] Although further research is required, these results suggest that TUG-891 may stimulate osteogenesis in the MC3T3-E1 cells through activation of the β-arrestin 2 signalling pathway while inhibiting osteogenesis in the MG-63 cells through the Gα pathway.

5.2 Integration with current knowledge

The identification of GPCRs for FFA at the beginning of the twenty-first century altered the perception of FFAs as merely building blocks for triglycerides, phospholipids, and cholesteryl esters.[144] FAs can also function as endogenous ligands that mediates inflammatory responses.[10] GPR120 is the only lipid sensing GPCR which is highly expressed in a variety of cells including adipose tissue, pro-inflammatory CD11c+ macrophages, mature adipocytes, and monocytic

RAW 264.7 cells.[10] TUG-891 is an orally available, potent and selective agonist for GPR120[16] which has been shown to display similar signalling properties to the long chain FFA α-linolenic acid at human GPR120 across various assay end.[15,144]

GPR120 signalling has been shown to have beneficial effects in bone. In a study by Ahn *et al.,* it was suggested that GPR120 mediated omega-3 FA-stimulated bone formation by regulating β-catenin activity while supressing osteoclast formation by inhibiting NF-kB activity.[13] In the study, mouse calvariae were locally injected with DHA (50-250 μM) and broader width of total calvariae in DHA treatment was noted compared to PBS treatment, DHA stimulated osteoblast differentiation by increasing ALP and OCN mRNA expressions as well as β-catenin activity *in vivo*.[13] Kishikawa *et al.,* showed that using DHA, GPR120 mediated the inhibition of inflammation-induced osteoclast formation and bone resorption *in vivo* by inhibiting TNF-α production in macrophages.[99] We have previously reported that GPR120 inhibits RANKL-induced osteoclastogenesis by decreasing reactive oxygen species formation in RAW264.7 macrophages.[100] TUG-891 (40-100 μM) significantly decreased osteoclast formation, increased expression of proteins involved in reactive oxygen species formation and decreased reactive oxygen species formation.[100] Gao *et al.*, used BMMSCs and demonstrated that high concentrations (30, 50, and 100 μM) of TUG-891 play a positive role in inducing osteogenesis by increasing ALP activity, calcium mineralization, ERK phosphorylation and expression osteogenic markers.[12] In the current study, the highest concentration of TUG- 891 used was 100 μM. However, using human MG-63 cells in this study showed that TUG-891 decreased osteogenesis by decreasing osteoblastic gene expression while increasing ALP activity. Therefore, we report that the GPR120 agonist, TUG-891, inhibits the expression of genes involved in osteoblast differentiation and function. However, no significant changes were recorded for gene expression, preliminary data was presented.

The role of ERK and ERK inhibitors in osteoblast formation is controversial. Different studies have reported both increased and decreased cell differentiation after ERK inhibitor treatment.[138] Higuchi *et al.* has previously shown that MC3T3-E1 cells exposed to 3 days of PD98059 lowers ERK phosphorylation, increase ALP activity, and promote early osteoblastic differentiation and mineralized nodules.[140] On the contrary, ERK inactivation has also been reported to block osteogenic differentiation in the same osteoblastic cell line, MC3T3-E1.[145] The controversial findings regarding the ERK signalling pathway in osteoblast differentiation are thought to be related to the cell type,[138] extracellular stimulus types, degree and duration of activation.[60,146] It has been suggested that in instances where ERK activation enhances survival, the ERK activation occurs rapidly and more transient; in instances where it is apoptotic, ERK activation tends to be delayed and sustained.[146] However, this current study could not show significant changes in ERK phosphorylation. Nevertheless, TUG-891 significantly decreased alizarin red staining and reduced gene expression at 14 days. These results may indicate that TUG-891 inhibited osteogenesis through ERK and independent pathways.

Although AKT has been shown to play a role in osteogenesis,[143] in this present study, preliminary data was reported. GPR120 agonists however, have been shown to stimulate AKT phosphorylation through Gα pathways,[84] suggesting that TUG-891 signal through the Gα pathway in the MG-63 cells. Contrary to this finding, Kasonga *et al.* have shown that TUG-891 can stimulate osteoblast through β-arrestin 2 signalling pathways in MC3T3-E1 cells.[24] As previously mentioned in an article by Talukdar *et al.,* GPR120 agonists may stimulate different signalling pathways depending on the cell types.[11] Moreover, TUG-891 and GW9508 have been demonstrated to use different signalling pathways in Caco-2 colon carcinoma cells.[83] Therefore, to fully understand the biological function of GPR120, it is very important to generate selective biased agonists and assess their signalling in different cell types.

Chapter 6 Conclusion

The study investigated the effects of TUG-891, a GPR120 agonist, on MG-63 osteosarcoma cells. The study tested the effects of TUG-891 on cell viability, ALP activity, calcium mineralisation, gene expression and protein expression. The aim of this study was to further understand the mechanisms of action of GPR120 agonists in osteoblast formation.

TUG-891 had no effects on cell viability at the tested concentrations. TUG-891 significantly increased ALP activity at day 7 and day 21 but not day 14. However, no significant differences were observed between the baseline and negative control indicating a possibility that osteoblast differentiation did not occur. A significant decrease in alizarin red S staining at 14 and 21 days of differentiation was observed after TUG-891 exposure compared to the baseline control. Once again, no difference was seen in alizarin red S staining between cells exposed to osteogenic media and those without. However, studies investigating the ability of the MG-63 osteosarcoma cells to mineralise have been controversial. Here we show that the MG-63 cells may not be able to mineralize.

The MG-63 cells are known to express high levels of osteoblast-specific genes in the presence of osteogenic media. However, MG-63 cells exposed to osteogenic media were not able to increase the expression of the tested genes after 14 days. These results suggest that TUG-891 may exert a negative effect on osteoblast differentiation. The role of ERK in osteoblast differentiation is controversial with some studies suggesting ERK negatively regulates osteoblast differentiation, while others have shown ERK stimulates osteogenesis. This current study could not make conclusive findings due to time constraints. Only two biological repeats were conducted for qPCR and western blotting.

In conclusion, TUG-891 may show negative changes in osteoblast differentiation, without affecting the cell viability. In the contrary, TUG-891 positively affected

ALP activity. Furthermore, ERK and AKT pathways may not be concluded to play a role in the reported changes. Nevertheless, these findings suggest that GPR120 may not be a suitable target for the treatment of bone degenerative diseases. However, further research is required to confirm this as previous studies have shown GPR120 agonists to cause a positive effect in osteogenesis.

6.1 Limitations of the study

The current study may provide further information when designing future studies based on this study. The major limitation of this study was the use of the MG-63 osteosarcoma cell line and time. MG-63 osteosarcoma cells could be blocked at a pre-osteoblastic state, making them unsuitable for ALP experiments and mineralisation assays. Although some studies have shown MG-63 cells can express ALP and mineralise in the presence of OM, others have shown the opposite. The MG-63 cells used in this present study were unable to mineralise in the presence of OM. The lack of mineralisation by the MG-63 cells made it difficult to access the effect of TUG-891 on osteoblast activity. Future studies should make use of other osteoclast cell lines such as the SaOS-2 osteosarcoma cells or the MC3T3-E1 pre-osteoblasts. More biological repeats are needed.

For the qPCR and western blot experiments, only two successful technical repeats were completed. Due to limited time and resources, further technical repeats could not be completed before submission of this dissertation. This may explain why no statistical differences were not seen in some of the experiments. Completing further technical repeats for the PCR and western blot experiments may increase the strength of the comparisons and conclusions made in this study. Furthermore, the genes tested in this study could be determined for 21 days of differentiation to test whether the genes are expressed in the late stages of osteoblast differentiation.

6.2 Recommendations for future studies

There are a few studies being conducted on the effects of GPR120 in bone health with more mechanisms still to be explored and understood. Potential cost-effective therapeutic drugs could be discovered for bone diseases.

Future studies could use alternative cell line models for cell culture, such as SaOS-2 cells, bone marrow MSCs, human foetal osteoblasts (hFOB 1.19) or MC3T3-E1 cells. Late osteoblast-specific markers such as OCN and OPN could also be carried out to assess their expression.

Several studies revealed that ERK activation inhibit osteoblast formation, while other studies reveal contradicting results. The role of ERK signalling pathway is poorly understood in osteogenesis. Future studies could further explore the relationship between the ERK pathway and osteogenesis.

The effects of TUG-891 in AKT signalling pathways could be investigated further to better understand their association in osteoblast differentiation.

Using TUG-891, future studies could use this study as a foundation to continue the research on GPR120 by including *in vivo* experiments to evaluate the effects of GPR120 on osteoblast activity and ultimately bone health.

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APPENDIX A
Ethical approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

18 September 2020

Approval Certificate
New Application

Ethics Reference No.: 582/2020
Title: Modulation of osteoclast and osteoblast activity and differentiation by selective G-protein coupled receptor agonists and antagonists

Dear Miss CN Sithole

The **New Application** as supported by documents received between 2020-08-26 and 2020-09-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2020-09-09 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-09-18.
- Please remember to use your protocol number (582/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

13 May 2021

Approval Certificate
Amendment

Dear Miss CN Sithole

Ethics Reference No.: 582/2020
Title: In vitro effects of G-Protein Coupled Receptor 120 agonist on osteoblast differentiation and activity in osteosarcoma cells.

The **Amendment** as supported by documents received between 2021-04-28 and 2021-05-12 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-05-12 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Please remember to use your protocol number (582/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

30 July 2021

Approval Certificate
Annual Renewal

Dear Miss CN Sithole

Ethics Reference No.: 582/2020
Title: In vitro effects of G-Protein Coupled Receptor 120 agonist on osteoblast differentiation and activity in osteosarcoma cells.

The **Annual Renewal** as supported by documents received between 2021-06-29 and 2021-07-28 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-07-28 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2022-07-30.
- Please remember to use your protocol number (582/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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APPENDIX B
Statistical clearance

Date: 19 / 05 / 2020

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that,

Name(s): Ms Cynthia Navela Sithole

from the University of Pretoria

discussed with me the study titled: *In vitro* effects of GPR120 agonist on osteoblast differentiation and activity in MG-63 osteosarcoma cells

I hereby confirm that I am aware of the project and also undertake to assist possible, with the Statistical analysis of the data generated from the project.

The analytical tool(s) that will be used is (are) Data will be analysed using oneway ANOVA test of variance to compare TUG-891 concentrations. To avoid false positives the Bonferroni correction will be applied when doing multiple comparisons , pairwise or in contrasts . The standard deviation used to derive confidence intervals follow as the square root of the residual mean square. At least four biological repeats will be done at each TUG-891 concentration, each following from triplicate (technical) repeats for resazurin and Alizarin red S staining. Western blot and qPCR will be done in duplicates. Testing will be done at the 0.05 level of significance, i.e. a p -value ≤ 0.05 denotes a statistically significant result **to achieve the objective (s) of the study.**

Signature 

Name: PJ Becker (Tel: 012-319-2203**)**

Research Office, Faculty of Health Sciences

APPENDIX C

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Jul 08, 2021

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Expected presentation date	Feb 2022
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		Expected presentation date	2022-02-01
Instructor name	Cynthia Sithole		

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APPENDIX D

Declaration

DECLARATION OF ORIGINALITY

UNIVERSITY OF PRETORIA

The Department of ……Physiology……. places great emphasis upon integrity and ethical conduct in the preparation of all written work submitted for academic evaluation.

While academic staff teach you about referencing techniques and how to avoid plagiarism, you too have a responsibility in this regard. If you are at any stage uncertain as to what is required, you should speak to your lecturer before any written work is submitted.
You are guilty of plagiarism if you copy something from another author's work (e.g. a book, an article or a website) without acknowledging the source and pass it off as your own. In effect you are stealing something that belongs to someone else. This is not only the case when you copy work word-for-word (verbatim), but also when you submit someone else's work in a slightly altered form (paraphrase) or use a line of argument without acknowledging it. You are not allowed to use work previously produced by another student. You are also not allowed to let anybody copy your work with the intention of passing if off as his/her work.

Students who commit plagiarism will not be given any credit for plagiarized work. The matter may also be referred to the Disciplinary Committee (Students) for a ruling. Plagiarism is regarded as a serious contravention of the University's rules and can lead to expulsion from the University.

The declaration which follows must accompany all written work submitted while you are a student of the Department of ……Physiology…….. No written work will be accepted unless the declaration has been completed and attached.

Full names of student: Cynthia Navela Sithole……

Student number: 15039499……

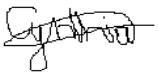
Topic of work: *In vitro* effects of GPR120 agonist on osteoblast differentiation and activity in osteosarcoma cells

Declaration

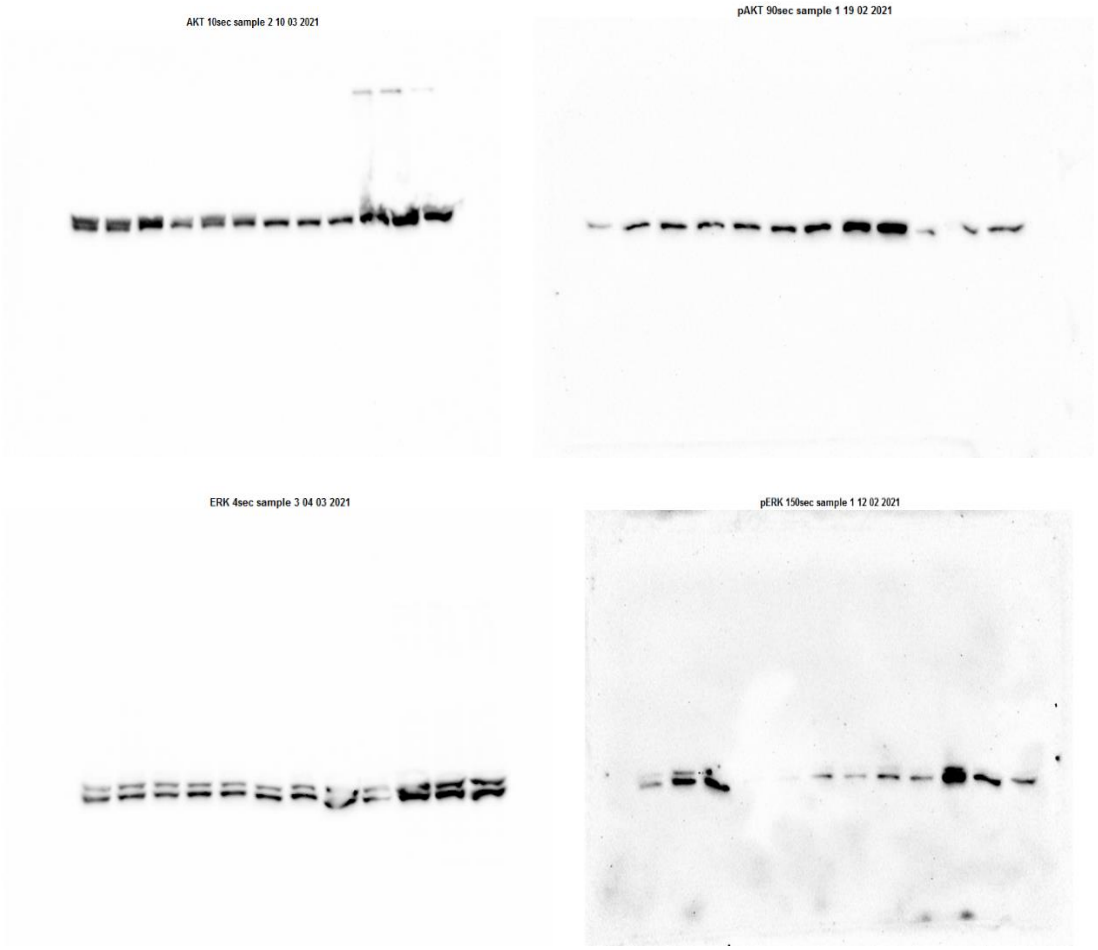
1. I understand what plagiarism is and am aware of the University's policy in this regard.

2. I declare that this ……Dissertation…… (e.g. essay, report, project, assignment, dissertation, thesis, etc.) is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.

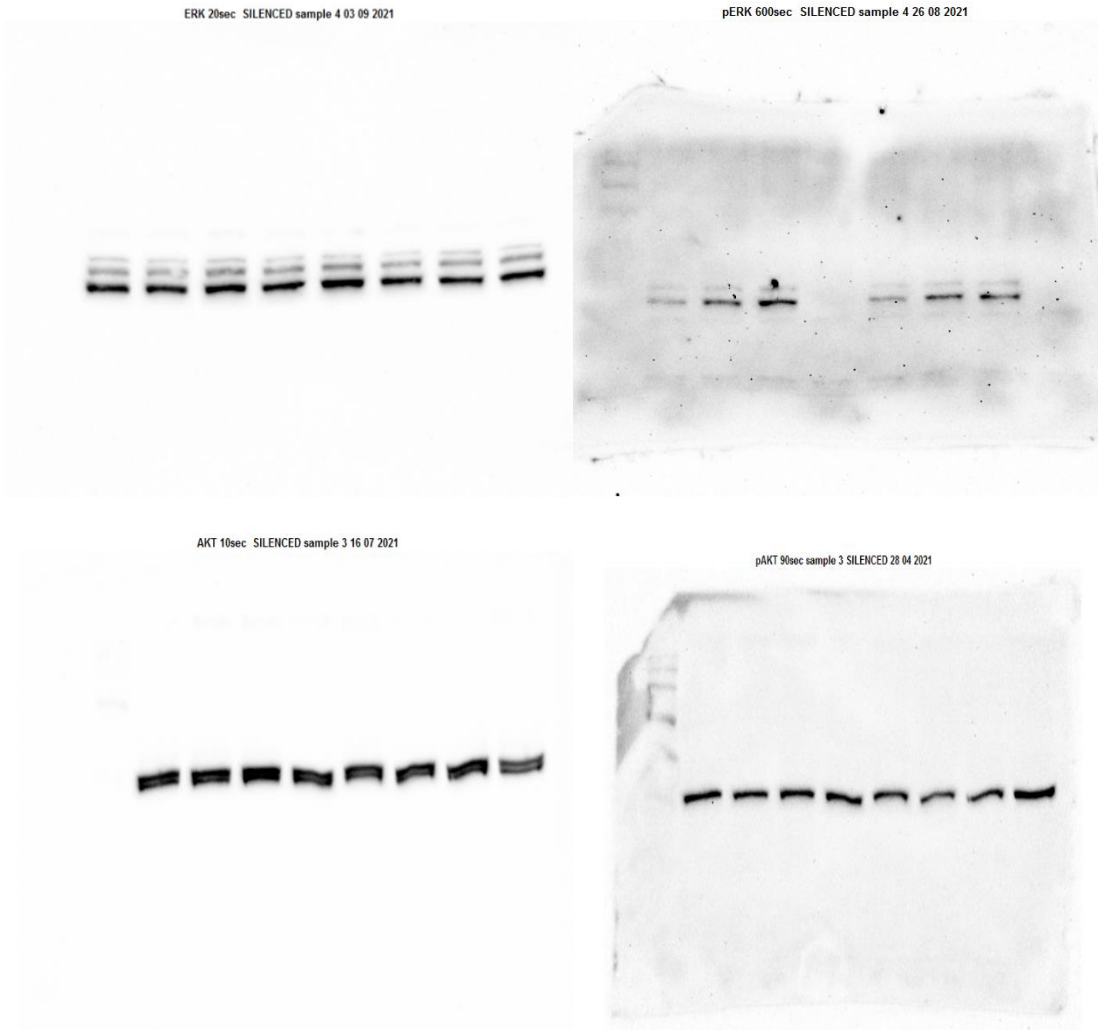
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

SIGNATURE 

APPENDIX E
Western blots



Blots for western blotting for ERK, pERK, AKT and pAKT for 15 minutes, 60 minutes, 4 hours, and 24 hours.



Blots for western blotting for ERK, pERK, AKT and pAKT for 15 minutes in the control siRNA and GPR120 siRNA cells, please note that bands for PD184352 for AKT and pAKT were included in these images whilst the bands were cut out in the results section.

APPENDIX F

Data for the primers used

Primer-BLAST » JOB ID:5e86dUhqRcJi_ED5TZlkyzeCdfkakW7kGw

Primer-BLAST Results
Help

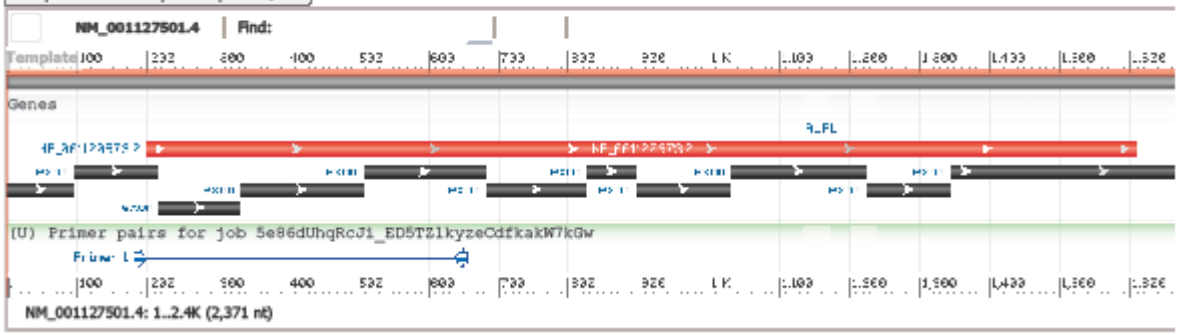
Input PCR template
NM_001127501.4 Homo sapiens alkaline phosphatase, biomineralization associated (ALPL), transcript variant 2, mRNA

Range
1 - 2371

Specificity of primers
Primers may not be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens, Mus musculus)...help on specific primers

Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit
Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ACGTGGCTAAGAATGTCATC	Plus	20	180	199	55.56	45.00	5.00	3.00
Reverse primer	CTGGTAGGCGATGTCCTTA	Minus	19	655	637	55.92	52.63	4.00	2.00
Internal oligo		Plus							
Product length	476								
Product Tm									
Product Tm									
min(OLIGO Tm)									

Primer-BLAST » JOB ID:CwHUm55Sk_q0xJbBm6Gy8-G6o8HMqbjczQ

Primer-BLAST Results
Help

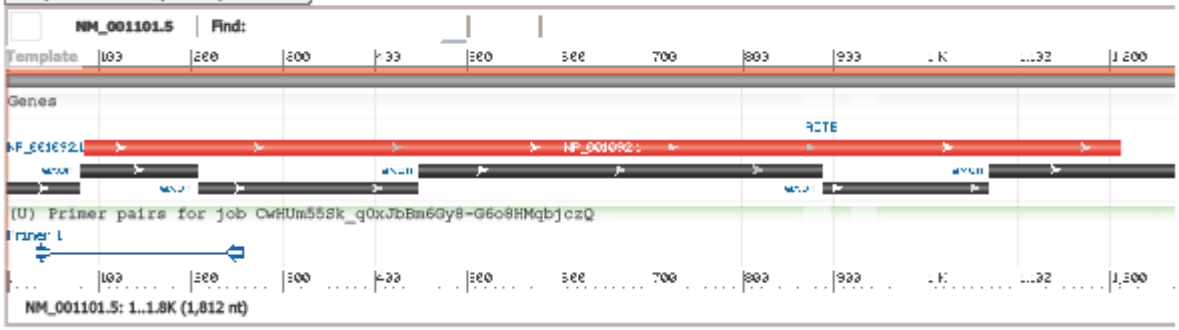
Input PCR template
NM_001101.5 Homo sapiens actin beta (ACTB), mRNA

Range
1 - 1812

Specificity of primers
Primers may not be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers

Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit
Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAGAGCCTCGCCTTTGC	Plus	17	31	47	58.39	64.71	5.00	2.00
Reverse primer	CTCGTCGCCCACATAGGA	Minus	18	255	238	58.48	61.11	3.00	0.00
Internal oligo		Plus							
Product length	225								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon junction									
Total intron size									

Primer-BLAST » JOB ID:OjDlq5zwkVi2YgFnDAclVXYcNGdbDy96Wg

Primer-BLAST Results
Help

•

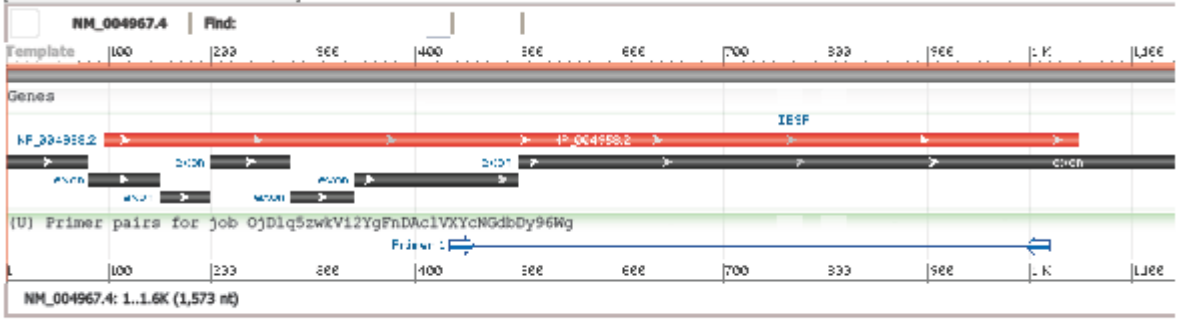
Input PCR template
NM_004967.4 Homo sapiens integrin binding sialoprotein (IBSP), mRNA

Range
1 - 1573

Specificity of primers
Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

Other reports
Search Summary

Graphical view of primer pairs + −



Detailed primer reports + −

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit

Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTATGGAGAGGACGCCACGCCTGG	Plus	24	433	456	68.64	66.67	6.00	6.00
Reverse primer	CATAGCCATCGTAGCCTTGTCCT	Minus	23	1019	997	62.12	52.17	4.00	0.00
Internal oligo		Plus							
Product length	587								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon junction									

Primer-BLAST » JOB ID:__Ugm0luRMZj-F79U516zymGa_0ElXDgBQ

Primer-BLAST Results
Help

▪

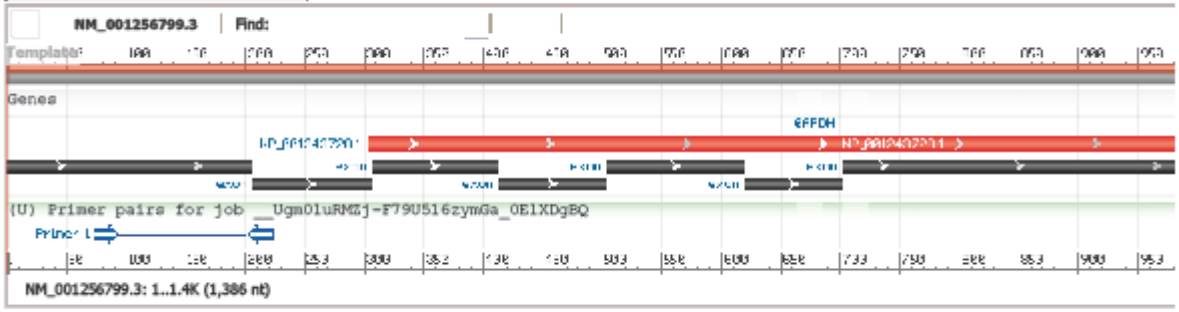
Input PCR template
NM_001256799.3 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 2, mRNA

Range
1 - 1386

Specificity of primers
Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

Other reports
Search Summary

Graphical view of primer pairs ✚ ━



Detailed primer reports ✚ ━

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit

Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAAAGCCTGCCGGTGACTAA	Plus	20	74	93	60.32	55.00	4.00	1.00
Reverse primer	GCCCAATACGACCAAATCAGAG	Minus	22	223	202	59.39	50.00	3.00	1.00
Internal oligo		Plus							
Product length	150								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon junction									

Primer-BLAST » JOB ID:s7ls1wVuCMYv-BL9H502z2WGJ_1IlTzgSQ

Primer-BLAST Results
Help

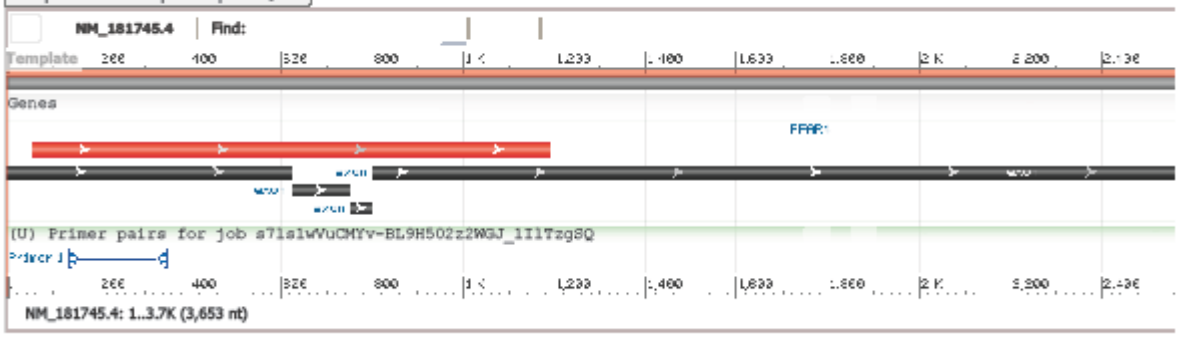
Input PCR template
NM_181745.4 Homo sapiens free fatty acid receptor 4 (FFAR4), transcript variant 1, mRNA

Range
1 - 3653

Specificity of primers
Primers may not be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers

Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit

Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TCTTCTCCGACGTCAAGGGC	Plus	20	136	155	61.87	60.00	6.00	2.00
Reverse primer	GGCCAGCACCAGAGGGATAG	Minus	20	347	328	62.05	65.00	4.00	0.00
Internal oligo		Plus							
Product length	212								
Product Tm									
Product Tm - min(OLIGO Tm)									

Primer-BLAST » JOB ID:uLJnKCpVJ_0AwyLGL6YG9FW9F8Z4rgzbeQ

Primer-BLAST Results
Help

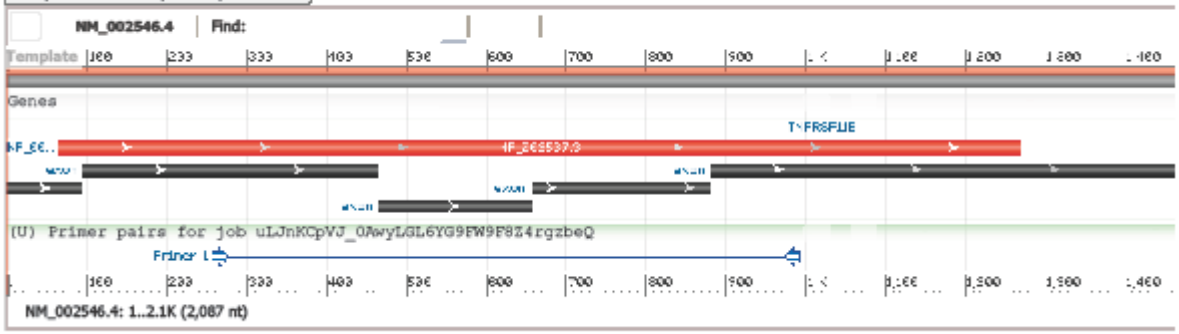
Input PCR template
NM_002546.4 Homo sapiens TNF receptor superfamily member 11b (TNFRSF11B), mRNA

Range
1 - 2087

Specificity of primers
Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit

Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCCCTGACCACTACTACACA	Plus	20	258	277	58.74	55.00	3.00	0.00
Reverse primer	TCTGCTCCCACTTTCTTTCC	Minus	20	993	974	57.43	50.00	2.00	0.00
Internal oligo		Plus							
Product length	736								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon									

Primer-BLAST » JOB ID:S0GU2-Zq68LM_O7545nKy5mC2_m0kcDktQ

Primer-BLAST Results
Help

Input PCR template
NM_001300837.2 Homo sapiens Sp7 transcription factor (SP7), transcript variant 3, mRNA

Range
1 - 3190

Specificity of primers
Primers may not be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens, Mus musculus)...help on specific primers

Other reports
Search Summary

Graphical view of primer pairs

Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit

Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCAGCTAGAAGGGAGTGGTG	Plus	20	1037	1056	60.11	60.00	4.00	0.00
Reverse primer	GCAGGCAGGTGAACTTCTTC	Minus	20	1395	1376	59.12	55.00	5.00	3.00
Internal oligo		Plus							
Product length	359								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon									

Primer-BLAST » JOB ID:JC77tIlqhMKj_IH5jJmly_aCtPnbka_k2g

Primer-BLAST Results
Help

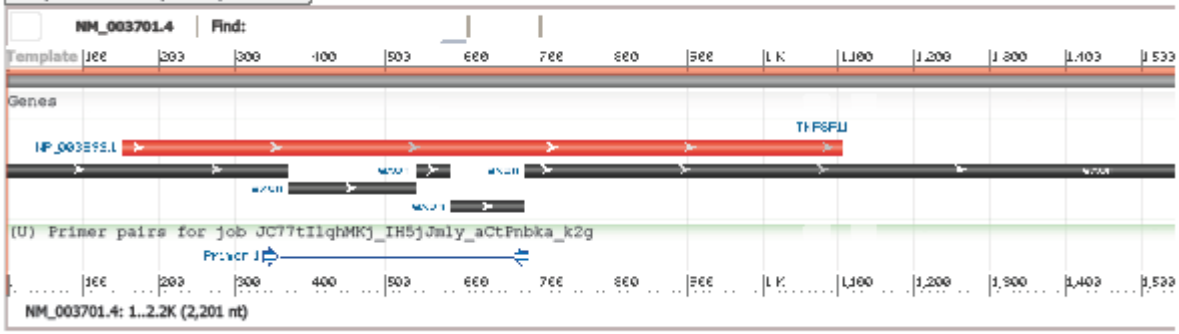
Input PCR template
NM_003701.4 Homo sapiens TNF superfamily member 11 (TNFSF11), transcript variant 1, mRNA

Range
1 - 2201

Specificity of primers
Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit

Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCGTCGCCCTGTTCTTCTAT	Plus	20	340	359	60.18	55.00	5.00	2.00
Reverse primer	GGAACCAGATGGGATGTCGG	Minus	20	689	670	60.18	60.00	4.00	1.00
Internal oligo		Plus							
Product length	350								
Product Tm									
Product Tm - min(OLIGO Tm)									

Primer-BLAST » JOB ID:Bw3Yl6trpsOB_aP4rpiHytSDlvj5kI3l-A

Primer-BLAST Results
Help

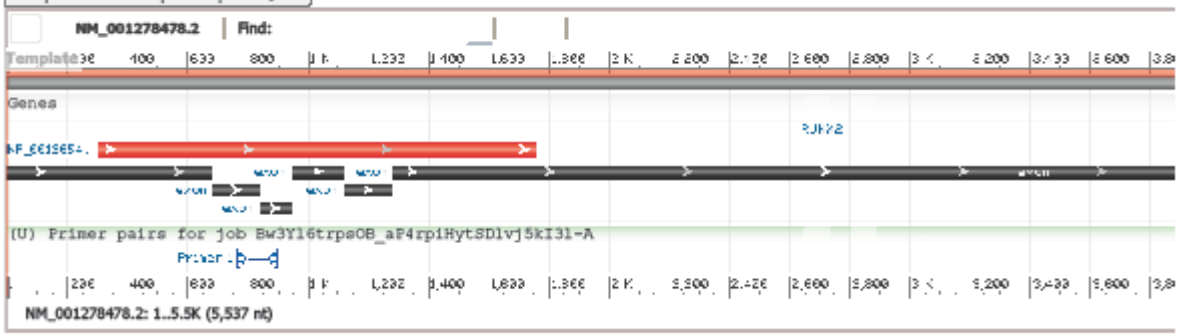
Input PCR template
NM_001278478.2 Homo sapiens RUNX family transcription factor 2 (RUNX2), transcript variant 4, mRNA

Range
1 - 5537

Specificity of primers
Primers may not be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens, Mus musculus)...help on specific primers

Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit
Help

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCTGTTATGAAAAACCAAGT	Plus	20	777	796	52.24	35.00	4.00	1.00
Reverse primer	GGGAGGATTTGTGAAGAC	Minus	18	884	867	52.20	50.00	2.00	1.00
Internal oligo		Plus							
Product length	108								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon									