

**STUDIES OF ANGIOGENESIS IN OSTEOCYTES: IMPLICATIONS FOR PATHOGENIC
MECHANISMS OF OSTEONECROSIS IN CHILDREN WITH ACUTE LYMPHOBLASTIC
LEUKAEMIA**

Dr Madeleine Adams

Thesis submitted for examination for the award of an MD degree

April 2015

Declaration 1.

Except where indicated by specific reference, the work in this thesis is the result of my own investigation and the views expressed are those of myself.

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Declaration 2.

No portion of the work presented has been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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Acknowledgements

I express my extreme gratitude for the support and expertise I have experienced from my supervisors Dr Bronwen Evans and Professor John Gregory during both the practical and writing stages of this work. I would also like to thank Mrs Carole Elford and Dr Nicole Scully for their practical help in the laboratory, Professor Linda Bonewald for the kind donation of the MLO-Y4 and IDG-SW3 cell lines and Dr Deborah Mason and Dr Marisol Vasquez for a number of primer sequences. Thank you also to Dr Meriel Jenney who has supported me not only in this MD project but throughout my career so far and has been my inspiration to become a paediatric oncologist.

THESIS SUMMARY

The work presented in this thesis is the result of 2 years of investigation into pathogenic mechanisms resulting in the development of osteonecrosis in children and young people treated for acute lymphoblastic leukaemia (ALL). This was a study using *in vitro methods* to investigate the effects of corticosteroids (namely dexamethasone which is used in the treatment of ALL) on osteocyte angiogenesis with particular focus on vascular endothelial growth factor (VEGF) and markers of bone remodelling. Interactions between dexamethasone and vitamin D were investigated in order to identify potential preventative or therapeutic strategies for osteonecrosis that could be studied *in vivo*. The actions of sex steroids on osteocyte biology and their interactions with dexamethasone were also studied in order to begin to explain the increased susceptibility to osteonecrosis that exists in both adolescent and female patients.

Results demonstrate a number of novel findings including; i) significant interactions between dexamethasone and vitamin D on osteocyte VEGF gene expression and protein secretion as well as the RANKL: OPG ratio which is crucial to bone remodelling, ii) dexamethasone treatment leading to significant alterations in expression levels of an array of genes expressed by osteocytes that are involved in angiogenesis pathways and iii) significant effects of sex steroids on osteocyte VEGF production and modulation of the effects of dexamethasone by oestradiol.

Osteonecrosis is an extremely disabling side effect of the treatment for ALL which in the vast majority of cases is now a curable disease. The results of this thesis contribute to the current understanding of the pathogenesis and have identified a number of potential therapeutic pathways to target.

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ABBREVIATIONS

+ve	Positive
-ve	Negative
%	Percent
°C	Degrees centigrade
μL	Microlitre
μg	Microgram
μM	Micromole
1α25(OH)₂D₃	1α,25-dihydroxy-vitamin D ₃ (calcitriol)
3D	Three dimensional
11β-HSD	11β-hydroxysteroid dehydrogenase
25-OHD₂	25-hydroxy-vitamin D ₂ (ergocalciferol)
25-OHD₃	25-hydroxy-vitamin D ₃ (calcidiol)
ACP1	Acid phosphatase 1 gene
ALL	Acute lymphoblastic leukaemia
Akt	Protein kinase B
ALP	Alkaline phosphatase
Ang-1	Angiopoeitin 1
AR	Androgen receptor
bFGF	Basic Fibroblast growth factor
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BSA	Bovine serum albumin
CA	Capture antibody
Cbfa1	Core binding factor alpha1 subunit protein
CD	Cluster of differentiation

cDNA	Complementary DNA
cm	Centimetre
CM	Conditioned medium
CNS	Central nervous system
CO₂	Carbon dioxide
CT	Computed tomography
C_t	Threshold cycle
CX43	Connexin 43
CYP24A1	24-hydroxylase enzyme
DA	Detection antibody
DAB	3, 3'-diaminobenzidine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DFO	Desferrioxamine
DMP1	Dentin matrix protein 1
dsDNA	Double stranded DNA
E11	Podoplanin
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked Immunosorbant assay
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	fibroblast growth factor
FoxO	Redox-sensitive forkhead box subgroup O
g	Gram
<i>g</i>	Gravitational force

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC(s)	Glucocorticoid(s)
GCR	Glucocorticoid receptor
GH	Growth hormone
gp	Glycoprotein
GPR30	G-coupled protein receptor 30
H₂O₂	Hydrogen peroxide
HCF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HRE	Hormone response element
HRP	Horse radish peroxidase
HSPG	Heparin sulphate proteoglycans
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IL6	Interleukin 6
IU	International units
JAK	Janus kinases
kDa	Kilodalton
LRP	Lipoprotein receptor-related proteins
M	Mole
m²	Metres squared
MEM	Minimal essential medium
mg	Milligram
mL	Millilitre
MLO-Y4	Murine long bone osteocyte Y4

mM	Millimole
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
ng	Nanogram
nm	Nanometre
nM	Nanomole
M-CSF	Macrophage colony stimulating factor
MEPE	matrix extracellular phosphoglycoprotein with ASARM motif
MRD	Minimal residual disease
MSC(s)	Mesenchymal stem cell(s)
NADP	Nicotinamide adenine dinucleotide phosphate
NaHCO₃	Sodium bicarbonate
NaOH	Sodium hydroxide
NCS	Newborn calf serum
NRP	Neuropilin
OCN	Osteocalcin
OD	Optical density
ON	Osteonecrosis
OPG	Osteoprotegerin
OPN	Osteopontin
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
pg	Picogram
PG	Prostaglandin
PHEX	Phosphate regulating gene with homologies to endopeptidases on the X-chromosome

PLGF	Placenta growth factor
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related peptide
Pyk2	Proline rich tyrosine kinase 2
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Real-time quantitative PCR
R²	Correlation coefficient for primer optimisation
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RD	Reagent diluent
RNA	Ribonucleic acid
RR	Relative risk
SOST	Sclerostin
VEGF	Vascular endothelial growth factor
VEGF_{Fl}	Full length isoform of vascular endothelial growth factor
VEGF_{xxx}	Vascular endothelial growth factor isoforms (xxx = amino acid length)

CHAPTER 1: INTRODUCTION

1.1. Childhood acute lymphoblastic leukaemia

1.1.i Epidemiology

Childhood cancer is relatively rare with an overall incidence of 150.5/1,000,000 (Cancer Research UK, 2012). Acute lymphoblastic leukaemia (ALL) is the commonest malignant condition affecting children, accounting for approximately one third of all cancer diagnoses in children aged 0-14 years (Figure 1)

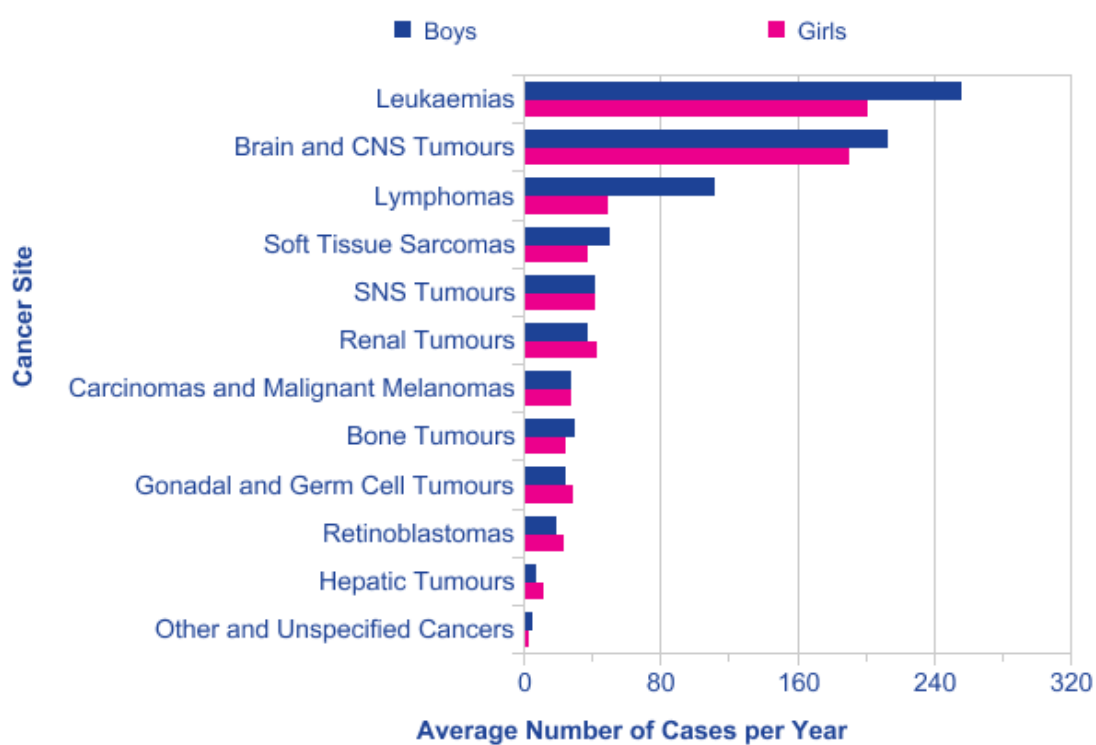


Figure 1: Incidence of childhood cancers by site (2006-2007). (Reproduced with permission from Cancer Research UK statistics, 2012)

In the UK approximately 500 children are diagnosed with ALL each year accounting for 78% of all leukaemia diagnoses. Incidence peaks in boys and girls at two and three years

respectively and decreases with age thereafter (Cancer Research UK statistics, 2012).

(Figure 2)

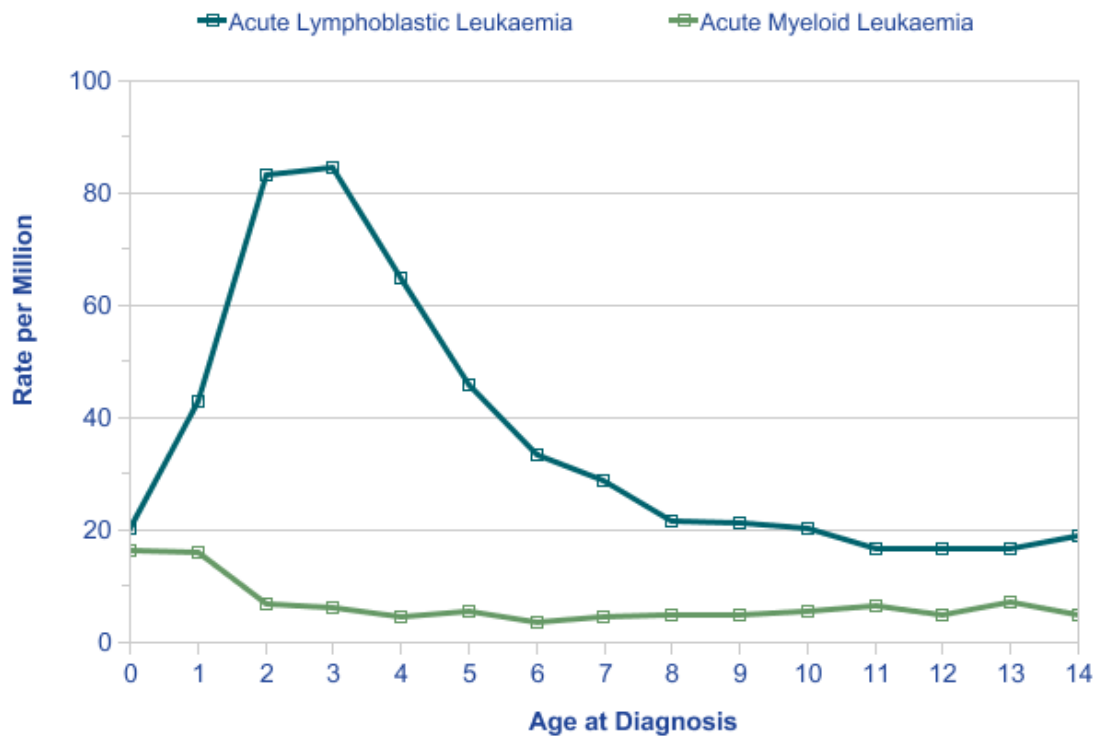


Figure 2. UK childhood leukaemia diagnoses 1996-2005: Incidence rates per million population. (Reproduced with permission from Cancer Research UK Statistics, 2012).

Although a few cases of ALL are associated with inherited genetic conditions such as Down syndrome, Bloom syndrome or Fanconi anaemia the cause remains largely unknown. Many environmental factors (*e.g.* parental smoking, exposure to radiation) as well as exposure to viral infections have been implicated but in most cases no direct cause can be found (Wiemels, 2012).

1.1.ii Survival rates

Optimal use of chemotherapeutic agents and supportive care in the context of national and multi-national clinical trials has meant that overall five year survival rates for childhood ALL

now exceed 85% in developed countries (Pui et al, 2012). For “low risk” patients this figure approaches 90% (Vrooman and Silverman, 2009). Low risk prognostic indicators include age less than 10 years at presentation (excluding infant ALL), low presenting white blood cell count, favourable cytogenetics (t(12:21), hyperdiploidy) and good response to treatment (measured by minimal residual disease - MRD). Despite the overall improvement in survival rates, there remains a subgroup of patients with high-risk disease for whom the prognosis is poor. These patients tend to be older with unfavourable cytogenetics (*e.g.* iAMP 21, t(9:22) Philadelphia chromosome, MLL rearrangement).

Teenagers and young adults aged 16 years and over, until recently were treated on different protocols depending on whether their treatment was conducted in a paediatric or adult oncology centre. Recent evidence has shown that these patients have better survival outcomes if treated on paediatric protocols (de Bont et al., 2004, Boissel et al., 2003, Ramanujachar et al., 2006). The recently completed UKALL 2003 trial was therefore extended to include patients aged up to 24 years and 364 days at diagnosis and this has been continued into the current trial, UKALL 2011.

1.1.iii Effects of ALL on bone

Bony morbidities including reduced bone mineral density (BMD) leading to osteopenia or osteoporosis, fractures and osteonecrosis (ON) are all prevalent complications of childhood ALL but distinguishing the effects of the leukaemia itself and treatment agents on bone is difficult. Leukaemic invasion, chemotherapy (particularly with glucocorticoid agents (GCs)), malnutrition and extended periods of limited physical activity have all been implicated but the individual impact of these factors has not been determined (Rayar et al., 2012).

Markers of bone formation (procollagen type 1 and alkaline phosphatase) have been shown to be low both at diagnosis as well as at certain time points during treatment (Davies et al., 2005). There is also evidence of acquired growth hormone deficiency at

diagnosis with subsequent reduction in bone metabolism (Davies et al., 2005). Children treated for ALL are often overweight during treatment and remain so at follow up. Increased adiposity and osteopenia as measured by dual energy X-ray absorptiometry (DXA) scanning of the hips and spine has been documented in children treated for ALL, regardless of whether or not they received cranial irradiation (Davies et al., 2004). Of particular importance is that the presence of symptomatic bone disease often leads to reductions in chemotherapy dosing which may adversely impact the chances of cure. One study has shown musculoskeletal pain to be present in 36% of ALL patients at diagnosis and that >50% of patients have skeletal abnormalities (including metaphyseal lucency, dense bands, sclerotic lesions, osteopenia and fractures) visible on plain radiographs (Halton et al., 1995). Low BMD scores and older age at diagnosis have been shown to correlate with low BMD scores during later phases of treatment and dexamethasone and low BMD scores are independent risk factors for fractures (Rayar et al., 2012).

1.2 Treatment of ALL

1.2.i Treatment overview

The treatment for childhood ALL has been delivered and optimised over the past 25 years through the use of national and international clinical trials (Stiller et al., 2012). These trials have enabled improvements in survival rates, while reducing overall treatment toxicity. Treatment consists of a combination of cytotoxic chemotherapy agents including asparaginase, vincristine, daunorubicin and methotrexate along with GCs and central nervous system (CNS) prophylaxis in the form of intrathecal methotrexate. Cranial irradiation is now reserved for patients with proven persistent CNS disease only. Treatment is delivered in intensive blocks during the first five weeks (induction) followed by

consolidation and then a further delayed intensification block during the first six months. The maintenance phase of treatment then follows with daily oral chemotherapy and week-long pulses of dexamethasone and vincristine every four weeks. Treatment continues for a total of three years in boys and two years in girls. The current treatment that a child receives is risk stratified at diagnosis depending on age and white blood cell count. Recent advances allow further stratification depending on early response to treatment. This is known as MRD (Brisco et al., 1994) and is measured by quantitative polymerase chain reaction (qPCR) on bone marrow aspiration at day 28. Patients with low levels of MRD have a reduction in treatment and those with high risk MRD are given more intensive therapy.

UKALL 2011 aims to build on this by additional reductions in treatment for good risk patients thus reducing overall burden of therapy without compromising survival rates. All patients aged 1-24 years 364 days with a diagnosis of ALL in the UK are eligible for entry into UKALL 2011.

1.2.ii Dexamethasone

Dexamethasone has been the gold standard drug of choice in UK protocols since results of the UKALL 97 randomised trial showed it to be statistically superior to prednisolone in both improving event free survival and reducing the risk of CNS relapse (Mitchell et al., 2005). It is administered in high doses, traditionally $6\text{mg}/\text{m}^2$ for 28 days in induction followed by week long pulses every month during maintenance therapy. UKALL 2011 has two randomisations involving dexamethasone. The overall aim of these randomisations is to reduce overall steroid related toxicity and improve quality of life for patients on therapy while maintaining survival rates.

The initial randomisation takes place at the start of therapy where children are randomly assigned to receive either the standard induction arm as described above or the

experimental arm of 10mg/m² dexamethasone for 14 days. The primary outcome measures for this randomisation are steroid induced morbidity, defined as all serious adverse events and treatment related mortality during induction (events subcategorised as steroid related and steroid contributory). Secondary outcome measures are rate of remission, overall and event free survival. The second randomisation involving dexamethasone takes place at the start of maintenance therapy. Patients are assigned either to receive maintenance with (standard arm) or without (experimental arm) dexamethasone pulses. Primary outcome measures for this randomisation are bone marrow relapse, defined as any relapse with bone marrow involvement and quality of life measured by PedsQL™. Secondary outcome measures are event free and overall survival and treatment related morbidity and mortality.

1.3 Dexamethasone

1.3.i Glucocorticoids

GCs are synthesized and released by the adrenal glands in response to stress and regulate numerous physiological effects in a wide range of tissues. Cortisol release from the adrenal glands is under the control of the hypothalamic – pituitary axis. It is the immunosuppressive qualities of GCs and the fact that they induce apoptosis of many cell types including T-cells, macrophages and blast cells that make them an important component of chemotherapy regimens used to treat ALL as well as many other haematological malignancies. Approximately 250,000 adults in the UK take long term GCs (Kanis et al., 2007). Long term treatment with GCs is associated with many side effects, including increased risk of cataracts and glaucoma, hypertension, hyperglycaemia, hyperlipidaemia, weight gain, easy bruising, susceptibility to infection, impaired healing,

myopathy and adrenal insufficiency. Patients receiving long term GCs should carry a steroid therapy card to alert medical practitioners in the event of an emergency. In addition prolonged steroid exposure causes dramatic reduction in bone mineralisation and strength, similar to endogenous elevation of GCs (Newell-Price et al 2006, Weinstein 2001, van Staa, 2006). This bone loss results in osteoporosis and increased risk of fractures. Clinically, most of the bone loss is seen in the initial period of GC exposure (Mazziotti et al, 2006).

There are many preparations of synthetic GCs available and these have varying potencies as shown in Table 1. GCs can be administered to patients via many routes including enteral, intravenous and topical to both skin and mucous membranes.

Glucocorticoid	Relative Potency
Hydrocortisone	1
Prednisolone	4
Methylprednisolone	5
Dexamethasone	25
Betamethasone	25

Table 1. Relative potencies of commonly used GC preparations (British National Formulary, 2013).

1.3.ii Mechanism of action in ALL

It was first discovered that cortisone caused tumour regression in a mouse model of lymphosarcoma (Canalis et al., 2002). Tissue culture studies showed that lymphoid cells were particularly sensitive to the effects of GCs and responded to treatment with decreases in DNA, RNA and protein synthesis (Rosen et al., 1970). In particular GC treatment of lymphoid cells leads to G1 phase cell cycle arrest and apoptosis (Laane et al.,

2009). Studies of proliferating human leukaemic lymphoblasts supported the hypothesis that GCs have preferential lymphocytolytic effects. The mechanism of action was initially thought to be caused by impaired energy use via decreased glucose transport and/or phosphorylation; it was later discovered that GCs induce apoptosis in certain lymphoid cell populations (Baxter et al., 1989, Cohen, 1989). There is also emerging evidence that dexamethasone induces a process known as autophagy prior to apoptosis within leukaemic blast cells (Laane et al., 2009). Autophagy is a form of non-apoptotic programmed cell death and is characterised by the formation of autophagocytic vesicles which engulf cytoplasmic organelles such as mitochondria and endoplasmic reticulum. These autophagosomes eventually fuse with lysosomes and their contents are degraded by lysosomal enzymes (Gozuacik and Kimchi, 2004).

Early clinical studies in childhood ALL patients treated with prednisolone alone showed that 50% of children responded and achieved remission (Vietti et al., 1965). However, after a time period of approximately one year relapse and steroid resistance was inevitable. This led to the evolution of multi-drug therapies used in modern treatment protocols.

Dexamethasone has been shown to be more effective than prednisolone particularly in reducing the risk of CNS relapse and therefore is the steroid of choice in current therapy.

Both *in vivo* and *ex vivo* steroid sensitivity is an important prognostic factor in ALL (Kaspers et al., 1998, Schrappe et al., 2000).

1.3.iii Pharmacokinetics

Chemically dexamethasone is 9-Fluoro-11 β , 17, 21-trihydroxy-16 α -methylpregna-1, 4-diene-3, -20-dione. It is a potent GC and oral absorption is found to be 90%. Volume of distribution is 0.58l/Kg and plasma protein binding is 66-77%. It has a half-life of approximately four hours (Aarden et al, 2012). Pharmaceutical studies have shown that

liquid preparations used for young children have bioequivalence when compared to tablets (Queckenberg et al., 2011). There is inter-patient variation in dexamethasone clearance and high dexamethasone clearance and thus reduced exposure to dexamethasone has been associated with higher risk of relapse in ALL (Kawedia et al., 2012). Poor dexamethasone clearance on the other hand is associated with an increased risk of developing ON (Kawedia et al., 2011). Two isoenzymes of 11 β -hydroxysteroid dehydrogenase (11 β -HSD1 and 11 β -HSD2) catalyse the interconversion of active GCs (such as cortisol or prednisolone) and inactive GCs (such as cortisone and prednisone). 11 β -HSD1 is an activator and 11 β -HSD2 is an inactivator (Cooper et al., 2002). GCs differ in their sensitivity to 11 β -HSD2, the inactivation enzyme. In dexamethasone a fluorine atom at the 9 α position of the β ring extends its potency and increases its resistance to 11 β -HSD2, a fact which may at least partially explain the increased rates of osteoporosis and ON with dexamethasone compared with other GCs such as prednisolone (Weinstein, 2012a).

1.3.iv Effects on bone

GCs are well recognised as being “bad for bones”. Long term exposure results in reduced BMD which leads to osteopenia and/or osteoporosis and increased fracture risk.

Osteopenia is a common complication following treatment for childhood cancer. Many of the chemotherapeutic agents used in childhood malignancies have been shown to reduce osteoblast cell numbers and alter collagen type 1 and alkaline phosphatase (ALP) activity within human osteoblasts in vitro (Davies et al., 2003). ON is a specific pathological process related to GC exposure. It is more commonly seen in younger patients with the adolescent population being at highest risk. In patients receiving GC therapy long term 30-50% fracture and 9-40% develop ON (Weinstein, 2012b).

Studies have shown that increased prevalence of apoptosis of osteocytes (and osteoblasts) is associated with the so-called GC-induced bone fragility syndrome (Weinstein et al., 1998, O'Brien et al., 2004, Gohel et al., 1999). GCs have been demonstrated to induce apoptosis of both osteoblasts and osteocytes (Weinstein et al., 1998, Weinstein et al., 2000), promote osteocyte autophagy as well as prolonging osteoclast life span (Jia et al., 2011, Xia et al., 2010) tipping the balance of bone turnover in favour of bone loss. Transgenic mice overexpressing 11 β -HSD2 (the enzyme that inactivates GCs – as described above) are protected from GC induced bone fragility (O'Brien et al., 2004).

GCs bind to the GC receptor and cause conformational change and nuclear translocation of the ligand bound receptor, followed by cis or trans interactions with DNA resulting in induction or repression of gene transcription (Necela and Cidlowski, 2004, Rhen and Cidlowski, 2005). GCs also exert actions independently of gene transcription. One of these actions is to activate intracellular kinases. Proline rich tyrosine kinase 2 (Pyk2) is a member of the focal adhesion kinase (FAK) family of non-receptor tyrosine kinases. Pyk2 and FAK, although highly homogenous, exhibit opposite effects on cell fate. FAK activation leads to survival and Pyk2 to apoptosis (Avraham et al., 2000, Plotkin et al., 2007). Plotkin et al have shown that GCs promote osteocyte apoptosis by activating Pyk2, hence opposing FAK-induced survival (Plotkin et al., 2007). This leads to cell-detachment induced apoptosis (anoikis). It is also thought that the pro-apoptotic actions of GCs may involve suppression of locally synthesized anti-apoptotic factors including Insulin-like growth factor 1 (IGF-1) and interleukin-6 (IL-6) as well as matrix metalloproteinases (MMPs) (Canalis et al., 2002). GCs also reduce osteoblast differentiation by attenuating Akt (protein kinase B) phosphorylation and increasing activation of the redox-sensitive forkhead box subgroup O transcription factor family (FoxOs), which inhibit wingless (Wnt)/ β -catenin signalling, which is a critical pathway for the generation of osteoblasts (Weinstein, 2012b, Almeida et al.,

2011). GCs also enhance the expression of Dickkopf-1, an antagonist of the Wnt pathway and suppress bone morphogenetic proteins (BMPs), factors required to induce osteoblast differentiation (Ohnaka et al., 2004). In addition, GCs increase production of peroxisome proliferator-activated receptor γ , a transcription factor that induced terminal adipocyte differentiation, while suppressing osteoblast differentiation and thus possibly leading to increased marrow fat and reduced osteoblasts (Lecka-Czernik et al., 1999).

Histological studies of human bone biopsies from patients receiving GCs show increased bone resorption and decreased bone formation (Dalle Carbonare et al., 2001). The predominant feature however, is decreased bone formation. This is in contrast to post-menopausal osteoporosis where increased bone resorption is predominantly seen (Canalis et al., 2002). The increased bone resorption that is seen in GC-induced osteoporosis has been shown to involve activation of Receptor activator of nuclear factor kappa-B ligand (RANKL) which induces osteoclastogenesis and inhibition of osteoprotegerin (OPG), which binds and inactivates RANKL (Udagawa et al., 1999). GCs have also been shown to reduce osteoblast replication and prevent differentiation of cells into mature, functioning osteoblasts (Pereira et al., 2001). In addition GCs enhance osteoblast apoptosis by mechanisms described above. The net effect is an overall reduction in bone forming cells. GCs also reduce the ability of osteoblasts to produce type I collagen, the major component of bone extracellular matrix resulting in a decrease in matrix available for mineralisation (Canalis et al., 2002).

Recent laboratory research has shown that GCs may exhibit their adverse effects on osteocytes via the mechanism of autophagy (Xia et al., 2010). As previously stated, autophagy is a lysosomal degradation pathway that is essential for cell growth, survival, differentiation, development and homeostasis. During autophagy parts of the cytoplasm and intracellular organelles are enclosed within autophagocytic vacuoles that are delivered

to lysosomes for bulk degradation. Autophagy can protect the cells from apoptosis by removing oxidatively damaged organelles. On the other hand, excess autophagy can destroy cellular components. Autophagy therefore can preserve viability or, alternatively, can be a self-destructive process that leads to cell death. Xia et al used acridine orange staining of acidic vesicular organelles within dexamethasone treated MLO-Y4 (murine long bone osteocyte Y4) cells, followed by flow cytometry as well as monodansylcadavarine, a fluorescence marker to detect autophagic vacuoles to show that dexamethasone induces autophagy in osteocytes. They also showed that inhibition of autophagy leads to a further reduction of normal cell numbers in response to dexamethasone. This in vitro study concluded that dexamethasone activates autophagy in osteocytes which is a major mechanism used by osteocytes in self-protection against the detrimental effect of GCs that results in bone loss (Xia et al., 2010).

1.4 Glucocorticoid–induced bone disease

1.4.i Osteoporosis

GCs are the most common cause of secondary osteoporosis (Weinstein, 2012b). Bone loss in GC-induced osteoporosis is biphasic, with a rapid 6-12% reduction in BMD within the first year of treatment followed by a slower 3% annual loss thereafter with continued GC administration (LoCascio et al., 1990). The relative risk (RR) of fracture increases dramatically by up to 75% in the first three months of therapy, often before a significant decline in BMD can be measured (Van Staa et al., 2003). Several large case-controlled studies have shown clear associations between GC exposure and fracture (Van Staa et al., 2003, Steinbuch et al., 2004). The combination of higher dose, longer duration and continuous use gives the greatest risk of fractures, with 10mg/day of prednisolone given

continuously for more than 90 days being associated with a seven-fold increase in hip fractures and 17-fold increase in vertebral fractures (Steinbuch et al., 2004). Risk factors for GC-induced osteoporosis are advancing age, prolonged treatment duration, increased daily dosage, cumulative dose, low body mass index, previous fractures, frequent falls, underlying chronic disease and polymorphisms in the GC receptor (Weinstein, 2012b). Histological studies in patients with GC-induced osteoporosis show reduced numbers of osteoblasts and diminished wall width (Weinstein, 2011, O'Brien et al., 2004). The reduced osteoblast number is caused by GCs decreasing the production of new osteoblast precursors and causing apoptosis of mature osteoblasts (Weinstein, 2012b). Increased osteocyte apoptosis is also seen and is associated with decreases in vascular endothelial growth factor (VEGF), skeletal angiogenesis, bone interstitial fluid and bone strength (Weinstein et al., 2010b). GCs also reduced the water content of bone (usually 20-25%) which contributes to reducing stress to bone during dynamic loading (Weinstein, 2012b, Weinstein et al., 2010b). Transmission of fluid shear stress to the lacunar-canalicular network is also vital for the mechanosensory function of osteocytes and the mechanical adaptation of bone to mechanical force. It has therefore been hypothesised that GC-induced osteocyte apoptosis could account for the loss of bone strength that occurs before a measurable loss in BMD (Weinstein, 2012b, Seeman and Delmas, 2006). GC excess also reduced osteoclast production but in contrast with osteoblasts, the lifespan of osteoclasts is prolonged. As a result, during long term GC therapy osteoclast numbers usually remain within normal limits whereas osteoblast numbers and rate of bone formation decrease (Weinstein et al., 1998, Jia et al., 2006). These particular histological features are different from those found in other forms of osteoporosis (Weinstein, 2012b).

GC excess has adverse effects on all three bone cell types. This has been demonstrated in experiments in transgenic mice over expressing the GC-inactivating enzyme 11 β -HSD2.

Mice with over expression of 11β -HSD2 in osteoblasts and osteocytes were protected from prednisolone-induced apoptosis and reduced osteoblast number and bone formation but they still lost bone as the osteoclasts were exposed to prednisolone. Bone strength however was conserved in these animals suggesting an independent contribution of the osteocyte to maintaining bone strength (O'Brien et al., 2004). In similar experiments, over expression of 11β -HSD2 in osteoclasts preserved bone but did not prevent the prednisolone-induced decrease in osteoblast number and bone formation (Jia et al., 2006).

Prior to commencing a patient on long term GCs, circulating serum levels of 25-hydroxy-vitamin D₃ (25-OHD₃, cholecalciferol) should be measured. This is because GC use has been independently associated with low serum 25-OHD₃ levels and therefore in patients with pre-existing low levels of 25-OHD₃, GC administration can result in severe vitamin D deficiency (Skversky et al., 2011). It remains unclear whether this association is due to a direct causal effect of GCs or as a result of underlying disease, nutritional status or sun exposure of the patients (Skversky et al., 2011). One study has demonstrated that dexamethasone treatment of osteoblasts *in vitro* potentiated the transcription of 24-hydroxylase (CYP24A1), the enzyme responsible for metabolism of 25-OHD₃ and $1\alpha,25$ -dihydroxy-vitamin D₃ ($1\alpha,25(\text{OH})_2\text{D}_3$, calcitriol) (Dhawan and Christakos, 2010).

Current treatments for the prevention of GC-induced osteoporosis include vitamin D (cholecalciferol) and calcium supplementation, hormone-replacement therapy and bisphosphonates. Until recently, treatment of GC-induced osteoporosis was restricted to bisphosphonates, but newer agents include teriparatide (parathyroid hormone; PTH) and denosumab (RANKL monoclonal antibody) (Saag et al., 2009, Rizzoli et al., 2012). There is less evidence for bisphosphonate therapy in GC-induced osteoporosis than in post-menopausal cases because GCs inhibit their effects on osteoclast cells (Weinstein, 2012b).

However a meta-analysis has concluded that bisphosphonate therapy is cost-effective for high risk patients on GC therapy (Kanis et al., 2007).

1.4.ii Osteonecrosis

ON is also termed avascular or ischaemic necrosis. It most commonly affects the large joints, particularly the hip and the most common cause is trauma. GCs are the second most common cause with 9-40% of patients on long term GC therapy being affected (Weinstein, 2012a, Weinstein, 2011). Other causes include alcohol, sickle cell disease, metabolic and idiopathic causes. Like osteoporosis, the risk of ON increases with higher doses and prolonged treatment. However ON may also occur with short-term exposure to high doses as used in the treatment of ALL and ON can occur without co-existing osteoporosis (Weinstein, 2012b).

1.5 Osteonecrosis (Overview)

1.5.i Epidemiology

The annual incidence for all types of ON is 3.0/100,000 population (Weinstein, 2012a). This incidence has increased since the 1980s probably due to increased use of MRI scans (Cooper et al., 2010) and therefore better detection rates. Known risk factors for developing GC-induced ON are outlined in Table 2;

Risk factors
<ul style="list-style-type: none">• Dose and duration of therapy• Intra-articular administration• polymorphisms in VEGF, GC receptor, 11β-HSD2, collagen type II PA1 (COL2A1), plasminogen activator inhibitor 1 (PA1) and P-glycoprotein• Underlying disorders; renal insufficiency, transplantation, inflammatory bowel disease, HIV, ALL• GC used (dexamethasone greater risk than prednisolone)

Table 2. Risk factors for GC-induced ON. (Adapted from Weinstein, 2012a)

GC-induced ON, although seen in older patients, is more commonly seen in the adolescent population particularly those treated for malignant conditions such as ALL and lymphoma, where patients are given GCs in combination with cytotoxic chemotherapy agents. ON in ALL is discussed in further detail in section 1.6.

1.5.ii Pathophysiology

Previous hypotheses for the pathogenesis of ON include fat embolism, increased marrow fat resulting in increased intra-osseous pressure and reduced bone perfusion, vascular thrombosis, hypercoagulability reducing blood flow to the femoral head and fatigue fractures (Weinstein, 2012a, Weinstein, 2011, Mankin, 1992, Drescher et al., 2011b). More recently however, attention has shifted to direct effects of GCs on bone cells particularly osteocytes and many recent studies have focussed on the role of osteocyte apoptosis (Weinstein et al., 2000, Kabata et al., 2000, Eberhardt et al., 2001, Calder et al., 2004, Youm et al., 2010). A study of mice treated with prednisolone showed increased apoptosis of osteocytes, decreased VEGF and skeletal angiogenesis as well as reduced bone interstitial fluids and bone strength compared with placebo (Weinstein et al., 2010b). Osteocytes and the lacunar-canalicular network provide the strain-sensing properties of bone and communicate with other bone cell types to control the re-modelling that occurs following load or damage to bone. It is thought that osteocyte apoptosis induced by GCs and the resulting disruption in bone vascularity leads to the development of ON (Weinstein, 2012a). One study has shown that there is connection between the systemic vasculature system, canalicular processes and osteocytes by demonstrating that low molecular weight tracers can move from the venous system into the lacuna-canalicular space within minutes of injection (Weinstein et al., 2010b). There is increasing evidence that GCs cause disruption to this vascular connection. A study using fluorescent imaging of the osteocyte-lacunar-canalicular system in mice shows a marked decrease in bone interstitial fluid following

administration of GCs. This study also used X-ray microtomography (micro-CT) imaging of decalcified bones to demonstrate reduced vertebral and femoral vessel volume and surface area (SA) in mice treated with prednisolone (Weinstein et al., 2010b).

Interestingly, histological bone sections from patients undergoing total hip replacement for GC-induced ON have been shown to contain large numbers of apoptotic osteocytes whereas in alcohol-induced ON there were much smaller numbers and in traumatic ON they were absent (Weinstein et al., 2000). Additionally the typical cell swelling and inflammation usually seen in necrosis was not present in GC-induced ON suggesting differences in the underlying pathogenesis. The apoptotic osteocytes within these specimens were mainly located in the subchondral area and adjacent to fracture sites (Weinstein et al., 2000, Weinstein, 2012a) (Figure 3). The histological differences between GC-induced ON and ON due to other causes has led Weinstein R.S, a well published researcher of ON and author of the above study to go as far as to say that GC-induced ON is actually a misnomer and that “GC-induced ON is actually osteocyte apoptosis” (Weinstein, 2012a).

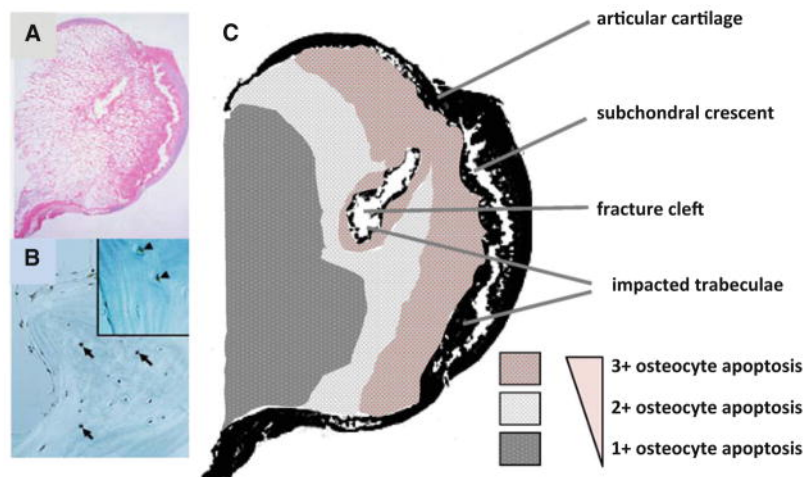


Figure 3. Glucocorticoid-induced osteonecrosis is osteocyte apoptosis. Abundant apoptotic osteocytes are present in sections of whole femoral heads obtained during total hip replacement for glucocorticoid-induced osteonecrosis (a, b). In c is a prevalence map of osteocyte apoptosis made from the section of the femoral head shown in (a). Osteocyte apoptosis was most prevalent (3) adjacent to the subchondral crescent and fracture cleft and decreased (1) as the examination progressed more distally. Osteocyte apoptosis was anatomically juxtaposed to the osteonecrotic fracture. Adapted from Weinstein 2012a. Reproduced with permission from Springer Publishing.

The role of vascular factors in GC-induced ON is supported by studies that show that GCs affect both endothelial angiogenesis and the production and action of VEGF (Drescher et al., 2011b, Weinstein et al., 2010b). Recent *in vitro* studies show that dexamethasone treatment reduced VEGF mRNA levels in both osteoblasts and MLO-Y4 cells stimulated with desferrioxamine (DFO) that mimics hypoxia. Dexamethasone also decreased DFO-stimulated hypoxia-inducible factor 1 (HIF-1) expression. This study concluded that at least some of the adverse effects of GCs on bone are due to impaired angiogenesis, caused by suppression of HIF-1 and VEGF production by both osteoblasts and osteocytes (Weinstein, 2012a). High levels of VEGF are found in osteonecrotic bones where it contributes to the healing process (Radke et al., 2006). VEGF is discussed in further detail in section 1.9.

1.5.iii Diagnosis

Persistent hip, knee or shoulder pain in a patient receiving GC therapy requires investigation for ON. Plain radiographs do not show stage 0-1 ON and may not show any abnormalities even in more advanced disease (Medscape, 2012). The gold standard for imaging is an MRI scan which typically shows loss of marrow fat due to oedema and the pathognomonic “crescent sign” caused by subchondral fracture. Eventually collapse of the femoral head occurs. Typical MRI features of ON are demonstrated in Figure 4. (Weinstein, 2012a)

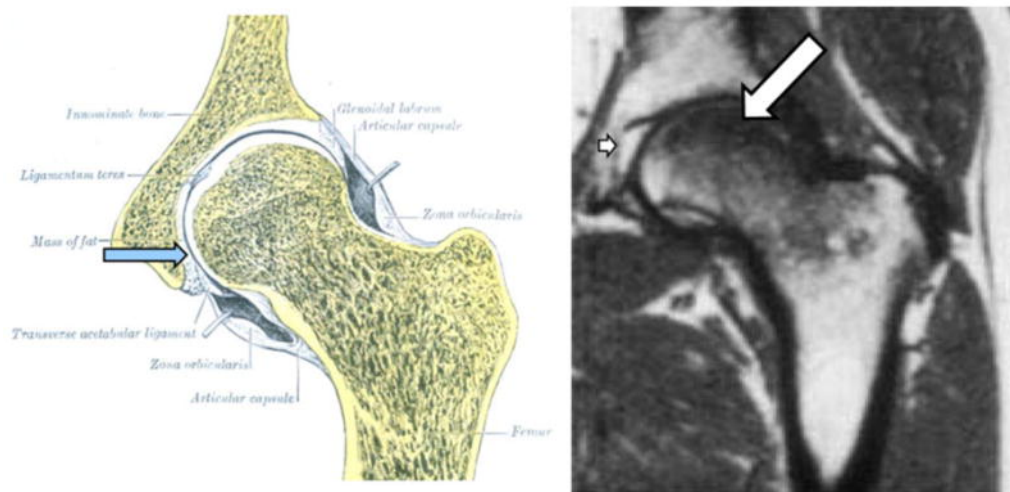


Figure 4. MRI of osteonecrosis. The acetabular fat pad (*blue arrow*) shown in the *left panel* gives an intense white signal with MRI as shown in the *right panel* (*small arrow*), while the loss of marrow fat (*large arrow*) gives a dark signal typical of edema with T1 MRI imaging. Reproduced with permission from Springer publications (Weinstein, 2012a).

1.5.iv Management

Unlike osteoporosis there is no proven preventative therapy for GC-induced ON. As ON typically occurs in younger patients a wide range of joint-preserving surgical techniques are used in the earlier stages of the disease, in order to try and delay the need for joint

replacement. Core decompression is the commonest operation performed for early stage disease. Bone grafts and stem cell therapies are more recently developed surgical options (Zalavras 2014). For advanced disease however, joint replacement is usually required and some young patients treated for conditions such as ALL require bilateral hip replacement in adolescence. Hip replacements typically have a 10-year life span resulting in multiple procedures during a patient's life time.

Recently a potential role of bisphosphonates has been identified. One study in adults with established ON has shown that alendronate treatment significantly delayed disease progression and reduced the need for hip replacement when compared to control subjects over a 24 month period (Lai et al., 2005). Importantly both this study and a second study reporting the 10-year follow-up of patients treated with alendronate for ON demonstrated significant improvements in pain and mobility scores (Lai et al., 2005, Agarwala and Shah, 2011). At present however there are no published clinical guidelines for the use of bisphosphonates in ON.

1.6 ON in children with ALL

1.6.i Epidemiology

Symptomatic ON is present in 9-17% of children and adolescents treated for ALL (Mattano et al., 2000, Kawedia et al., 2011). Known risk factors for ON include female sex (Mattano et al., 2000), white race (Mattano et al., 2000), high body mass index (Niinimaki et al., 2007) and adolescent age between 10-20 years (Mattano et al., 2000, Kawedia et al., 2011, Relling et al., 2004, Ribeiro et al., 2001). One long term follow up study of childhood ALL patients showed the highest rates of ON in 15-20 year olds (29% at 10 years) compared with 8% in >20 year olds (Patel et al., 2008), whilst a large study (n=1409) of childhood ALL

patients on treatment showed an overall incidence of ON of 9.3%, again with the adolescent population being at greatest risk. The estimated incidence of ON was 0.9% in patients <10 years, 13.5% in those aged 10-15 years and 18% in patients 16-20 years. Symptoms were chronic in 84% of patients with 39% of patients with ON requiring surgical intervention. Rates were higher in patients randomised to two courses of dexamethasone rather than one (Mattano et al., 2000). Several other studies have shown similar results, demonstrating that adolescents are at highest risk of ON (Vrooman and Silverman, 2009, Burger et al., 2005). Interestingly, results from the UKALL XII study treating patients aged 15-55 years showed significantly higher rates in the 15-20 year-old age group (29% at 10 years) than the >20 year-old age group (8%) $p=0.0004$ (Patel et al., 2008). All of the above studies have documented the incidence of symptomatic ON but a more recent study has demonstrated radiological evidence of ON in 71% of ALL patients, >60% of whom were asymptomatic (Kawedia et al., 2011). The long term implications of these findings are yet to be determined.

1.6.ii Pathophysiology

Children and adolescents with ALL receive high doses of dexamethasone and this is thought to lead to ON via the mechanisms outlined in section 1.5.ii. However, some treatment protocols for ALL have much higher frequencies of ON than others, suggesting that some non-GC drugs such as methotrexate or asparaginase may modify the risk of developing ON (Sala et al., 2007, Kawedia et al., 2011). Recently it has been demonstrated that low serum albumin, high serum cholesterol, poor dexamethasone clearance and polymorphisms of the ACP1 (acid phosphatase 1) gene which regulates lipid levels and osteoblast differentiation are independently associated with the development of ON in patients with ALL (Kawedia et al., 2011). Hypoalbuminaemia is a marker of asparaginase treatment and is associated with greater plasma exposure to dexamethasone. This led the authors to hypothesise that

asparaginase treatment potentiates the development of ON via this mechanism (Kawedia et al., 2011). ACP1 polymorphisms have been shown in other studies to be associated with serum cholesterol and triglyceride levels strengthening the evidence for its role in ON risk (Bottini et al., 2002). Polymorphisms at other gene loci such as SERPINE1 (French et al., 2008), which encodes the principal inhibitor of tissue plasminogen activator, VDR (Relling et al., 2004) and CYP3A4 (Asano et al., 2003) have been studied with conflicting results (Kawedia et al., 2011, French et al., 2008, Hadjigeorgiou et al., 2008).

1.6.iii Management

It is not current practice in the UK to routinely screen patients for ON during treatment for ALL. However a low threshold exists for investigation of symptoms, particularly joint pain and restricted mobility. Current management of established, symptomatic ON is mainly restricted to surgical intervention, as described in section 1.5.iv. During early stages of disease, patients are encouraged to non-weight bear and physiotherapy input is often required.

1.7. Bone architecture and cell types

1.7.i Gross structure

At birth there are over 270 bones in the human body but as the child grows many of these bones fuse leaving a total of 206 within the adult skeleton (Steele, 1988). Histologically there are two types of bone; cortical (compact) bone and trabecular (cancellous) bone. Cortical or compact bone forms the dense, hard outer layer of bones and gives the smooth, white, solid appearance. It accounts for 80% of the total bone mass of the adult skeleton. Trabecular (cancellous or spongy) bone fills the interior of long bones. This is an open, porous cell network which is composed of a network of rod and plate like elements that

make the overall bone lighter and allow space for the blood vessels and bone marrow. The histological difference between compact and cancellous bone is that compact bone consists of Haversian sites (osteons), while cancellous bones do not. Also, bone surrounds blood in the compact bone, while blood surrounds bone (bone marrow) in the cancellous bone (Marieb, 2004).

1.7.ii Molecular structure

The majority of bone comprises the matrix which has both organic and inorganic parts. It is the hardening of this matrix that entraps osteoblasts which subsequently differentiate into osteocytes. The inorganic bone mineral is formed from carbonated hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) with lower crystallinity (Legros et al., 1987). The matrix is initially produced as unmineralised osteoid which is manufactured by osteoblasts. Mineralisation subsequently occurs following secretion of ALP containing vesicles by osteoblasts. This cleaves the phosphate groups leading to calcium and phosphate deposition (Marieb, 2004). The organic component of bone is mainly composed of type 1 collagen as well as various proteins including osteocalcin, osteopontin, osteonectin and glycosaminoglycans (Marieb, 2004). Histologically bone can be described as “woven” which is found during foetal bone development, the initial stages of fracture healing and in Paget’s Disease and is characterised by irregular organisation of collagen fibres, or “lamellar” which has parallel alignment of collagen into sheets. Lamellar bone is mechanically strong and is the structure of healthy adult bones.

1.7.iii Bone formation

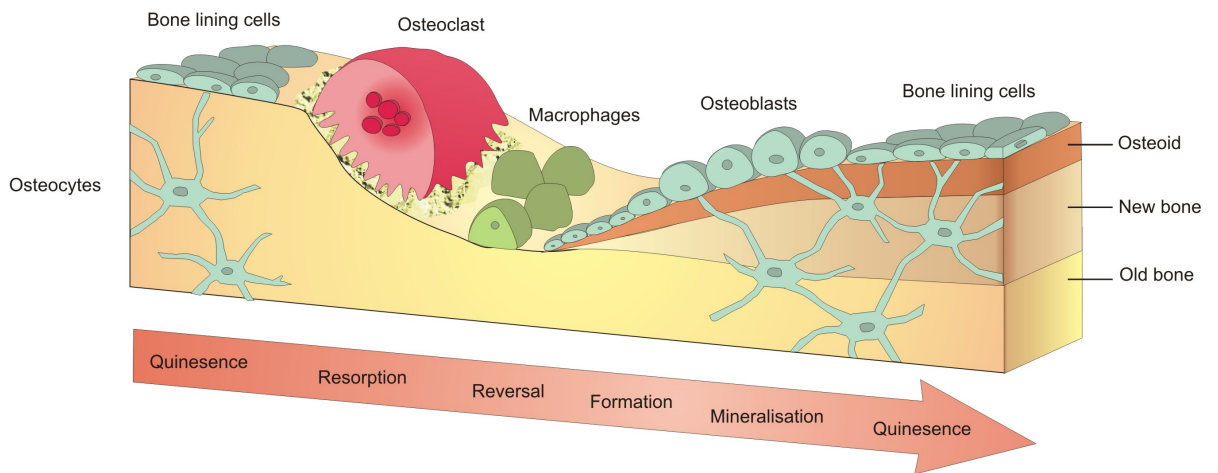
During embryonal development bone formation occurs via two principal mechanisms, namely intramembranous ossification and endochondral ossification. Intramembranous ossification occurs in the flat bones of the skull, mandible, maxillae and clavicles and bone

is formed from mesenchymal tissue rather than from cartilage. The four stages of intramembranous ossification are i) formation of ossification centre, ii) calcification, iii) trabecular formation and iv) development of periosteum. Endochondral ossification occurs in other bone types including the long bones and involves the formation of an initial hyaline cartilage. The steps in endochondral ossification are therefore; development of the cartilage model, growth of cartilage model, development of the primary ossification centre, development of the secondary ossification centre and formation of articular cartilage and the epiphyseal plate. Endochondral ossification is also known as “bone modelling” and occurs during the first two decades of life. During puberty there is an active period of bone modelling which results in growth and lengthening of the skeleton (Beaupre et al., 2005). This is in contrast to “bone remodelling” which occurs throughout life following microdamage to bone.

1.7.iv Bone remodelling

Bone remodelling or bone turnover refers to the process by which bone is resorbed and subsequently replaced with little change in the overall shape. This is a continuous process that occurs throughout life and is a complex process involving all three cell types under the control of various circulating factors including calcium and phosphate and parathyroid hormone (PTH). Remodelling occurs in a sequential way known as the Activation-Resorption-Formation cycle (Henriksen et al., 2009). Bone remodelling takes place in unique temporary structures within the bone called basic multicellular units (BMU) containing osteoclasts at the front which resorb the old bone and osteoblasts in the rear which fill the cavities with new bone. The BMU also contains a central vascular capillary, its own nerve supply and connective tissue. Once the remodelling is complete the BMU disappears by apoptosis (usually after 6-9 months). Activation of a BMU is thought to be triggered by osteocyte cell death in a neighbouring area of micro-fracture which stimulates

osteoclast activity (Seeman, 2008). Mature osteoclasts adhere to bone and remove it by acidification and proteolytic digestion and release minerals – this process is known as resorption. Osteoclasts then leave the resorption site and die by apoptosis. Osteoblast precursors then enter the excavated area and differentiate into osteoblasts which begin secreting osteoid, which is subsequently mineralised to new bone. The lifespan of an osteoclast is two weeks, after which they are replaced by new pre-osteoclasts that originate in the bone marrow and travel via the circulation to the site of resorption. Osteoblasts have a longer lifespan (approximately 3 months). They form most rapidly initially, slowing progressively with some becoming embedded as osteocytes, some dying and the remainder assuming the shape of lining cells (Manolagas, 2000). Overall bone mass is determined by the balance between bone resorption and production within each remodelling cycle. If this process is unbalanced then bone pathology appears, either in excess (osteopetrosis) or deficiency (osteoporosis). Sex steroid deficiency and GC excess cause derangement in this normal process of regeneration and result in loss of this balance. Studies have shown that the balance between bone resorption and bone formation depends more on overall numbers of osteoblasts and osteoclasts rather than on the individual cell's capacity or function (Manolagas et al., 2002). The process of bone remodelling is outlined in Figure 5.



The bone remodelling process.

Bone is continuously remodelled at discrete sites in the skeleton in order to maintain the integrity of the tissue. During this process, old bone is resorbed by osteoclasts and replaced with new osteoid, secreted by osteoblasts. First osteoclasts are activated, and the resorption phase takes approximately 10 days. Following resorption, unclassified macrophage-like cells are found at the remodelling site in the intermediate, or reversal phase. Osteoblast precursors are then recruited, which proliferate and differentiate into mature osteoblasts, before secreting new bone matrix. The matrix then mineralises to generate new bone and this completes the remodelling process. Copyright BTR©

Figure 5. Bone remodelling. Reproduced with permission from Biomedical Tissue Research.

Bone remodelling is facilitated by a series of factors which allow osteoblasts to regulate the rate of both differentiation and activity of osteoclasts (Ninomiya et al., 1999). The relationship between osteoblasts and osteoclastogenesis was hypothesised when Komori et al. discovered that lack of Core-binding factor alpha 1 (Cbfa1) transcription led not only to a deficit in osteoblast maturation but also to reduced osteoclast cell number (Komori et al., 1997). Two years later Kong and co-workers reported that osteoblasts express RANKL which is essential for osteoclastogenesis. Haemopoietic stem cells differentiate in response to macrophage-colony stimulating factor (M-CSF) into pre-osteoclasts that express receptor activator of nuclear factor kappa-B (RANK) (Kong et al., 1999), which binds to RANKL and induces osteoclast maturation, fusion and then bone resorption (Manolagas, 2000, Shiotani et al., 2002). OPG is also produced by osteoblasts and acts as a decoy receptor for RANKL, negatively regulating its function by blocking the binding of RANKL to RANK on osteoclast precursor cells. It is widely accepted that the ratio of RANKL: OPG is the main determinant

and regulator of bone resorption at the cellular level (Lacey et al., 1998b, Simonet et al., 1997, Yasuda et al., 1998).

1.7.v Bone cell subtypes

There are 3 main cell types present in bone: osteoblasts, osteoclasts and osteocytes. The main focus of this thesis is osteocytes and therefore these will be discussed in most detail.

Osteoblasts: Osteoblasts are mononuclear bone forming cells that derive from mesenchymal stem cells (MSCs) within the bone marrow. These stem cells also differentiate into adipocytes, chondrocytes and myocytes depending on cell signalling markers that they encounter (Friedenstein et al., 1987, Long et al., 1995). Commitment of a progenitor stem cell to the osteoblast lineage is initiated by BMPs which stimulate Cbfa1, an osteoblast transcription factor. Subsequent gene modifications result in a cascade of events which has three principal periods; proliferation, extracellular matrix maturation and mineralisation (Lian and Stein, 1992). *In vitro* studies on osteoblast cultures derived from the calvaria of foetal rats show that during the first 10-12 days following osteoblast isolation, there is a period of active proliferation with high levels of mitotic activity and expression of cell cycle (*e.g.* histone) and cell growth (*e.g.* c-myc, c-fos and AP-1) regulated genes. During this proliferation phase several genes (collagen type 1, fibronectin and TGF- β) essential to the development of the bone cell phenotype are expressed. These genes are then subsequently down-regulated during subsequent phases of osteoblast differentiation (Lian and Stein, 1992).

Osteoblasts are located on the surface of Haversian systems (the sites of active bone formation) and create a protein mixture known as osteoid, which then mineralises to form bone. Osteoblasts also produce prostaglandins that act on the bone itself, ALP as a marker of bone turnover and many bone matrix proteins. Osteoblasts begin as immature cells,

which differentiate over time, eventually becoming trapped within the bone matrix as osteocytes. An osteoblast phenotype-related gene associated with extracellular matrix maturation is ALP. However with the onset of mineralisation, osteopontin (OPN) and osteocalcin (OCN) as well as bone proteoglycans I and II are highly expressed (Lian and Stein, 1992). Platelet-derived growth factor (PDGF), IGFs and some members of the fibroblast growth factor (FGF) family also activate committed osteoblast differentiation. *In vitro* development of the osteoblast phenotype is dependent on factors in foetal calf serum (FCS)(Aronow et al., 1990) and in addition dexamethasone, retinoic acid (Bellows et al., 1986, Zhou et al., 1991), vitamin D (Owen et al., 1991) and insulin (Gopalakrishnan et al., 2006).

Osteoclasts: Osteoclasts are derived from haematopoietic precursors of the monocyte macrophage lineage. They are large cells that contain 15-20 oval shaped nuclei. They are found in pits at the bone surface that are the result of their own resorptive activity known as Howship's Lacunae. Their function is bone resorption and under the regulation of osteoblasts and osteocytes they control continuous bone turnover (Ninomiya et al., 1999). Osteoclasts are characterized by high expression of tartrate resistant acid phosphatase (TRAP) and cathepsin K. Osteoclast cytoplasm has a foamy appearance due to the presence of multiple vesicles and vacuoles which are lysosomes filled with acid phosphatase. As described above the formation of osteoclasts from precursor cells requires the presence of RANKL and M-CSF. Osteoclast differentiation is inhibited by OPG. RANKL is produced by osteoblasts, osteocytes and stromal cells in response to several inducers such as vitamin D, thyroid hormone (Miura et al., 2002), PTH (Lee and Lorenzo, 1999), GCs (Chung et al., 2001a), PGE-2 (Li et al., 2002), IL-1,6 and 11(Aubin and Bonnelye, 2000, Hofbauer, 1999, O'Brien et al., 1999, O'Brien et al., 2000), FGF-2 (Chikazu et al., 2001), histamine (Deyama et al., 2002) and IGF-1 (Rubin et al., 2002). Oestradiol (Aubin and Bonnelye, 2000) and TGF- β (Quinn et al., 2001) on the other hand, are RANKL inhibitors. Although OPG is a soluble

decoy receptor for and acts to inhibit RANKL, many of the above named factors that induce RANKL production also inhibit OPG. Other genes vital for osteoclastogenesis are PU.1, M-CSF and c-fos. Deletion of these genes leads to absence of osteoclast formation (Tondravi et al., 1997, Felix et al., 1990, Grigoriadis et al., 1994).

Osteocytes: Osteocytes originate from osteoblasts that have terminally differentiated and then migrated into and become trapped and surrounded by the forming osteoid tissue that they themselves produce. Overall, 10-20% of osteoblasts will differentiate into osteocytes. During this process osteoblasts are buried, firstly into newly formed osteoid material and then under enzymatic control this osteoid matrix becomes mineralised (Suzuki et al., 1996, Aubin and Turksen, 1996).

Osteocytes are the most abundant cell type within bone with approximately $25,000/\text{mm}^2$ or approximately a 10 times greater number than osteoblasts (Kato et al., 1997). The fact that they are buried in the mineralised matrix means that until recent years, osteocytes have been relatively inaccessible and difficult to study. The spaces they occupy are known as lacunae. Osteocytes share markers with osteoblasts but differ in their level of expression. For example ALP is expressed at lower levels in osteocytes than osteoblasts (Palumbo et al., 1990). Osteocytes differ morphologically from osteoblasts in that they have dendritic processes (approximately 50/osteocyte) that reach out to meet other cell types (including osteoblasts, other osteocytes and periosteal fibroblasts) for the purposes of communication. These dendritic processes are enclosed in channels known as canaliculi, hence the term “lacuna-canalicular network” which is a descriptive phrase derived from the osteocytes’ satellite character (Aarden et al., 1994). These canaliculi also make contact with the bone marrow and endothelial cells (Bellido, 2014). These dendritic processes allow direct communication between cells. The lacuna-canalicular network also provides a huge

surface area for the osteocyte which is estimated to be more than 100 times greater than the trabecular bone surface (Teti and Zallone, 2009).

An osteocyte lacuna measures $9 \times 20 \mu\text{m}$ in humans (almost 2 times bigger than in mice). It has been described that certain molecules released by osteocytes, migrate through the proteoglycan and non-collagenous protein rich extracellular fluid. The relative size of the lacunae and canaliculi allows a detectable space between the mineralised bone surface and the osteocyte surface (Mullender et al., 1996, Kogianni and Noble, 2007). This structure also allows nutrients and oxygen to be supplied to osteocytes via the extracellular fluid (Talmage and Talmage, 2007). It is also now well established that there are functional gaps (connexions) between osteocytes, osteoblasts and bone-lining cells (Civitelli, 2008, Stains and Civitelli, 2005, Donahue et al., 2000).

Osteocyte functions include, to varying degrees, formation of bone, matrix maintenance and calcium homeostasis. They have also been shown to act as mechano-sensory receptors, regulating the bone's response to stress and mechanical load. Sensors and/or transducers on osteocytes respond to load induced strain. Glucose 6-phosphate dehydrogenase activity increases transiently in osteocytes following loading and loading also induces prostaglandin (PGE_2 and PGI_2) production by these cells. Isolated osteocytes, but not osteoblasts respond to pulsating fluid flow by releasing PGE_2 . Osteocytes also respond to mechanical loading with an increase of IGF-1 mRNA expression (Kato et al., 1997). Osteocytes share many markers with osteoblasts, though the two cell types differ in their level of expression. For example ALP is expressed at lower levels in osteocytes than in osteoblasts whereas osteocytes express greater amounts of casein kinase and OCN, the latter being important in maintaining an unmineralised area around the osteocyte cell body and its processes (Kato et al., 1997). Osteocyte markers include dentin matrix protein 1 (DMP1), Podoplanin (E11), phosphate regulating gene with homologies to endopeptidases

on the X-chromosome (PHEX), matrix extracellular phosphoglycoprotein with ASARM motif (MEPE) and sclerostin (SOST). Expression of these genes varies depending on the stage of osteocyte differentiation. For example, E11 is a pre-osteocyte marker necessary for elongation of dendritic processes whereas SOST is only expressed in mature osteocytes (Atkins et al., 2011).

Osteocytes are now recognised as the main controller of overall bone turnover and they do this by both direct (via gap junctions and hemi channels in the dendritic processes) and indirect communication with both osteoblasts and osteocytes (Bellido, 2014). One mechanism by which osteocytes control bone remodelling is to reduce bone formation via production of SOST which binds to LRP4, 5 and 6 and prevents the actions of Wnt signalling which usually promotes osteoblast proliferation and differentiation (Bellido, 2014).

Osteocytes are also the major producer of RANKL and OPG, which allows them to control osteoclastogenesis (Nakashima et al 2011., Xiong et al 2011.). More recently osteocytes have been shown to express and secrete FGF-23, a hormone which controls phosphate reabsorption in the kidney and by changing circulating phosphate levels, affects bone mineralisation (Yoshiko et al., 2007)(Riminucci et al., 2003, Weber et al., 2003). FGF-23 has also been shown to suppress osteoblast differentiation and matrix mineralisation *in vitro* (Wang et al., 2008, Sitara et al., 2008), suggesting that it plays a role not only in the regulation of systemic phosphate levels, but also in the local control of bone mineralisation.

1.7.vi MLO-Y4 cell line

The MLO-Y4 early osteocyte cell line was first identified and cloned by Kato et al in 1997 (Kato et al., 1997). This immortalised cell line was isolated and cloned from transgenic mice, overexpressing T-antigen driven by the OCN promoter. The cells were identified using cellular morphology (the presence of dendritic processes) as the initial criteria and then

their properties were compared with known properties of primary osteocytes, osteoblasts and other cells. Initial studies showed MLO-Y4 cells to have low ALP and type 1 collagen activity, similar levels of OPN and CD44 expression and high OCN secretion when compared to osteoblasts. They were also shown to express high levels of connexin 43 (Cx43), a gap junction protein, whereas they did not express osteoblast-specific factor 2, a primary osteoblast marker (Kato et al., 1997). MLO-Y4 cells express E11, an early osteocyte marker but not the mature osteocyte marker SOST. E11 was first detected on the forming dendritic processes and is thought to play a role in both mechanical strain and dendrite elongation (Zhang et al., 2006, Wetterwald et al., 1996, Schulze et al., 1999). Due to the relative inaccessibility of osteocytes resulting from them being buried in mineralised matrix, the osteocyte was until recently difficult to access. Since its isolation and culture, the MLO-Y4 cell line has been a widely used tool for *in vitro* studies of osteocyte activity and has greatly contributed to the understanding of osteocyte biology.

Many studies have been carried out using the MLO-Y4 cell line for example; conditioned medium (CM) from MLO-Y4 cells has been shown to promote proliferation of bone marrow MSCs and their differentiation into osteoblasts (Heino et al., 2004). Apoptotic CM from MLO-Y4 cells has been shown in separate experiments to contain high levels of RANKL and to promote osteoclast precursor migration and differentiation and up-regulate both osteoclast number and size (Al-Dujaili et al., 2011). MLO-Y4 cells also produce large amounts of M-CSF and OPG as well as RANKL providing further evidence of their control of osteoclastogenesis (Zhao et al., 2002, You et al., 2008). These studies provide further evidence of osteocytes' leading role in controlling overall bone turnover.

More recently it has been discovered that 17β -oestradiol up-regulates Cx43 expression and enhances gap junction intercellular connection via the classical oestrogen receptor (ER) pathway in MLO-Y4 cells (Ren et al., 2012). Both oestrogen and CD40 ligand, a molecule

expressed by activated T-cells have been shown to protect MLO-Y4 cells from GC-induced apoptosis, having potential clinical implications for both post-menopausal and GC-induced bone loss (Bonewald, 2004). MLO-Y4 cells have been demonstrated to express both the classical oestrogen receptors ER α and ER β as well as the G-coupled protein receptor 30 (GPR30) protein which is thought to exert non genomic functions of oestrogen within osteocytes, via the intracellular release of calcium stores (Ren and Wu, 2012). CM from apoptotic MLO-Y4 cells has been demonstrated to promote endothelial cell proliferation and migration, form endothelial tubule networks with longer and more branches than non-apoptotic osteocyte CM. Apoptotic osteocyte CM was shown to contain higher levels of VEGF than non-apoptotic osteocyte CM. Results from this study suggest that osteocyte apoptosis promotes angiogenesis in a VEGF-mediated manner (Cheung et al., 2011). The role of VEGF as a coupling factor between angiogenesis and osteogenesis is discussed in more detail in chapter 1.8.

1.7.vii Immortomouse/Dmp1-GFP-SW3 (IDG-SW3) cell line

This clonal cell line has been recently developed and cloned from mouse long bone chips carrying a Dmp1 promoter driving GFP crossed with the Immortomouse, which expresses a thermolabile SV40 large T antigen regulated by interferon- γ (IFN- γ). IDG-SW3 cells can be cultured in different conditions (temperature +/- the presence of IFN- γ) to either allow proliferation of the osteoblast phenotype or differentiation from an osteoblast to a late osteocyte. The differentiation process takes place over 21 days and different levels of gene expression are seen for specific markers at each stage of differentiation. DMP1-GFP is expressed during osteocyte differentiation. IDG-SW3 cells are Dmp1-GFP-negative and T-antigen-positive under immortalizing conditions but Dmp1-GFP-positive and T-antigen-negative under osteogenic conditions. Like osteoblasts, they express ALP and produce a type I collagen matrix containing calcospherulites. Like early osteocytes, they express E11,

DMP1, MEPE, and PHEX. Like late osteocytes, they develop a dendritic morphology and express SOST and FGF-23, regulated by PTH and $1,25(\text{OH})_2\text{D}_3$. The different levels of gene expression in these cells at progressive stages of differentiation are outlined in figure 6 (Woo et al., 2011). When cultured on 3D matrices, they express Dmp1-GFP and SOST (Woo et al., 2011). More recently studies of this cell line have shown a relationship between extracellular phosphate levels and vitamin D metabolism within osteocytes, an effect mediated by FGF-23 (Ito et al., 2012). The development of the IDG-SW3 cell line, has for the first time allowed studies of the osteoblast to osteocyte differentiation process to take place using a single template and within this thesis I have compared the response to varying treatments, of IDG-SW3 cells at different stages of differentiation to that of the MLO-Y4 cells.

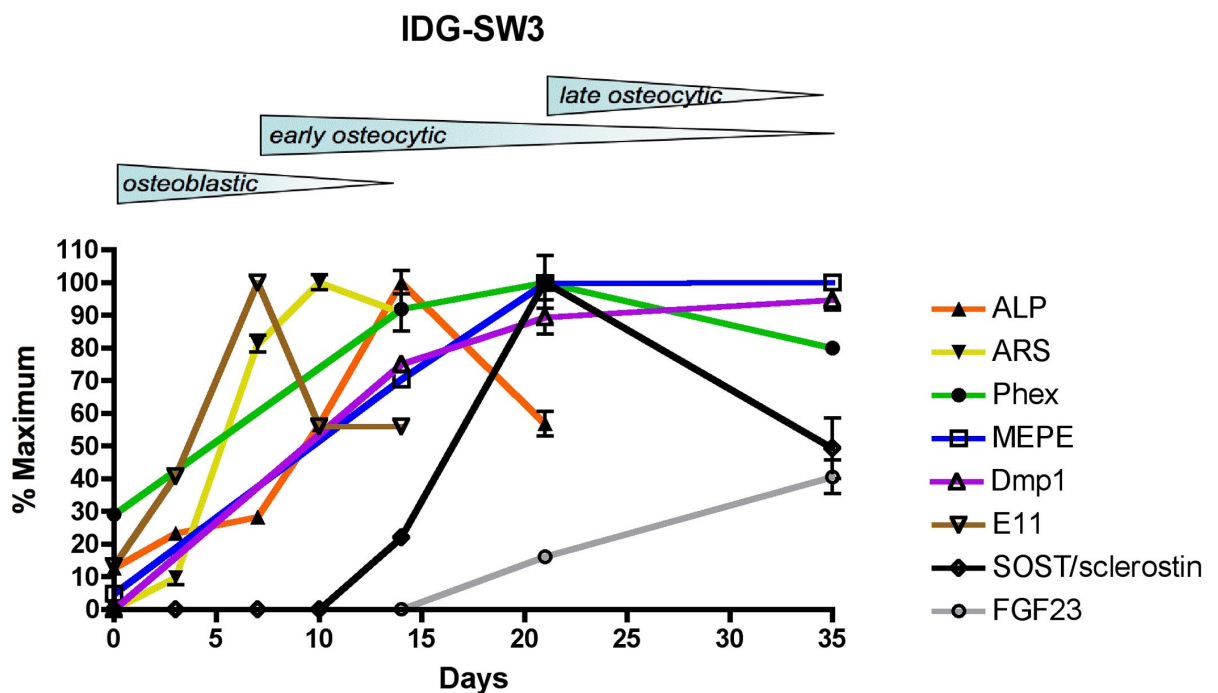


Figure 6. Schematic diagram summarizing osteoblastic and osteocytic markers in IDG-SW3 cells over time. ARS (alizarin-red staining) (Woo et al., 2011). Reproduced with permission from Wiley Publications Ltd.

1.8. Angiogenesis and bone

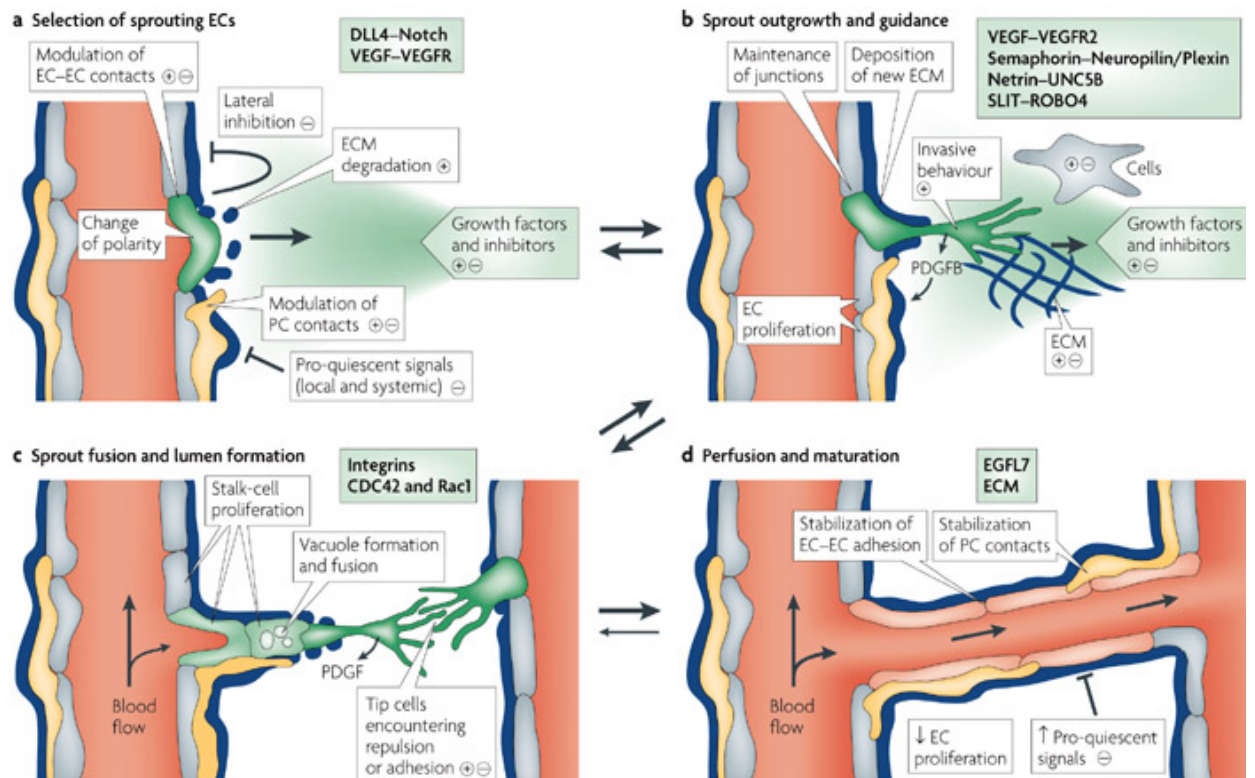
1.8.i Embryonal development

The importance of blood vessels in skeletal development was documented as early as the 1700s (Haller, 1763, Hunter, 1794). Skeletal development in the embryo is in molecular terms a similar process to fracture healing (Kanczler and Oreffo, 2008). It involves the coordination of multiple events including migration, differentiation, and activation of multiple cell types and tissues (Colnot, 2005). The development of a microvasculature is crucial for the homeostasis and regeneration of living bone. Without this microcirculation, bone tissue would degenerate and die (Schmid et al., 1997). In 1963 Trueta and Buhr published data proposing that there was a vascular stimulating factor (later defined as VEGF) that was released at fracture sites in bone (Trueta and Buhr, 1963). This study revived an interest in vasculogenesis in osteogenesis and triggered several lines of both *in vitro* and *in vivo* research that have provided a better understanding of this process (Brandi and Collin-Osdoby, 2006).

The growth and development of a mature vascular structure is one of the earliest processes in organogenesis (Coultas et al., 2005). In mammalian embryonic development, the vascular networks develop by aggregation of *de novo* forming angioblasts into a primitive vascular plexus, a process known as vasculogenesis. This undergoes a complex remodelling process in which sprouting, bridging and growth from existing vessels (angiogenesis) leads to the onset of a functional circulatory system (Jain, 2003). These events that occur in the embryo are mirrored in situations of neoangiogenesis (for example in bone healing following a fracture) in the adult (Kanczler and Oreffo, 2008). There are a number of factors involved in angiogenesis including VEGF, basic fibroblast growth factor (bFGF, FGF2), several members of the TGF β family and hypoxia (via activation of hypoxia-inducible factor

- HIF α). Other factors that have angiogenic properties include the angiopoietins, (Ang-1); hepatocyte growth factor (HGF); PDGF; IGF-1 and 2) and the neurotrophins (Madeddu, 2005).

The process of normal angiogenesis is outlined in Figure 7.



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Figure 7. Normal process of angiogenesis is controlled by a balance of pro-angiogenic factors such as VEGF and factors which promote quiescence such as certain extracellular matrix molecules or VEGF inhibitors. Reproduced from (Adams and Alitalo, 2007) with permission from Nature Publishing Group.

VEGF and their receptors are key regulators of the events that lead to vasculogenesis, angiogenesis and formation of the lymphatic vascular system (Zelzer et al., 2002, Gerber et al., 1999, Haigh et al., 2000). VEGF has also been shown to play an important role in skeletal growth (Zelzer et al., 2002, Gerber et al., 1999, Haigh et al., 2000), influencing two stages in particular (Zelzer et al., 2002). Initially VEGF is involved in the recruitment of blood vessels into the perichondrium and later it controls invasion of vessels into the

primary ossification centre, demonstrating a significant role of VEGF at both an early and late stage of cartilage vascularisation (Zelzer et al., 2002). This study outlines a two-step model of VEGF-controlled vascularisation of the developing skeleton, and also demonstrates that VEGF is highly expressed in the perichondrium and surrounding tissue of cartilage templates of future bones, well before blood vessels appear in these regions. Blocking VEGF in embryonic mice results in enlarged areas of hypertrophic cartilage, loss of metaphyseal blood vessels and impaired trabecular bone formation (Zelzer et al., 2002, Gerber et al., 1999, Haigh et al., 2000). Zelzer et al also provide evidence for a possible role of VEGF in chondrocyte maturation, and document that VEGF has a direct role in regulating osteoblastic activity based on *in vivo* evidence and organ culture experiments (Zelzer et al., 2002). This shows that during embryonic development VEGF is essential for normal growth plate morphogenesis, including blood vessel invasion and cartilage remodelling. In endochondral ossification, the coupling of chondrogenesis and osteogenesis to determine the rate of bone ossification is dependent on the level of vascularisation of the growth plate (Gerber and Ferrara, 2000). VEGF isoforms (described below) are utilised in this process to coordinate metaphyseal and epiphyseal vascularisation, cartilage formation and ossification (Maes et al., 2004).

The vascularisation of cartilage occurs at different stages of long bone development. Initially, in early embryonic development, blood vessels that originate from the perichondrium incorporate into the cartilage structures. Secondly, during postnatal growth, capillaries invade the growth plate of long bones and thirdly, in adulthood, angiogenesis can be periodically switched on during bone remodelling in response to bone trauma or pathophysiological conditions such as rheumatoid arthritis and osteoarthritis (Gerber and Ferrara, 2000).

The VEGF receptor, VEGFR2 is also an important molecule involved in embryonic bone vascularisation (Kanczler and Oreffo, 2008). It is secreted by hypertrophic chondrocytes, which subsequently leads to recruitment of osteoblasts, osteoclasts and haemopoietic cells. Histological studies show that osteoblasts and osteoprogenitor cells always develop alongside endothelial cells in the newly formed blood vessels at the sites where new bone is formed (Deckers, 2000). This sequence of events leads to the formation of the primary ossification centres, in which the recruited osteoblasts lay down trabecular bone which forms the bone marrow cavity (Hall et al., 2006, Gerber and Ferrara, 2000, Collin-Osdoby, 1994). In the developing long bone, VEGF is expressed before any blood vessels are detected and this expression has been shown to be linked to the bone formation process (Carano and Filvaroff, 2003, Zelzer et al., 2002). The HIF- α pathway is responsible for the expression of VEGF in this process. The increase in bone formation is due to enhanced angiogenic activity, which is mediated by high levels of VEGF in osteocytes over expressing HIF- α (Wang et al., 2007). It is this process that has led to the labelling of HIF- α and VEGF as “coupling factors” between angiogenesis and osteogenesis during bone formation and repair (Kanczler and Oreffo, 2008).

1.8.ii Bone healing and repair

There is an abundance of evidence for the role of angiogenesis and particularly VEGF in bone healing and repair (Street et al., 2002, Chu et al., 2002, Peng et al., 2002). Many of the same molecules including VEGF, FGF, TGF- β , BMP and PDGF are expressed and active during both (Glowacki, 1998, Einhorn, 1995, Einhorn, 1998, Mandracchia et al., 2001, Gerstenfeld et al., 2003). Inhibition of angiogenesis in animal models leads to a clinical picture similar to non-union of fractures (Hausman et al., 2001). Blocking VEGF activity has also been shown to delay bone repair in mice (Street et al., 2002), as well as reduced blood flow and non-union of fractures in rabbits (Chu et al., 2002). It is thought therefore that

during bone repair VEGF is required not only for blood vessel formation but also for normal callus volume and mineralisation. These studies indicate that normal angiogenesis is essential for bone repair and that VEGF may be the major signal to couple angiogenesis and osteogenesis during bone repair (Chu et al., 2002, Peng et al., 2002, Colnot et al., 2003). Serum levels of VEGF as well as bFGF and PDGF are elevated in patients following fractures compared with healthy controls. Peak levels of these cytokines are reached during early fracture healing (Weiss et al., 2009). Levels are highest in patients with delayed or non-union of fractures compared to those with normal physiological fracture healing (Sarahrudi et al., 2009).

1.8.iii Osteonecrosis

Angiogenesis plays a multifactorial role in the pathogenesis of ON, in that an interruption in vascular supply is implicated as a potential mechanism for its development (Wang et al., 2010, Weinstein et al., 2010b, Varoga et al., 2009), but in addition, elevated VEGF levels are found in osteonecrotic bones suggesting that it contributes to the repair process (Radke et al., 2006). Following necrosis of bone, the repair process begins with blood vessel invasion into the necrotic area, followed initially by bone resorption and then subsequent new bone formation (Kerachian et al., 2009). This process is influenced by various circulating factors, including VEGF and any dysregulation of these can result in impaired angiogenesis and defective bone repair. *In vivo* studies by Yang have shown that gene transfection can be used to enhance the process of ON repair in a rabbit model (Yang et al., 2003b, Yang et al., 2003a). Polymorphisms within the VEGF gene may also affect an individual's risk of development of GC-induced ON (Lee et al., 2012). GCs are thought to influence bone angiogenesis via multiple mechanisms and factors, many of which may contribute to the pathogenesis of ON (Kerachian et al., 2009).

As well as affecting bone cells, GCs also directly affect endothelial cell function (Kerachian et al., 2009). GCs can injure endothelial cells and lead to hypercoagulability (Kerachian et al., 2006, Boss and Misselevich, 2003). An early study by Jacobs et al suggested that damaged reticular vessels are the underlying mechanism of ON (Jacobs, 1978) and although the mechanisms have subsequently been shown to be more complex and multifactorial, more recent studies have shown that damage to endothelial cells can lead to abnormal coagulation and thrombus formation with ON formation occurring distal to the site of arterial occlusion (Kerachian et al., 2006). Further evidence for the role of endothelial damage in ON is the fact that levels of 6-ketone prostaglandin $F_{1\alpha}$, a marker of endothelial cell injury (Li et al., 2004b), were significantly altered in a rabbit model where ON was induced using a combination of GCs and endotoxin (He et al., 2004).

Effects on the coagulation pathway are another proposed mechanism in the pathogenesis of ON (Kerachian et al., 2009). High doses of GCs have been demonstrated to inhibit fibrinolysis (Gray et al., 1996, van Giezen and Jansen, 1992, van Giezen et al., 1994), which occurs as a result of reduced tissue plasminogen activator (t-PA) activity and increased plasma levels of plasminogen activator inhibitor-1 (PAI-1) antigen (Gray et al., 1996, Minneci et al., 2004). Decreased fibrinolytic activity has been described in patients with ON (Smith, 1997) and furthermore, plasma fibrinogen levels were elevated in an animal model of ON treated with “mega dose” steroids (Drescher et al., 2004). As well as GCs, cartilage components have also been identified as potential inhibitors of angiogenesis (Kerachian et al., 2009). Following a subchondral fracture the fracture cleft exposes the cartilage components to the on-going repair process. It is thought therefore that cartilage must play a role in the development or on-going disease process in ON and may explain the localisation of ON to subchondral bone (Smith, 1997).

1.9 Vascular endothelial growth factor

1.9.i Subtypes and function

1.9.i.a VEGF(A)

VEGF (also known as VEGFA, vascular permeability factor or vasculotrophin) is a homodimeric 34-42 kDa protein with potent pro-angiogenic properties that regulates both normal and pathological angiogenesis (Senger et al., 1993). It is a member of the subfamily of growth factors, namely the PDGF family of cysteine knot growth factors and its amino acid sequence contains some homology with that of the A and B chains of PDGF. VEGFA was initially identified in 1989 as an endothelial-specific growth factor (Ferrara and Henzel, 1989), but has subsequently been shown to be produced by multiple cell types including fibroblasts, smooth muscle cells, hypertrophic chondrocytes and osteoblasts as well as neoplastic cells (Gerber et al., 1999, Ferrara, 2001, Gerber and Ferrara, 2003). Its normal function is to create new blood vessels. This can be during embryonal development, a process known as vasculogenesis or angiogenesis where new blood vessels are formed from existing vasculature in areas of ischaemia or wound healing. VEGFA has been shown to play a key role in tumour angiogenesis and metastasis, allowing tumours to maintain their blood supply and develop a new blood supply at a distant site.

VEGFA is now understood to be one of a family of VEGFs that also includes VEGFs B,C,D,E,F and placenta growth factor (PLGF) (Veikkola and Alitalo, 1999, Ferrara et al., 2003, Roy et al., 2006). All members share a common structure of eight cysteine residues. VEGFA is the most well studied of the VEGF subtypes and is the focus of studies of VEGF in this thesis. VEGFA will from now on be denoted as VEGF, unless otherwise specified.

VEGF mediates both physiological and pathological angiogenesis. Structurally it is an antiparallel dimer with receptor binding sites at each pole (Dai and Rabie, 2007). The VEGF

gene is located on chromosome 6p21.3 in humans (Vincenti et al., 1996) and consists of eight exons. Two families of proteins are described according to the terminal exon (exon 8) splice site. Gene products with a proximal splice site are pro-angiogenic and are expressed during angiogenesis. These isoforms are named VEGF_{xxx}, where xxx is a 3 digit number describing the amino acid length. Gene products with a distal splice site have antiangiogenic properties and are expressed in normal tissues. They are named VEGF_{xxx_b}. Alternate splicing of exons 6 and 7 alters the amino acid number and mediates certain properties including interactions with heparin sulphate proteoglycans (HSPG) and neuropilin (NRP) co-receptors on the cell surface, affecting their ability to bind and activate VEGF receptors (Houck et al., 1991). Human forms of VEGF include VEGF₁₂₁, VEGF_{121b}, VEGF₁₄₅, VEGF₁₆₅, VEGF_{165b} and VEGF₂₀₆. (Tischer et al., 1991). The rodent forms of VEGF have one fewer amino acid. The VEGF₁₆₅ is the best studied isoform and therefore most well understood in terms of its properties. The VEGF isoforms are represented schematically in Figure 8.

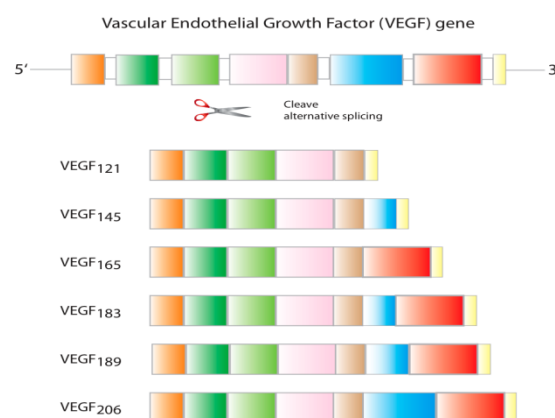


Figure 8; VEGF isoforms as formed by alternative splicing of exons 6 and 7 (Reproduced with permission from Alexandre, 2010).

VEGF₁₂₁ is the shortest isoform and is mostly freely diffusible with very little remaining membrane bound. It lacks exons 6 and 7 which encode the HSPG and NRP binding domains and therefore does not bind these molecules. It functions as a mitogenic and chemoattractant for endothelial cells during both angiogenesis and vasculogenesis (Houck et al., 1991). VEGF₁₄₅ is partly membrane bound although some is secreted (Ferrara & Davis-Smyth., 1997). By binding to HSPG it has been shown to induce endothelial cell proliferation and angiogenesis (Poltorak et al., 1997). VEGF₁₆₅ is the most abundant isoform and is the most well studied. It lacks exons 6a and 6b (Tischer et al., 1991), and binds to all the VEGF receptors (VEGFR1, VEGFR2, HSPGs, NRP1 and NRP2 – these receptors are described in more detail below) (Walterberger et al., 1994). It is thought that VEGF₁₆₅ and VEGF₁₂₁ are the isoforms involved in bone formation (Dai and Rabie, 2007), but their exact roles in this process have yet to be established. VEGF₁₈₉ and VEGF₂₀₆ contain all the exons and are fully membrane bound (Houck et al., 1991). These two isoforms are much less active than the others and in fact VEGF₂₀₆ expression is restricted to embryonal tissues (Ferrara & Davis-Smyth., 1997). A summary of the VEGF isoform functions is outlined in Table 3.

Isoform	Structure	Binding ability	Functions	References
VEGF ₁₂₁	Lacks exon 6 & 7	VEGFR-1, 2	↑EC proliferation, migration, osteoclastogenesis	Houck et al 1991, Wise et al, 2003
VEGF ₁₄₅	Lacks exons 6b & 7	VEGFR-1,-2, HSPG, NRP-2	↑EC proliferation	Poltorak et al., 1997
VEGF ₁₆₅	Lacks exons 6a&6b	VEGFR-1,-2, HSPG, NRP-1,-2	↑EC proliferation, permeability, osteoclastogenesis	Wise and Yao, 2003, Cramer et al., 2004)
VEGF ₁₈₉	Contains all exons	HSPG, NRP-1,-2	↑EC proliferation, migration	(Herve et al., 2005)
VEGF ₂₀₆	Contains all exons	HSPG, NRP-1,-2	Not determined	{Ferrara & Davis-Smyth., 1997)

Table 3. Structure and function of the VEGF isoforms (Adapted from Dai and Rabie, 2007)

1.9.i.b VEGFB, C, D, E, F and PLGF

VEGFB (also known as VEGF related factor) is located on chromosome 11 and has two splice variants (Li et al., 2001). Large amounts of VEGFB are found in the heart, skeletal muscle and pancreas and it is required for vascularisation of skeletal muscle (Enholm et al.,

1997). It binds to VEGFR1 and has been shown to induce angiogenesis associated with the activation of Akt and eNOS-related pathways (Silvestre et al., 2003). VEGFB has more recently been identified to have a potential role in tumour survival and progression (Jayasinghe et al., 2013).

VEGFC (also referred to as VEGF-related protein) is located on chromosome 4 and binds to VEGFR3, which is associated with the lymphatic system (Paavonen et al., 1996, Karkkainen et al., 2004). It does promote endothelial cell migration and proliferation but it less potent than VEGFA (Bhardwaj et al., 2003). It may also play a role in the pathogenesis of rheumatoid arthritis, (Wauke et al., 2002) and recently evidence is emerging for a role of VEGFC as a serum marker for cancer prognosis (Cheng et al., 2013).

VEGFD (also called c-fos induced growth factor) is located on chromosome X and activates VEGFR2 and VEGFR3 in humans but only VEGFR3 in mice (Baldwin et al., 2001). It is expressed in limb buds, teeth, liver and heart, lung and kidney mesenchyme and vertebrae (Avantaggiato et al., 1998). It has angiogenic properties both *in vitro* and *in vivo* (Marconcini et al., 1999) and contributes to lymphatic vessel development in tumours (Miyata et al., 2006).

VEGFE exclusively binds to VEGFR2 and has very similar actions and potency to VEGF₁₆₅, in terms of promoting endothelial cell growth, proliferation and migration and angiogenesis (Ogawa et al., 1998).

VEGFF was identified in 2005 from snake venom. It consists of two proteins and binds to VEGFR2 (231). Little is known about its function but it appears to block VEGF₁₆₅ activity and play a role in the development of vascular fenestrations (Yamazaki et al., 2005, Matsunaga et al., 2009).

PLGF was the first of the VEGF family proteins to be discovered and it shares homology with the PDGF-region of VEGF (Nissen et al., 1998). It is located on chromosome 14, has four splice variants named PLGF1-4 and is chemotactic for monocytes and macrophages (Roy et al., 2005). The role of PLGF in bone is not well described but it has been described in one study to mediate cartilage turnover and bone remodelling during fracture repair (Maes et al., 2006).

1.9.1.c VEGF Receptors

The VEGFRs belong to a subfamily of PDGF receptors and are classified as receptor tyrosine kinases (Dai and Rabie, 2007). The three main receptors are VEGFR1, VEGFR2 and VEGFR3 but VEGF also binds to co-receptors NRP1 and NRP2 and HSPGs (Ferrara et al., 2003). The biological effects of VEGF are mediated by more than one VEGF receptor which is thought to ensure balanced signalling (Dai and Rabie, 2007).

VEGFR1 (also known as fms-like tyrosine kinase receptor 1 or flt-1) is a 180kDa transmembrane protein, which is important for vascular maintenance and the recruitment of endothelial precursors during vasculogenesis (Shibuya, 2006). It binds all isoforms of VEGF, VEGFB and PLGF (Park et al, 1994, Olofsson et al, 1998). Soluble VEGFR1 can function as a VEGF inhibitor (decoy receptor) which suggests that it can act as both a negative and positive regulator of VEGF activity (Gille et al., 2001). VEGFR1 activity is also thought to be influenced by interaction with VEGFR2 (Dai and Rabie, 2007). VEGFR1 is expressed by osteoblasts and mice that are deficient in its' signalling, have reduced osteoblast and osteoclast cell number and defective bone marrow cavity formation (Niida, 2005). *In vitro* osteoblast differentiation is also reduced when VEGFR1 is blocked suggesting that VEGFR1 plays an important role in this process (Liu et al.). Both VEGFR1 and VEGFR2 are expressed

by osteocytes and expression increases in the first 14 days following distraction osteogenesis (Byun et al., 2007).

VEGFR2 (or kinase insert domain-containing receptor or KDR/Flk 1) is a 230kDa glycoprotein and is thought to mediate almost all of the endothelial cell responses to VEGF (Neufeld et al., 1999). It binds to VEGFA, C, D and F but not VEGFB or PLGF (Ferrara et al., 1997). It is located predominantly on the surface of endothelial cells and induces cell migration, proliferation and angiogenesis (Hutchings et al., 2003). VEGFR2 mediated cell migration is enhanced in the presence of NRP1 suggesting that NRP1 acts as a co-receptor for VEGFR2 (Shraga–Heled et al., 2007). VEGFR2 is extremely important for embryonic vasculogenesis as demonstrated by the fact that in knock-out mice this process is severely impaired and results in embryonic death (Shalaby et al, 1995).

VEGFR3 (or Flt 4) is a 170kDa glycosylated protein and is a receptor for VEGFC and VEGFD but not VEGFA (Dai and Rabie, 2007). It is required for blood vessel formation especially during early development as well as formation of lymphatic vessels (Lohela et al., 2003). Later, however its expression is mainly restricted to the lymphatic system (Takahashi., 2012), although it may play a role in the pathogenesis of osteoarthritis where it appears to be over expressed in chondrocyte cells (Shakibaei et al., 2003).

NRP1 and NRP2 are VEGF co-receptors and are non-tyrosine kinase transmembrane receptors that have a small cytoplasmic domain and multiple extracellular domains (Soker et al., 1998). NRPs cannot act alone to mediate VEGF effects and instead associate with both VEGFR1 and VEGFR2 by binding to the different VEGF subtypes and enhancing phosphorylation of the VEGF receptors (Zelzer et al., 2001). NRP1 has been shown to bind to VEGF₁₆₅ in MC3T3-E1 osteoblasts and its expression is down-regulated during the differentiation of these cells into osteocytes (Deckers, 2000). In keeping with these

findings, an *in vivo* study has shown that NRP1 is expressed by osteoblasts where it mediates the effects of VEGF but not by osteocytes (Harper et al., 2001). The role of NRP2 in bone is currently unknown.

1.9.ii VEGF and osteocytes

Until recently most of the research into VEGF and its coupling effect on angiogenesis and osteogenesis was restricted to osteoblasts but recently there is increasing evidence that osteocytes secrete VEGF and that levels of secretion vary under certain conditions suggesting an important role of the osteocyte in bone angiogenesis (Juffer et al., Cheung et al.). Verborgt et al in 2000 showed that osteocyte apoptosis is induced by bone fatigue secondary to mechanical loading and that this apoptosis is localized to regions of bone containing micro-cracks and also demonstrated that osteoclastic reabsorption after fatigue is localized to the same areas as osteocyte apoptosis. A subsequent study showed an exponential decrease in VEGF production with distance from fatigue induced micro-cracks within bone, suggesting that VEGF production by osteocytes may induce osteoclastic activity via a paracrine mechanism (Kennedy et al., 2012).

Cheung et al in 2011 also showed that osteocyte apoptosis is mechanically mediated. Osteocytes were exposed to oscillatory fluid flow or no flow conditions with or without TNF α . Flow protected osteocytes from apoptosis regardless of the presence of TNF α . CM from apoptotic and non-apoptotic osteocytes were added to endothelial cells to assess effect of osteocyte apoptosis on angiogenesis. Apoptotic CM caused more endothelial cell proliferation and migration as well as tubule networks with longer branches than non-apoptotic CM. Apoptotic osteocyte CM contained higher concentrations of VEGF than non-apoptotic CM. This study suggests that osteocyte apoptosis is flow related and promotes angiogenesis in a paracrine VEGF-mediated manner (Cheung et al 2011.). As well as

influencing angiogenesis, apoptotic osteocytes are also thought to be responsible for inducing osteoclastogenesis and several studies have suggested that VEGF contributes to this process (Gupta et al 2010., Kennedy et al 2014.).

1.9.iii VEGF and acute lymphoblastic leukaemia

VEGF is known to be secreted by many tumour types and in many malignancies contributes to tumour angiogenesis and development of metastases and therefore conveys a poor prognosis (Neufeld et al, 1999, de Mello et al., 2012., Schoenleber et al, 2009, Sawhney et al, 2008). As a result a number of anti-VEGF pathway agents, the most well know of which is bevacizumab (Avastin®), have been developed and are used in the treatment of many cancers including colorectal and renal cell carcinoma. In adult leukaemias (lymphoid and myeloid) there are reports of increased bone marrow vascularity and a role of angiogenesis and VEGF in the pathophysiology (Aguayo et al, 2000., Faderl et al, 2005). In childhood ALL there are fewer studies and results are conflicting with some studies showing that high serum VEGF levels are predictive of poor outcome (increased relapse risk or reduced survival) (Koomagi et al, 2001., Schneider et al, 2003., Schneider et al, 2007., Avramis et al, 2006., Stachel et al, 2007) and others showing inconclusive results or no correlation between VEGF levels and prognosis (Yetgin et al, 2001., Lyu et al, 2007., Pule et al, 2002., Leblebisatan et al, 2012). The difference in results may be due to small patient numbers (the maximum cohort was 53), different methods of measuring VEGF (serum, plasma, urine or bone marrow levels) and circadian variations in normal serum VEGF levels (Schneider et al.).

VEGF and other angiogenic factors have been shown to be produced by lymphoblasts as well as other cell types within the bone marrow microenvironment. It is thought that the relationship between the leukaemia cells and the bone marrow endothelium allows

angiogenic factors including VEGF to promote survival of leukaemic cells by maintaining the expression of anti-apoptotic genes (Veiga et al, 2011).

To date there have been no trials of anti-angiogenic agents in the treatment of childhood ALL although there has been one study of bevacizumab in refractory acute myeloid leukaemia in adults with some clinical benefit seen (Karp et al, 2004). Evidence that high serum VEGF levels are present in relapsed or refractory ALL (Lyu et al, 2007, Schneider et al, 2007), may prove a useful tool for identifying cohorts of patients in whom anti-VEGF therapy could have potential benefits. Another possible use for VEGF in the future management of ALL is to use serum VEGF levels to monitor response to treatment as there is evidence that levels are higher than in healthy controls at diagnosis and decrease following treatment (Avramis et al, 2006).

1.10. IL-6

1.10.i Structure and function

IL-6 is a pro-inflammatory cytokine, secreted by T-cells and macrophages in order to mediate an immune response, for example during an infection or following trauma. It is encoded by the IL-6 gene which is found on chromosome 7 in humans (Ferguson-Smith et al, 1988). One of its primary functions is to mediate the “acute phase response” which occurs in response to infection and results in an initial increase in inflammation followed by switching off of this process once the infecting micro-organism has been cleared. The IL-6 receptor is a cell surface type 1 cytokine receptor which has a binding domain (unique to the receptor) and a signal transducing domain (gp130) which is common to several other cytokines and is expressed in nearly all tissue types. Other cytokines (such as IL-11, IL-27,

cardiotrophin 1 and leukaemia inhibitory factor) who mediate their effects via gp130 are often referred to as IL-6 like or gp130 utilising cytokines (Heinrich et al, 2003). The intracellular cascade of events that occurs after IL-6 binds to its receptor occurs via Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) (Heinrich et al, 1998., O'Brien et al, 1999).

1.10.ii IL-6 and bone

IL-6 is produced by osteoblasts but most of its documented biological effects have been on osteoclast cells. IL-6 up-regulates RANKL production by binding to its receptor on osteoblasts and thus indirectly promotes osteoclastogenesis and bone resorption (Udagawa et al, 1995). Paradoxically the direct effects of IL-6 on osteoclasts are inhibitory (Yoshitake et al, 2008, Axmann et al, 2009). IL-6 is thought to contribute to bone loss in osteoporosis (oestrogen deficiency leads to a mild increase in pro-inflammatory cytokines) and structural damage to bone in rheumatoid arthritis (Terauchi 2011., Jilka et al, 1992). This has led to the development of anti-IL-6 receptor agents (tocilizumab) which has been used in patients with moderate to severe rheumatoid arthritis (Agarwal, 2011). As stated above, it is now recognised that the osteocyte appears to be the primary controlling cell of bone turnover and re-modelling. Osteocytes, like osteoblasts are known to produce IL-6 and apoptotic osteocytes secrete higher amounts than non-apoptotic osteocytes. IL-6 from these apoptotic osteocytes promotes osteoclast precursor adhesion to endothelial cells, an important step in the initiation of bone remodeling (Cheung et al.). In previous studies, co-culture of MLO-Y4 cells with spleen or bone marrow derived precursor cells also promoted osteoclastogenesis *in-vitro*. However, this study concluded that the effects seen were secondary to RANKL and M-CSF production (Zhao et al., 2002). It is likely therefore that osteocytes control osteoclastogenesis via multiple pathways of which IL-6 secretion is an

important element. As well as effects on osteoclast cells, IL-6 also acts to inhibit osteoblast formation (Peruzzi et al.).

1.10.iii IL-6 and acute lymphoblastic leukaemia

Serum and CSF IL-6 concentrations as well as serum concentrations of another pro-inflammatory marker (TNF α) have been shown to be raised at diagnosis in children with ALL (Giordano et al, 2010, Protas et al, 2011). The effect of treatment on IL-6 levels remains largely inconclusive with one study demonstrating a significant reduction in levels following induction therapy (Giordano et al, 2010) and others showing persistently elevated levels throughout treatment (Protas et al.), or acute elevations during episodes of mucositis following the administration of methotrexate (Morales-Rojas et al., 2012.). Although adult survivors of childhood ALL have been shown to have raised levels of some inflammatory markers, which is thought to contribute to their increased risk of cardiovascular morbidity, levels of IL-6 in these patients appear to return to normal (Sulicka et al., 2013).

1.11. Bone during puberty

1.11.i Bone accrual during puberty

Skeletal development begins *in utero* and continues throughout childhood and adolescence until skeletal maturity is reached (Mughal and Khadlikar, 2011). As bones “grow”, they increase in overall size as well as in bone mineral content and strength (Mughal and Khadlikar, 2011). Studies have shown that similar to overall growth, bone growth also follows a centile so that an individual will stay in the same position in the overall population distribution throughout childhood and adolescence (Kalkwarf et al., 2013., Ferrari et al, 1998). This centile is established during the first year of life (Wang and Seeman, 2008).

Puberty contributes to more than half of adult bone mass. During puberty an increase in height precedes the increase in bone mineral content and bone width. At the end of skeletal growth, males have a higher bone mass than females, due to their larger skeleton. This is due to a longer period of prepubertal growth in boys (Euling et al., 2008), and the fact that in early puberty, oestrogens inhibit, while androgens stimulate periosteal bone formation (Seeman, 2003). "Peak bone mass", defined as the amount of bone accrued at the end of skeletal maturity is achieved by early adulthood and contributes to the risk of osteoporotic fractures in later life. Factors influencing peak bone mass include genetic and epigenetic factors, body composition (obesity appears to be detrimental) physical activity and nutrition (particularly calcium, phosphate and vitamin D intake).

1.11.ii Vitamin D and bone development

It is well recognised that even in countries where sunshine is abundant, there is widespread vitamin D insufficiency and deficiency throughout all ages of the population (Mithal et al, 2009., Johnson et al, 2013., Lips 2010), including children and adolescents (Das et al, 2006., Rovner and O'Brien, 2008., Gordon et al, 2008). Recently it has become apparent that intrauterine and early postnatal vitamin D status may be important for bone mass accrual with studies showing reduced bone mineral content in infants and children of mothers with low 25-OHD₃ levels and/or less sunlight (UVB) exposure compared to those with levels in the normal range (Viljakainen et al, 2011., Javaid et al, 2006., Sayers et al, 2009). These findings remained present in children when bone mineral content was measured at age nine years, suggesting that maternal vitamin D status has a significant positive effect on neonatal bone status, which appears to continue up to the prepubescent period (Viljakainen et al, 2011, Javaid et al, 2006).

Adequate vitamin D status is important for calcium absorption as well as mineralisation of the growth plate and skeleton. Studies have shown positive correlation between serum 25OHD₃ levels and BMD in children and adolescents (Outila et al, 2001, Marwaha et al, 2005). However studies into whether or not vitamin D supplementation either in infancy or later in childhood influences BMD have yielded conflicting results (Zamora et al, 1999., El-Hajj Fuleihan et al, 2006., Viljakainen et al, 2006., Molgaard et al, 2010), and a large scale randomised controlled trial is probably required to establish any potential benefit of this.

1.11.iii Differences between the adult and growing skeleton

Bone mass development during puberty depends more on sexual maturation than chronological age. The effects of oestrogen on the growing skeleton are different from those of the mature skeleton and vary according to gender. There is evidence that short-term exposure to oestrogen during foetal life affects bone growth during postnatal life suggesting a possible imprinting mechanism that acts on bone cells *in utero* (Migliaccio et al., 1996). Skeletal development is also influenced by sex steroids on the initiation of the pubertal growth spurt and the closure of epiphyses at the end of puberty (Frank et al., 2000). Linear skeletal growth is controlled by chondrocytes of the growth plate. The effects of sex steroids on pubertal growth and epiphyseal closure result from direct actions of sex hormones on these chondrocytes which express classical receptors for both oestrogens and androgens (Manolagas et al., 2002).

True BMD (the amount of mineral per volume of bone in gr/cm³) does not increase with size or age. DEXA scans measure areal BMD in gr/cm². Therefore the 40-50 fold increase in bone mass that occurs during the time period from birth to maturity is a result of growth rather than a true increase in density. During growth, bone mass and density may change in opposite directions. This is in contrast to the mature skeleton where there is no change in

bone size and therefore mass and density always change in the same direction. The growing and mature skeletons also differ in both the spatial and temporal organisation of bone cell function.

Lack of oestrogen on the growing skeleton prolongs the duration of growth which eventually leads to increases in height and long bone length (Simpson, 1998., Grumbach and Auchus, 1999). These longer bones have accrued more bone than normal and the increased rate of remodelling in these subjects is a normal physiological response due to the fact that they have not reached sexual maturity. This is in contrast to the pathological increase in bone remodelling caused by loss of sex steroids in the mature skeleton post menopause (Manolagas et al., 2002).

1.11.iv Sex steroids

Oestrogens and androgens are present in both male and females though the relative levels of these differ according to sex. Androgens are produced by the adrenal glands as well as the gonads in both sexes and the enzyme aromatase that converts androgens to oestrogens is present in both sexes. The higher levels of oestrogen in females are partly due to aromatase activity but also to direct oestrogen production by the ovaries. The presence of both androgens and oestrogens in both sexes has made it difficult to separate the individual effects of these compounds on skeletal development. However, recent studies of patients with androgen insensitivity syndrome and similar oestrogen insensitivities have enabled a greater understanding of the effects of oestrogen and androgens individually (Frank, 2003).

1.11.v Oestrogen Receptors

ERs are intracellular receptors that are activated upon binding of oestrogens including 17β -oestradiol. They are members of the nuclear receptor family (which also includes the VDR, GCR and androgen receptors - ARs), so called due to their ability to bind directly to DNA and regulate the expression of adjacent genes. These receptors are also therefore described as DNA-binding transcription factors (Evans, 1988, Olefsky, 2001). Activated upon binding of specific ligands, nuclear receptors control expression of target genes at the transcriptional level (Imai et al, 2013). The ER is a type I nuclear receptor located in the cytoplasm of cells (type II nuclear receptors are located in the nucleus). Upon binding of 17β -oestradiol, the receptor moves to the nucleus and undergoes conformational change which in turn allows it to bind specific sequences of DNA known as hormone response elements (HREs) (Mangelsdorf et al., 1995). There are two forms of ER, ER α and ER β which are encoded by the ESR1 and ESR2 genes respectively. Activated ERs dimerise to form homodimers (ER $\alpha\alpha$ or ER $\beta\beta$) or heterodimers (ER $\alpha\beta$) (Li et al., 2004a). Nuclear oestrogen-bound ERs mediate the genomic actions of oestrogens through oestrogen response element (ERE)-dependent transcription of target genes (Carroll and Brown, 2006, Carroll et al., 2005, Carroll et al., 2006). This is in contrast to immediate oestrogen responses defined as non-genomic pathways (Imai et al, 2013}.

1.11.iv Oestrogen and androgens in normal puberty

Oestrogen appears to have two distinct roles during puberty; firstly during the growth spurt, low levels of oestrogen promote skeletal growth and then subsequently high levels during late puberty result in growth plate fusion and cessation of growth (Chagin et al, 2006, Juul 2001). The two nuclear ERs (ER α and ER β) as well as the membrane G-protein coupled GPR30 and the AR are all expressed in the growth plate cartilage during puberty

(Chagin et al, 2007., Kusec et al, 1998., Martensson et al, 2009., Nilsson et al, 1999., Nilsson et al, 2003). There is no difference in receptor expression between the sexes, although GPR30 expression decreases as puberty progresses suggesting that it contributes to longitudinal bone growth rather than growth plate fusion {Nilsson, 2003 #118;Chagin, 2007 #113}. ER α is the main receptor responsible for skeletal growth though the role of ER β is not yet fully understood. Oestrogen also exerts some of its effects indirectly by modulation of the GH/IGF-1 axis (Jansson et al, 1985., Ohlsson et al, 2009., Veldhuis et al, 1997).

1.11.vii Oestrogenic and androgenic effects on the male and female skeleton

In patients with androgen insensitivity syndrome, the peak height velocity is within the normal range (closer to that seen in normal girls than boys), suggesting that oestrogen alone is sufficient to support normal skeletal growth (Zachmann et al, 1986) In contrast, a patient with aromatase deficiency and therefore high levels of androgens and low oestrogens has demonstrated that androgens alone are incapable of producing this effect and it has therefore been concluded that it is oestrogens rather than androgens that are responsible for the pubertal growth spurt and epiphyseal maturation in females (Conte et al, 1994). There is some evidence however that androgens do have a direct effect on the female skeleton (rather than via conversion to oestrogen), which contributes to BMD (Wakley et al, 1991., Goulding et al, 1993).

It had until recently been thought that testosterone is responsible for the growth spurt and epiphyseal maturation in males. However, recent evidence suggests that many of the effects are mediated by secondary aromatisation of androgens to oestrogens and occur via the ERs (Frank, 2003). Androgens, in the absence of oestrogen are capable of sustaining

linear growth but normal epiphyseal maturation and growth plate fusion does not occur (Frank, 2003).

1.11.viii Oestrogen and osteocytes

Overall, oestrogens slow the rate of bone remodelling and help maintain a balance between bone formation and bone resorption. They do this by slowing the production of new osteoblast and osteoclast progenitor cells in the bone marrow and by exerting a pro-apoptotic effect on osteoclasts and an anti-apoptotic effect on osteoblasts and osteocytes (Manolagas et al, 2002., Riggs et al, 2002., Gohel et al, 1999). Osteocytes, including MLO-Y4 cells express all 3 ERs (ER α , ER β and GPR30) and oestrogen has been demonstrated to protect MLO-Y4 cells from apoptosis (Tomkinson et al., 1997, Mann et al., 2007). Blocking of the ERs in mice has demonstrated reduced response to mechanical loading in osteocytes (Aguirre et al, 2007., Damien et al, 1998). Studies on MLO-Y4 cells have shown that oestrogen increased Cx43 expression and intercellular communication via gap junctions, as well as enhanced the mechanosensitivity of osteocytes to mechanical loading (Ren et al, 2013).

1.11.ix Oestrogen and VEGF

Oestrogen is well known to promote angiogenesis and increase VEGF production by many tissues including the pituitary gland (Lawnicka et al, 2013), endometrial tissue (Walter et al, 2010) and the thyroid gland (Kamat et al, 2011). In bone, the effects of oestrogen are less well understood but oestrogen has been shown to up-regulate VEGF expression in growth plate chondrocytes both *in vitro* and *in vivo*. In the same study VEGF protein production increased as puberty progressed, suggesting a link between oestrogens and local VEGF

production in the growth plate (Emons et al.). VEGF levels have been shown to be reduced in the vertebrae of ovariectomised pigs as well as to be up-regulated by oestrogen in osteoblasts (Pufe et al., 2007). In contrast to this study, serum VEGF levels were elevated in ovariectomised mice and this resulted in increased osteoclast formation (Kodama et al, 2004).

Mice with increased angiogenesis due to activation of the HIF α pathway have been shown to be protected from ovariectomy-induced bone loss suggesting that VEGF may be protective against bone loss due to oestrogen deficiency (Zhao et al.). In support of these findings, serum VEGF levels positively correlate with circulating oestrogen concentrations in post-menopausal women. However, there was no correlation between VEGF levels and BMD in this study. There was however, an association between two VEGF gene polymorphisms and lumbar spine BMD (Costa et al, 2009). These results suggest a definite association between oestrogen and angiogenesis during bone turnover and in preserving BMD but the exact relationship requires further understanding and as far as we are aware, there have been no studies to date of the relationship between VEGF and oestrogen in osteocytes.

1.11.x Oestrogen and ON

Although adolescent females appear to be the highest risk group for the development of GC-induced ON, the relationship between oestrogen and ON has not been well studied. A case of bilateral femoral head ON has been reported in a patient with aromatase deficiency and therefore congenital oestrogen deficiency (Balestrieri et al, 2003), and a polymorphism of the aromatase gene has been shown to be predictive of the risk of developing bisphosphonate-induced ON of the jaw (La Ferla et al, 2012). In terms of any therapeutic

interventions, phyto-oestrogenic compounds (plant-derived compounds with oestrogenic activity) have been shown to reduce the risk of steroid induced ON in rabbits and rats (Qin et al, 2008, Bitto et al, 2009).

1.11.xi Selective Oestrogen Receptor Modulators (SERMs)

SERMs is a name given to a family of compounds that bind to ERs and either exert agonistic or antagonistic activity that are tissue-specific (Draper et al, 1996., Yang et al, 1996). The main clinical use of SERMs is in ER +ve breast cancer treatment where they are used as an adjuvant agent to chemotherapy. Until recently tamoxifen was the most common agent used but more recently this has been replaced in many cases by aromatase inhibitors such as anastrozole (Arimidex®). Many of the SERMs including tamoxifen are antagonist in breast tissue but have partial agonistic activity in bone, leading to a protective effect in postmenopausal bone loss. Tamoxifen binds to both ER α and ER β but exerts its partial agonistic effects via ER α . Fulvestrant (ICI-182780, Faslodex®) is a pure antagonist to both ER α and ER β meaning that it has antagonistic effects in all tissues including bone. Its main clinical use is in hormone receptor positive metastatic breast cancer where previous anti-oestrogen therapy has failed.

1.12 Vitamin D

1.12.i Structure and function

The term vitamin D encompasses a family of steroid compounds that exert actions on many tissue types within the body. Over recent years, large epidemiological studies have examined the relationship between vitamin D and many health issues including cardiovascular disease, cancer and psychological diagnoses such as depression with

conflicting results and the definitions of adequate circulating vitamin D levels, insufficiency and deficiency remain controversial. There is currently no evidence from randomised controlled trials that maintaining vitamin D within the accepted “normal” limits, prevents chronic disease (NICE expert opinion).

In humans, vitamin D can be produced from 7-dehydrocholesterol by the action of sunlight in the skin. This vitamin D is then metabolised in the liver to Vitamin D₂ (also known as 25-hydroxy-vitamin D, 25(OH)D₂ or ergocalciferol). In the kidney 25(OH)D₂ is converted to its active form 1,25(OH)₂D₃. Both 25(OH)D₂ and 1,25(OH)₂D₃ are inactivated by the enzyme 24-hydroxylase which is encoded by the CYP24A1 gene. Vitamin D can also be sourced from the diet or supplementation (in either form) (Holick et al, 2007). Dietary sources of vitamin D include oily fish, milk and cereals, the latter of which are fortified.

1.12.ii Serum levels

25(OH)D₂ levels are thought to be the most reliable indicator of vitamin D status and it is this that is routinely measured in clinical settings. Levels of 1,25(OH)₂D₃ show greater variability as they are influenced by many other factors including PTH, calcium and phosphate levels and FGF-23 (Pramyothin, and Holick, 2012).

Currently there is consensus that serum levels of 25(OH)D₂ of less than 25nmol/L (10ng/mL) qualify as “deficient” (NICE expert opinion, Pearce and Cheetham, 2010). There is currently no standard definition of optimal vitamin D levels (Lanham-New et al, #176}, however some sources suggest that levels above 50nmol/L (30ng/mL) are ‘sufficient’, whereas 70–80nmol/L (28-32ng/mL) is ‘optimal’ (Bischoff-Ferrari et al, 2006., Dawson-Hughes et al, 2005). There are UK Department of Health (England, Wales, Scotland and NI) guidelines for the recommended dietary intake of vitamin D for certain groups within the population and those with increased risk of deficiency (pregnant women, people lacking

sun exposure) are recommended to take 10µg daily supplementation. A NICE guideline for the management of vitamin D status in the UK population was completed in 2014.

1.12.iii Vitamin D and bone

Vitamin D increases intestinal calcium absorption and deficiency in adults can lead to osteomalacia and in children to rickets. At the cellular level vitamin D has multiple effects which are exerted via the VDR. The importance of vitamin D in normal bone homeostasis is illustrated by the fact that VDR null mice display mineral and hormonal abnormalities typically seen in osteomalacia and rickets (Erben et al, 2002, Yoshizawa et al, 1997, Van Cromphaut et al, 2001). More specifically vitamin D is responsible for osteoblast control of bone remodelling, increasing bone formation and decreasing resorption (Gardiner et al, 2000), as well as regulating bone and phosphate metabolism in chondrocytes (Masuyama et al, 2006). Vitamin D appears to have different effects depending on the stage of differentiation down the osteoblast to osteocyte phenotype pathway, with more effects of vitamin D seen in early osteoblasts than more mature osteoblasts or osteocytes (Lieben and Carmeliet, 2012).. Studies using targeted deletion of the VDR in mature osteoblasts and osteocytes have revealed that VDR signalling is crucial to suppress bone matrix mineralisation only during episodes of negative calcium balance, in order to maintain normocalcaemia. However, when enough calcium is available from the diet, vitamin D activity in these mature osteoblasts/osteocytes is not essential for normal bone remodelling to occur (Lieben and Carmeliet, 2012). Vitamin D also influences calcium and phosphate homeostasis in osteocytes by stimulating the production of FGF-23 (Ito et al, 2012.).

Vitamin D is also able to stimulate bone resorption during periods of negative calcium balance but it exerts its effects indirectly by increasing osteoblast production of RANKL

rather than by direct actions on osteoclasts (Lieben and Carmeliet, 2012). When there is insufficient calcium intake, there is resultant increase in both circulating $1,25(\text{OH})_2\text{D}_3$ and PTH levels both of which act to stimulate bone resorption. The effects of $1,25(\text{OH})_2\text{D}_3$ on RANKL production by osteoblasts are well studied. Recent evidence suggests that osteocytes (including MLO-Y4 cells) are the predominant source of RANKL during bone remodelling, producing greater than 40 times higher levels of RANKL *in vitro* than osteoblasts (Xiong et al, 2011., Nakashima et al, 2011., Zhao et al., 2002). MLO-Y4 cells when co-cultured *in vitro* with haematopoietic precursor cells have been demonstrated to promote osteoclastogenesis in the absence of vitamin D, but the addition of $1,25(\text{OH})_2\text{D}_3$ enhanced this effect. CM from MLO-Y4 cells however, did not promote osteoclastogenesis suggesting that cell to cell contact is necessary (Zhao et al, 2002). This study showed that MLO-Y4 cells exert their effects on osteoclasts via M-CSF and RANKL production.

1.12.iv Vitamin D and ALL

Reports of vitamin D status at diagnosis in ALL patients vary considerably from normal levels to >80% of patients being vitamin D insufficient (Halton et al., 1995) (Arikoski et al., 1999, van der Sluis et al., 2002). It may be that the wide variation in vitamin D status within the normal population, as previously described accounts for this inconsistency. Levels during therapy for ALL have been less well studied, although one study of 40 patients showed that despite normal vitamin D status as defined by 25-hydroxyvitamin D levels, more than 70% of patients had low circulating levels of $1,25(\text{OH})_2\text{D}_3$ throughout treatment (Halton et al., 1996).

At present it is not routine practice to monitor serum vitamin D levels during ALL therapy or to administer supplementation. Current practice varies according to treatment centre but

overall investigation and treatment is restricted to patients with bony symptoms such as pain or limited mobility or fractures.

Reports of vitamin D status in long term survivors of childhood cancer survivors have demonstrated levels of insufficiency or deficiency that vary from 29-62% and vary according to the initial cancer type (Choudhary et al, 2013, Rosen et al 2013, Sinha et al, 2011). For ALL survivors specifically, 53% were vitamin D insufficient and 12% deficient in vitamin D (Simmons et al, 2011). These levels do not differ significantly from those seen in the healthy population but as these patients often have other long term health implications following chemotherapy (cardiovascular risk, changes in body composition, endocrinopathies etc.), it may be that vitamin D status may play a more significant role that is not yet fully understood.

1.12.v Vitamin D status and GC therapy

Possible interactions between GC therapy and vitamin D status have been proposed with one study showing that GC use was independently associated with 25-OHD₂ deficiency (Skversky et al, 2011.). A recent meta-analysis has demonstrated lower serum 25-OHD₂ levels in adults receiving GC therapy when compared with healthy controls but not when compared to disease matched controls suggesting that it is difficult to distinguish the effects of chronic disease from GC therapy (Davidson et al, 2011.). However this study concluded that the suboptimal concentrations of vitamin D seen in patients on GC therapy are inadequate for prevention of GC-induced osteoporosis. The author recommended that adjustments be made to the guidelines for supplementation to ensure a minimum dose of 1800 IU/day (45 mg/day) vitamin D supplementation be prescribed for adults on GC therapy. There are no similar studies including children on GC therapy. Further evidence

for an interaction between GCs and vitamin D comes from *in vitro* studies demonstrating that dexamethasone treatment of osteoblasts potentiates the transcription of 24-hydroxylase (CYP24A1), the enzyme responsible for metabolism of 25 OHD₃ and 1,25(OH)₂D₃ (Dhawan and Christakos, 2010).

1.13 PTH

1.13.i Structure and function

PTH is produced by the parathyroid glands as a polypeptide containing 84 amino acids. It acts to increase serum calcium levels by indirectly enhancing calcium absorption from the intestines (by increasing the activity of the 1 α -hydroxylase enzyme which converts 25OHD₂ to the active 1,25(OH)₂D₃ increasing tubular reabsorption of calcium via the kidneys and by promoting bone resorption. Secretion of PTH is mainly controlled by serum calcium levels that act via negative feedback. However, PTH release is also stimulated by decreases in serum magnesium or increases in serum phosphate. PTH exerts its effects via the PTH 1 and 2 receptors. PTH 1 receptor is the predominant subtype found in bone. Average serum concentrations of PTH are 10-60 pg/mL.

1.13.ii PTH and bone

There is widespread evidence that the actions of PTH on bone remodelling vary depending on the mode of administration, with continuous PTH therapy having catabolic effects, (as demonstrated in patients with hyperparathyroidism who have increased rates of bone remodelling and overall loss of bone) and intermittent or pulsatile therapy having an anabolic effect (Ishizuya et, 2012., Lombardi et al, 2011., Bellido, et al, 2013). The anabolic effect of intermittent PTH administration has led to daily injections of recombinant PTH (1-

34) (Teriparatide®) being used to treat both post-menopausal as well as GC-induced osteoporosis (Yamamoto et al, 2013., Hashimoto et al, 2007). Studies show that PTH administration results in increases in both cortical and trabecular bone formation (Datta et al, 2012). In particular, PTH stimulates periosteal bone expansion as evidenced by the fact that patients with hyperparathyroidism have 2-3 fold higher rates of periosteal bone formation than controls (Christiansen et al, 1993), and when PTH is given to osteoporotic patients, it increases bone formation in the periosteal areas (Lindsay et al, 2007). PTH also stimulates periosteal bone formation in rodents and rabbits (Kneissel et al, 2001., Hirano et al, 2000) and some studies have suggested that the mechanism may involve IGF-1 signalling (Bikle et al, 2002., Miyakoshi et al, 2001). Despite this evidence, there are some studies showing that PTH does not affect bone formation in the periosteum but instead causes intracortical bone formation (Recker et al, 2009, Burr et al, 2001). The differences in the findings of these studies have yet to be explained, but it is likely that PTH acts via multiple mechanisms.

The mechanism of the anabolic effect of intermittent PTH therapy on osteoblast cells is via several mechanisms, both direct and indirect. Firstly intermittent treatment *in vitro* promotes osteoblast proliferation and differentiation (Hock et al, 1992) as well as reducing osteoblast apoptosis. PTH also inhibits SOST, and thus stimulates the Wnt- β catenin pathway that is central to osteogenesis and bone formation and stimulates IGF-1 production which in turn promotes osteoblast differentiation and survival (Lombardi et al, 2011).

PTH is well recognised to influence the ratio of RANKL: OPG in osteoblasts (Lee and Lorenzo, 1999, Huang et al., 2004). PTH treatment of osteoblasts during different stages of differentiation demonstrates that PTH, when administered for 2 hours out of a 24 hour

cycle, significantly reduces OPG gene expression at all stages of osteoblast differentiation and significantly increases RANKL expression by osteoblasts particularly in the later stages of maturity. PTH also increased osteoclastogenesis when these cells were co-cultured with osteoclast precursors (Huang et al., 2004).

1.13.iii PTH and osteocytes

Evidence that osteocytes express the PTH/PTH-related peptide (PTHrP) receptor was first demonstrated by autoradiography studies showing binding of radiolabeled PTH in osteocytes of growing rats. Less is known about the effects of PTH on osteocytes than osteoblasts though a recent study, using mice that express the PTH receptor exclusively in osteocytes has shown that PTH administration leads to increased cortical bone area and an elevated rate of both periosteal and endocortical bone formation. There was also an increase in intracortical remodelling thought to be mediated via the Wnt pathway (Rhee et al, 2011.). Chronic elevation of PTH in mice has been shown to reduce SOST production by osteocytes, suggesting a potential mechanism for osteocyte control of osteoblastogenesis (Bellido et al, 2005). Recent studies have shown that PTH (as well as Vitamin D) controls FGF-23 secretion by osteocytes and thus influences phosphate metabolism (Bellido et al, 2012.). This is illustrated by the fact that patients with chronic kidney disease and hyperphosphataemia have elevated levels of both FGF-23 and PTH (Yu & White, 2005, Silver et al, 2000).

In relation to bone angiogenesis both PTH (Wang et al, 1996, Schlaeppli et al, 1997., Esbrit et al, 2000) and PTHrP (Isowa et al, 2010) have been shown to increase VEGF secretion by osteoblasts *in vitro* but there have been no such studies in osteocytes.

1.13.iv PTH and ON

Little is known about any role of PTH in the pathogenesis of ON but recently an emerging role has been identified for Teriparatide in the treatment of bisphosphonate-induced ON of the jaw. This role of Teriparatide was first identified following two case reports of its use in ON of the jaw that was unresponsive to conventional therapies. Both cases showed resolution of disease (Lau and Adachi, 2009, Harper and Fung, 2007). The exact mechanism of action is unknown but it thought to be that anabolic actions of Teriparatide counter acts the suppressed bone turnover that is seen in patients with long term bisphosphonate use (Narongroeknawin et al, 2010). Any potential role of PTH therapy in GC-induced ON has yet to be investigated.

1.14 Hypotheses

Based on the evidence and literature outlined above, I hypothesised the following;

1. Dexamethasone may influence areas of osteocyte biology relevant to the pathogenesis of GC-induced ON; specifically osteocyte cell number, mRNA expression and protein secretion of a number of angiogenic factors including VEGF isoforms and VEGFRs as well as IL-6 expression and secretion.
2. $1\alpha(\text{OH})_2\text{D}_3$ may have interactions with GCs and may in fact modulate the effects of dexamethasone on the above, which could provide evidence of a potential therapeutic role for vitamin D in GC-induced ON.
3. There may be differences in response of the MLO-Y4 cells depending on culture environment, namely monolayer vs. 3D collagen gels.
4. Dexamethasone may influence both intracellular VEGF levels as well as that secreted into the medium.
5. Dexamethasone may influence a number of factors involved in the angiogenesis pathway. It is predicted that dexamethasone treatment of MLO-Y4 cells will decrease the expression of pro-angiogenic factors and increase the expression of anti-angiogenic factors.
6. Dexamethasone treatment of osteocyte-like cells may influence expression and secretion of bone-remodelling factors, namely RANKL and OPG.
7. RANKL may have an autocrine effect on osteocytes and influence cell proliferation as measured by cell number and angiogenesis as measured by VEGF gene expression.
8. PTH treatment of MLO-Y4 cells may also influence a number of factors relevant to the pathogenesis of GC-induced ON; specifically osteocyte cell number, mRNA expression and protein secretion of angiogenic factors including VEGF and the

bone remodelling agents RANKL and OPG. PTH may also modulate the effects of dexamethasone in osteocytes.

9. There may be interactions between GCs (dexamethasone) and sex steroids (namely oestradiol, DHEA and testosterone) which could improve understanding of the adolescent female susceptibility to developing ON.

1.15 Aims

In order to investigate the above hypotheses, I have designed experiments with the following aims;

1. To investigate the effects of dexamethasone and/or $1,25(\text{OH})_2\text{D}_3$ on:
 - i. Proliferation of osteocyte cell lines as measured by cell number.
 - ii. Osteocyte cell line VEGF₁₆₄ secretion and VEGF isoform gene expression.
 - iii. IL-6 secretion.
2. To assess whether $1\alpha,25(\text{OH})_2\text{D}_3$ treatment can modulate the effects of dexamethasone on the above.
3. To compare findings between cells treated in monolayer and 3D collagen gels.
4. To compare intracellular VEGF₁₆₄ levels with that secreted into the CM and to assess the effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on intracellular VEGF₁₆₄ levels (Chapter 3).
5. To investigate the MLO-Y4 expression profile of 84 genes involved in angiogenesis, and secondly to assess the effect of dexamethasone treatment on the expression of these genes (Chapter 4)
6. To investigate the effects of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on:
 - i. Osteocyte sRANKL and OPG secretion.
 - ii. Osteocyte RANKL and OPG gene expression.
 - iii. The distribution of RANKL within the osteocyte.
7. To investigate any potential autocrine effect of RANKL in osteocytes and specifically on:
 - i. Osteocyte cell number.

- ii. VEGF gene expression and VEGF₁₆₄ secretion (Chapter 5).
- 8. To investigate the effects of PTH (1-34) on:
 - i. osteocyte-cell line proliferation as measured by cell number
 - ii. osteocyte-cell line VEGF₁₆₄ and IL-6 secretion
 - iii. RANKL and OPG gene expression (Chapter 6).
- 9. To investigate the effects of:
 - i. oestradiol, testosterone and DHEA on osteocyte cell number and VEGF₁₆₄ secretion
 - ii. the addition of ER antagonists (tamoxifen/fulvestrant) or aromatase inhibitor (anastrozole) to the above
 - iii. the addition of dexamethasone to i. and ii. Above
 - iv. dexamethasone and 1,25(OH)₂D₃ on ER expression (Chapter 7).
- 10. To use the above findings to draw conclusions relevant to the pathogenesis of GC-induced ON.

Chapter 2: Materials and Methods

2.1 Cell culture

2.1.i General measures

All cell culture procedures were carried out in Class II Biological Safety Cabinets (BIOQUELL Medical Ltd, Weston Supermare, UK or Microflow Peroxide Class II ABS Cabinet (Andover, Hants, UK) using sterile precautions to prevent microbial contamination. Cells were cultured in a humidified 5% CO₂: 95% air incubator at 37 °C (NuAire Direct Heat DH Auto Flow CO₂ Automatic Air-Jacketed Incubator, Aylesbury, Buckinghamshire, UK) or 33 °C (Hera Cell 240 incubator, Thermo Fisher Scientific, Loughborough, UK). Cell lines were stored in liquid nitrogen in cryovials (Nunc™ Brand Products, Fisher Scientific, Loughborough, UK) prior to use. Penicillin 50 IU and Streptomycin 50 µL/ 100 mL (Life Technologies, Paisley, UK) were added to all media prior to use.

All sterile culture plates and flasks were purchased from Nunc, Thermo Fisher Scientific, Loughborough, UK. All other cell culture materials were purchased from Lonza Biologics PLC, Slough, UK and Life Technologies Ltd, Paisley, UK. One batch of FCS and one batch of newborn calf serum (NCS) were used throughout the study.

2.1.ii Cell storage and handling in vitro

Following removal from liquid nitrogen cells were thawed and transferred into 75 cm² flasks with 10 mL culture medium pre-incubated to 37 °C, which was replaced with fresh medium the following day.

Cells were cultured in flasks and plates coated for at least 1 hour with rat tail type I collagen (BD Biosciences, Oxford, UK) prepared in 0.02 N acetic acid at a concentration of 0.15 mg/mL. This collagen solution was stored at 4 °C and reused a maximum of 20 times. This

coating ensures that the cells maintain their dendritic morphology. Cells were maintained in α -MEM (minimal essential medium alpha) without ribonucleotides and deoxyribonucleotides, containing serum (types and amounts vary according to cell type). Cells were cultured to 80% confluency and used between passages 29 and 50.

Cells were observed regularly using a light microscope (Olympus CK2, Olympus Optical Co Ltd, London, UK) and maintained in 75 mL flasks with 10 mL culture medium. Medium was changed every 2-3 days. When being sub-cultured, cells were initially washed with phosphate buffered saline (PBS) (Life Technologies Ltd) and removed using 1 mL 0.025% Trypsin/0.025% EDTA (Worthington Biochemical Corp. distributed by Lorne Laboratories Ltd, Reading, Berkshire, UK).

In order to set up cells for an experiment, the removed cell suspension was centrifuged for 3 minutes at 1700 rpm at room temperature using a refrigerated centrifuge (Sanyo Harrier 18.80, MSE UK Ltd, London, UK). The supernatant was discarded and cells re-suspended in a known volume of medium, counted with a haemocytometer (see below) and seeded at a known density into 6, 12 or 24 well plates for monolayer and 48 well plates for 3D collagen gels.

2.1.iii MLO-Y4 cell culture

The MLO-Y4 cell line was kindly donated by Professor Lynda Bonewald, University of Missouri, Kansas City, USA. This cell line was isolated from the long bones of 2-week-old transgenic mice, in which the targeted osteocytes expressed SV40 large T-antigen oncogene under the control of the OCN promoter (Kato et al., 1997). MLO-Y4 cells are early osteocytes – they demonstrate satellite morphology and have long dendritic processes (Figure 9). The biological characteristics of MLO-Y4 cells are discussed in further detail in the Introduction (Section 1.7.vi).



Figure 9. MLO-Y4 cell stained with crystallised violet in order to demonstrate the morphological appearance (Rosser and Bonewald, 2011). Reproduced with permission from Springer Science and Business Media

Cells were frozen using 1mL freezing tubes in a Mr Frosty™ Freezing Container (Thermo scientific Ltd) at -80°C in 50% α -MEM, 40% heat inactivated FCS and 10% Dimethyl Sulfoxide (DMSO) (all from Life Technologies Ltd) for 24 hours and then stored in liquid nitrogen until use. Cells were cultured in α -MEM containing 2.5% each of heat inactivated FCS and heat inactivated NCS (both from Life Technologies Ltd), the former of which maintains cell proliferation and the latter, differentiation. Both sera were heat inactivated at 56°C for 30 minutes.

2.1.iv IDG-SW3 cell culture

The IDG-SW3 cell line was also kindly donated by Professor Lynda Bonewald. This cell line was isolated from the long bones of 3-month-old Immortomouse^{+/-}/Dmp1-GFP^{+/-} mice. (Woo et al., 2011). These mice express a thermolabile SV40 large T antigen regulated by interferon- γ (IFN- γ). Cells from these mice can be expanded at 33 °C in the presence of IFN- γ and then allowed to resume their original phenotype at 37 °C in the absence of IFN- γ . IDG-SW3 cells are Dmp1-GFP⁻ and T antigen⁺ under immortalizing conditions but Dmp1-GFP⁺ and T antigen⁻ under osteogenic conditions. Biologically these cells are able to differentiate and therefore express late osteoblast, early osteocyte and late osteocyte gene profiles at different stages of differentiation. The biology of these cells is discussed in more detail in the Introduction (section 1.7.vii).

Cells were frozen at -80 °C in 60% α -MEM, 30% heat-inactivated FCS and 10% DMSO at $1-2 \times 10^6$ cells/mL/vial for 24 hours and then transferred into liquid nitrogen until use.

Cells were cultured in rat tail type 1 collagen coated flasks and plates and maintained in different conditions depending on whether they were proliferating or differentiating (Woo et al., 2011). For proliferation of cells they were maintained in “permissive conditions” in α -MEM with 10% heat inactivated FCS and 50 U/mL Interferon- γ (IFN- γ) at 33 °C in 5% CO₂: 95% air. Cells were cultured to 80% confluency and used between passages 19 and 30. In order to induce differentiation (osteogenesis) cells were transferred at a seeding density of 80000 cells/cm² to a temperature of 37 °C with 50 μ g/mL ascorbic acid and 4 mM β -glycerophosphate in the absence of IFN- γ .

2.1.v Preparation of charcoal stripped medium

In experiments using sex steroids (17 β -oestradiol, DHEA or testosterone), phenol red free α -MEM (Life Technologies Ltd) was used to prevent any oestrogenic effects of phenol red

from influencing results. Any endogenous sex steroids were also removed from the serum by charcoal stripping prior to use. 100mL serum (NCS or FCS) was acidified to pH 4.2 with 5M hydrochloric acid (HCL) and then allowed to equilibrate for 30 minutes at 4 °C. A charcoal solution (18mL distilled water, 2g activated charcoal 250 mesh and 0.01g Dextran T70 – all from Sigma Aldrich Co Ltd) was prepared. 5mL charcoal solution was added to 100mL serum and incubated at 4 °C for 16 hours with gentle agitation. The charcoal was then removed by centrifugation at 12000 *g* for 30 minutes at 4 °C. The pH was then readjusted to 7.2 using sodium hydroxide (NaOH). Prior to use the serum was filtered through a 0.25u Millipore filter to sterilise and remove any tiny remaining charcoal particles. The serum was then stored in aliquots at -20 °C.

2.1.vi Cell lysis

Cells were washed twice with PBS and then lysed (250 µL per well of a 6 well plate) in radioimmunoprecipitation assay (RIPA) buffer which comprised 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCL, pH 7.5, 150 mM sodium chloride (NaCl), 1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxycholate. A protease inhibitor cocktail (chymostatin, leupeptin, antipain and pepstatin all at 10 µg/mL) was added to the buffer immediately prior to use. Plates containing lysates were stored overnight at -80 °C and lysates were then transferred into 1.5 mL centrifuge tubes for ELISA analysis as described in section 2.4. Lysates were clarified by centrifugation (3 minutes at 6500G) and protein levels determined using the Bio-Rad protein assay (Bio-Rad laboratories LTD, Hertfordshire, UK) as described in section 2.5.

2.2 Culture of MLO-Y4 cells in 3D collagen gels

The collagen gels containing cells were prepared by Nicole Scully, a PhD student in the laboratory. Rat tail tendon type 1 collagen (Sigma-Aldrich Co Ltd) was used at a concentration of 2.5 mg/mL in 7mM acetic acid. The solution was placed on a roller mixer for at least 3 hours prior to use to aid reconstitution. To set up the cells in 2mg/mL collagen gels, equal volumes of 10 X MEM (Sigma-Aldrich Company Ltd) and sodium bicarbonate (NaHCO_3) were mixed on ice and the pH adjusted to 7.0 using 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris Base, pH 11.5; 1M). MLO-Y4 cells (375,000/mL) were then added to the collagen solution and evenly dispersed. 250 μL of the gel solution containing the cells was then added to each well in a 48 well plate and placed at 37°C in 5% CO_2 : 95% air. After 30 minutes, 750 μL medium was added gently to the top of each gel. The plate was then returned to the incubator and cells were treated with various experimental agents after a further 24 hours.

2.3 Assessment of cell number

2.3.i Cell counting using a haemocytometer

In order to determine the seeding density of cells, the cell number in suspension was counted using a haemocytometer (Camlab Ltd, Cambridge, UK). Cell suspensions were diluted in a known volume of medium to give 10-50 cells per square of the haemocytometer chamber. 8 μL was transferred into each chamber of the haemocytometer. The central and 4 outer squares were counted on both chambers of the haemocytometer giving a total of 10 counted squares, using x100 magnification. The mean cell number was calculated as follows:

Cell number per ml =

Mean cell count x total cell suspension volume (ml) x 10^4 (haemocytometer factor)

For all experiments cells were seeded in 2 mL medium per well for 6 well plates, 1 mL for 12 well plates, 500 μ L for 24 well plates and 100 μ L for 96 well plates. The outer wells of 96 well plates were not seeded and instead contained medium only to prevent any change in the volume of medium that occur by evaporation and thus lead to inaccuracy of results.

When seeding cells into multi-well plates, cells were aliquoted randomly and a multichannel pipette was used for 96 well plates.

2.3.ii Promega cell number assay

MLO-Y4 cells were seeded (4.6×10^3 cells/cm²) in 96-well culture plates and after 24 hours treated with various experimental treatments. Cell number was determined after 72 hours of treatment using the Celltiter96® AQueous One Solution Cell Proliferation assay (Promega, Southampton, UK). This is a quantitative colorimetric assay which was developed in 1983 by Mosmann. (Mosmann, 1983) This assay will detect viable but not dead cells and it is used for studying cellular growth and survival. The viable cells are detected using a tetrazolium compound [3-(4,5-diamethylthiazol-2-yl) – 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; MTS] and an intermediate electron acceptor; (phenazine ethosulfate; PES). MTS and PES are supplied as a single solution and form an enhanced stable compound. In metabolically viable cells, the MTS is reduced to a water soluble formazan compound by nicotinamide adenine dinucleotide phosphate (NADP) or its reduced form NADPH which are mitochondrial dehydrogenase enzymes. (Ganguly et al., 2006, Marshall et al., 1995, Cory et al., 1991) The dye produced has maximal absorbance at 490-500 nm and the optical density (OD) is

directly proportional to the viable cell number and time period of incubation. (Cory et al., 1991)

All cell number assays were undertaken in 96 well plates. MTS solution (stored in aliquots at -20 °C) were protected from light and brought to room temperature. 20 µL of this solution was added to each well containing cells and 3 wells where there were no cells to act as blanks. Following addition of the MTS, the plates were covered in foil to protect from light and transferred into the incubator at 37°C. Absorbance at 490 nm was measured after 2 hours incubation using a Spectracount™ Microplate Photometer (Packard Instrument Company LTD, Cambridge, UK) or MRX^{TC} Revelation (Dynex Technologies LTD, Worthing, UK).

2.3.iii Cell counting using Trypan blue

For cells cultured in 3D collagen gels a different method of assessing cell viability was used. This technique allows assessment of whether cells counted are alive or dead and therefore % cell viability can be determined. The trypan blue test is an “exclusion test” of cell viability. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes including trypan blue. The cell suspension is mixed with dye and then visually examined to determine whether cells take up or exclude the dye. When examined using a light microscope a viable cell will have a clear cytoplasm where as a no- viable cell will appear blue. {Strober, 2001}. For this method the collagen gels were digested using 1mg/mL collagenase (Sigma-Aldrich Co Ltd) at 37 °C in a water bath until the collagen dissolved (approximately 10 minutes). Cells were then centrifuged (1700G, ALC micro centrifuge 4204) briefly to form a pellet, washed in PBS to remove the collagenase and resuspended in 1 mL medium. An aliquot of 20 µL was removed and 2 µL trypan blue added before cells were counted using a haemocytometer as outlined above. Live and dead cells were counted separately and then the % of live (or viable) cells was calculated as follows:

$$(\text{Mean live cell count} / \text{Mean total cell count}) \times 100$$

2.4 Enzyme linked Immunosorbant assay (ELISA)

ELISA is a test that uses enzymes and a colour change to detect specific substances. In general, there are three parameters to the test; a solid phase (usually a plastic microtiter plate) in which an antibody against the antigen to be measured is adsorbed and then a number of bound and free reagents are added in turn. Results are obtained via development of colour, the intensity of which can be measured and used to quantify the amount of substance present. There are 3 main ELISA types; Direct, Indirect and Sandwich ELISA. The ELISA method used in this thesis is a sandwich method (Gaastra, 1984, Crowther, 2000).

2.4.i VEGF ELISA

The DuoSet® mouse VEGF₁₆₄ ELISA Kits (R&D Systems Europe Ltd, Abingdon, UK) provide all the reagents for this test unless otherwise stated. All reagents were reconstituted to their working concentration and stored in aliquots at -80 °C as recommended by the supplier. The streptavidin-horse radish peroxidase (HRP), substrate and stop solutions were stored at 2-8 °C. All reagents were brought to room temperature prior to use and the working dilutions were made and used immediately. The capture antibody and Tween®20 were both diluted in sterile PBS. All other reagents were diluted in reagent diluent (RD; 1% bovine serum albumin (BSA) Sigma Aldrich Co Ltd) in PBS. All of the incubation steps were carried out at room temperature and all steps apart from the addition of the substrate solution to determine the colour reaction at the end of the experiment were followed by a washing step. Washing was carried out thoroughly using a washing buffer (Tween®20

0.05%, Fisher Scientific, Loughborough, UK). Each well was washed with a squeeze bottle a total of 3 times, with complete removal of all liquid by blotting on clean towel followed by aspiration of residual bubbles in between each wash.

A 96 well ELISA plate (Nunc, Thermo Fisher Scientific, Loughborough, UK) was initially coated with capture antibody (CA) (100 μ L/well) at a concentration of 0.4 μ g/mL in PBS. The plate was then covered with an adhesive strip and left overnight. The plate was then blocked by adding 300 μ L RD for 1 hour. 100 μ L of samples or standards were then added to the wells, covered and incubated for 2 hours. A seven-point standard curve was created using serial dilutions. The highest standard was 1000 pg/mL which contained 95 ng/mL of recombinant mouse VEGF₁₆₄. Two wells for each point of the standard curve were prepared. RD was used to fill two wells and used as blanks. The next step was to add 100 μ L detection antibody (DA), which was reconstituted to a working concentration of 100ng/mL. The plate was again covered and incubated for 2 hours. 100 μ L Streptavidin-HRP was then added and the plate covered and protected from light while being incubated for 20 minutes. A substrate solution was prepared using an equal mixture of colour reagent A (H₂O₂) and colour reagent B (Tetramethylbenzidine) and 100 μ L added to each well, protected from light and incubated for 20 minutes. The reaction was stopped using stop solution (2N sulphuric acid), which was added at 50 μ L/well. The plate was tapped gently to ensure thorough mixing and then read at 450 nM using a microplate reader. The resulting OD was measured at 450nm and then again at 540 nm, with the latter reading being subtracted from that obtained at 450 nm in order to correct any optical imperfections in the plate.

The standard curve was created using I-SMART software (Canberra Packard Ltd, Berkshire, UK) which reduces the data by generating a four parametric logistic (4-PL) curve fit. This produces a curve of best fit for the given data and by analysis regression it linearises the

mean absorbance by plotting the log of VEGF concentrations versus the log of the OD.

Blank values were subtracted from all samples.

2.4.ii IL-6 ELISA

DuoSet® mouse IL-6 ELISA kit (R&D systems) was used and the methodology was identical to the VEGF ELISA described above in terms of plate preparation, protocol steps, washing procedures, duration of incubations as well as the reading and interpretation of results.

The working concentration of CA was 2.0 µg/mL and the DA 400 ng/mL. The standard curve was created using 70 ng/mL recombinant mouse IL-6 and the top standard was again 1000 pg/mL.

2.4.iii RANKL ELISA

Quantikine® mouse RANKL ELISA (R&D Systems) kit was used. This kit contains a single pre-coated microplate, mouse RANKL conjugate (a polyclonal antibody against mouse RANKL conjugated to hRP), a mouse RANKL standard (10 ng lyophilised recombinant mouse RANKL), assay diluent (a buffered protein solution), calibrator diluent (a buffered protein solution), wash buffer concentrate (a 25-fold concentrated solution of buffered surfactant), colour reagents A (hydrogen peroxide) and B (chromogen/tetramethylbenzidine) and stop solution (hydrochloric acid solution). All reagents were stored at 2-8 °C apart from the reconstituted standard which is aliquoted and stored at -20 °C.

This method also employed the quantitative sandwich ELISA technique but differs from the DuoSet® in that an affinity purified polyclonal antibody specific for mouse RANKL has been pre-coated on to a microplate. A 7 point standard curve was created using serial dilutions as described above using a top standard of 2000 pg/mL recombinant mouse RANKL reconstituted in calibrator diluent. Calibrator diluent was used as the 0 pg/mL standard. 50 µL assay diluent was added to each well. 50 µL standards or samples were then pipetted

directly into the wells containing the assay diluent and any mouse RANKL present was bound by the pre-bound antibody. The plate was then covered and incubated at room temperature for 2 hours. The wells were then washed a total of 5 times using a squirt bottle with wash solution diluted 25-fold in distilled water. After each wash the wells were blotted and aspirated as described previously for the VEGF ELISA. 100 μ L mouse RANKL conjugate was then added to each well and the plate again covered and incubated at room temperature for 2 hours. The plate was then washed as described above and 100 μ L substrate solution (equal quantities of colour reagents A and B) were added to each well, the plate was then covered to protect from light and incubated for 30 minutes. 100 μ L stop solution was then added to each well and the optical density determined by reading at 450 nm and 540 nm as described for the VEGF ELISA.

2.4.iv OPG ELISA

The Quantikine® mouse OPG ELISA kit (R&D systems) was used for these assays. The kit contents, storage and methodology are identical to the Quantikine® mouse RANKL kit but in this assay the pre-coated and conjugated antibodies are specific to mouse OPG. A seven point standard curve was created using 4 ng lyophilised recombinant mouse OPG diluted in calibrator diluent to a top standard of 2000 pg/mL.

2.5 Protein assay

All VEGF, IL-6, RANKL and OPG ELISA values were corrected for cellular protein concentrations in order to eliminate any effect that changes in cell number may have.

2.5.i Collection of cell protein

Following removal and storage of medium from 24 well plates, cells were washed with PBS and then fixed with 300 μ L of 100% methanol (Fisher Scientific, Loughborough, UK) for 1 minute. The methanol was then removed and cells allowed to dry at room temperature before the addition of 300 μ L of 0.5 M NaOH, (Sigma-Aldrich Company Ltd). The plates were then sealed with autoclave tape and stored at -20 °C. Prior to performing the protein assay the plates were thawed and the NaOH containing the dissolved protein was removed from each well by thorough scraping and pipetting.

2.5.ii Quantification of cell protein

In order to quantify the cellular protein content, a Bio-Rad protein assay (Bio-Rad Laboratories Ltd, Hertfordshire, UK) was used. This is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein (Bradford, 1976). The basis of the assay is that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs (Reisner et al., 1975, Sedmak and Grossberg, 1977). There is a resulting spectral change of dye from reddish brown to blue and the change in the reagents colour is directly proportional to the amount of protein present. The advantages of this test are that it is easy to perform and highly sensitive (it can detect less than 1 μ g of albumin) and the adsorption of the solution formed is stable at room temperature for 1 hour.

To carry out the assay, a standard curve (2.5-20 μ g/mL) was prepared using BSA dissolved in distilled water. All standards, blanks and samples were prepared in a total volume of 800 μ L using distilled water, in polypropylene test tubes. 50-100 μ L of each sample was used to ensure that values were within the threshold of the standard curve. 200 μ L of Bio-Rad reagent was added to each tube and then mixed thoroughly. After 10 minutes the optical

density was measured at 595 nm using a GENESYS 10UV (ThermoSpectronic, Madison, USA) spectrophotometer. Distilled water was used for the blank values and the total protein concentration in each sample was determined using the standard curve.

2.6 RANKL Immunohistochemistry

In this experiment MLO-Y4 cells were plated onto cover slips in 24 well plates. After 72 hours of incubation with or without $1,25(\text{OH})_2\text{D}_3$ and dexamethasone, RANKL content of the cells was qualitatively assessed using immunohistochemistry.

Immunohistochemistry is an umbrella term that encompasses many methods used to determine tissue constituents (antigens – in this case, mouse RANKL) with the use of specific antibodies that can be visualised through staining (Brandtzaeg, 1998, Haines and West, 2005). This method employs the Avidin-Biotin Complex (ABC) technique and uses an immunoperoxidase system which is highly sensitive due to the fact that the avidin-biotin complex allows irreversible antibody binding (Matos et al., 2010). The general procedure for staining involves three steps; firstly an unlabelled primary antibody is applied, followed by biotinylated secondary antibody and then a preformed avidin and biotinylated HRP macromolecular complex. The HRP is visualised by the development of a peroxidase substrate that produces a colour.

For this assay, the following items were used; 1) Anti-RANKL antibody (Abcam Plc, Cambridge, UK) 0.5mg/mL. This antibody was aliquoted and stored at $-20\text{ }^{\circ}\text{C}$ as per company instructions. 2) Vectastain® Universal Elite® ABC kit (Vector Laboratories Ltd, Peterborough, UK), which contains blocking serum (normal horse serum), biotinylated secondary antibody (1% anti mouse IgG in normal horse serum) and Vectastain® Elite® ABC reagents A (Avidin DH) and B (Biotinylated enzyme). 3) DAB peroxidase substrate kit (Vector Laboratories Ltd) which contains 3, 3'-diaminobenzidine (DAB), a reagent which turns a

brown colour allowing visualisation of the substrate being investigated. The kit contains a buffer stock solution, DAB stock solution and a hydrogen peroxide solution. All of the reagents were made up in wash buffer (0.1% Tween®20 in PBS) and each step carried out at room temperature unless stated otherwise. A 5-minute washing step using wash buffer was carried out prior to each incubation. Mouse IgG1 was used as the negative control.

For the experiment, medium was removed and the cells washed in PBS and fixed with 500 µL 4% formaldehyde for 10 minutes. The formaldehyde was then removed, the cells again washed with PBS and the cover slips removed from the wells and placed on a glass slide.

After the cover slips were drawn around with a hydrophobic marker to aid washing a series of washes was then carried out using a glass dropper as follows. Wash buffer for 5 minutes, 0.3% H₂O₂ in wash buffer for 5 minutes and then wash buffer again for 5 minutes. Each washing reagent was removed by gentle tipping of the slides and blotting. Slides were then incubated overnight at 2-8 °C with anti-RANKL antibody (5 and 10 µg/mL in wash buffer) or negative control (mouse IgG1 at the same concentration). The following day, the antibodies were removed and slides were incubated for 20 minutes with diluted blocking serum (50 µL stock in 5mL wash buffer), followed by 30 minutes with diluted biotinylated secondary antibody (100 µL of stock normal blocking serum and 100 µL biotinylated secondary antibody stock in 5 mL wash buffer) and a further 30 minutes with diluted Vectastain® Elite® ABC reagent (2 drops of reagent A and 2 drops of reagent B added to 5 mL wash buffer followed by immediate mixing and allowed to stand for 30 minutes prior to use). The DAB substrate solution was then made using thorough mixing in 5 mL distilled water of 2 drops of the buffer stock, 4 drops of DAB stock and 2 drops of H₂O₂ solutions provided in the kit. Slides were then incubated in this solution until the desired staining intensity was reached (approximately 10 minutes). Slides were washed in tap water and counterstained for 1 minute with Mayer's Haematoxylin. A further wash in distilled water was carried out followed by dehydration of the slides by placing them twice into 70% followed by 95% and

then 100% ethanol. Cover slips were then mounted to the slide using Vectamount, allowed to air dry overnight and then imaged using an bright field microscope (Olympus Provis ax70 with an Olympus U-RFL-T burner). Any differences in the amounts of RANKL present and its distribution within the osteocyte cells between treatment groups was assessed qualitatively by comparing colour intensity.

2.7 RNA extraction

RNA was extracted from MLO-Y4 cells using two methods and the RNA yield and purity was compared. The initial technique used TRI-reagent® and the second used a binding column, the GenElute™ Mammalian Total RNA Miniprep kit (both from Sigma-Aldrich Company Ltd). Higher RNA yields without any significant difference in purity was obtained using TRI reagent® and therefore this methodology was adopted for the majority of the experiments.

2.7.i RNA extraction using TRI-reagent®

TRI-reagent® is a single step total RNA isolation reagent similar to that first developed by Chomczynski in 1987 (Chomczynski and Sacchi, 1987). This method is widely used for isolating RNA from a variety of different sources (Chomczynski and Mackey, 1995). The principle of the method is that RNA is separated from DNA after extraction with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform, followed by centrifugation. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol (Chomczynski and Sacchi, 2006).

For isolation of RNA from cells in a 6 well plate the methodology was as follows. All incubations took place at room temperature unless stated otherwise. Medium was removed and 1 mL TRI-reagent® was added to each well, pipetted up and down several

times to improve cell lysis and incubated for 5 minutes. If the full RNA extraction procedure could not be carried out immediately, lysed cells were stored at this stage in TRI-reagent at -80°C for up to 1 month. The lysed cells were then transferred into 1.5 mL tubes and 200 µL chloroform (Fisher Scientific Ltd) was added. The contents of the tube were shaken vigorously for 15 seconds and then incubated for 10 minutes followed by centrifugation at 12,000 G in a MSE microcentaur (MSE UK Ltd, London, UK) or an ALC® micro-centrifuge 4204 (Camlab Ltd, Cambridge, UK) mini centrifuge for 15 minutes. The top aqueous layer (containing the RNA) was then carefully transferred to a new 1.5mL tube, with the interphase and lower layer being discarded. 2 µL glycogen (Life Technologies Ltd) was added to each tube followed by 500 µL of isopropanol (Fisher Scientific Ltd), the contents mixed by inversion 10 times and incubated for 10 minutes to allow precipitation of RNA. The tubes were then centrifuged at 12000 *g* as above for 10 minutes following which the pellet of RNA could be visualised at the bottom of the tube. The supernatant was discarded and the pellet washed in 1 ml 75% nuclease- free ethanol. Following a further 5 minute centrifugation the ethanol was removed and the pellet allowed to air dry for a maximum of 10 minutes. The pellet was then dissolved in 25 µL nuclease-free water (Life technologies Ltd) and incubated at 55 °C in a water bath for 10 minutes to aid dissolving. RNA could be stored at this stage at -80 °C. For other size multi-well plates the methodology was identical to that described above but the following modifications to reagent volumes was made.

Plate size	TRI-reagent® (µL)	Chloroform(µL)	Isopropanol(µL)	75% ethanol(µL)
12 well	400	80	200	400
24 well	200	40	100	200
48 well	100	20	50	100
96 well	35	7	17.5	35

Prior to use the RNA was re-precipitated using sodium acetate to improve purity and removal of any contaminating DNA was carried out using a DNA-free™ kit (Life technologies Ltd), the methodologies of which are outlined below.

2.7.ii RNA extraction using the GenElute™ Columns

This method of RNA extraction uses a silica based column system to extract RNA, which comprises a number of centrifugation steps using a microcentrifuge tube with an in-built silica membrane (Ausubel, 1995, Farrell, 1998). Cells are lysed and homogenised in a buffer containing guanidine thiocyanate which ensures thorough denaturation of macromolecules and inactivation of RNases. The addition of ethanol causes RNA to bind when the lysate is spun through the silica membrane. An elution solution is then used to elute the RNA, ready for use. The main advantage to column extraction over TRI-reagent® is that the use of hazardous organic compounds such as phenol and chloroform is avoided.

For RNA used in gene arrays the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Company Ltd) was used. This kit contains a lysis solution, 2-mercaptoethanol (2-ME), wash solution 1 and wash solution 2 concentrate, elution solution, GenElute™ Filtration Columns in tubes, GenElute™ Binding Columns in tubes and collection tubes. All reagents were stored and steps carried out at room temperature. All centrifugation steps were carried out in an ALC® micro-centrifuge 4204 (Camlab Ltd, Cambridge, UK) mini centrifuge at 12,000 *g*.

The initial stage of extraction was to prepare the lysis solution. 250µL of lysis solution (10 µL 2-ME added to 1 mL lysis solution) was required for each well of a plate (this volume does not vary according to well size unless greater than 5×10^6 cells are present which was never the case in this work). Medium was removed from the cells and 250 µL lysis solution was added. The plate was rocked and tapped for a few seconds to ensure all the cells were

covered and then incubated for 2 minutes to allow cell lysis to occur. The lysate was then transferred into a 1.5 mL tube and 250 μ L 70% nuclease-free ethanol was added followed by thorough vortexing to mix. The lysate/ethanol mixture was then loaded into the GenElute™ binding column and centrifuged for 15 seconds. The flow through liquid was discarded and the first column wash performed by adding 500 μ L Wash Solution 1 into the column and centrifuging for a further 15 seconds. The binding column was then transferred into a new clean collection tube and the second column wash performed. Wash solution 2 concentrate (2.5 mL) was diluted with 10 mL 100% ethanol prior to use. 500 μ L of this ethanol containing wash solution 2 was transferred to the binding column and centrifuged for 15 seconds. This step was then repeated to complete the 3rd column wash but the column was centrifuged for 2 minutes to ensure it is fully dry. A further 1 minute centrifugation was carried out if any residual wash solution could be visualised on the column. The final step was to elute the RNA. The binding column was transferred into a clean collection tube and 50 μ L elution solution was added. This was centrifuged for 1 minute and purified RNA was present in the flow through eluate. RNA was stored at -80 °C until used. Prior to use, RNA was treated with the DNA-free™ kit (Life Technologies Ltd) as outlined below.

2.7.iii Precipitation of RNA using sodium acetate

In order to improve the purity of RNA extracted using TRI-reagent, re-precipitation with sodium acetate was carried out prior to DNase treatment. 3 M sodium acetate was added to the RNA to a final concentration of 0.3 M (for example 2.5 μ L 3 M sodium acetate was added to 25 μ L RNA). 2.5 volumes of 95% nuclease-free ethanol was then added along with 1 μ L glycogen. This solution was then stored at -20 °C for at least 30 minutes and then centrifuged at 12000 *g* in a microcentrifuge for 15 minutes. The supernatant was discarded and the RNA pellet washed with ice cold 75% nuclease-free ethanol to remove any residual

salt. This ethanol was then removed and the pellet re-dissolved in 25 μL nuclease-free water by incubation at 55°C in a water bath for 10 minutes.

2.7.iv DNase treatment of RNA to remove any residual contaminating DNA

Prior to use of RNA for qRT-PCR, residual contaminating DNA should be removed in order to improve results (Bustin, 2002). For this purpose the Ambion® DNA-free™ kit (Life Technologies Ltd) was used. This kit digests DNA to below the level of detection by qPCR and also removes divalent cations such as calcium and magnesium which can catalyse RNA degradation. The kit contains a 10 x DNase 1 buffer (100 mM tris-HCl pH 7.5, 25 mM MgCl_2 , 5 mM CaCl_2), DNase inactivation reagent and rDNase I enzyme (2 Units/ μL). All reagents were stored at -20 °C and thawed on ice prior to use.

For the method, 0.1 volume of 10x DNase 1 buffer and 1 μL rDNase 1 were added to the RNA sample and mixed gently. The mixture was incubated at 37 °C for 40 minutes using a Thermal cycler (DNA Thermal Cycler 480, PerkinElmer, Cambridge, UK). 5 μL DNase inactivation reagent was added and mixed 2-3 times during incubation at room temperature for 2 minutes. The mixture was centrifuged at 12,000 g using a micro-centrifuge for 2 minutes and the purified RNA transferred into a separate tube. This purified RNA was then ready for purity analysis.

2.7.v Measuring the purity of extracted RNA

Real-time quantitative PCR (qRT-PCR) has become the benchmark for detection and quantification of nucleic acids in both research and diagnostic settings. However, a number of factors including poor assay design and inappropriate data analysis lead to the concern that published results using qRT-PCR may be inconsistent, inaccurate and sometimes misleading (Bustin, 2010). This has led to the development of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines which must

be adhered to in order to ensure that any results produced using qRT-PCR are reliable and robust (Bustin et al., 2009). These guidelines state that it is essential for nucleic acid quantification and DNA contamination data to be provided with any submitted manuscript and that purity data (A_{260}/A_{280}) is desirable (Bustin et al., 2009).

The RNA sample purity was assessed using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific Ltd). This uses light absorbance to provide information on sample concentration, purity ratios and full spectral data using 1 μ L of RNA sample. Absorbance measurements made include the absorbance of all molecules within the sample that absorb at the wavelength of interest. Since nucleotides, RNA, ssDNA and dsDNA all absorb at 260 nm, they will contribute to the total absorbance of the sample which is why the purification techniques described above are carried out.

The ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is used to assess the purity of RNA. A ratio of ~ 2.0 is accepted as pure for RNA. If the ratio is lower, it may indicate the presence of protein, phenol or other contaminants that absorb at or near to 280 nm. A secondary measure of purity is the A_{260}/A_{230} ratio which in pure RNA is usually higher than the A_{260}/A_{280} at ~ 2.0 - 2.2 . Lower values than this may indicate contamination with substances such as phenol or carbohydrates.

Prior to use in qRT-PCR purity of all RNA samples was checked using the Nanodrop™ 1000 and samples considered to be impure based on the A_{260}/A_{280} and A_{260}/A_{230} ratios were discarded. The Nanodrop™ reading of RNA quantity (measured in ng/ μ L) was used to calculate the volume of RNA to use in the Reverse Transcription (RT) assay outlined below.

2.8 Reverse transcription of RNA to cDNA

RT reaction, also known as first strand complementary (cDNA) synthesis is a process by which single-stranded RNA is reverse transcribed into cDNA by using RNA, a RT enzyme, a primer and an RNase inhibitor. The resulting cDNA can be used in a qRT-PCR reaction.

Three types of primer can be used for RT reactions; Oligo (dT) primers, random (hexamer) primers and gene specific primers. The methodology employed in this thesis used Oligo (dT) primers. The general principles for an RT reaction are that 200 ng-2 µg RNA are used depending on the individual protocol, RNA is denatured by heating and then added to a cocktail containing the other reagents. The reaction then takes place at 42°C over the course of approximately 1 hour followed by rapid heating to inactivate the enzyme (DPProtocol, 2013).

The following reagents were used for 20 µL reactions (all from Promega Ltd. Southampton, UK); Maloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) enzyme, 5x M-MLV RT Reaction buffer, Oligo (dT) primer, RNasin® Plus RNase inhibitor, 10 nM DNTP mix (containing 10 nM dATP, 10 nM dCTP, 10 nM dGTP and 10 nM dTTP). 500 ng RNA was used in each reaction, the volume of which was calculated using the Nanodrop® reading described in section 2.7.v. Nuclease free water was used to make the total volume to 20 µL. A “no RT” control reaction was also made for each treatment group tested. In this reaction all reagents were included apart from the M-MLV RT enzyme, the volume of which was replaced with nuclease free water. This no RT reaction acts as a purity control, as any amplification seen when qRT-PCR is performed will be from contaminating genomic DNA rather than cDNA. Table 4 outlines the quantities of each reagent used in each RT reaction.

Reagent	Quantity per reaction (μL)
5X M-MLV RT buffer	4
Oligo (dT)	0.2
DNTP mix	1
RNasin® plus	0.2
5X M-MLV RT	0.5 (replaced with water in no RT reaction)
RNA	500 ng (volume calculated from Nanodrop® reading)
Nuclease free water	To make total of 20 μL

Table 4. RT reaction reagents

For the procedure, the RNA was initially denatured by heating to 65 °C for 10 minutes in a DNA Thermal cycler 480 (PerkinElmer, UK). During this time the above RT reaction “master mixture” was prepared and aliquoted into the desired number of reaction tubes. The denatured RNA was added to the mixture and incubated at 42 °C for 1 hour, followed by rapid heating to 95 °C For 1 minute to stop the reaction. The cDNA samples obtained were kept on ice for immediate use or stored at -20 °C.

2.9 qRT-PCR.

2.9.i General Principles

qRT-PCR is a method used for quantifying gene expression in cells and tissues and has been used extensively to measure biological responses to various stimuli (Heid et al, 1996., Bieche et al, 1998., Funato et al, 1998). It is a widely used tool in both the research and clinical settings as a method for amplifying nucleic acid from samples as small as 1 cell (Heid et al, 1996). There have been several methods developed for qPCR (Kellogg et al, 1990.,Heid et al, 1996) but essentially all involve the amplification of specific sequences within a DNA or cDNA template using specific oligonucleotides, heat stable DNA

polymerase and thermal cycling. In traditional (end point) PCR, detection and quantification of the amplified sequence are performed at the end of the reaction, after the last PCR cycle and involve post PCR analysis such as gel electrophoresis in order to quantify. The advantage of real time PCR is that the quantity of PCR product is measured at each cycle using fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated. This allows highly accurate quantification of the amount of starting material in samples. These fluorescent markers may be double stranded DNA (dsDNA) -binding dyes or dye molecules attached to primers or probes that hybridise with the PCR product during amplification. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning. By plotting fluorescence against the cycle number, the qPCR instrument generates an amplification plot which represents the accumulation of PCR product over the course of the reaction. The cycle number at which the PCR product is first detected is known as the C_t (threshold cycle) value which is inversely proportional to the starting amount of target and therefore allows comparison of gene expression between samples. For example a starting sample with twice the amount of target DNA will have a C_t value one cycle earlier compared to another containing half the amount. Essentially therefore, a difference of 1 C_t value between samples represents a doubling of the target DNA sequence. Figure 10 shows an example of amplification plots from serial dilutions of a target sample

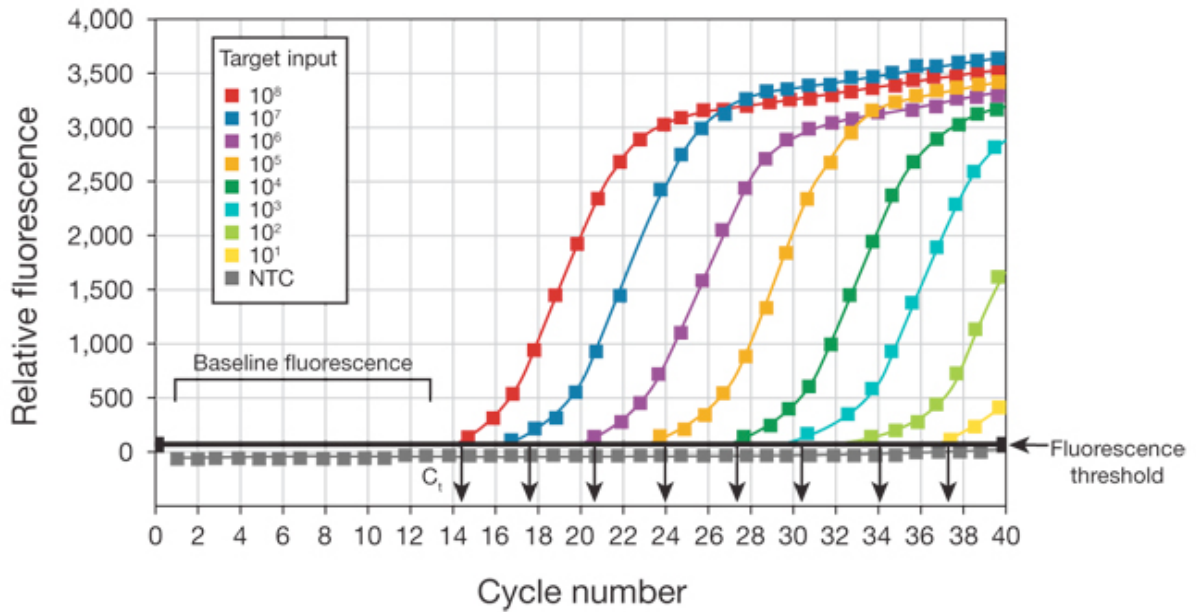


Figure 10. Relative fluorescence vs. cycle number and formation of an amplification plot.

2.9.ii Overview of qRT-PCR procedure

Using sequence-specific primers, qRT-PCR allows the number of copies of a particular DNA sequence to be determined. There are three major steps that make up the reaction and although specific conditions such as temperature and number of cycles may vary according to the enzymes and instruments used, the basic principles of each step are as follows;

1. **Denaturation.** In this step high temperature incubation is used to denature the double stranded DNA structure into single strands. The highest temperature that the DNA polymerase enzyme can withstand (usually 95°C) is used.
2. **Annealing.** During this step, complimentary sequences are able to hybridise which allows the primers to bind to their position on the DNA template. The temperature for this step is based on the melting temperature of the primers.
3. **Extension.** This step allows optimal activity of DNA polymerase and primer extension occurs.

2.9.iii Primer design

Optimal primer design is required to ensure good quality qRT-PCR results. A variety of online primer design tools exist and primer sequences used in this MD project were either obtained from previously published work or designed using the Primer-BLAST (Primer3 and BLAST) online tool from the National Center for Biotechnology Information (NCBI). General principles for good primer design (Bustin et al., 2009) were adhered to. These include ensuring that the amplicon length is between 50-200 base pairs in length as longer products do not amplify as efficiently, designing primers that are 18-24 nucleotides in length which allows for practical annealing temperatures and ensuring that the sequences only recognise the target of interest. The latter was done by performing a BLAST search of the sequences against public data bases, to ensure no other targets are identified. Forward and reverse primers that have compatible melting temperature (within 5°C) and contain approximately 50% GC content were chosen, in order to try and prevent the formation of primer dimers (where complementary primer sequences hybridise to each other). Primers were also designed so that they anneal to exons on both sides of an intron (or span an exon/exon boundary of the mRNA) to allow differentiation between amplification of cDNA and potential contaminating genomic DNA by melting curve analysis.

2.9.iv Primer Optimisation

In order to optimise the efficiency of the qRT-PCR reaction two primer optimisation techniques were utilised. The first is to test varying concentrations of primers and choose the most efficient forward and reverse primer concentration combination. For this method combinations of forward and reverse primers were tested at 50, 100, 400 and 900 nM. The target cDNA used for primer optimisation was a known positive control (either MLO-Y4 cell cDNA or cDNA obtained from a mouse femur). For all primers tested the 400nM

concentration of both forward and reverse sequences was found to be the most effective as determined by C_t value and optimal amplification curve shape.

The second optimisation technique is to create a standard curve for each primer pair using serial dilutions of known target DNA concentrations. The standard curve is created by plotting the log of each known concentration in the dilution series (X-axis) versus the C_t value for that concentration (Y-axis). From the resulting curve information regarding the performance of the reaction (including the slope and correlation co-efficient) can be obtained. The concentrations chosen for the standard curve should encompass the expected concentration range of the target in the experimental samples. The correlation coefficient (R^2) represents how well the data fits the standard curve and should be close to 1. The slope is a measure of reaction efficiency and should be as close to 100% as possible which is equivalent to a slope of -3.32. If the reaction efficiency falls outside of the 90-110% efficiency range, modifications are required prior to use of those primers. Examples of standard curves for primer pairs used in this thesis can be found in Appendix 1.

All qRT-PCR reactions were carried out using the MxPro™ Stratagene Mx3000P® instrument and the R^2 coefficient and slopes calculated using the MxPro™ software (both from Agilent Technologies UK Ltd, Stockport, Cheshire, UK).

2.9.v QRT-PCR using Platinum®SYBR® Green

Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies, Ltd) was used for all qRT-PCR reactions. This is a “ready to use cocktail” of all components required for the amplification and detection of DNA in qRT-PCR reactions, except for the primers. It uses the “hot start” technology of platinum® *Taq* polymerase which prevents the reaction from starting during the set up stage. The SYBR cocktail contains; platinum® *taq* DNA polymerase which is a recombinant *taq* polymerase complexed with an antibody that prevents

polymerase activity at ambient temperatures, SYBR® green 1 fluorescent dye that binds directly to dsDNA and therefore the signal generated by this dye is directly proportional to the DNA concentration, DNTPs and uracil DNA glycosylase (UDG) which destroys any contaminating uracil residues.

Experiments were carried out using 96 well PCR plates. All reagents were mixed thoroughly prior to use and all pipetting done on ice. A master mixture was made for each primer pair used, containing all of the reagents apart from the template cDNA which was pipetted separately at the end. For 1 reaction the following components were used:

Reagent	Volume (µL)
SYBR® Green SuperMix	12.5
Forward Primer	1
Reverse Primer	1
Nuclease free water	9.5
cDNA	1 (pipetted separately)
Total reaction volume	25

Although care was taken to pipette the contents to the bottom of each well, the plate was centrifuged at 1600 G for 3 minutes prior to loading it into the qRT-PCR instrument. qRT-PCR was carried out using the following cycling conditions

- 50 °C for 2 minutes (UDG incubation)
- 95 °C for 2 minutes
- 40 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds
- Melting curve analysis

When comparing one treatment versus another, a no RT reaction and water blank (also referred to as a no template control, containing 1 µL nuclease free water in place of cDNA) were prepared for each treatment group. Relative expression of the gene of interest was compared with a house keeping gene glyceraldehyde 3-phosphate dehydrogenase

(GAPDH). All analysis was carried out using the MxPro® software which calculates relative gene expression in all samples and compares this to one of the control samples, known as the calibrator.

2.10 Mouse angiogenesis gene array

Gene arrays are single PCR plates that are pre-loaded with a number of primers to detect genes from a particular biological pathway. They provide a profile of expression of a panel of genes using qRT-PCR. The advantage is that expression of a large number of genes within a particular pathway can be analysed without the need to design and optimise primers for them all. Any gene whose expression is up or down regulated by a treatment can be identified by the array and further qRT-PCR experiments performed to verify the results.

For this experiment the RT² Profiler mouse angiogenesis PCR array (SABiosciences, A Qiagen Company, West Sussex, UK) was used. This array is a single 96 well PCR plate that comprises 84 genes that function within the angiogenesis pathway, 5 housekeeping genes, a genomic DNA control, 3 reverse transcription controls and 3 positive PCR controls. As the manufacturers only guarantee results of the array if materials used for the RT reaction and SYBR green are from their company, the materials were purchased and methods followed as outlined below.

2.10.i RNA Extraction

RNA extraction was performed using the column method with GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Company Ltd), the methodology of which is outlined in section 2.7.ii.

2.10.ii Reverse Transcription

cDNA synthesis was performed using the RT² First Strand Kit (SABiosciences Ltd). This kit has an in-built step for removal of genomic DNA so RNA was not pre-treated with DNase. The RT² First Strand Kit comprises a buffer GE2 (genomic DNA elimination buffer), a 5x buffer BC3, a control P2 and a RE3 reverse transcriptase mix. The procedure has 2 steps, firstly to eliminate any residual genomic DNA and then secondly to perform the cDNA synthesis using a master mix of hexamers, oligoDT primers, MMLV enzyme and magnesium chloride. All incubation steps were performed using a DNA Thermal Cycler 480 (PerkinElmer, UK).

For the first step a genomic elimination mix was formed for each RNA sample. This comprised 500ng RNA (volume calculated from Nanodrop™ 1000 reading), 2 µL GE2 buffer and nuclease free water to a total volume of 10 µL. This mixture was incubated at 42 °C for 5 minutes and then placed immediately on ice for at least 1 minute.

For the second step, the RT mix was prepared as outlined in the table below

Component	Volume for 1 reaction (µL)
5x Buffer BC3	4
Control P2	1
RE3 Reverse Transcriptase Mix	2
Nuclease free water	3
TOTAL	10

10 µL of this RT mix was then added to the 10 µL genomic DNA elimination mix and mixed by gentle pipetting to give a total reaction volume of 20 µL. This was then incubated at 42

°C for 15 minutes followed by rapid heating and incubation at 95 °C for 5 minutes to stop the reaction. 91 µL nuclease-free water was then added to each reaction tube to give a final volume of 111 µL. These cDNA samples were stored at -20 °C or placed on ice for immediate use in the PCR array.

2.10.iii RT² Profiler mouse angiogenesis PCR array

For this array the RT² SYBR Green Mastermix (SABiosciences, UK) was used. The 96 well PCR array plate was kept in its protective packaging until just before use. All reaction components were kept on ice and vortexed thoroughly prior to use but the reaction was prepared at room temperature according to the manufacturer's instructions. The following PCR components mix was made.

Component	Volume (µL)
2x RT ² SYBR Green Mastermix	1350
cDNA synthesis reaction	102
Nuclease free water	1248
TOTAL	2700

The PCR array was then carefully removed from its packaging and 25 µL PCR components mix was added to each well using a single channel pipette. The PCR array plate was then sealed using the Optical Thin-Wall 8-Cap Strips provided and then centrifuged for 1 minute at 1000g at room temperature to remove any bubbles and ensure all components were at the bottom of each well. The PCR array was then placed on ice while setting up the following thermal cycle programme using the MxPro™ Stratagene Mx3000P® Instrument (Agilent Technologies Ltd).

- 95 °C for 10 minutes (1 cycle)
- 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute
- Melting analysis

The C_t values for each well were then entered into a Microsoft Excel® spreadsheet and the SABiosciences PCR Array Data Analysis Template (SABiosciences, 2013) used to determine whether the expression of each gene within the array had been up or down-regulated. This software uses a 2-fold change in expression as a significant change, when analysing differences in expression between two plates.

2.11 Preparation of agarose gels

In order to confirm presence the of the correct PCR product size gel electrophoresis was used. 100 mL 10 x Tris/Borate/EDTA (TBE) buffer was diluted to 1000 mL using double distilled water. A 2% agarose gel was prepared by adding 1g agarose (Sigma Aldrich Co Ltd) in 50mL of the TBE buffer. The solution was heated on a hot plate to 100 °C to dissolve the agarose. The gel mixture was then cooled to 60 °C and 2.5 µL ethidium bromide was added before pouring into a casting plate. The gel was then left to cool until solid with an electrophoresis comb *in situ* to create wells at one end of the gel. Once solidified the remaining TBE buffer was poured over the gel to completely submerge it. 2 µL loading dye (Life technologies Ltd) was added to each qRT-PCR product and then 10 µL was loaded into each well of the gel. 50 bp DNA ladder (5µL, Life technologies LTD) was loaded into one well of the gel in order to determine the amplicon length of the PCR product. The electrodes were then connected and 100V current applied to the gel for 1 hour. The gels were removed from the buffer solution and viewed and photographed using a UV transilluminator BioDoc-It™ (Ultra-Violet Products Ltd, Cambridge, UK).

2.12 Data analysis and statistics

All results were analysed using Microsoft Excel® spreadsheets. Unless otherwise stated all experiments were performed three times and results combined during the final analysis. To allow for differences between assays all cell number and ELISA results were expressed relative to the mean value of the control samples on that plate. For certain experiments, control values were normalised to 1 to allow easier interpretation of results. Statistical comparison between treatment groups was undertaken using IBM SPSS Statistics®. SPSS® was used to carry out normality tests as well as Q-Q plots to ensure normal distribution prior to analysis. The paired students T-test was used for single comparisons and one-way ANOVA for multiple comparisons (i.e. 3 or more treatment groups). A p value of <0.05 was considered significant and significance is expressed within figures using the following format; *p<0.05, **p<0.01, ***p<0.001.

CHAPTER 3

The effects of dexamethasone and/or 1,25(OH)₂D₃ on osteocyte proliferation, VEGF and IL-6 secretion and VEGF isoform gene expression

3.1 Introduction

The work in this chapter outlines investigation into the effects of dexamethasone and 1,25(OH)₂D₃ on specific areas of osteocyte biology thought to be involved in the development of GC-induced ON, namely osteocyte number and angiogenesis. Recent studies suggest that the pathogenesis of ON has two main mechanisms; firstly osteocyte apoptosis (Weinstein et al., 2000, Kabata et al., 2000, Eberhardt et al., 2001, Calder et al., 2004) and secondly interruption to normal bone angiogenesis (Weinstein, 2012a). Osteocytes are found in close proximity to blood vessels and it is thus possible that they influence angiogenesis. Indeed the influence of osteocyte apoptosis on VEGF secretion and endothelial cell proliferation and migration suggest that osteocyte apoptosis induces angiogenesis and supports the now well recognised role of VEGF as a coupling factor between angiogenesis and osteogenesis in bone healing (Cheung et al., 2011, Filvaroff, 2003). Several studies have shown VEGF to play a role in the pathogenesis of ON (Weinstein, 2012b, Weinstein et al., 2010b, Wang et al., 2010, Varoga et al., 2009), and high levels of VEGF are found in osteonecrotic bones where it contributes to the healing process (Radke et al., 2006). Polymorphisms within the VEGF gene may also influence development of GC-induced ON (Lee et al., 2012).

The VEGF and VEGF receptor family in mammals is well described (Olsson et al., 2006, Dai and Rabie, 2007), and the potency of VEGF is known to be splice variant specific. The human isoforms of VEGF are VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆, whereas

mouse isoforms are one amino acid shorter (VEGF₁₂₀ etc.) (Dai and Rabie, 2007). VEGF is expressed by osteocytes and its expression is influenced by mechanical loading (Juffer et al., 2012). As well as influencing angiogenesis, apoptotic osteocytes are also thought to be responsible for inducing osteoclastogenesis and several studies have suggested that VEGF contributes to this process (Kennedy et al., 2012, Gupta et al., 2010). Recent *in vitro* studies show that dexamethasone treatment reduces VEGF mRNA levels in both osteoblasts and MLO-Y4 cells stimulated with desferrioxamine (DFO) to mimic hypoxia (Weinstein, 2012a).

High serum levels of IL-6, which correlate with episodes of mucositis, are found in children with ALL (Morales-Rojas et al., 2012), but the relationship between IL-6 and development of ON has not been investigated. IL-6 is known to inhibit osteoblast formation (Peruzzi et al., 2012) and promote osteoclastogenesis. It has been recently demonstrated that apoptotic MLO-Y4 cells produce IL-6 and that this is required for osteoclast precursor adhesion to endothelial cells at sites of bone remodelling (Cheung et al., 2012). This suggests that IL-6 plays an important role in osteocytic control of bone turnover, and may have implications at sites of osteocyte apoptosis in osteonecrotic bones.

3.2 Aims

11. To investigate the effects of dexamethasone and/or 1,25(OH)₂D₃ on:

- iv. Proliferation of osteocyte cell lines as measured by cell number.
- v. Osteocyte cell line VEGF₁₆₄ secretion and VEGF isoform gene expression.
- vi. IL-6 secretion.

12. To assess whether 1 α 25(OH)₂D₃ treatment can modulate the effects of dexamethasone on the above.

13. To compare findings between cells treated in monolayer and 3D collagen gels.
14. To compare intracellular VEGF₁₆₄ levels with that secreted into the CM and to assess the effect of dexamethasone and 1,25(OH)₂D₃ on intracellular VEGF₁₆₄ levels.

3.3 Materials and methods

For all experiments, the concentrations of dexamethasone chosen for cell treatments (10^{-8} and 10^{-7} M) are representative of serum concentrations achieved during treatment for ALL in children (Yang et al., 2008). The concentration of 1,25(OH)₂D₃ (10^{-8} M) used is representative of normal serum levels (Jin et al., 2013).

Higher concentrations of 1,25(OH)₂D₃ were used in some experiments in order to investigate a potential dose-dependent effect. Results of some experiments are compared between cells cultured in monolayer and cells cultured in 3D collagen gels, which more closely represents the normal physiological state of osteocytes *in vivo*. The rationale for the experiments carried out in this chapter is outlined in the Introduction chapter of this thesis (sections 1.3-1.6 and 1.11-1.12).

3.3.i Cell number assays

MLO-Y4 cells were seeded (4.6×10^3 cells/cm²) into 96 well culture plates and after 24 hours were treated with dexamethasone (10^{-9} - 10^{-6} M), 1,25(OH)₂D₃ (10^{-11} - 10^{-8} M), dexamethasone (10^{-7} M) and 1,25(OH)₂D₃ (10^{-8} M) in combination or medium containing vehicle (ethanol). Cells were then incubated at 37 °C for a further 72 hours and cell number measured using the [®] AQueous One Solution Cell Proliferation assay as outlined in Chapter 2.2.

3.3.ii ELISA

MLO-Y4 cells were seeded (2.4×10^4 cells/cm²) into 24 well plates. After 24 hours cells were treated with dexamethasone (10^{-8} and 10^{-7} M), $1,25(\text{OH})_2\text{D}_3$ (10^{-11} - 10^{-8} M), dexamethasone (10^{-7} M- 10^{-8} M) and $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) in combination or medium containing vehicle (ethanol). After a further 24 hours incubation at 37°C medium was removed and VEGF₁₆₄ secretion was measured by ELISA as outlined in Chapter 2.4. Total cellular protein in each well was measured using the Bio-Rad protein assay as outlined in Chapter 2.5. Results were expressed as total VEGF₁₆₄ concentration in pg/ µg protein and expressed relative to a control value of 1.

3.3.iii qRT-PCR

MLO-Y4 cells were seeded (4.6×10^3 cells/cm²) into 12 well plates. After 24 hours cells were treated with dexamethasone (10^{-7} M), $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M), dexamethasone (10^{-7} M) and $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) in combination or medium containing vehicle (ethanol). Cells were then further incubated for 72 hours at 37°C followed by RNA extraction, sodium acetate precipitation, DNase treatment (methodology outlined in chapter 2.7) and reverse transcription (methodology outlined in chapter 2.8). Expression of the following VEGF isoforms; VEGF_{full length (FL)}, VEGF₁₈₈, VEGF₁₆₄ and VEGF₁₂₀ was measured by qRT-PCR as outlined in chapter 2.9. Gene expression was normalised to the expression of the housekeeping gene GAPDH. Results are expressed as a ratio compared to control samples, one of which was calibrated to 1. The presence or absence of the expression of VEGF receptors VEGF-R1, VEGF-R2 and neuropilin 1 (NP1) was also assessed by qRT-PCR and gel electrophoresis to measure PCR product size. The primer sequences used are outlined in Table 5.

Gene	Sense (5'-3')	Antisense (5'-3')	Product size (bp)	Source
VEGF _{FL}	GTCCGAGCCGGAGAGGG AGC	CGTGGGTGCAGCCTGGGAC	143	(Zhao et al., 2004)
VEGF ₁₈₈	TGCAGGCTGCTGTAACGA TG	CTCCAGGATTTAAACCGGGAT T	243	(Ruiz de Almodovar et al., 2010)
VEGF ₁₆₅	TGCAGGCTGCTGTAACGA TG	GAACAAGGCTCACAGTGATTT TCT	190	(Ruiz de Almodovar et al., 2010)
VEGF ₁₂₀	TGCAGGCTGCTGTAACGA TG	CCTCGGCTTGTCACATTTTCT	192	(Ruiz de Almodovar et al., 2010)
VEGF receptor 1 (R1)	TGTCCCCGCGCTCAGATC A	ACGGCCCCCTTCTGGTTGGT	162	Primer3
VEGF receptor 2 (R2)	AGATGCCCGACTCCCTTTG	CCAGAGCAACACACCGAAAG A	114	Primer3
Neuropilin 1 (NP1)	GGCTGCCGTTGCTGTGCG CCA	ATAGCGGATGGAAAACCTG C	404	Primer3

Table 5. Primer sequences for VEGF isoforms and VEGF receptors.

In separate assays IDG-SW3 cells were plated (4.0×10^4 cells/cm²) in 12 well plates and incubated at 33° C in proliferating conditions. Once cells reached 80% confluence, they were transferred into differentiating conditions and incubated at 37° C with replenishment of media every 2-3 days. Cells were treated on days 7, 14 and 21 of differentiation with dexamethasone (10^{-7} M), $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) individually and in combination or medium containing vehicle (ethanol). After 72 hours incubation VEGF₁₆₄ gene expression was measured and results normalised to GAPDH using identical methodology to MLO-Y4 cells. This assay was only performed once in order to provide a comparison to MLO-Y4 cells. As a result statistical analysis was not carried out on these results.

3.3.iv 3D collagen gels

MLO-Y4 cells were set up (375000 cells/gel) in type 1 collagen gels (2mg/mL) using rat tail collagen as outlined in chapter 2.2. After 24 hours cells were treated for 72 hours with either dexamethasone (10^{-7} M), $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} M) or a combination of both of these agents. Control cells were treated with vehicle (ethanol) only. Cells were counted using a haemocytometer following removal from the gel by collagenase digestion, and cell viability was also assessed using the Trypan Blue exclusion method (for full methodology see Chapter 2.3). In addition, VEGF₁₆₄ and IL-6 concentrations in conditioned media were measured by ELISA and corrected for cellular protein using the methodology outlined for monolayer cultures in chapters 2.4 and 2.5.

3.3.v Cell lysis

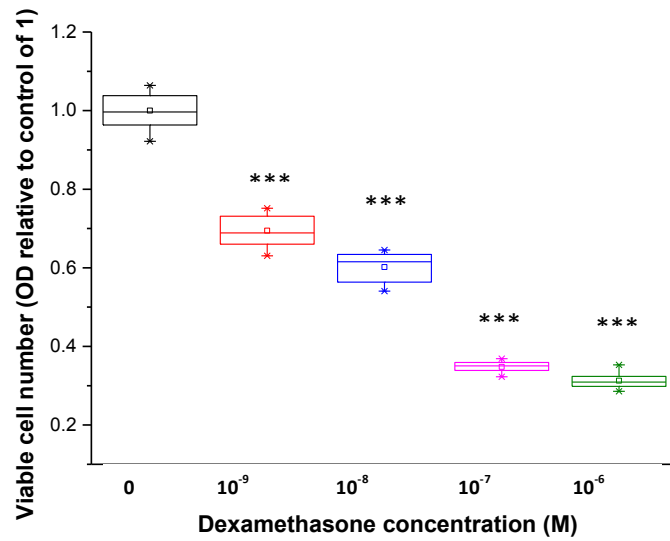
MLO-Y4 cells were seeded (2.4×10^4 cells/cm²) into 24 well plates. After 24 hours cells were treated with dexamethasone (10^{-7} M), $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M), dexamethasone (10^{-7} M) and $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) in combination or medium containing vehicle (ethanol). After 24 hours incubation, medium was collected for measurement of secreted VEGF₁₆₄ concentrations by ELISA. Cells were then lysed using 50 mM Tris, 150 mM sodium chloride, 1% Nonidet-P40, 0.1% SDS, 0.5% sodium deoxycholate and 1% protease inhibitor cocktail (as outlined in chapter 2.1.iv) and VEGF₁₆₄ concentrations were measured in the cell lysates, using the same ELISA assay. This allowed direct comparison of intracellular and secreted VEGF₁₆₄ levels as well as the effects of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on these. This assay was only performed once (n=3 in each treatment group).

3.4 Results

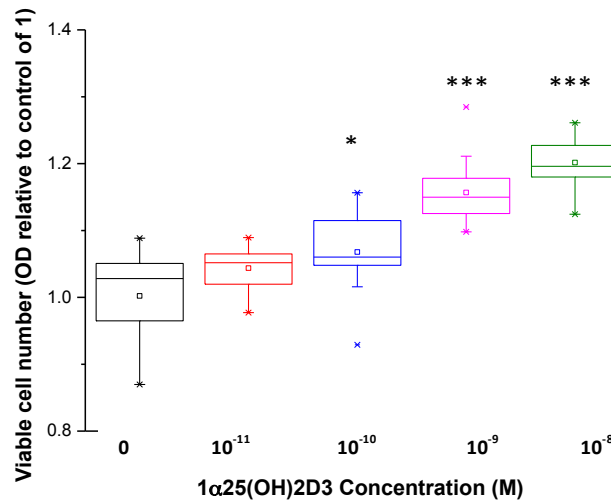
3.4.i Effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on osteocyte cell number in monolayer

Dexamethasone significantly ($p < 0.001$) reduced MLO-Y4 cell number at all concentrations tested after 72 hrs and in a dose dependent manner (30.6% and 68.8% reduction at 10^{-8}M and 10^{-7}M respectively, $p < 0.001$) (Figure 11A). $1\alpha25(\text{OH})_2\text{D}_3$ treatment resulted in a 6.8% (10^{-10}M , $p = 0.03$) – 20.2% (10^{-8}M , $p < 0.001$) increase in cell number over the same time period (Figure 11B). When given in combination $1\alpha25(\text{OH})_2\text{D}_3$ significantly modulated the effects of dexamethasone on cell number (Figure 11C). (35.5% reduction in combination versus 53.3% reduction with dexamethasone alone; $P < 0.001$)

A



B



C

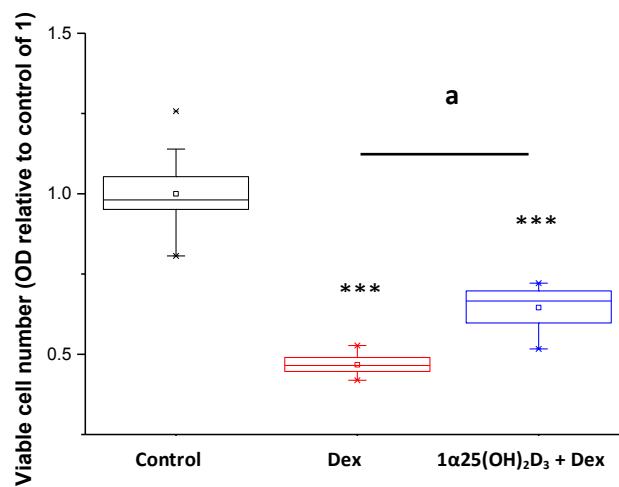


Figure 11. Effect of increasing concentrations of (A) dexamethasone (DEX) and (B) $1\alpha25(\text{OH})_2\text{D}_3$ as well as (C) a combination of dexamethasone (10^{-7} M) and $1\alpha25(\text{OH})_2\text{D}_3$ (10^{-8} M) on viable osteocyte cell number *in vitro* (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control, ^a $p < 0.001$ dexamethasone alone vs. combination of dexamethasone and $1\alpha25(\text{OH})_2\text{D}_3$) (Each assay performed 3 times; $n = 36$)

3.4.ii Effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on osteocyte VEGF₁₆₄ secretion.

Dexamethasone treatment significantly reduced VEGF₁₆₄ secretion by MLO-Y4 cells by 36.7% at 10^{-8} M and 47.6% at 10^{-7} M ($p < 0.001$) (Figure 12A). $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} M and 10^{-9} M), however, significantly increased VEGF₁₆₄ secretion by 41.8% ($p = 0.007$) and 29.6% ($p = 0.04$) respectively (Figure 12B). Although results were not significantly different to controls at lower concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$, the effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on VEGF₁₆₄ secretion by MLO-Y4 cells appears to be dose dependent. When $1\alpha,25(\text{OH})_2\text{D}_3$ was given in combination with dexamethasone it inhibited, at least in part, the reduction in VEGF secretion caused by dexamethasone, and at the 10^{-8} M concentration of dexamethasone, VEGF concentrations were similar to control values (Figure. 12C).

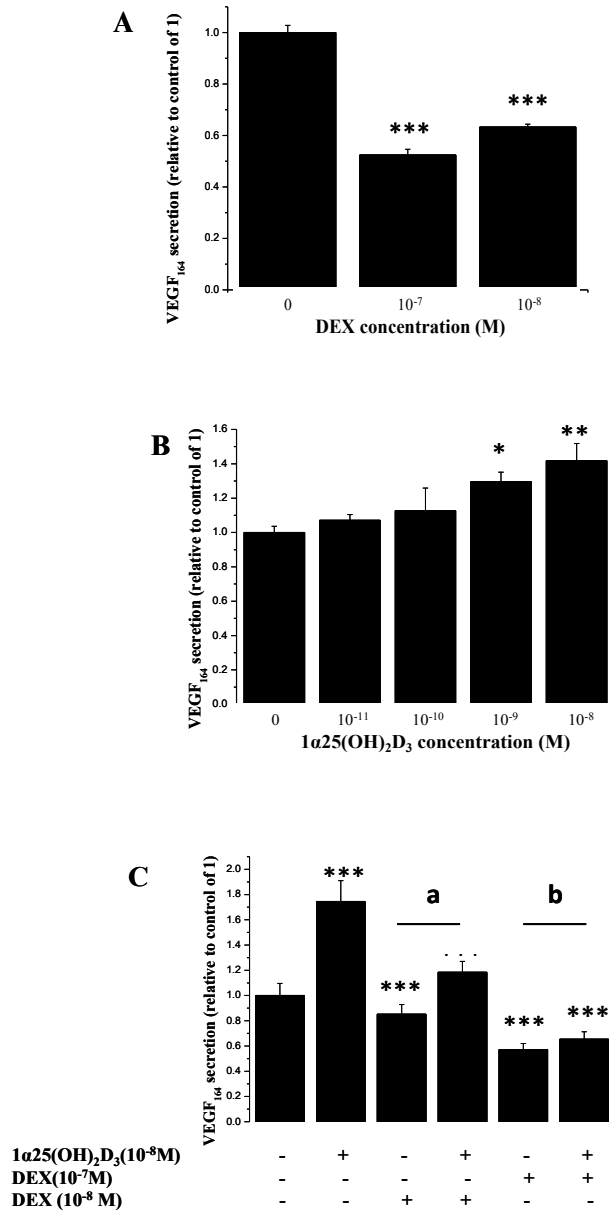


Figure 12. Effect of (A) dexamethasone and (B) 1α25(OH)₂D₃ as well as (C) a combination of dexamethasone and 1α25(OH)₂D₃ on VEGF₁₆₄ secretion by MLO-Y4 cells *in vitro*. (*P<0.05, **p<0.01, ***p<0.001 vs. control, ^{a+b}p<0.001 1α25(OH)₂D₃ and dexamethasone in combination vs. dexamethasone alone) Each experiment performed 3 three times (n=9 in each treatment group. Data represents mean VEGF secretion (+/- SEM) relative to control of 1.

3.4.iii Effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on expression of VEGF isoform genes by MLO-Y4 cells

MLO-Y4 cells were shown by RT-PCR to express VEGF_{FL} VEGF₁₈₈ VEGF₁₆₄ and VEGF₁₂₀ isoforms, as well as NP1 (Figure. 13A). VEGFR2 expression was not detected. A band was obtained with VEGFR1 primers but this was not of the expected size. cDNA derived from mouse aorta was used as a positive control for the VEGF receptor primers (Figure 13B).

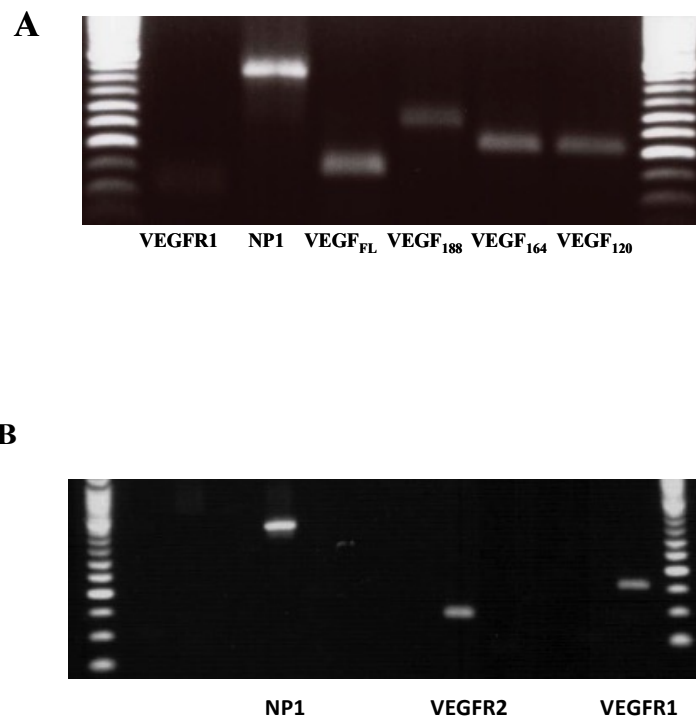


Figure 13. Effects of dexamethasone (DEX) (10^{-7} M) or $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} M) alone or in combination on VEGF_{FL} and VEGF₁₈₈, VEGF₁₆₄ and VEGF₁₂₀ isoform expression. (A) Agarose gel showing presence of NP1, VEGF_{FL} and VEGF isoforms in MLO-Y4 cells. (B) Agarose gel showing VEGFR1, VEGFR2 and NP1 in mouse aorta as positive control.

After treatment with dexamethasone (10^{-7} M) the relative expression of VEGF_{FL} and the 3 VEGF isoforms (VEGF₁₈₈, VEGF₁₆₄ and VEGF₁₂₀) was down-regulated by 54.0%, 70.0%, 58.7% and 65.5% respectively (Figure 14A). In the presence of $1\alpha 25(\text{OH})_2\text{D}_3$ (10^{-8} M) however, there was up-regulation of these genes by 1.5, 1.88, 1.86 and 1.71 fold respectively (Figure 14A). There were no significant differences in the expression of the VEGF isoforms between treatment with dexamethasone alone and treatment with dexamethasone in combination with $1\alpha 25(\text{OH})_2\text{D}_3$.

In IDG-SW3 cells the same pattern was observed, with dexamethasone causing down regulation and $1\alpha 25(\text{OH})_2\text{D}_3$ causing up regulation of VEGF₁₆₄ expression on day 7, 14 and 21 of differentiation (Figure 14B).

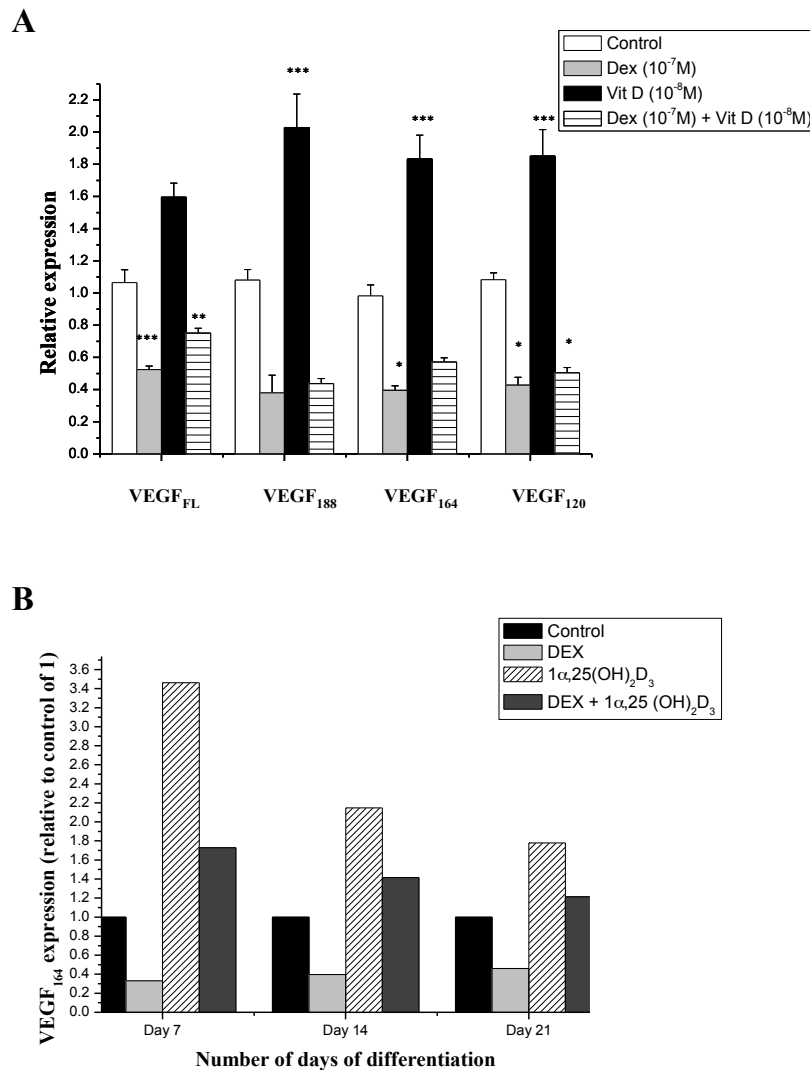


Figure 14. Effect of $1\alpha,25(\text{OH})_2\text{D}_3$ and dexamethasone on VEGF expression by osteocyte cell lines. (A) VEGF gene expression measured by qRT-PCR. Relative expression of gene of interest to GAPDH is shown and one of the control samples in each case has been normalised to a value of 1. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control samples. (B) Relative VEGF₁₆₄ gene expression in IDG-SW3 cells on days 7, 14 and 21 following treatment with DEX and $1\alpha,25(\text{OH})_2\text{D}_3$ alone or in combination. Each experiment performed three times ($n=9$ in each treatment group). Data represents mean VEGF isoform expression (\pm SEM) relative to control of 1.

Effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on osteocyte IL-6 secretion.

Dexamethasone treatment resulted in a 51.8% (10^{-8} M; $p < 0.001$) and 73.4% (10^{-7} M; $p < 0.001$) reduction in IL-6 secretion by MLO-Y4 cells (Figure 15A). $1\alpha,25(\text{OH})_2\text{D}_3$ also reduced IL-6 secretion by 23.0% ($p = 0.03$) at 10^{-8} M and 32.5% ($p = 0.002$) at 10^{-7} M (Figure 15B). Dexamethasone (10^{-7} M) and $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} M) in combination seemed to further reduce IL-6 secretion, although this was not significantly different from either treatment alone (Figure 15C).

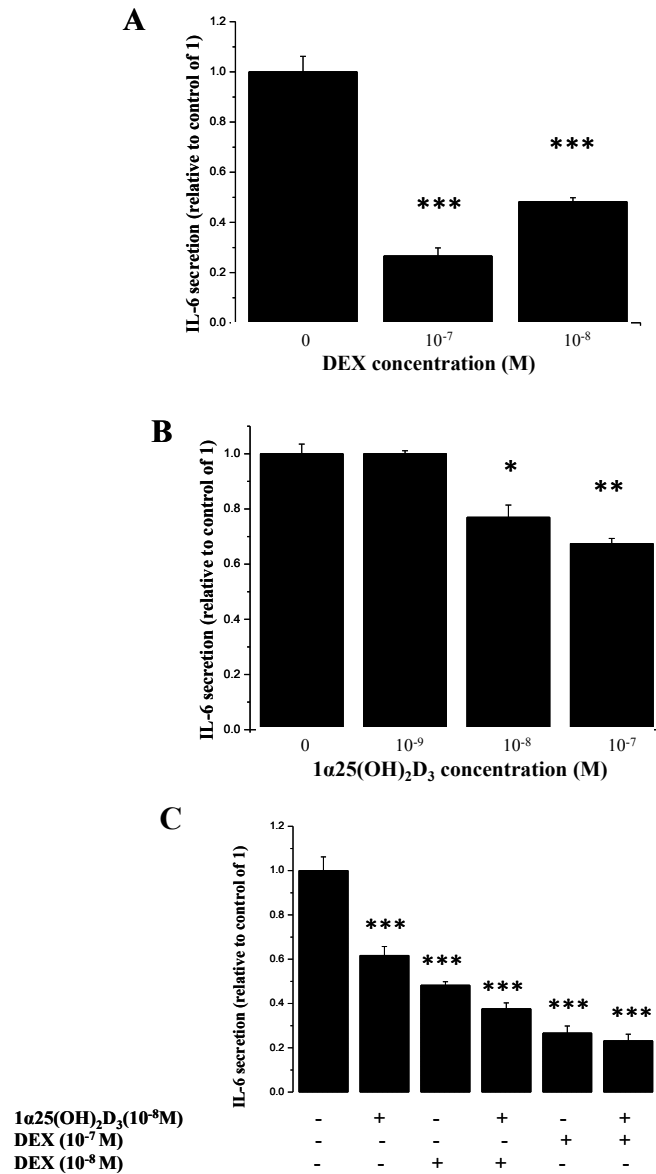


Figure 15. Effect of increasing concentrations of (A) Dexamethasone (DEX), (B) $1\alpha25(\text{OH})_2\text{D}_3$ as well as (C) a combination of dexamethasone and $1\alpha25(\text{OH})_2\text{D}_3$ on MLO-Y4 IL-6 secretion *in vitro*. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control) Each experiment performed three times ($n=9$ in each treatment group). Data represents mean IL-6 secretion (+/- SEM) relative to control of 1.

3.4.v Effects of dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$ on MLO-Y4 cell number and VEGF₁₆₄ and IL-6 secretion in 3D collagen gel cultures.

VEGF₁₆₄ and IL-6 concentrations in conditioned media collected from MLO-Y4 cells maintained in 3D collagen gels for 72 hours showed similar patterns to those observed in monolayer cultures – Dexamethasone (10^{-7}M) reduced both VEGF₁₆₄ (32.5% reduction, $p<0.01$) and IL-6 (73.0% reduction, $p<0.001$) concentrations and $1\alpha 25(\text{OH})_2\text{D}_3$ increased VEGF₁₆₄ (18.4% increase, $p<0.05$) secretion and decreased IL-6 secretion (29.2% decrease, $p<0.05$) (Figure 16A, B). However, when maintained in 3D collagen gels, although there was some reduction in cell number with dexamethasone and an increase with $1\alpha 25(\text{OH})_2\text{D}_3$ treatment these differences did not reach statistical significance (Figure 16C). No differences in % cell viability were seen between the different treatment groups (Figure 16D).

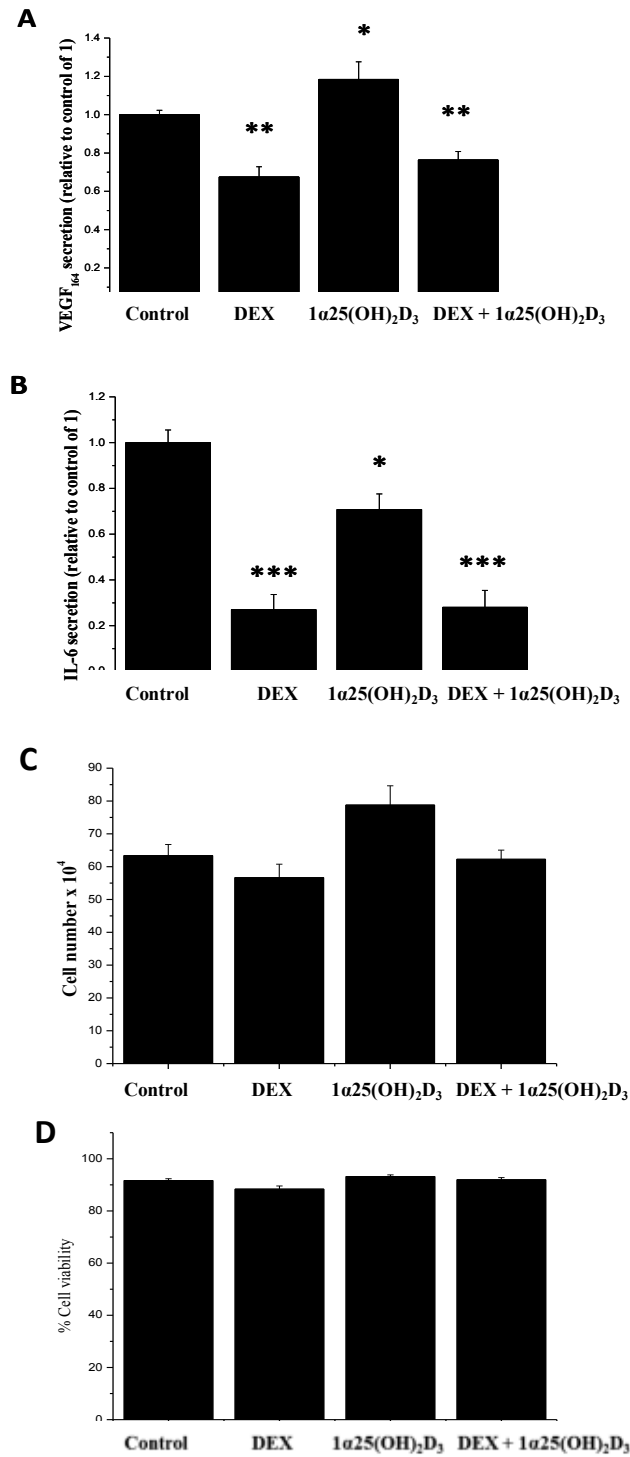


Figure 16. Effects of dexamethasone (DEX) (10^{-7} M) and $1\alpha 25(\text{OH})_2\text{D}_3$ (10^{-8} M) individually and in combination on MLO-Y4 VEGF₁₆₄ secretion (A), IL-6 secretion (B), cell number (C), and % viability (D) in 3D collagen gels after 72 hours. ELISA results expressed relative to a control of 1 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control). Each experiment performed three times ($n=9$ in each treatment group). Error bars demonstrate mean \pm SEM.

3.4.vi Effect of dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$ on intracellular MLO-Y4 cell VEGF_{164} levels

VEGF_{164} levels within cell lysates were lower than those in the conditioned medium removed from the same cells, however the observed effects of dexamethasone (decreasing) and $1,25(\text{OH})_2\text{D}_3$ (increasing) VEGF_{164} levels was the same. Mean VEGF_{164} concentrations in the cell lysates were 0.20, 0.09, 0.30 and 0.19 $\text{pg}/\mu\text{g}$ protein for control, dexamethasone, $1\alpha 25(\text{OH})_2\text{D}_3$ and dexamethasone plus $1\alpha 25(\text{OH})_2\text{D}_3$ respectively compared to 1.43, 0.76, 2.66 and 1.3 $\text{pg}/\mu\text{g}$ protein respectively in the media removed from those cells. (Figure 17)

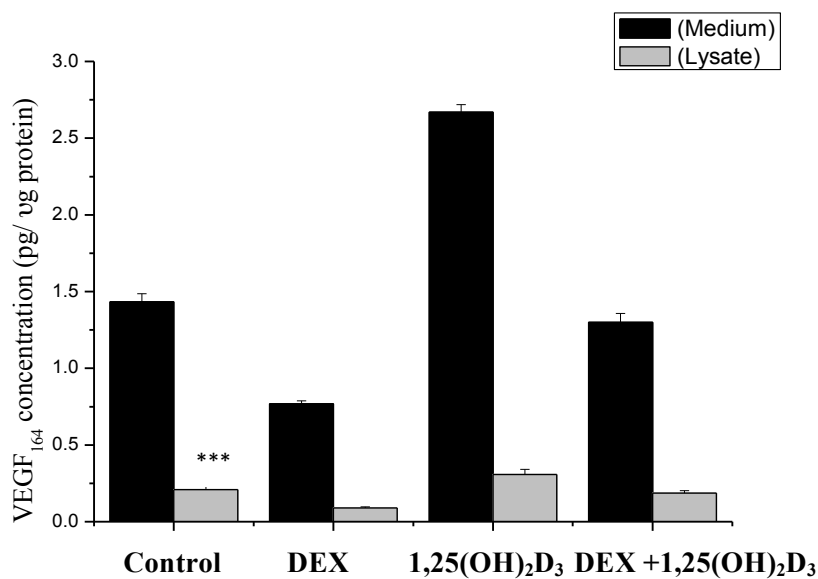


Figure 17. Effects of dexamethasone (DEX) (10^{-7}M) and $1\alpha 25(\text{OH})_2\text{D}_3$ (10^{-8}M) individually and in combination on MLO-Y4 VEGF_{164} secretion into conditioned medium compared with levels within cell lysates. (***) $p < 0.001$ medium vs. lysate). Each experiment performed three times ($n=9$ in each treatment group). Error bars demonstrate mean \pm SEM.

3.5 Discussion

The influence of dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$ both individually and in combination on osteocyte cell number was investigated in this chapter. Results demonstrate opposing effects of the above agents with dexamethasone decreasing and $1\alpha 25(\text{OH})_2\text{D}_3$ increasing MLO-Y4 cell number when measured by MTS assay. Although morphologically, there did not appear to be vast numbers of dead cells this methodology does not allow confirmation of whether the resultant decrease in cell number caused by dexamethasone is due to decreased proliferation or an increase in cell death. Although apoptosis of osteocytes following exposure to dexamethasone is well documented as a major pathogenic mechanism of ON (Plotkin et al., 2007, Weinstein, 2012a), more recently dexamethasone has been demonstrated to exert dose dependent effects on osteocyte autophagy (Jia et al., 2011) suggesting that other mechanisms in the surviving osteocytes may also influence the development of ON. The 3D collagen gel cultures were used in order to more closely replicate the natural *in vivo* environment of an osteocyte within the bone matrix. When in 3D, cells have a much larger surface area for communication with neighbouring cells and this is important in ON where apoptosis of osteocytes at the subchondral site, leads to signalling and bone remodelling at adjacent sites (Weinstein 2012a). When MLO-Y4 cells in the collagen gels were exposed to dexamethasone, there was not the same reduction in osteocyte number. This may be because proliferating cells in monolayer may be more susceptible to the effects of dexamethasone, or it may be that the 3D organisation of osteocytes makes them more robust. It appears that the dexamethasone was able to penetrate the collagen as effects were demonstrated on VEGF secretion (see below).

It has previously been shown that dexamethasone reduces MLO-Y4 number, partly due to increased apoptosis (Plotkin et al., 2007, Jia et al., 2011) whereas it has not been previously documented that $1\alpha 25(\text{OH})_2\text{D}_3$ increases early osteocyte number. This result is intriguing

since $1\alpha 25(\text{OH})_2\text{D}_3$ has previously been documented to reduce osteoblast numbers (Shi et al., 2007). One potential explanation for this finding is that rather than increasing proliferation of MLO-Y4 cells that instead the $1\alpha 25(\text{OH})_2\text{D}_3$ merely prevented apoptosis of a proportion of the cells that naturally may occur over 72 hours incubation in the control assay. One way to account for this would have been to measure the % cell viability in a 24, 48 and 72 hour control incubation. The limitation of the MTS reagent only detecting viable cells means that it may be unable to distinguish between an early apoptotic and a fully live cell and it would have been interesting to use apoptotic staining of cells to try and investigate this further.

The above experiments also investigate the effect of dexamethasone exposure on osteocyte VEGF and IL-6 secretion as well as the potential modulating effects of $1\alpha 25(\text{OH})_2\text{D}_3$. The results reveal novel *in vitro* findings in relation to the interaction between dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$ on osteocyte function, specifically in terms of VEGF and IL-6 secretion. I have demonstrated the expression of the 3 commonly studied VEGF isoforms, VEGF₁₈₈, VEGF₁₆₄ and VEGF₁₂₀ by MLO-Y4 cells and have shown that the level of expression is influenced by both dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$. Results show opposing effects of dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$ on osteocyte VEGF production as well as modulation of dexamethasone effects by $1\alpha 25(\text{OH})_2\text{D}_3$. A potential explanation for this is the effects of these two agents on the cell cycle. $1\alpha 25(\text{OH})_2\text{D}_3$ binds to the VDR and exerts its actions via vitamin D response elements (VDREs) which are present in many genes and result in regulation of a number of vital factors involved in cell growth. $1\alpha 25(\text{OH})_2\text{D}_3$ is well documented to inhibit proliferation (by preventing progression from the G1 to S phase of the cell cycle) and promote differentiation of a number of different cell types (Samuel and Sitrin, 2008). Similarly dexamethasone exerts its actions following binding to the GR via glucocorticoid response elements (GREs). Again the cell cycle is arrested at the G1-S phase transition but rather than promoting differentiation like

1 α 25(OH) $_2$ D $_3$, cells exposed to dexamethasone activate a number of pro-apoptotic molecules that results in cell death (Greenstein et al, 2002). Studies have demonstrated an up-regulation of the VDR when cells are dually treated with 1 α 25(OH) $_2$ D $_3$ and dexamethasone compared with 1 α 25(OH) $_2$ D $_3$ alone (Bernardi et al, 2001), a finding that was also seen in experiments carried out by this research group on MLO-Y4 cells .

A similar interaction between dexamethasone and 1 α 25(OH) $_2$ D $_3$ was seen on VEGF_{FL} and well as VEGF_{180, 164} and ₁₂₀. In addition the same trends were recorded with VEGF secreted into the medium, compared with those concentrations in cell lysates, although the latter concentrations were considerably lower than those in conditioned media (0.2pg/ μ g protein versus 1.43pg/ μ g protein in control cultures). These observations are important in relation to recently published work indicating that intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation (Liu et al., 2012), thus raising the question whether intracellular VEGF is functional in osteocytes?

It could be argued that although the 1.7-1.9 fold increase in VEGF gene expression caused by 1 α 25(OH) $_2$ D $_3$ reached statistical significance that it is less than the 2-fold increase which is widely regarded as a true increase. However the fact that an increase in VEGF was also demonstrated at the protein level by ELISA lends weight to the validity and significance of the findings.

The fact that an identical pattern of VEGF gene expression was seen when MLO-Y4 cells are cultured in 3D collagen gels as well as in a second osteocyte cell line (IDG-SW3) following treatment with dexamethasone and 1 α 25(OH) $_2$ D $_3$, adds further validity to the findings and also demonstrates that osteocyte VEGF expression is susceptible to the effects of these agents at various stages of differentiation, from a late osteoblast through to a fully differentiated, SOST expressing osteocyte.

VEGF is known to play an important role in coupling angiogenesis and osteogenesis during bone formation and repair (Cheung et al., 2011) and the down regulation of its expression by dexamethasone may be involved in the pathogenic effects of corticosteroids on the osteocyte. The reduction in osteocyte VEGF secretion caused by dexamethasone may prevent the usual repair processes that occur and contribute to the formation of osteonecrotic lesions. $1\alpha 25(\text{OH})_2\text{D}_3$ in contrast to dexamethasone increases VEGF secretion by MLO-Y4 cells. A previous study reported that vitamin D treatment increased VEGF gene expression in both normal and pathological (osteoporotic and osteoarthritic) osteoblasts (Corrado et al., 2013) and others have shown enhanced VEGF expression in growth plate chondrocytes and osteoblasts of juvenile mice treated systemically with $1,25(\text{OH})_2\text{D}_3$ as well as increased expression of the VEGF₁₂₁ and ₁₆₅ isoforms in a human chondrocyte cell line (Lin et al., 2002). Interestingly, outside of bone biology $1\alpha 25(\text{OH})_2\text{D}_3$ has been shown to up regulate VEGF secretion by smooth muscle cells (Cardus et al., 2009).

In terms of VEGF signalling pathways in MLO-Y4 cells it is shown that NP1 is expressed, and even though it has not been possible to demonstrate the presence of VEGFR1 or VEGFR2, the band observed on gels following amplification with primers for VEGFR1 indicates that a splice variant might be present. Several VEGFR1 splice variants have previously been described (Jebbink et al., 2011, Sela et al., 2008) and it would be interesting to study this further in osteocytes.

IL-6 is a pro-inflammatory cytokine that is raised in the serum of children undergoing induction chemotherapy for ALL and is correlated with episodes of mucositis (Morales-Rojas et al., 2012). CSF levels have also been shown to be elevated (Protas et al., 2011). Most ON in ALL patients occurs during the first 6 months of treatment but it is currently unknown whether IL-6 contributes to its development. Interestingly, a recent study of patients with ON of the jaw following bisphosphonate therapy showed elevated IL-6 levels

within the oral mucosa compared to the control population without ON (Mozzati et al., 2013). The results in this chapter demonstrate a reduction in IL-6 secretion by MLO-Y4 cells in response to both dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$. It is not surprising that dexamethasone reduced IL-6 secretion as it is well known to have potent anti-inflammatory effects. The fact that $1\alpha 25(\text{OH})_2\text{D}_3$ also reduced IL-6 secretion again is significant for two reasons; firstly it highlights the complex role that $1\alpha 25(\text{OH})_2\text{D}_3$ plays in bone turnover – by increasing one important promoter of osteoclastogenesis (RANKL – see results from chapter 4) and decreasing another (IL-6) and secondly it adds further support to the role of $1\alpha 25(\text{OH})_2\text{D}_3$ in the prevention and management of GC-induced bone diseases.

The results in this chapter reveal definite actions of dexamethasone on osteocyte VEGF activity and led to the thought that glucocorticoids may influence other pathways involved in angiogenesis. There are very few published studies on this subject to date and triggered further investigation of this using a PCR gene array. The rationale for and results from these experiments is outlined in Chapter 4 of this thesis.

Chapter 4

The effects of dexamethasone on the production of angiogenesis factors by osteocytes

4.1 Introduction

Interruption to bone angiogenesis is thought to be a major contributory factor to the pathophysiology of GC-induced osteonecrosis (see section 1.5 of the introduction to this thesis). (Weinstein, 2012b, Weinstein, 2012a) One of the key angiogenic factors known to be secreted by osteocytes is VEGF, and as outlined in Chapter 3 of this thesis I have demonstrated the reduction in gene expression of the VEGF isoforms (VEGF₂₀₈, ₁₆₄ and ₁₂₀), as well as a reduction in VEGF₁₆₄ secretion, in osteocytes treated with dexamethasone. This reduction in VEGF mRNA expression and protein secretion correlates with published *in vitro* findings (Weinstein, 2010, Weinstein et al., 2010b) as well as *in vivo* studies demonstrating decreased VEGF and skeletal angiogenesis in mice treated with prednisolone (Weinstein et al., 2010b) and is hypothesised to be an important pathogenic mechanism in the development of ON in children treated for ALL.

The activity of VEGF and its receptors are the most widely studied angiogenic agents in all tissues but there are multiple other factors and pathways including PDGFs, FGFs and downstream signaling molecules (Ellis et al., 2001, Limaverde-Sousa et al., 2013) whose expression and activity in osteocytes is largely unstudied. In bone, PDGF and FGF have been shown to be important in differentiation of bone marrow MSCs into osteoblasts (Ng et al., 2008). In order for angiogenesis to be initiated in damaged bone, endothelial cells must penetrate into the surrounding tissue. This process is initiated by factors such as MMPs, which are activated by VEGF, and the plasminogen activator protease system (*urokinase* and *tissue* plasminogen activators – uPA and tPA). New blood vessel growth is then facilitated by the migration and differentiation of endothelial cells. Reduced basement

membrane turnover by MMPs (Chen et al, 2012) and reduced cleavage of extracellular proteins by t-PA have been described in patients with ON (Glueck et al, 2001).

Many osteogenic factors are known to stimulate angiogenesis (Carano and Filvaroff, 2003) during bone repair highlighting how closely these two pathways are related. The table below adapted from a review by Carano and Filvaroff (Carano and Filvaroff, 2003) outlines these factors and demonstrates that a number of them are known to induce VEGF *in vitro* and/or *in vivo*.

Treatment	Osteoblastic ¹	Angiogenic ²	Induction of VEGF ³
Activins	+	? ⁵	ND ⁴
BMPs 2 and 4	+	+	+
Osteogenic Protein-1	+	+	+
FGF 1 and 2	+	+	+
Growth and Differentiation Factor-5	+	+	ND
GH	+	+	ND
IGF-1	+	+	+
PTH	+	+	+
PDGF	+	+	+
Prostaglandins	+	+	+
TGF β	+	+	+

Table 6. Summary table of bone-derived cytokines/ treatment factors that enhance bone repair. ¹ osteoblastic defined as having actions on (pre)osteoblasts, ²angiogenic defined as being able to form new blood vessels *in vivo*, ³ induction of VEGF demonstrated *in vitro* and/or *in vivo*, ⁴ activity not yet determined (ND), ⁵ activins are possibly anti-angiogenic in cancer. (Adapted from (Carano and Filvaroff, 2003)).

The Qiagen® RT² Profiler mouse angiogenesis PCR array profiles the expression of 84 genes involved in modulating the process of angiogenesis. These include the above agents as well as adhesion molecules, matrix proteins, chemokines and their inhibitors and transcription factors. The use of such an array provides a relatively quick and broad screening tool for

the action of dexamethasone on specific factors within the angiogenesis pathways, but as it is essentially a single PCR reaction, results then require validation using other methods outlined in this thesis (RT-PCR, ELISA etc.).

The use of this particular array has been documented in a number of studies (Ligi et al., 2011, Nguyen et al., 2011, Jensen et al., 2011).

4.2 Aims and hypothesis

Hypothesis:

In view of the findings outlined in Chapter 3 of this thesis regarding the effects of dexamethasone on osteocyte VEGF secretion, it is hypothesised that dexamethasone would have a modulating effect on the expression of angiogenic factors within the MLO-Y4 cell. In view of the negative influence of dexamethasone on VEGF expression, it is predicted that dexamethasone would decrease the expression of other pro-angiogenic factors and increase the expression of anti-angiogenic factors.

Aims:

The aim of the work in this chapter was firstly to investigate the MLO-Y4 expression profile of 84 genes involved in angiogenesis, and secondly to assess the effect of dexamethasone treatment on the expression of these genes.

4.3 Materials and methods

MLO-Y4 cells were seeded (2.4×10^4 cells/cm²) into 6 well plates. After 24 hours, cells were treated with either dexamethasone (10^{-7} M) or vehicle (ethanol) and incubated for a further 72 hours at 37° C. RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit as outlined in chapter 2.7.ii. Reverse transcription and RT-PCR using the Qiagen® RT² Profiler mouse angiogenesis PCR array was carried out as described in Chapter 2.10. Control and dexamethasone treated samples were analysed using separate array plates and so in order to minimise inter-plate variation both arrays were carried out sequentially during the same afternoon. The following genes, outlined in table 7 were measured using the array. Appendix 2 demonstrates the full gene names and expression profile in the MLO-Y4 cells.

Angiogenic factors

Growth factors and receptors: Angpt1 (Agpt), Bai1, Col18a1, Ctgf, Ereg, Fgf1, Fgf2, Fgf6, Fgfr3, Figf (Vegf-d), Flt1, Fzd5, Gna13, Itgav, Jag1, Kdr, Nrp1, Pgf, Stab1, Tek, Tnfsf12 (APO3L), Vegfa, Vegfb, Vegfc.

Adhesion molecules: Col18a1, Ctgf, Eng, Itgav, Nrp1, Stab1, Tek, Thbs1.

Proteases, inhibitors and other matrix proteins: Anpep, Col4a3, Mmp19, Serpinf1, Tmprss6.

Transcription factors and others: Angpt2 (Agpt2), Epas1, Hand2, Mapk14, Smad5, Tbx1, Tbx4, Tnfaip2.

Other factors involved in angiogenesis:

Cytokines and chemokines: Ccl11, Ccl2, Cs3, Cxcl1 (GRO), Cxcl2 (GRO2), Cxcl5 (CHEMOKINE), Ifng, Il1b, Il6, Tnf.

Other growth factors and receptors: S1pr1, Egf, Ephb4, Hgf, Igf1, Itgb3, Lep, Mdk, Npr1, Nrp2, Pdgfa, Plxdc1, Tgfa, Tgfb1, Tgfb2, Tgfb3, Tgfbr1.

Adhesion molecules: Cdh5, Itgb3, Lama5, Nrp2, Pecam1, Thbs2.

Proteases, inhibitors and other matrix proteins: Tymp, F2 (CF-2), Mmp2, Mmp9, Plau, Plg, Timp1, Timp2.

Transcription factors and others: Efna1 (Ephrin A1), Efnb2, Hif1a, Lect1, Ptgs1, Sphk1.

Table 7. Genes measured by the Qiagen® RT² Profiler mouse angiogenesis PCR array.

4.4 Results

4.4.i Dexamethasone modulated the expression of 16 genes involved in angiogenesis in MLO-Y4 cells

In accordance with manufacturer guidance, genes with a C_t value of 35 or less were classed as being expressed in the array. Using this criterion, 65 of the 84 genes in the array were detectable in the control samples (Appendix 2). There were a total of 19 genes that were undetectable which may reflect either a low transcript copy number or complete lack of expression of these genes in MLO-Y4 cells.

Expression of 13 genes in the array by MLO-Y4 cells was significantly altered (defined as a 2-fold change or greater) by dexamethasone treatment, with the expression of 6 genes increased and 7 genes decreased. The results are outlined in table 8.

Gene	Change in expression	Pro/anti-angiogenesis
Angiogenin (ang)	5.4-fold increase	Pro
Endothelin (edn1)	4.6-fold increase	Pro and anti
Fibroblast growth factor 2 (FGF2)	4.3-fold increase	Pro
C- fos induced growth factor (Figf)	4.3-fold increase	Pro
endothelial plasminogen activator inhibitor (Serpine1)	3.3-fold increase	Anti
Sphingosine kinase-1 (Sphk1)	7.7-fold increase	Pro
Aminopeptidase N (anpep)	3.1-fold decrease	Pro
Chemokine ligand 5 (CXCL5)	3.1-fold decrease	Pro
Interleukin-6 (IL-6)	16.3-fold decrease	Anti
Matrix metalloproteinase-2 (MMP2)	3.5-fold decrease	Pro
Urokinase-type plasminogen activator (Plau)	22.1-fold decrease	Pro
sphingosine-1-phosphate receptor 1 (S1pr1)	6.5-fold decrease	Pro
Transforming growth factor- β (Tgf β 2)	3.8-fold decrease	Anti

Table 8. 13 Angiogenesis-related genes whose expression in MLO-Y4 cells was significantly altered by dexamethasone (10^{-7} M). Data collected from a single control and single dexamethasone-treated assay. Expression defined at a Ct value of 35 or less.

4.5 Discussion

These results indicate that MLO-Y4 cells express a large number (64) of genes involved in angiogenesis pathways and that the expression of 13 of these genes is significantly altered by dexamethasone treatment. This is the first time that the expression profile of angiogenesis genes as well as the influence of GCs has been shown in osteocyte-like cells. The validation of the above results using RT-PCR has subsequently been carried out in studies following on from this thesis.

Angiogenin

Results of the array demonstrate a 5.4 fold increase in angiogenin expression following treatment with dexamethasone. Angiogenin is a potent inducer of neovascularisation *in vivo* – it acts to form a complex with actin that enhances the activity of tissue plasminogen activators. This generates plasmin which in turn facilitates destruction of the basement membrane which is necessary for the migration of endothelial cells during angiogenesis (Hu et al, 1991). Angiogenin is known to be elevated in various inflammatory disease states and is thought to form part of the acute phase response. Dexamethasone has been shown to enhance IL-6-induced production of angiogenin by hepatoma cells (Verselis et al., 1999) but there has been no published literature regarding angiogenin and bone cells and its potential role in ON is currently unstudied.

Endothelin 1

Dexamethasone treatment resulted in a 4.6 fold increase in endothelin 1 expression. Endothelin is a potent vasoconstrictor which stimulates endothelial cell proliferation, migration and invasion *in vitro* (Salani et al, 2000). Endothelin is known to be expressed by osteoblasts and osteocytes and has been shown to stimulate mitogenesis in osteoblasts as well as decreasing osteoclast activity and motility in mice (Yin et al, 2003). Long-term GC treatment in two studies (in pigs and rats) demonstrated enhanced endothelin-1 induced

vasoconstriction of the epiphyseal arteries in the femoral heads (Drescher et al, 2006, Drescher et al, 2006). It may be that in the development of ON the enhanced endothelin-activated vasoconstriction caused by GCs plays an important pathogenic role.

Basic fibroblast growth factor (FGF-2)

FGF-2 is a similar agent to VEGF in that it is activated by heparin sulfate and is a potent mediator of angiogenesis. It is found in tissue basement membranes and in the subendothelial extracellular matrix of blood vessels. In its inactivated form it is membrane bound and following activation by heparan sulfate-degrading enzymes it stimulates new blood vessel development. Although they induce angiogenesis via different signalling pathways cross-talk between VEGF and FGF-2 signalling has been reported (Mandriota et al, 1997). In pre osteoblasts, FGF-2 binds to fibroblast growth factor receptor 1 and increases proliferation and FGF-2 has been shown to contribute to the differentiation of osteoblasts to osteocytes (Kuhn et al., 2012) . FGF-2 is also thought to inhibit SOST and therefore exerts anabolic effects on bone formation, a finding that is consistent with evidence that FGF-2 knockout mice have decreased bone mass and bone formation (Montero et al, 2000). In terms of ON, FGF-2 expression has been shown to be decreased in pathological specimens of both traumatic and non-traumatic ON of the femoral head (Wang et al., 2011). Another study has shown that injection of recombinant FGF-2 into the femoral heads of rabbits with steroid-induced ON resulted in new bone formation in the femoral head and prevented the femoral head from collapsing. There was also a reduction in the severity of secondary osteoarthritis in the FGF-2 treated group. This study concluded that FGF -2 may be a promising strategy for the treatment of ON (Kuroda et al., 2010). In light of these studies the fact that our results show that dexamethasone caused a 4.3 fold increase in FGF-2 expression in MLO-Y4 cells is intriguing and highlights the complex actions of GCs on bone. FGF-2 and dexamethasone have been shown to act synergistically in

promoting cartilage formation by chondrocytes (Yokoyama et al, 2007) and dexamethasone has been shown to up-regulate FGF-2 expression in other tissues including brain astrocytes (Molteni et al, 2001).

C-fos induced growth factor (FIGF)

FIGF is also known as VEGFD and is therefore a member of the PDGF/VEGF family. FIGF binds to VEGFR2 and VEGFR3 and as well as promoting angiogenesis, it plays an important role in lymphangiogenesis, particularly in lymphatic tumour metastasis (Dai et al, 2007., Achen et al, 1998). In bone FIGF/VEGFR3 signalling pathways play a key role in the maturation of osteoblasts. The treatment of osteoblasts with recombinant FIGF induces the production of OCN and the formation of mineralised nodules (Orlandini et al, 2006). The array demonstrated that FIGF expression is increased 4.3 fold by dexamethasone which is in contrast to the down-regulation of VEGFA expression outlined in Chapter 3 of this thesis. As far as I am aware there have been no previous studies of dexamethasone on FIGF expression in bone or any other tissues and any potential role of FIGF in ON is also unstudied.

Serpine 1

Results from the array show a 3.3 fold increase in serpine1 expression following treatment with dexamethasone. Serpine 1 encodes the protein PAI-1. PAI-1 inhibits fibrinolysis via inhibition of t-PA and hence modulates angiogenesis by regulating plasmin-mediated proteolysis (Chen et al., 2012). The PA pathway has been described to be involved in bone remodelling (Martin et al., 1993) and dexamethasone is known to up-regulate Serpine1 in primary bone adipocytes (Hozumi et al., 2010). In terms of ON, several studies have shown

that certain polymorphisms of the Serpine1 gene are associated with increased risk of GC-induced ON of the femoral head, (Liang et al., 2013, Zhang et al., 2013b) with one study suggesting that the rs6092; AA/GA genotype is associated with a 2.8 fold increased risk of ON in children treated for ALL (French et al., 2008). It was suggested that the increased PAI-1 levels associated with this polymorphism may lead to reduced fibrinolysis, increased intraosseous venous pressure, compromised blood flow and bone death. However, an attempt to validate this finding in the UK population treated on UKALL 2003 failed to show the same association (Bond et al., 2011).

Sphingokinase 1

Dexamethasone treatment resulted in a 7.7 fold increase in Sphk1 expression in MLO-Y4 cells. Sphk1 is one of two enzymes that produce the enzyme sphingosine-1-phosphate (S1P). S1P exists as two isotypes, S1P1 and S1P2. S1P exerts both pro- and anti-angiogenic effects through S1P1 and S1P2 respectively (Takuwa et al., 2008). S1P has been described to be involved in both osteoblast and osteoclast migration (Quint et al., 2013) as well as promoting bone formation (Lotinun et al., 2013). There has not been any published research regarding Sphk1 or S1P and ON.

Aminopeptidase N (Anpep)

The expression of Anpep was up regulated 3.1 fold by dexamethasone treatment. Anpep is a metalloproteinase that degrades extracellular matrix. In angiogenesis this allows migration and invasion of endothelial cells. (Fukasawa et al, 2006) Anpep is also known as CD13 which in haematopoiesis is a marker of myeloid lineage. CD13 positive bone marrow-derived myeloid cells have been shown to promote angiogenesis, tumour growth and

metastases in malignant conditions (Dondossola et al., 2013). Aberrant CD13 expression is a relatively uncommon finding in ALL but its presence has been demonstrated to infer a poor prognosis in adults (Dalal et al., 2013, Craddock et al., 2013, Van Vlierberghe et al., 2013) and be associated with reduced early response to treatment in children (Sobol-Milejska et al., 2013). There does not appear to be any published literature regarding Anpep and bone cells or ON.

Chemokine ligand 5 (CXCL5)

The results demonstrated a 5-fold decrease in CXCL5 expression in MLO-Y4 cells. CXCL5 is a cytokine belonging to the CXC chemokine family that is also known as epithelial-derived neutrophil-activating peptide 78 (ENA-78). It is produced following stimulation of cells with the pro-inflammatory cytokines IL-1 or TNF α (Chang et al., 1994). By binding to CXCR2 it induces endothelial cell migration and tubule formation thus promoting angiogenesis (Moldobaeva et al, 2010). Clinically, CXCL5 has been mainly associated with atherosclerosis risk but it has also been shown in mice to play a role in bone repair by stimulating chemotaxis of MSCs and aiding their differentiation into osteoblasts (Nedeau et al, 2008). CXCL5 may also be involved in new bone formation via the Wnt signaling pathway (Heilmann et al., 2013) and FGF2 has been demonstrated to differentially regulate CXCL5 in osteoblastic and endothelial cells, thus promoting haemopoietic stem cell migration (Yoon et al., 2012). CXCL5 has also been shown to stimulate RANKL expression in Paget's disease, a condition associated with localized areas of increased osteoclastic activity and thus accelerated bone turnover. (Sundaram et al., 2013). There have been no reports of CXCL5 influence in ON pathophysiology.

IL-6

The angiogenesis array demonstrated a 16.3 fold reduction in IL-6 expression following dexamethasone treatment. This is in keeping with the results of ELISA experiments which have been extensively outlined and discussed in Chapter 3 of this thesis. The fact that we have demonstrated a reduction in IL-6 at both the mRNA and protein level adds weight to the validity of these findings.

MMP-2

Results show a 3.5 fold reduction in MMP-2 expression following dexamethasone treatment. MMP-2 is a proteolytic enzyme that is secreted by endothelial cells. It degrades extracellular matrix, thus allowing endothelial cell migration and therefore promoting angiogenesis (Stetler-Stevenson et al, 1999). MMP-2 is mainly produced by osteoblast cells and it is known to regulate type 1 collagen turnover during bone remodelling (Martin, 1993). Bone strength and integrity is lower in mice lacking MMP-2 gene expression (Nyman et al., 2011) and these mice show defective osteoblast behaviour (Mosig and Martignetti, 2013). In humans polymorphisms of the MMP-2 gene have been associated with risk of lumbar disc degeneration (Zhang et al., 2013a). In a rat model of GC-induced ON MMP-2 levels were significantly increased, and this was attenuated by the administration of the bisphosphonate alendronate (Jianzhong W et al, 2011). A scoring system consisting of single nucleotide polymorphisms (SNPs) from 5 genes including MMP-2 has been developed as a predictive tool for ON of the jaw associated with bisphosphonate therapy (Katz et al., 2011). MMP-2 expression is associated with T-cell ALL (Scrideli et al., 2010) and hypoxia in T-cell ALL lines increases MMP-2 expression (via the notch1 pathway) which has been shown to increase tumour invasiveness (Zou et al., 2013).

Sphingosine-1-phosphate receptor 1 (S1PR1)

Dexamethasone treatment resulted in a 6.7 fold decrease in S1PR1 expression by MLO-Y4 cells. S1PR1 is also known as endothelial differentiation gene 1 (EDG1) and is a G coupled protein receptor which binds the S1P. S1PR1 is highly expressed by endothelial cells and it has an important pro-angiogenic role in regulating endothelial cell cytoskeletal structure, migration, capillary-like network formation and vascular maturation (Hla and Maciag, 1990, Lee et al., 1998, Liu et al., 1999). In embryogenesis S1PR1 is one of the major agents responsible for vascular growth and development and S1PR1 knockout mice die during development due to a defect in vascular stabilisation (Chae et al., 2004). In vascular endothelial cells the binding of S1P to S1PR1 induces migration, proliferation, cell survival and morphogenesis into capillary-like structures (Lee et al., 1999). S1P also exerts *in vivo* synergistic activity with other angiogenic agents including FGF-2 and VEGF (Garcia et al., 2001, Liu et al., 2000). In bone S1PR1 signalling has been linked to osteoblast differentiation (Sato et al, 2012) as well as controlling the migration of osteoclast precursors from the bone marrow during bone remodelling (Ishii and Kikuta, 2013, Kikuta et al., 2013). There appear to be no published studies investigating the role of S1PR1 in ON or reporting modulation by dexamethasone. Any potential relationship between S1PR1 and leukaemia remains unstudied.

Urokinase-type plasminogen activator (PLAU)

Dexamethasone treatment down-regulated the expression of PLAU, by MLO-Y4 cells by 22.1 fold. PLAU encodes the enzyme urokinase-type plasminogen activator (u-PA), which along with tissue plasminogen activator (t-PA), initiates fibrinolysis by activating plasminogen. This results in degradation of extracellular components and activation of

other proteinases (Chen et al, 2012). PLAU is known to contribute to the pathogenesis of many malignant conditions (Sudol, 2011) and circulating u-PA levels have been shown to be a negative prognostic marker in breast cancer (Annecke et al., 2008). In bone, PLAU is known to be expressed by both osteoblasts and osteoclasts and contributes to bone remodelling. It is known to influence osteoblast differentiation (Sato et al, 2012) and mice lacking PLAU expression display increased BMD, increased osteogenic potential of osteoblasts, decreased osteoclast formation, and altered cytoskeletal organisation (Furlan et al., 2007). U-Pa has more recently been shown to promote angiogenesis and remodelling of cartilage during fracture repair (Popa et al., 2013). In keeping with the results of the angiogenesis array, GCs have been demonstrated to down-regulate u-PA activity in other cell types including breast epithelial cells (Pew et al., 2008), adipocytes (Seki et al., 2001) and acute promyelocytic leukaemia cells (Mustjoki et al., 1998). Indeed, high expression of PLAU is associated with a poor prognosis in acute myeloid leukaemia (Graf et al., 2005) whereas ALL cells usually do not express PLAU (Mustjoki et al., 1999, Scherrer et al., 1999). The role of PLAU in ON remains unstudied.

Transforming Growth Factor Beta 2(TGFβ2)

Dexamethasone decreased TGFβ2 expression 3.8 fold in MLO-Y4 cells. TGFβ2 is one of three isoforms in the TGFβ family. TGFβ is produced by most human tissues and plays an important role in proliferation and differentiation in many cell types. It plays an important role in embryogenesis in forming structures including the heart (Penn et al., 2012), as well as contributing to extracellular membrane formation and cellular migration during wound healing (Finnsen et al., 2013a, Finnsen et al., 2013b). In terms of angiogenesis more is known about the TGFβ1 isoform which appears to have both pro and anti-angiogenic properties (Govinden and Bhoola, 2003), suggesting that it acts as a balancing agent in this

process. TGF β 2 does contribute to bone remodeling, particularly by control of osteoblast differentiation and overexpression in mice has been associated with progressive bone loss and a phenotype that closely resembles the bone abnormalities seen in human hyperparathyroidism and osteoporosis (Erlebacher and Derynck, 1996, Erlebacher et al., 1998). In terms of ON, a recent study has shown increased risk in people with a particular SNP of the TGF β gene (Samara et al., 2012). Expression of TGF β 1 has also been shown to be increased in a rat model of radiation induced ON where it contributes to fibrosis formation (Tovar-Vidales et al., 2013).

In summary, the above experiments have demonstrated novel findings in terms of the relationship between sex steroids and VEGF in osteocytes as well as interactions with dexamethasone. These findings may contribute to the understanding of the pathophysiology of GC-induced ON, particularly in adolescent females.

To summarise, in the MLO-Y4 cell line, dexamethasone treatment significantly decreased the expression of six genes that are considered to be pro-angiogenic (anpep, CXC5, IL-6, MMP2, PLAU, S1PR1), and one gene considered to be anti-angiogenic (TGF β 2). IL-6 and PLAU showed the greatest changes in mRNA expression after dexamethasone treatment (fold decreases of 16.6 and 20 respectively). Six genes were significantly up-regulated in the angiogenesis array, four of which are considered to be pro-angiogenic genes (ang, fgf2, figf, sphk1) and one is considered as an anti-angiogenic factor (serpine1). In addition Edn 1, a potent vasoconstrictor and regulator of vascular tone, was also down regulated. As this PCR array is a single assay statistical significance cannot be calculated but it certainly appears firstly that the MLO-Y4 cell expresses an angiogenic profile and secondly that dexamethasone modulates several genes within the angiogenesis pathways. This is the first time that this has been demonstrated in osteocyte-like cells. During the time since this array was performed, all of the gene changes have been confirmed by follow on studies in

the same laboratory, and using qRT-PCR to measure mRNA expression relative to two housekeeping genes (beta actin, GAPDH)

The sensitivity of this assay, as defined by the manufacturer states that only greater than a two-fold change in gene expression within the array is considered significant. As a result VEGF expression which showed a 1.96 fold reduction following dexamethasone treatment was not considered a significant change. However, experiments outlined in Chapter 3 of this thesis confirmed (by qRT-PCR and ELISA) that dexamethasone does down-regulate VEGF gene expression by 58.7% (approximately 2-fold) and protein secretion. Within the gene array results there are a further five genes whose expression changed between 1.7-2 fold, and it would be interesting to subject these to more sensitive testing.

The specific clinical relevance of these findings to GC-induced osteonecrosis remains to be fully understood, but it is likely that dexamethasone therapy influences the above angiogenic factors *in vivo* and therefore disrupts endothelial cell proliferation and migration during the bone repair process. The next step to investigate these findings further would be to measure *in vivo levels* within an animal model of GC-induced ON as well as in pathological specimens from patients requiring surgical intervention.

CHAPTER 5

The effects of dexamethasone and 1,25 dihydroxy-vitamin D₃ on osteocyte RANKL and OPG gene expression and protein production

5.1 Introduction

In this chapter the effects of dexamethasone and 1,25(OH)₂D₃ on RANKL and OPG as investigated. Both of these agents play an important role in controlling bone turnover by influencing osteoclastogenesis and consequently rates of bone resorption. Although this process is well documented to be under osteocyte control (Nakashima et al., 2011, Xiong et al., 2011), the effects of GCs and the role of the RANKL: OPG ratio in the development of ON has not been previously investigated. Genetic studies in mice have recently revealed that osteocytes produce the majority of the RANKL that, together with OPG, controls osteoclast formation (Xiong and O'Brien, 2012, Xiong et al., 2011). RANKL is produced as an integral membrane protein that is shed to produce a soluble form (sRANKL) (Lacey et al., 1998a). Although early studies suggested that cell to cell contact is required for osteoclastogenesis (Takahashi et al., 1988), more recently it has been shown that CM containing large amounts of RANKL from MLO-Y4 cells is sufficient to stimulate this process (Kurata et al., 2006). This suggests a role of sRANKL in promoting osteoclastogenesis (Xiong and O'Brien, 2012, Mizuno et al., 2002). Both vitamin D and PTH are potent stimulators of RANKL in osteoblasts (O'Brien et al., 2012, Kim et al., 2006, Lee and Lorenzo, 1999, Fu et al., 2006) and recent studies have alluded to a similar effect in osteocytes (Ito et al., 2012, Matsuo, 2012). Despite the fact that osteocytes are the main source of RANKL in bone, it has not previously been investigated whether RANKL, as well as exerting its well know actions on cells of the osteoclast lineage, has any autocrine action on the osteocyte itself.

Furthermore, it is not known whether RANKL and OPG concentrations influence the development of GC-induced ON. As previously outlined results have demonstrated both dexamethasone and $1,25(\text{OH})_2\text{D}_3$ to influence the VEGF pathway within the osteocyte (see chapter 3), it was hypothesised that dexamethasone may also influence RANKL and OPG levels and that $1,25(\text{OH})_2\text{D}_3$ may modulate this effect.

5.2 Aims

1. To investigate the effects of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on:

- 15. Osteocyte sRANKL and OPG secretion.
- 16. Osteocyte RANKL and OPG gene expression.
- 17. The distribution of RANKL within the osteocyte.

2. To investigate any potential autocrine effect of RANKL in osteocytes and specifically on:

- iii. Osteocyte cell number.
- iv. VEGF gene expression and VEGF_{164} secretion.

5.3 Materials and methods

The concentrations of dexamethasone (10^{-7} M) and $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) used are the same as those used in the previously outlined investigations (see Chapter 3). The concentrations of RANKL used (0-50 ng/mL) are those used to promote osteoclastogenesis *in vitro*.

5.3.i ELISA

MLO-Y4 cells were seeded (4.6×10^3 cells/cm²) into 24 well plates. After 24 hours cells were treated with i) dexamethasone (10^{-7} M) and ii) $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) individually and iii) in combination or iv) medium containing vehicle (ethanol). Following a further 72 hours incubation medium was removed and sRANKL and OPG levels measured by ELISA using Quantikine® kits as described in chapter 2.4.iii and 2.4.iv. Total cellular protein per well was measured using the Bio-Rad protein assay as outlined in Chapter 2.5. Results were expressed as total sRANKL or OPG concentration in pg/μg protein. The ratio of RANKL: OPG within the conditioned media was also calculated.

In separate experiments, cells were seeded (2.4×10^4 cells/cm²) in 12-well plates and after 24 hours treated with RANKL (0 – 50 ng/mL). Control cells were treated with media containing vehicle only (0.1% BSA/PBS). After 72 hours VEGF₁₆₄ concentrations in the medium were measured by ELISA as outlined in chapter 2.4.i. Total cellular protein in each well was measured using the Bio-Rad protein assay as outlined in Chapter 2.5. Results were expressed as total VEGF₁₆₄ concentration in pg/ μg protein and expressed relative to a control of 1.

5.3.ii qRT-PCR

MLO-Y4 cells were seeded (4.6×10^3 cells/cm²) into 12 well plates. After 24 hours cells were treated with i) dexamethasone (10^{-7} M), ii) $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M), iii) dexamethasone (10^{-7} M) and $1,25(\text{OH})_2\text{D}_3$ (10^{-8} M) in combination or iv) medium containing vehicle (ethanol). In separate experiments, cells were seeded (2.4×10^4 cells/cm²) in 12-well plates and after 24 hours treated with RANKL (0 – 50 ng/mL). Control cells were treated with media containing

vehicle only (0.1% BSA/PBS). Cells were then further incubated for 72 hours at 37°C followed by RNA extraction, sodium acetate precipitation, DNase treatment (methodology outlined in chapter 2.7) and reverse transcription (methodology outlined in chapter 2.8). Expression of the RANKL, OPG and VEGF₁₆₄ mRNA in those cells treated with RANKL was measured by qRT-PCR as outlined in chapter 2.9. Gene expression was normalised to the expression of the housekeeping gene GAPDH. Results are expressed as a ratio compared to control samples, one of which was calibrated to 1. The following primer sequences were used for each of the genes.

Gene	Sense (5'-3')	Antisense (5'-3')	Product size (bp)	Source
VEGF ₁₆₄	TGCAGGCTGCTGTAAACGATG	GAACAAGGCTCACAGTGATTTCT	190	(Ruiz de Almodovar et al., 2010)
RANKL	TGGAAGGCTCATGGTTGAT	CATTGATGGTGAGGTGTGCAA	75	Marisol Vasquez
OPG	GAGTGTGAGGAAGGGCGTTAC	GCAAACTGTGTTTCGCTCTG	111	Dr Deborah Mason
GAPDH	GACGGCCGCATCTTCTTGATGCA	TGCAAATGGCAGCCCTGGTGAC	114	Marisol Vasquez (Primer-BLAST)

Table 9. Primer sequences

In further experiments MLO-Y4 cells were set up (375,000 cells/gel) in type 1 collagen gels (2mg/mL) using rat tail collagen as outlined in chapter 2.2. After 24 hours cells were treated for 72 hours with either i) dexamethasone (10^{-7} M), ii) $1\alpha 25(\text{OH})_2\text{D}_3$ (10^{-8} M) or iii) a combination of both of these agents. Control cells were treated with vehicle (ethanol) only. After a further 72 hours, the gels were collagenase digested (methodology outlined in Chapter 2.3.iii) and RANKL and OPG expressions were measured by qRT-PCR as described

for monolayer cells. Gene expression was normalised to the expression of the housekeeping gene GAPDH.

In separate assays IDG-SW3 cells were plated (4.0×10^4 cells/cm²) in 12 well plates and incubated at 33° C in proliferating conditions (defined in Chapter 2.1.iv). Once 80% confluent, cells were transferred into differentiating conditions (defined in Chapter 2.1.iv) and incubated at 37° C with replenishment of media every 2-3 days. Cells were treated on days 7, 14 and 21 or differentiation with i) dexamethasone (10^{-7} M), ii) 1,25(OH)₂D₃ (10^{-8} M) individually and iii) in combination or iv) medium containing vehicle (ethanol). After 72 hours incubation, RANKL and OPG gene expressions were measured and results normalised to GAPDH using identical methodology to that described for MLO-Y4 cells. Since these assays were only performed once, in order to provide a comparison to MLO-Y4 cells, statistical analyses were not carried out on the results.

5.3.iii Immunohistochemistry

MLO-Y4 cells were plated onto cover slips in 24 well plates at a seeding density of 4.6×10^3 cells/ cm². After 72 hours of incubation with or without 1,25(OH)₂D₃ (10^{-8} M) and /or dexamethasone (10^{-7} M), RANKL content of the cells was visually assessed using immunohistochemistry as outlined in chapter 2.6

5.4 Results

5.4.i Detection of sRANKL secretion by MLO-Y4 cells

Initial test assays using the Quantikine® ELISA kit revealed that sRANKL and OPG could only be reliably detected in the CM of MLO-Y4 cells after culture for 72 hours. sRANKL was not detectable in control samples after culture for 24 hours. Very low levels (up to 0.09pg/mL) were detectable in samples treated with $1,25(\text{OH})_2\text{D}_3$. Levels were however detectable in all treatment groups after 72 hours (Figure 18). Similar results were obtained for the OPG assay (results not shown).

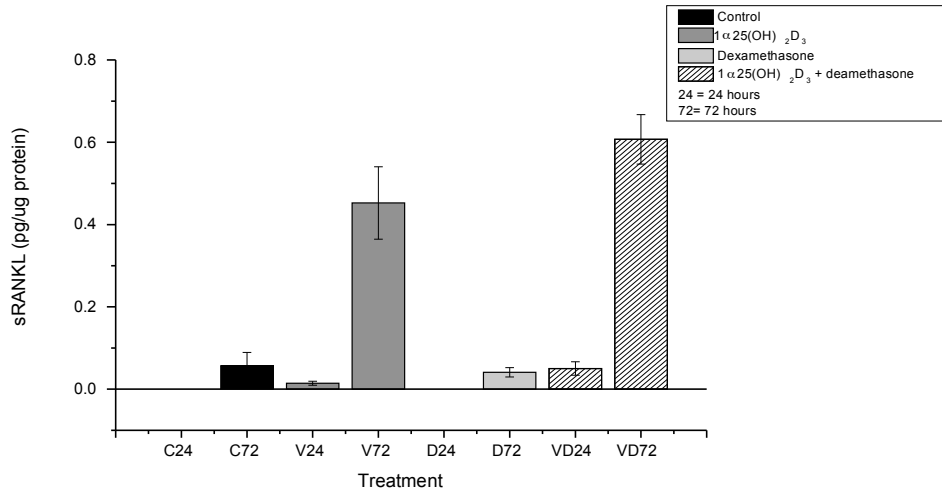


Figure 18. sRANKL levels in the CM of MLO-Y4 cells after 24 and 72 hours culture in the presence or absence of $1,25(\text{OH})_2\text{D}_3$ (V) and/or dexamethasone (D). C24/72 = control 24 and 72 hour assay. Each experiment performed three times (n=9 in each treatment group). Data represents mean sRANKL secretion (+/- SEM).

5.4.ii Effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on osteocyte RANKL and OPG secretion by osteocytes

Analysis of CM collected from untreated MLO-Y4 cells (after 72 hours incubation) by ELISA revealed very low (<10 pg/mL; <0.05 pg/ μ g total cellular protein) or undetectable concentrations of sRANKL. Results were similar following treatment with dexamethasone (10^{-7} M). However, in the cells treated with $1\alpha25(\text{OH})_2\text{D}_3$ (10^{-8} M), either alone ($p<0.01$) or in combination with dexamethasone (10^{-7} M, $p<0.001$), concentrations increased to 35-40 pg/mL (0.78 pg/ μ g total cellular protein) (Figure 16A). OPG protein concentrations in the CM were also very low. The highest concentrations were in the control samples (11-22 pg/mL; 0.19-0.29 pg/ μ g total cellular protein). However in the cells treated with $1\alpha25(\text{OH})_2\text{D}_3$ (10^{-8} M), either alone or in combination with dexamethasone (10^{-7} M), concentrations were reduced to a maximum of 6 pg/mL (0.1 pg/ μ g total cellular protein, $p<0.05$) (Figure 19A). In addition, $1\alpha25(\text{OH})_2\text{D}_3$ alone significantly increased (13.8 fold, $p<0.01$) the mean ratio of sRANKL: OPG secretion, and further increased this ratio (25 fold, $p<0.001$) when in combination with dexamethasone (Figure 19B). Dexamethasone treatment alone had no effect (Figure 19B).

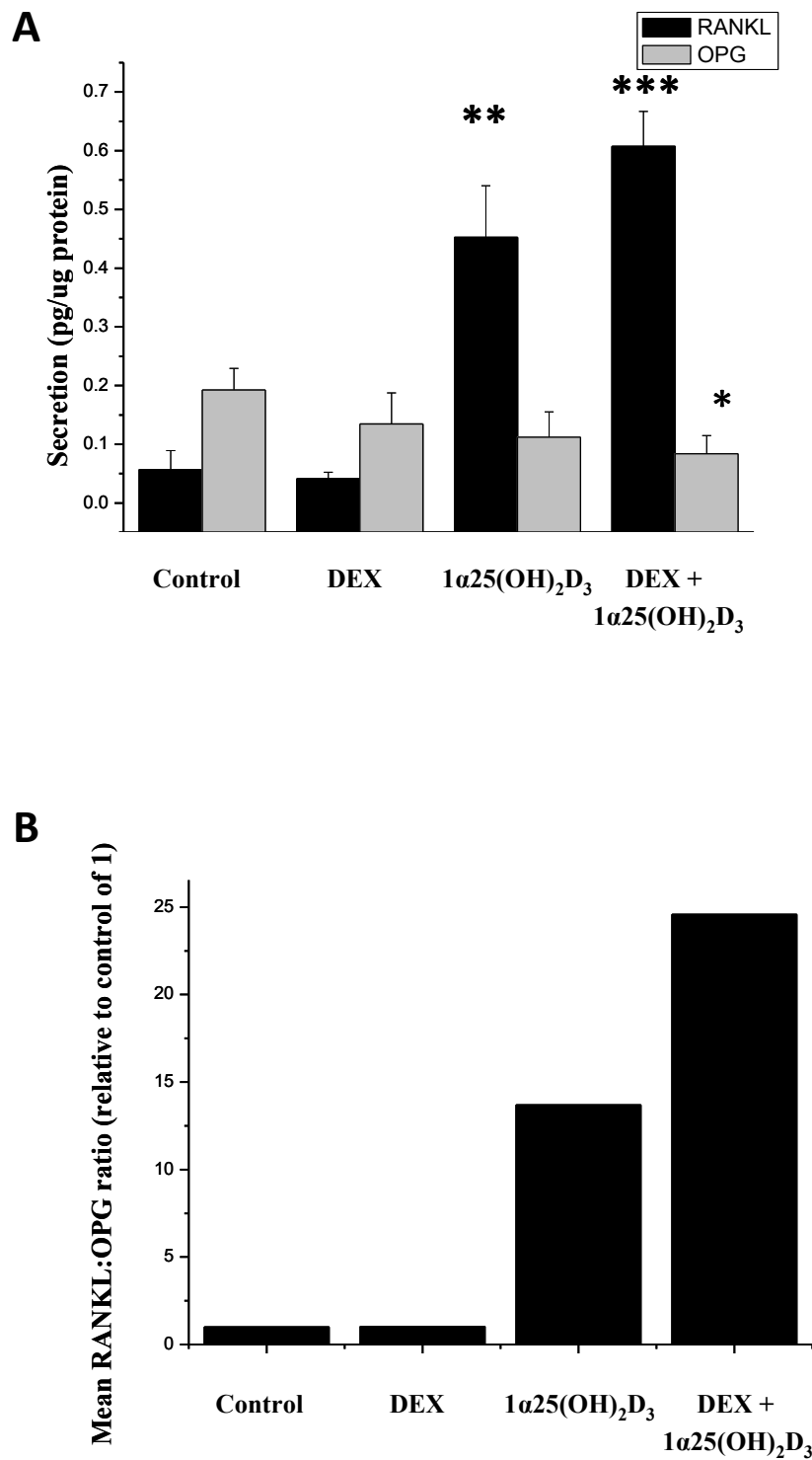


Figure 19. (A) Effects of dexamethasone (DEX) and 1α25(OH)₂D₃ alone and in combination on sRANKL and OPG secretion by MLO-Y4 cells (*p<0.05 **p<0.01 ***p<0.001 vs. control). Data represents mean sRANKL and OPG secretion (+/- SEM). (B) Ratio of sRANKL and OPG secretion by MLO-Y4 cells in monolayer. Ratio calculated by mean sRANKL concentration /mean OPG concentration. Each experiment was performed three times (n=9 in each treatment group).

5.4.iii Effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on osteocyte RANKL and OPG gene expression.

$1\alpha25(\text{OH})_2\text{D}_3$ (10^{-8}M) treatment of MLO-Y4 cells for 72 hours resulted in a 12.5 fold increase in RANKL expression ($p<0.001$). When cells were treated with a combination of dexamethasone and $1\alpha25(\text{OH})_2\text{D}_3$, there was a further increase in RANKL expression (33 fold, $p<0.001$) (Figure 20A). $1\alpha25(\text{OH})_2\text{D}_3$ treatment of MLO-Y4 cells also caused a significant 2.5 fold down-regulation of OPG expression ($p<0.01$), whereas dexamethasone had no effect. However, dexamethasone in combination with $1\alpha25(\text{OH})_2\text{D}_3$ resulted in a 5.1 fold down-regulation in OPG expression ($p<0.001$) (Figure 21A).

When MLO-Y4 cells were cultured in 3D collagen gels the same pattern was observed. Dexamethasone and $1\alpha25(\text{OH})_2\text{D}_3$ individually resulted in an up-regulation of RANKL gene expression (6.7 fold and 5.6 fold respectively, $p<0.05$) and a 12.0 fold up-regulation when present in combination ($p<0.001$) (Figure 20B). $1\alpha25(\text{OH})_2\text{D}_3$ alone (3.3 fold, $p<0.05$) and in combination with dexamethasone (3.2 fold, $p<0.05$) resulted in down-regulation in OPG expression. As seen in monolayer, no effect of dexamethasone alone on OPG expression was observed (Figure 21B).

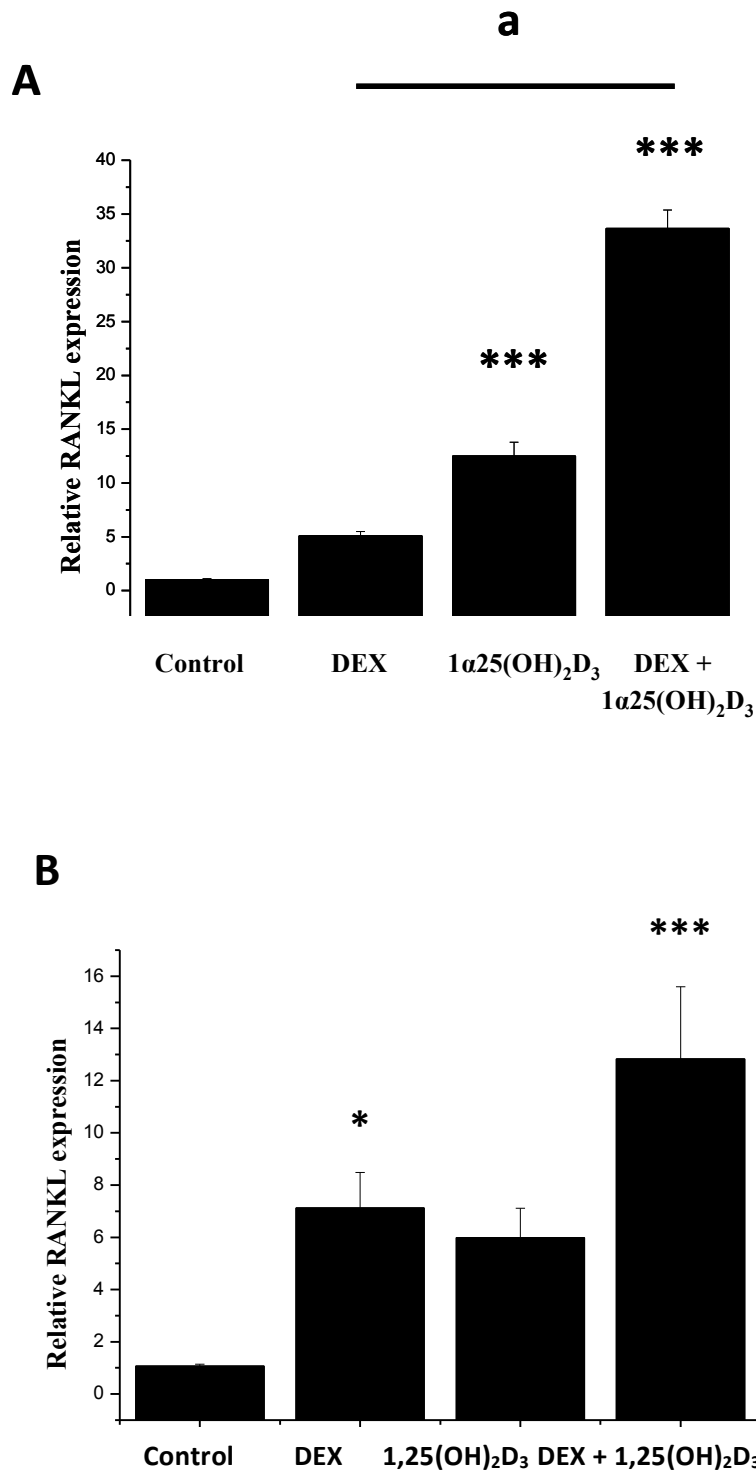


Figure 20. Effect of dexamethasone and 1 α 25(OH) $_2$ D $_3$ alone and in combination on RANKL expression in MLO-Y4 cells (A) in monolayer and (B) 3D collagen gels (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control. ^a $p < 0.001$ dexamethasone treatment alone vs. when treated in combination with 1 α 25(OH) $_2$ D $_3$). All experiments were performed three times (n=9 in each treatment group). Data represents mean RANKL expression (+/- SEM) relative to a control of 1.

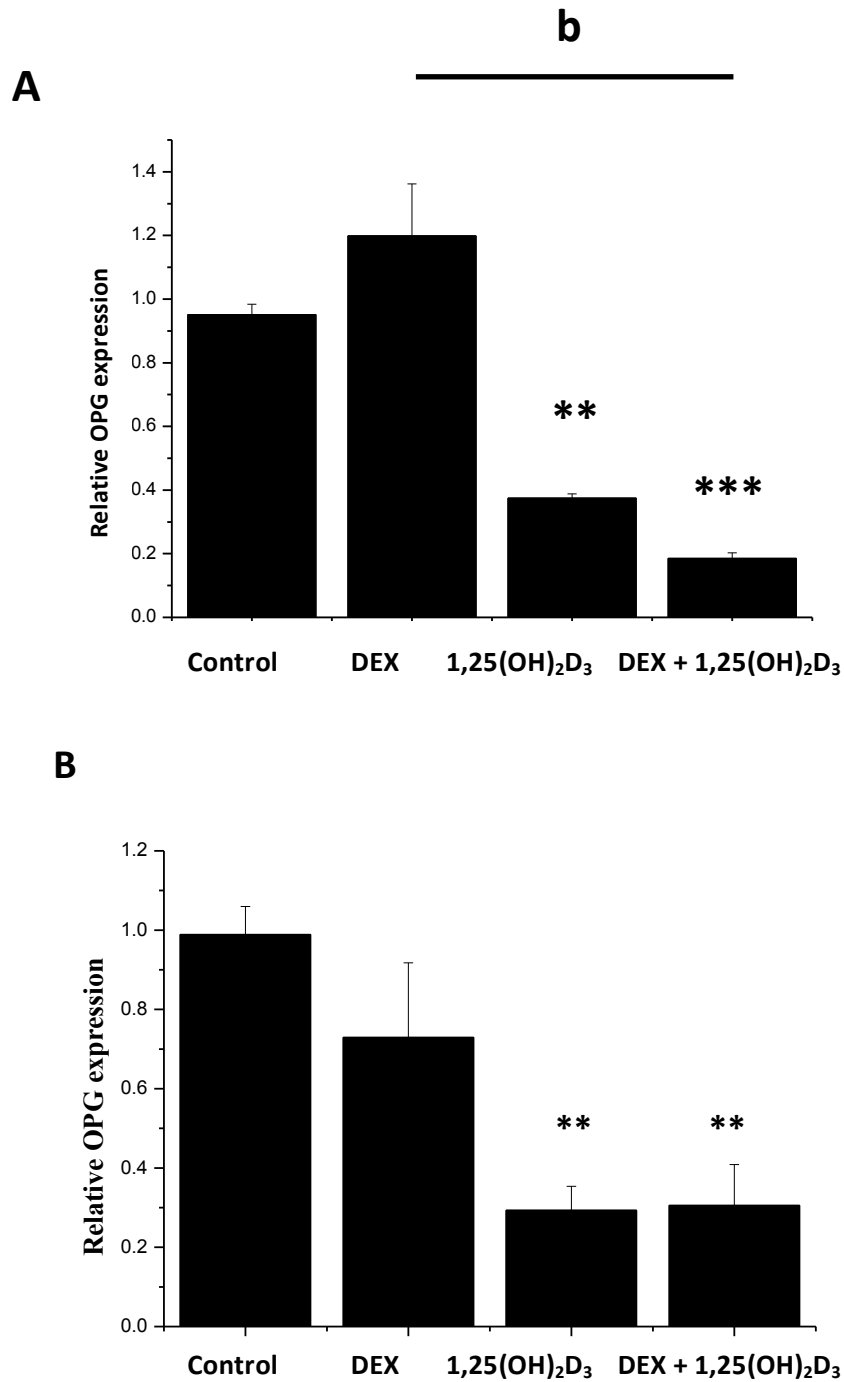


Figure 21. Effect of dexamethasone and $1\alpha,25(\text{OH})_2\text{D}_3$ alone and in combination on OPG expression in MLO-Y4 cells (A) in monolayer and (B) 3D collagen gels (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control. ^b $p < 0.001$ dexamethasone treatment alone vs. when treated in combination with $1\alpha,25(\text{OH})_2\text{D}_3$). All experiments were performed three times ($n=9$ in each treatment group). Data represents mean OPG expression (\pm SEM) relative to a control of 1.

The same increase in RANKL gene expression was seen after IDG-SW3 cells were treated with i) dexamethasone, ii) $1\alpha 25(\text{OH})_2\text{D}_3$ or iii) a combination of these two agents on days 7, 14 and 21 (Figure 22A). Treatment of IDG-SW3 cells demonstrated a varying pattern of OPG expression depending on the day of differentiation. Cells treated on day 7 and 14 showed the same pattern as observed in MLO-Y4 cells, but in cells treated on day 21, the down-regulation of OPG expression caused by $1\alpha 25(\text{OH})_2\text{D}_3$ alone and in combination with dexamethasone was not observed (Figure 22B). Measurement of relative gene expression in untreated control cells over the 21-day period showed that RANKL expression appears to increase 10 fold ($p=0.012$) over this time whereas there is no significant change in OPG expression during the same period (Figure 22C) These results were validated by comparison to expression of the house-keeping gene GAPDH whose expression did not change throughout this time period. This experiment was only performed once ($n=3$ replicates) and thus the data were not subjected to statistical analysis.

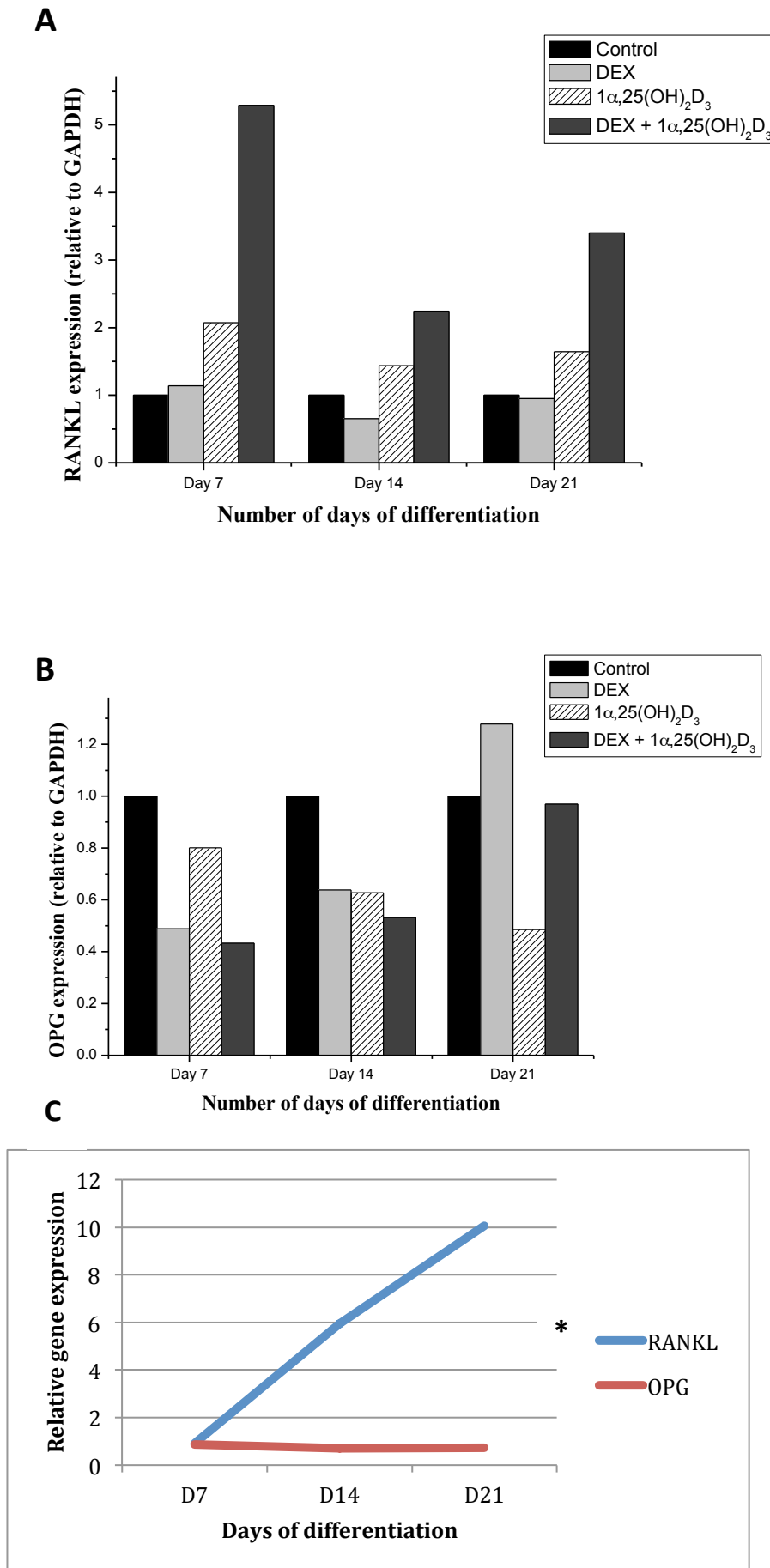


Figure 22. Effects of dexamethasone and 1 α 25(OH) $_2$ D $_3$ alone and in combination on (A) RANKL and (B) OPG gene expression in IDG-SW3 cells after 7, 14 and 21 days of differentiation. (C) Relative expression of RANKL and OPG gene expression in untreated

5.4.iv Effect of dexamethasone and 1,25(OH)₂D₃ on osteocyte RANKL production and distribution.

Following immunohistochemical staining using a RANKL antibody, RANKL appeared to be located within the nucleus and cytoplasm of MLO-Y4 cells (Figure 20). In cells treated with dexamethasone and 1,25(OH)₂D₃ the RANKL staining was more dense in colour although there did not appear to be any difference in distribution of RANKL within the cell (Figure 23).

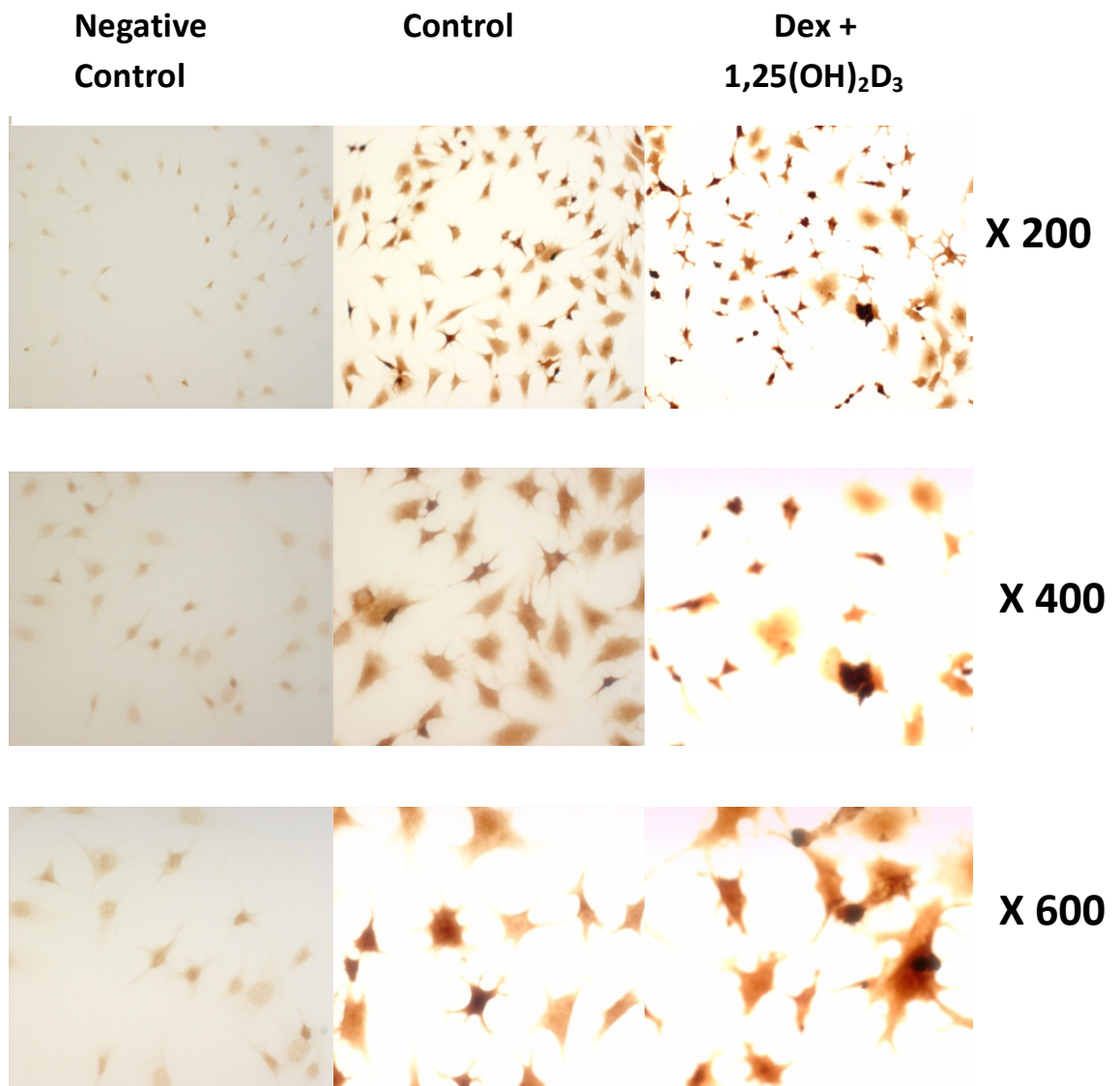


Figure 23. RANKL immunohistochemistry. MLO-Y4 cells following treatment with $1,25(\text{OH})_2\text{D}_3$ and dexamethasone. Photographs were taken at x200, x400 and x600 magnification.

5.4.v Effect of RANKL on MLO-Y4 cell number, VEGF₁₆₄ secretion and VEGF gene expression.

Treatment of MLO-Y4 cells with RANKL (0-50 ng/mL) did not affect cell number or VEGF mRNA expression and protein secretion (Figure 24 A and B)

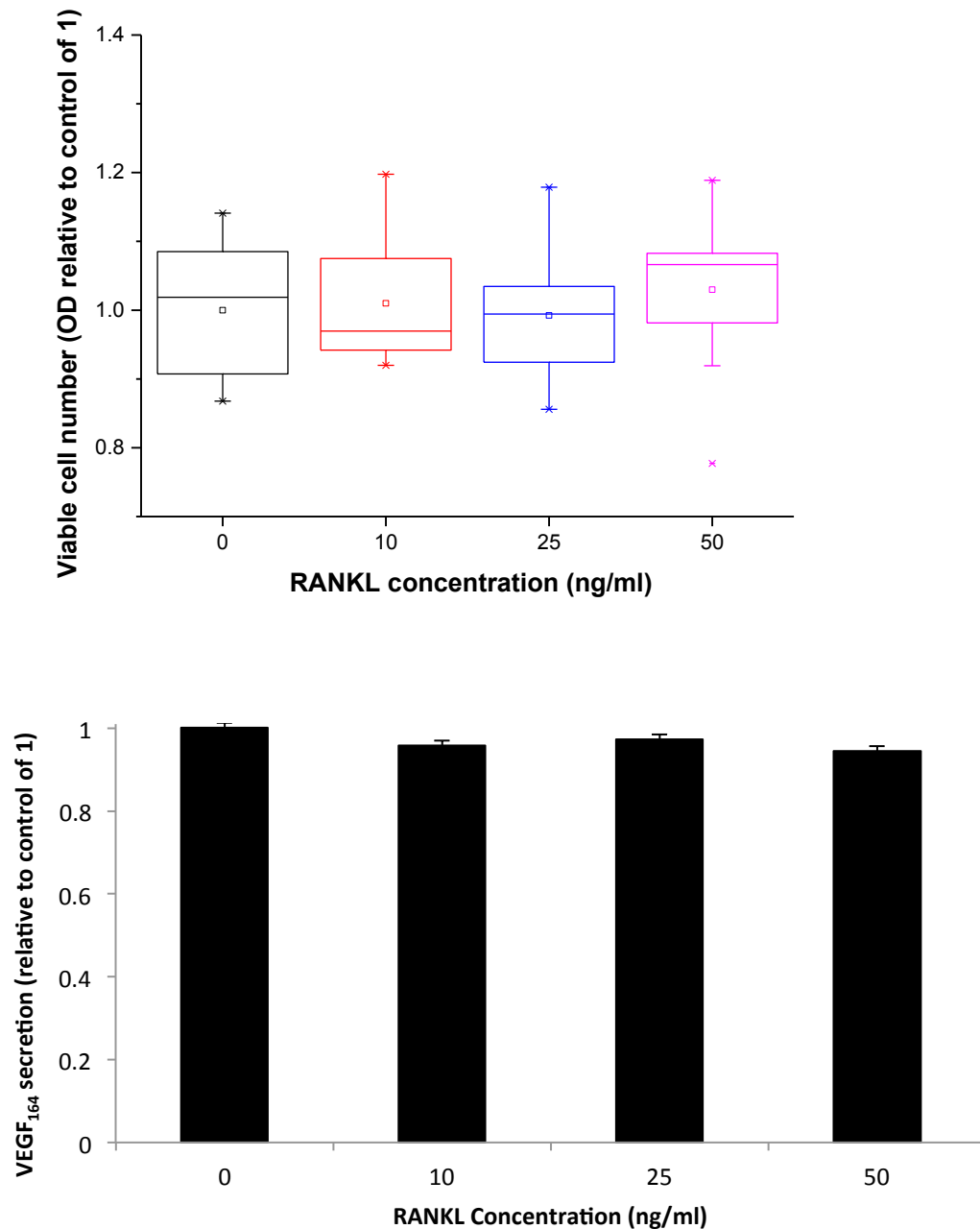


Figure 24. Effect of RANKL treatment on MLO-Y4 cell number (A) and VEGF₁₆₄ secretion (B). Each experiment performed 3 times. A = box and whisker plot (Band = median optical density (OD) relative to control of 1, box = first and third quartiles, whiskers = top and bottom values (n=36 in each treatment group)). B = Data representative of mean (+/- SEM) relative to a control of 1. (n=9 in each treatment group).

5.5 Discussion

Both MLO-Y4 and IDG-SW3 cells produce RANKL and OPG. These results are in keeping with previously published results showing that the osteocyte is the major source of RANKL within bone (Nakashima et al., 2011, Xiong et al., 2011) (Kurata et al., 2006). Both dexamethasone and $1,25(\text{OH})_2\text{D}_3$ alone and in combination resulted in an increase in osteocyte sRANKL secretion (as measured by ELISA), RANKL gene expression (q-RTPCR) and membrane bound RANKL protein production (immunohistochemistry). The effects on gene expression are demonstrated in MLO-Y4 and IDG-SW3 cell lines cultured in monolayer as well as MLO-Y4 cells cultured in 3D collagen gels. These results demonstrate a synergistic effect of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on RANKL production which contrasts to the opposing effects of these two agents on VEGF production as outlined in chapter 3.

The above results support existing literature that both dexamethasone and $1,25(\text{OH})_2\text{D}_3$ are promoters of osteoclastogenesis and bone resorption but this appears to be the first time that this synergistic relationship has been demonstrated in osteocyte cells. Early studies in mice calvaria showed that dexamethasone was able to enhance the effects of $1,25(\text{OH})_2\text{D}_3$ in promoting osteoclastogenesis (Nakagawa et al., 1999). A further study of bone marrow stromal cells again showed synergistic activity of corticosteroids and $1,25(\text{OH})_2\text{D}_3$ in stimulating osteoclast precursor differentiation. In this study deflazacort increased RANKL and decreased OPG mRNA in a dose dependent manner (Chung et al., 2001b). A study of human osteoblast cells has demonstrated that RANKL expression is related to differentiation state and that both $1,25(\text{OH})_2\text{D}_3$ and dexamethasone increased RANKL expression in pre-osteoblast cells where as little effect was seen in cells with a mature osteoblast phenotype (Atkins et al., 2003).

As expected a reciprocal down regulation of OPG secretion and gene expression is demonstrated in MLO-Y4 cells treated with $1,25(\text{OH})_2\text{D}_3$ alone or in combination with dexamethasone. This led to an overall increase in the sRANKL: OPG ratio measured by ELISA. Interestingly however, dexamethasone alone did not significantly influence the secretion or expression of OPG, suggesting that the down regulation seen when cells were treated with dexamethasone and $1,25(\text{OH})_2\text{D}_3$ in combination may be due to the influence of $1,25(\text{OH})_2\text{D}_3$ alone. In IDG-SW3 cells the down-regulation in OPG expression caused by dexamethasone is seen in the earlier stages of differentiation (day 7 and 14) but is not demonstrated once cells reach the mature osteocyte phenotype after 21 days of differentiation. The relationship between dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on the RANKL: OPG ratio appears complex and a previous study using neonatal mouse calvaria highlights this (Swanson et al., 2006). In this study, similarly to my results dexamethasone increased OPG as well as RANKL expression whereas vitamin D decreased OPG expression. Again a synergistic relationship on RANKL expression was demonstrated and the authors concluded that this offers an explanation of how glucocorticoids and $1,25(\text{OH})_2\text{D}_3$ interact to potentiate bone resorption (Swanson et al., 2006). The exact mechanism by which these two agents influence the RANKL: OPG pathway is not fully understood. Studies have shown that $1,25(\text{OH})_2\text{D}_3$ induces RANKL up-regulation primarily through actions initiated by the VDR (Kato, 1998) where as another study demonstrated the presence of a vitamin D response element within the mouse RANKL gene promoter (Kitazawa et al., 1999). There is also evidence for autocrine activity of $1,25(\text{OH})_2\text{D}_3$ on the RANKL:OPG pathway in osteocytes (Turner et al., 2013). A further study has demonstrated the presence of five regions on the mouse RANKL gene that are activated upon treatment with $1,25(\text{OH})_2\text{D}_3$ (Kim et al., 2006). One of these regions contained both the VDR and the GCR and was conserved within the human RANKL gene. These 5 regions may convey the synergistic

activity of dexamethasone and $1,25(\text{OH})_2\text{D}_3$ on RANKL activity but the exact mechanism requires further explanation.

The morphological distribution of RANKL within the nucleus and cytoplasm of the MLO-Y4 cell is consistent with previously published work (Kennedy et al., 2014, Kim et al., 2014a).

Although there was no demonstration of an autocrine effect of RANKL on the osteocyte in terms of change in cell number or VEGF production, there appears to be no other published studies that have investigated this. There is however evidence of the existence of RANK/RANKL autocrine activity in other cell types; one study of giant cell tumour (GCT) of bone, a histologically benign tumour of osteoclast-like cells has demonstrated a RANKL/RANK autocrine loop that determines sustained cell formation, (Avnet et al., 2013) and a further study of decidual stromal cells in early pregnancy also demonstrates a RANK/RANKL autocrine loop that contributes to the establishment of a viable pregnancy. (Meng et al., 2013) It would be interesting to investigate a potential autocrine effect further, in particular to assess whether the MLO-Y4 cells express RANK, the receptor for RANKL which is found on osteoclast precursors (Bellido, 2014).

In summary, results demonstrate that both dexamethasone and $1,25(\text{OH})_2\text{D}_3$ influence the RANKL: OPG pathway in both MLO-Y4 and ID3-SW3 cells. These two agents act independently as well as exerting synergistic activity on both RANKL gene expression and sRANKL secretion. These actions may be involved in the pathogenesis of ON, particularly as recent studies have demonstrated RANKL expression to be increased in the subchondral and necrotic areas of pathological specimens of femoral head ON (Wang et al., 2014,

Samara et al., 2014). This increase in RANKL expression suggests that GC treatment leads to increased bone remodelling in areas of ON, however what remains to be understood is whether this remodelling is pathogenic or part of the attempted repair process.

As PTH is also well known to influence RANKL in osteoblasts (Ezura and Noda, 2013, Greenfield, 2012) and osteocytes (Saini et al., 2013, Bellido et al., 2013) and a relationship between VEGF and PTH in osteoblasts (Deckers, 2000) as well as a potential role of PTH in the management of ON (Lau and Adachi, 2009) has been described, the next area of focus of this thesis (Chapter 5) is on PTH activity in MLO-Y4 cells as well as any interaction with dexamethasone.

CHAPTER 6

The effects of parathyroid hormone on osteocyte proliferation, VEGF and IL-6 secretion

6.1 Introduction

In this chapter the effects of PTH (1-34) on specific areas of osteocyte biology thought to be involved in GC-induced ON, namely osteocyte apoptosis and angiogenesis have been investigated. Despite the well documented effects of PTH (1-34), also known in its recombinant form as Teriparatide, on bone biology and its proven therapeutic use in osteoporosis (Warriner and Saag, 2013, Rizzoli et al., 2012, Murad et al., 2012), there have been very few studies investigating the role of PTH in GC-induced ON. As described in the introduction chapter there have been two case reports of the use of Teriparatide in treating ON of the jaw following bisphosphonate therapy (Harper and Fung, 2007, Lau and Adachi, 2009). It is only recently that the actions of PTH on the osteocyte are beginning to be documented, in particular its role in controlling FGF-23 secretion and phosphate metabolism, (Ito et al., 2012).

Although PTH has been shown to increase VEGF secretion by osteoblasts *in vitro* (Wang, 1996, Schlaeppli et al, 1997, Esbrit et al, 2000), very little is known regarding any potential influence of PTH on angiogenesis in bone. Studies have demonstrated that PTH enhances skeletal repair following fractures (Andreassen et al., 1999, Bukata and Puzas, 2010, Takahata et al., 2012) and in view of the fact that angiogenesis is vital for effective fracture healing, I hypothesised that PTH will influence osteocyte VEGF secretion.

IL-6 is known to promote osteoclast formation and thus bone resorption via RANKL independent processes (Blair and Athanasou, 2004, Masi and Brandi, 2001) and serum IL-6 levels have been shown to negatively correlate with BMD and grip strength in patients with osteopenia (Park et al., 2013). In the same study a relationship was demonstrated between circulating PTH levels and response to treatment as measured by the reduction in serum IL-

6. IL-6 is upregulated by PTH and is known to stimulate haematopoiesis in mouse bone marrow cultures (Pirih et al., 2010). Studies have shown PTH to increase soluble IL-6 receptor protein levels in primary osteoblast cultures *in vitro* as well as *in vivo* in haematopoietic cells of mice (Cho et al., 2013). This study concluded that the soluble IL-6 receptor protein is a mediator of hematopoietic and skeletal actions of parathyroid hormone. In view of these well-documented interactions between PTH and IL-6 in other bone cell types, it was hypothesised that PTH may influence osteocyte IL-6 secretion.

6.2 Aims

To investigate the effects of PTH (1-34) on:

18. osteocyte-cell line proliferation as measured by cell number
19. osteocyte-cell line VEGF₁₆₄ and IL-6 secretion
20. RANKL and OPG gene expression.

6.3 Materials and methods

The concentrations of PTH (1-34) used (10^{-6} – 10^{-9} M) are higher than circulating serum levels of PTH (Meulmeester et al., 1990, Bringham FR, 2011), but they are consistent with published work on PTH effects on bone cells *in vitro* (Saji et al., 2010, Midura et al., 2003, Woo et al., 2011). Initial experiments involved continuous treatment of cells with PTH for 24 hours, however in view of the clear evidence that continuous and pulsatile PTH exert different effects on bone (Bellido et al., 2012), the effects of pulsatile PTH (1-34) administration was also assessed.

6.3.i ELISA

In initial experiments MLO-Y4 cells were seeded (2.4×10^4 cells/cm²) into 24 well plates. After 24 hours cells were treated with human recombinant PTH (1-34) ($10^{-9} - 10^{-6}$ M) (Sigma Aldrich Co, UK) or medium containing vehicle (0.1% BSA in PBS). After a further 7 or 24 hours incubation at 37° C medium was removed and VEGF₁₆₄ and IL-6 measured by ELISA (methodology outlined in Chapter 2.4i and 2.4.ii). Total cellular protein in each well was measured using the Bio-Rad protein assay as outlined in Chapter 2.5. Results were measured as total VEGF₁₆₄ or IL-6 concentration in pg/μg protein and expressed relative to a control of 1.

Further experiments were then carried out to assess the effects of pulsatile vs. continuous PTH (1-34) administration for a total duration of 4 days in culture. For these assays MLO-Y4 cells were seeded (4.8×10^3 cells/ cm²) into 24 well plates. After 24 hours, cells were incubated in the presence of PTH (1-34) (10^{-7} M) for 1, 6 or 24 hours out of each 24 hour cycle. After PTH treatment cells were washed with PBS to remove the PTH and incubated for the remaining time in standard medium. Each treatment group had its own control wells which were treated with vehicle for the same duration. On day 4 medium was collected and VEGF₁₆₄ and IL-6 measured by ELISA and corrected for cellular protein as described above. Results were again measured as total VEGF₁₆₄ or IL-6 concentration in pg/μg protein and expressed relative to a control of 1.

6.3.ii Cell number assays

MLO-Y4 cells were seeded (4.8×10^3 cells/ cm²) into 96 well plates. After 24 hours cells were incubated in the presence of PTH (1-34) (10^{-7} M) for 1, 6 or 24 hours out of each 24 hour cycle for a total duration of 4 days. Each treatment group had its own control wells

which were treated with vehicle for the same duration. On day 4 cell number was measured using the [®] AQueous One Solution Cell Proliferation assay as outlined in Chapter 2.3.ii.

6.3.iii qRT-PCR

MLO-Y4 cells were seeded (4.8×10^3 cells/ cm²) into 12 well plates. After 24 hours cells were incubated in the presence of PTH (1-34) (10^{-7} M) for 1, 6 or 24 hours out of each 24 hour cycle for a total duration of 4 days. Each treatment group had its own control wells which were treated with vehicle for the same duration. On day 4 RNA was extracted using TRI-reagent[®] followed by sodium acetate precipitation, DNase treatment (methodology outlined in chapter 2.7) and reverse transcription (methodology outlined in chapter 2.8). Expression of RANKL and OPG was measured by qRT-PCR as outlined in chapter 2.9. Gene expression was normalised to the expression of the housekeeping gene GAPDH. Results are expressed as a ratio compared to control samples, one of which was calibrated to 1. The following primer sequences were used to measure mRNA expression.

Gene	Sense (5'-3')	Antisense (5'-3')	Product size (bp)	Source
VEGF ₁₆₄	TGCAGGCTGCTGAACGATG	GAACAAGGCTCACAGTGATTTTCT	190	(Ruiz de Almodovar et al., 2010)
RANKL	TGGAAGGCTCATGGTTGGAT	CATTGATGGTGAGGTGTGCAA	75	Primer BLAST
OPG	GAGTGTGAGGAAGGGCGTT AC	GCAAACGTGTTTCGCTCTG	111	Dr Deborah Mason
GAPDH	GACGGCCGCATCTTCTTGTC CA	TGCAAATGGCAGCCCTGGTGAC	114	Marisol Vasquez (Primer BLAST)

Table 10. Primer sequences

6.3.iv Assessing PTH activity on SOST expression in IDG-SW3 cells

IDG-SW3 cells were plated (4.0×10^4 cells/cm²) in 24 well plates and incubated at 33° C in proliferating conditions. Once 80% confluent, cells were transferred into differentiating conditions and incubated at 37° C with replenishment of media every 2-3 days. On day 21 PTH (1-34) (10^{-7} M) was added and cells were incubated for a further 48 hours. Control cells were treated with vehicle (0.1% BSA in PBS). RNA was extracted, using TRI-reagent® followed by sodium acetate precipitation, DNase treatment (methodology outlined in chapter 2.7) and reverse transcription (methodology outlined in chapter 2.8). Expression of SOST was measured by qRT-PCR as outlined in chapter 2.9. Gene expression was normalised to the expression of the housekeeping gene GAPDH. Results are expressed as a ratio compared to control samples, one of which was calibrated to 1.

6.4 Results

6.4.i Effect of PTH on VEGF₁₆₄ and IL-6 secretion

PTH did not significantly affect VEGF or IL-6 secretion in MLO-Y4 cells following 7 or 24 hours continuous incubation (Figure 25). Furthermore, PTH did not significantly modify VEGF₁₆₄ or IL-6 secretion when cells were cultured either continuously or with 1 and 6 hour pulses over a 4 day period (Figure 26).

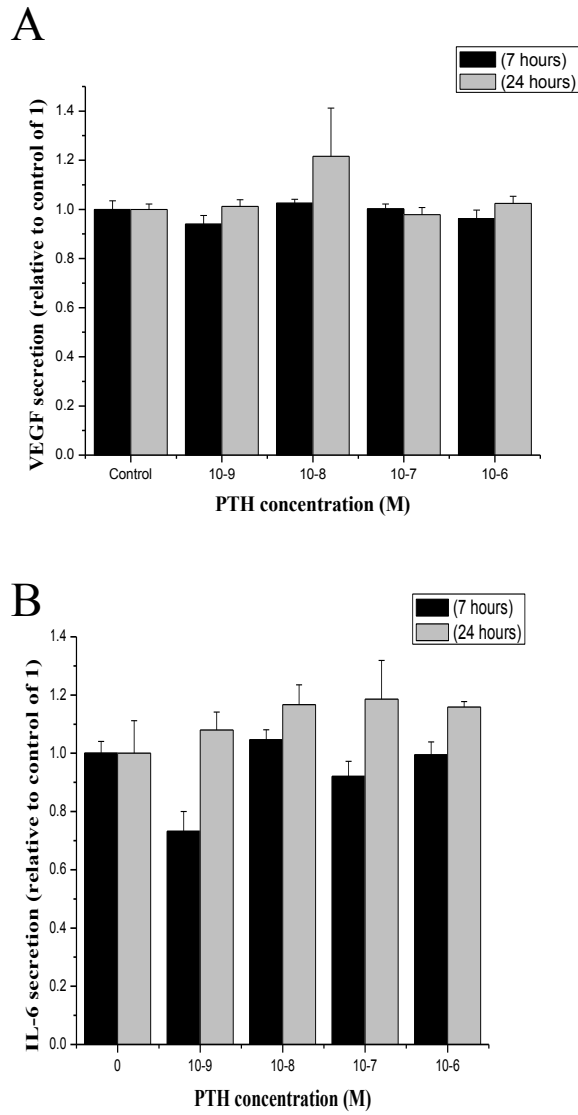


Figure 25. Effects of PTH (10^{-9} – 10^{-7} M) on (A) VEGF₁₆₄ and (B) IL-6 secretion by MLO-Y4 cells after 7 and 24 hours incubation. Each experiment performed three times (n=9 in each treatment group). Data represents mean VEGF and IL-6 secretion (+/- SEM) relative to control value of 1.

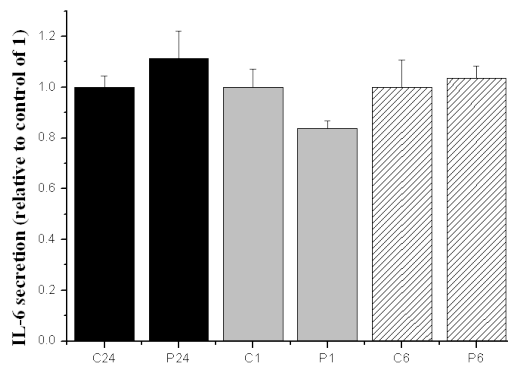
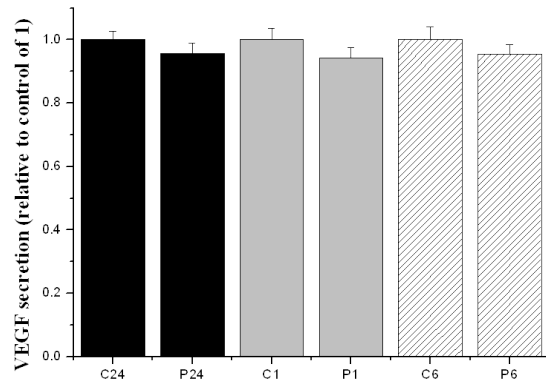


Figure 26. Effect of continuous (P24), 1 hour pulsatile (P1) and 6 hour pulsatile (P6) PTH (10^{-7} M) treatment of MLO-Y4 cells on (A) VEGF₁₆₄ and (B) IL-6 secretion compared to individual control samples for each treatment group (C24, C1 and C6). Each experiment performed three times (n=9 in each treatment group). Data represents mean VEGF and IL-6 secretion (+/- SEM) relative to control value of 1.

6.4.ii Effects of PTH on osteocyte cell number in vitro

Neither continuous nor pulsatile PTH administration had any significant effect on osteocyte cell number (Figure 27).

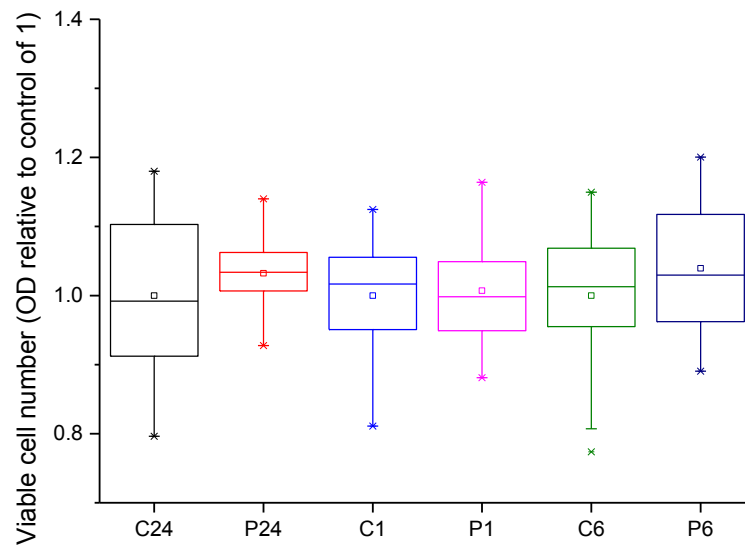


Figure 27. Effect of continuous (P24), 1 hour pulsatile (P1) and 6 hour pulsatile (P6) PTH (10^{-7} M) treatment of MLO-Y4 cells on cell number compared to individual control samples for each treatment group (C24, C1 and C6). The experiment was performed three times (n=36 in each treatment group). Band = median optical density (OD) relative to control of 1 (box = first and third quartiles, whiskers = top and bottom values)

6.4.iii Effects of PTH on osteocyte RANKL and OPG expression

A significant increase in OPG expression was seen after 6 hours of pulsatile treatment with PTH (41.0% increase, $P=0.009$) (Figure 28A) and although there was an apparent small increase with continuous treatment and 1 hour pulses, the results did not reach statistical significance. Neither continuous nor pulsatile PTH treatment affected RANKL expression by MLO-Y4 cells (Figure 28B).

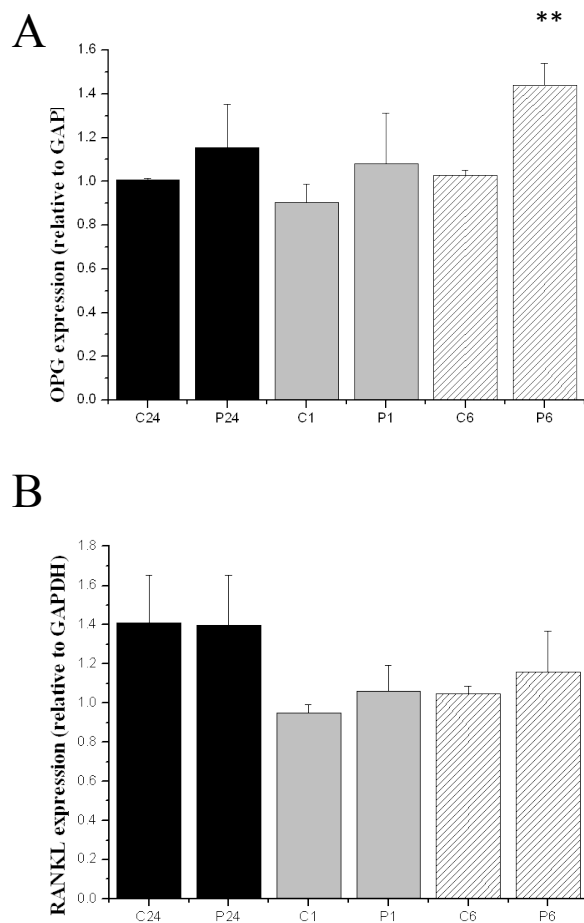


Figure 28. Effect of continuous (P24), 1 hour pulsatile (P1) and 6 hour pulsatile (P6) PTH (10^{-7} M) treatment of MLO-Y4 cells on (A) OPG and (B) RANKL gene expression compared to individual control samples for each treatment group (C24, C1 and C6). ** $p<0.01$ vs. control. Each experiment performed three times ($n=9$ in each treatment group). Data representative of mean OPG and RANKL expression (\pm SEM).

6.5 Effect of PTH on osteocyte SOST expression; positive control for PTH activity

As PTH is well documented to increase RANKL (Lee and Lorenzo, 1999, Huang et al., 2004, Greenfield et al., 1999, Horwood et al., 1998) and IL-6 (Dai et al., 2006, Feyen et al., 1989, Greenfield et al., 1993, Greenfield et al., 1996) activity in osteoblast cells, it was surprising to find that it did not affect RANKL gene expression by MLO-Y4 cells. In view of this and the fact that no significant effect of PTH was demonstrated in all but one of the above investigations, a positive control experiment was carried out using IDG-SW3 cells as outlined by Ito et al (Ito et al., 2012).

PTH treatment resulted in a significant 3.6 fold down-regulation ($p=0.004$) of SOST gene expression as previously reported (Figure 29) (Ito et al., 2012).

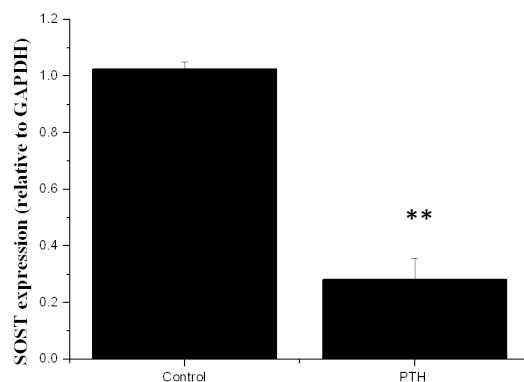


Figure 29. Effect of PTH (10^{-7} M) on SOST expression in IDG-SW3 cells, after 21 days of differentiation. The experiment was carried out three times ($n=9$ in each treatment group). ** $p<0.01$ vs. control . Data representative of mean SOST expression (\pm SEM) relative to control of 1.

6.6 Discussion

Normal serum circulating PTH levels are within the range of 11-54 pg/ ml (10^{-11} M) (Aloia et al., 2006a) although are often higher in people with vitamin D insufficiency (Aloia et al., 2006a, Aloia et al., 2006b). Experiments in this thesis have, wherever possible been designed to use treatment agents at concentrations that correspond to serum levels (although it is appreciated that this may differ from concentrations within the bone tissue itself). However, test experiments did not show any effect of PTH at 10^{-10} – 10^{-12} M and so a higher concentration was used in line with previously published studies of PTH on bone cells *in vitro* (Saji et al., 2010, Midura et al., 2003, Woo et al., 2011).

PTH treatment of MLO-Y4 cells either continuously or in pulses, did not significantly affect VEGF₁₆₄ or IL-6 secretion, cell number or RANKL expression. A significant increase in OPG expression was seen after 6 hourly pulses of PTH, but this is difficult to explain as no reciprocal effect on RANKL was seen and no significant effect was seen with the shorter 1 hour pulse. The lack of effect of PTH on the MLO-Y4 cell line is surprising as PTH has numerous, well-documented actions on both osteoblast proliferation (Hock et al, 1992) and the RANKL:OPG ratio (Lee and Lorenzo, 1999, Huang et al., 2004). In fact, in late osteoblast cells, PTH treatment resulted in an increase in RANKL and reduction in OPG mRNA expression (Huang et al., 2004a) which is contradictory to the increase in OPG expression seen in the above experiment.

The apparent lack of response of the MLO-Y4 cell to PTH seen in the above experiments may be explained by their expression of membrane voltage-operated calcium channels (VOCCs). Osteoblasts, osteoblast-like cell lines and bone marrow stromal cells all express VOCCs of which there are two types (L-type and T-type) as well as VOCC subunits (Gu et al., 2001, Amagai and Kasai, 1989, Chesnoy-Marchais and Fritsch, 1988, Preston et al., 1996) and VOCC expression has been shown in these cells to be induced by dexamethasone

(Publicover et al., 1994). These channels are believed to be involved in responses to hormonal stimuli including vitamin D and PTH (Gu et al., 2001, Barry et al., 1995, Lieberherr, 1987, Caffrey and Farach-Carson, 1989) as well as mechanical stimuli (Walker et al., 1999, Walker et al., 2000). An early study was unable to detect VOCC expression in MLO-Y4 cells unless they were stimulated with PTH, oestradiol or dexamethasone when VOCC activity was demonstrated in 5-10% of treated cells (Gu et al., 2001). Subsequent studies however have shown that osteocytes (including MLO-Y4 cells) do in fact express VOCCs but in contrast to osteoblasts which mainly express the L-type subunit (Ryder and Duncan, 2001, Meszaros et al., 1996, Thompson et al., 2011), they express predominantly the T-cell subunit (Shao et al., 2005, Thompson et al., 2011) which plays an important role in response to mechanical loading. It may be that this difference in VOCC expression between osteoblasts and osteocytes means that VOCC activity in the osteocyte predominantly responds to mechanical stimuli as demonstrated by Thompson et al (Thompson et al., 2011) rather than hormonal stimulation by PTH, but this has yet to be proven.

In order to confirm the activity of the PTH used, the well documented down regulation of SOST expression was demonstrated (Bellido et al., 2013, Pajevic, 2013, Ito et al., 2012). SOST, along with DMP1 and FGF-23 are expressed by mature osteocytes (Pajevic, 2013). MLO-Y4 cells are early osteocytes and therefore do not express SOST. Its expression in the IDG-SW3 cell line begins at day 14 and peaks at day 21 of differentiation (Woo et al., 2011). SOST, along with RANKL is one of the key agents in osteocyte control of bone remodelling. SOST inhibits bone formation by osteoblasts via blockade of the Wnt- β catenin signalling pathway (Bellido et al., 2013, Pajevic, 2013). Mice with targeted deletion of the SOST gene have high bone mass and SOST over expression in mice results in severe osteopenia (Winkler et al., 2003, Li et al., 2008, Rhee et al., 2011a). SOST expression is also highly regulated by mechanical forces and anti-SOST antibodies have been demonstrated in both

mice and rat models to prevent bone loss associated with disuse (Spatz et al., 2013, Li et al., 2009). These findings from animal studies have led to recent trials of monoclonal antibodies to SOST in the management of osteoporosis (Soen, 2013, Okazaki, 2011).

It is unclear therefore whether the lack of activity of PTH demonstrated in the initial experiments outlined above, was due to the choice of the MLO-Y4 cell line for the experiments and it would be interesting to repeat the above using IDG-SW3 cells. This work is on-going in the laboratory but is outside of the field of this thesis.

CHAPTER 7

The effects of sex steroids (oestradiol and testosterone) and dehydroepiandrosterone (DHEA) and their interaction with dexamethasone on osteocyte proliferation and VEGF₁₆₄ secretion.

7.1 Introduction

GC-induced ON is a condition that primarily affects the adolescent population in particular those aged between 10-20 years (Mattano et al., 2000, Kawedia et al., 2011, Relling et al., 2004, Ribeiro et al., 2001). Other risk factors include white race (Mattano et al., 2000), high body mass index (Niinimäki et al., 2007) and female sex (Mattano et al., 2000). Despite the well documented risk factors of adolescence and female sex, the mechanistic relationship between sex steroids, puberty and the development of ON remains unknown. With increasing numbers of adolescents being treated on paediatric protocols for ALL, survival rates are increasing (de Bont et al., 2004, Boissel et al., 2003, Ramanujachar et al., 2006), with the consequence of larger numbers of patients suffering long-term side effects of treatment. Many patients are requiring complex surgery to try and salvage their joints but inevitably large numbers of ALL survivors require joint (usually hip) replacements at a young age, which has significant implications for their long-term health.

Puberty and adolescence is a period of intense bone growth and accrual of peak bone mass (Mughal, 2011). Sex steroids play an important role in initiation of the pubertal growth spurt and the closure of epiphyseal growth plate at the end of puberty (Frank et al., 2000, Chagin et al., 2006, Juul et al., 2001). Osteocytes, including MLO-Y4 cells, express all three ERs (ER α , ER β and GPR30) and oestrogen has been demonstrated to protect MLO-Y4 cells from apoptosis (Tomkinson et al., 1997, Mann et al., 2007). In terms of VEGF signalling and

angiogenesis, oestrogen has been shown to up-regulate VEGF expression in growth plate chondrocytes both *in vitro* and *in vivo* (Emons et al., 2010). Relationships between VEGF concentrations and oestrogen have been demonstrated in both osteoblast cells (Pufe et al., 2007) and osteoclast formation (Kodama et al., 2004). VEGF gene polymorphisms have also been shown to influence lumbar spine BMD (Costa et al., 2009). These results suggest a definite association between oestrogen and angiogenesis during bone turnover and in preserving BMD but the exact relationship requires further understanding and as far as I am aware, there have been no studies to date of the relationship between VEGF and oestrogen in osteocytes or any relationship with the risk of developing ON.

In contrast to oestrogen, any relationship between testosterone and bone angiogenesis remains unknown. There is evidence that testosterone increases VEGF production in other cell types including myocardial cells following myocardial infarction (Chen et al., 2012b) and cells of the ventral prostate in rats (Montico et al., 2013). In contrast to these findings testosterone has been shown to reduce VEGF production by bone marrow mesenchymal stem cells (Ray et al., 2008).

Although there have been no studies of DHEA and ON, one study of human osteoblast cells has shown DHEA to have a steroid-sparing effect (Harding et al., 2006). In this study addition of DHEA prevented the increase in RANKL: OPG gene expression caused by dexamethasone. In the same study, no influence of DHEA on VEGF expression was demonstrated.

In this chapter, the effects of oestrogen, testosterone and DHEA on osteocyte cell number and VEGF₁₆₄ secretion were studied. The mechanism via which these effects take place were also investigated using SERMs (tamoxifen and fulvestrant) and the aromatase inhibitor (anastrozole). By using these agents to block the classical ERs and prevent

conversion of androgens to oestrogens respectively, both genomic and non-genomic pathways can be investigated. Both tamoxifen and fulvestrant have been shown to successfully antagonise ERs in previous *in vitro* studies of osteoblasts (Galea et al., 2013a) and a variety of aromatase inhibitors have also been demonstrated to effectively inhibit aromatase in bone cells *in vitro* (Miki et al., 2007b). The interaction between dexamethasone and oestradiol on osteocytes have been investigated, in order to identify effects that may be important in the pathogenesis of GC-induced ON in ALL.

7.2 Aims

To assess the effects of

1. oestradiol, testosterone and DHEA on osteocyte cell number and VEGF₁₆₄ secretion
2. the addition of ER antagonists (tamoxifen/fulvestrant) or aromatase inhibitor (anastrozole) to the above
3. the addition of dexamethasone to 1 and 2 above
4. dexamethasone and 1,25(OH)₂D₃ on ER expression.

7.3 Materials and methods

The concentrations of oestradiol used in these assays are representative of serum levels in pre and post-menopausal women, men (Vorvick, 2011) and adolescents in the later stages of puberty (Ruder et al., 2011, Ankarberg-Lindgren and Norjavaara, 2008). The concentrations of testosterone (Topiwala, 2012b, Ruder et al., 2011) and DHEA (Topiwala, 2012a) are also inclusive of normal serum levels. Concentrations of tamoxifen and fulvestrant used are consistent with published evidence of antagonistic activity in osteoblast cells (Galea et al., 2013a, Miki et al., 2007a). No *in vitro* studies have used anastrozole on bone cells but concentrations are consistent with antagonistic activity in breast cancer cells *in vitro* (Howell et al., 2000). Phenol red free α -MEM and charcoal stripped serum (methodology described in Chapter 2.1.v) were used in all assays to eliminate the oestrogenic effects of phenol red and any potential effects of endogenous sex steroids within the serum.

7.3.i Cell number assays

MLO-Y4 cells were seeded (4.6×10^3 cells/cm²) into 96 well culture plates and after 24 hours were treated with i) oestradiol (10^{-11} - 10^{-8} M), ii) testosterone (10^{-11} - 10^{-8} M), iii) DHEA (10^{-11} - 10^{-8} M) or iv) medium containing vehicle (ethanol). In separate assays cells were incubated with i) oestradiol (10^{-8} M) in the presence or absence of ER antagonists tamoxifen or fulvestrant (10^{-7} M) or ii) DHEA (10^{-8} M) in the presence or absence of anastrozole (10^{-7} M). Cells were then incubated at 37°C for a further 72 hours and cell number measured using the [®] AQueous One Solution Cell Proliferation assay as outlined in Chapter 2.3.ii.

6.3.ii VEGF₁₆₄ ELISA

MLO-Y4 cells were seeded (2.4×10^4 cells/cm²) into 24 well plates. After 24 hours cells were treated with i) oestradiol (10^{-11} - 10^{-8} M), ii) testosterone (10^{-11} - 10^{-8} M), iii) DHEA (10^{-11} - 10^{-8} M) or iv) medium containing vehicle (ethanol). In separate assays cells were incubated with i) oestradiol (10^{-8} M) in the presence or absence of oestrogen receptor antagonists tamoxifen or fulvestrant (10^{-7} M) or ii) DHEA (10^{-8} M) in the presence or absence of anastrozole (10^{-7} M). Assays where pre-treatment for 1 hour with tamoxifen, fulvestrant or anastrozole (10^{-7} M) prior to the addition of oestradiol or DHEA were carried out, in order to block the receptors and prevent competitive binding. A separate assay was also carried out where cells were treated with oestradiol (10^{-8} M) in the presence or absence of dexamethasone (10^{-7} M). After a further 24 hours incubation at 37°C medium was removed and VEGF₁₆₄ secretion was measured by ELISA as outlined in Chapter 2.4.i Total cellular protein in each well was measured using the Bio-Rad protein assay as outlined in Chapter 2.5. Results were measured as total VEGF₁₆₄ concentration in pg/ µg protein and expressed relative to a control of 1.

7.3.iii qRT-PCR

MLO-Y4 cells were seeded (4.6×10^3 cells/cm²) into 12 well plates. After 24 hours cells were treated with i) dexamethasone (10^{-7} M), ii) 1,25(OH)₂D₃ (10^{-8} M) or iii) dexamethasone (10^{-7} M) and 1,25(OH)₂D₃ (10^{-8} M) in combination or iv) medium containing vehicle (ethanol). Cells were then further incubated for 72 hours at 37°C followed by RNA extraction, sodium acetate precipitation, DNase treatment (methodology outlined in chapter 2.7) and reverse transcription (methodology outlined in chapter 2.8). Expression of ERα, ERβ and GPR30 were measured by qRT-PCR as outlined in chapter 2.9. Gene expression was normalised to the expression of the housekeeping gene GAPDH. Results are expressed as a ratio

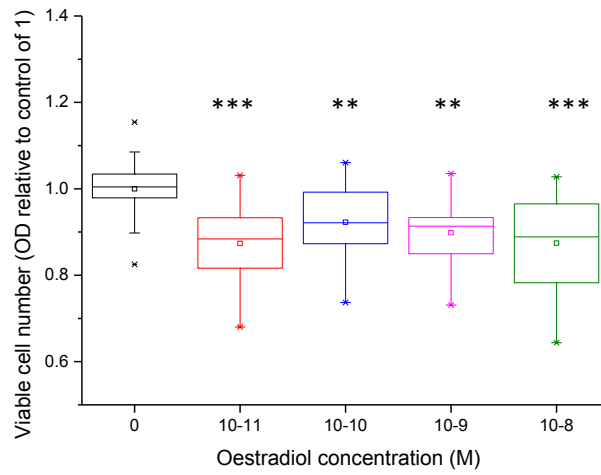
compared to control samples, one of which was calibrated to 1. The following primer sequences were used for each of the genes; ER α 5' GACCAGATGGTCAGTGCCTT and 3' ACTCGAGAAGGTGGACCTGA, ER β 5' CAGTAACAAGGGCATGGAAC and 3' GTACATGTCCCACTTCTGAC (Tachibana et al., 2000) and GPR30 5' GTCACGCCTACCCCTTGACA and 3' CCTGAAGGTCTCTCCAGGAA (Otto et al., 2009).

7.4 Results

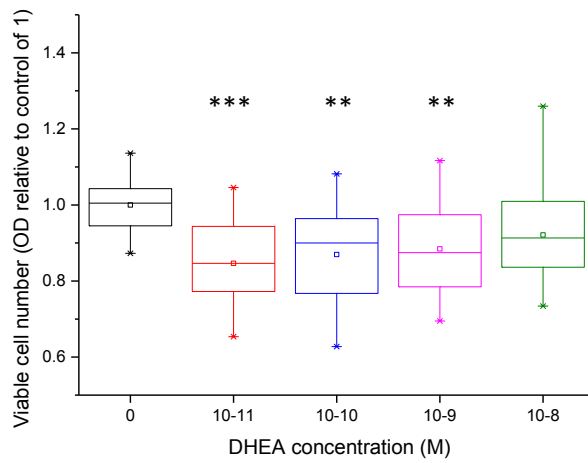
7.4.i Effects of oestradiol, testosterone and DHEA on MLO-Y4 cell number

Oestradiol significantly ($p=0.02$ - <0.001) decreased MLO-Y4 cell number at all concentrations tested (7.2-12.7% reduction) (Figure 30A). DHEA also significantly ($p=0.005$ - <0.001) decreased MLO-Y4 cell number (11.6 – 15.4 % reduction at 10^{-9} M and 10^{-11} M respectively) (Figure 30B). Testosterone did not affect cell number. (Figure 30C)

A



B



C

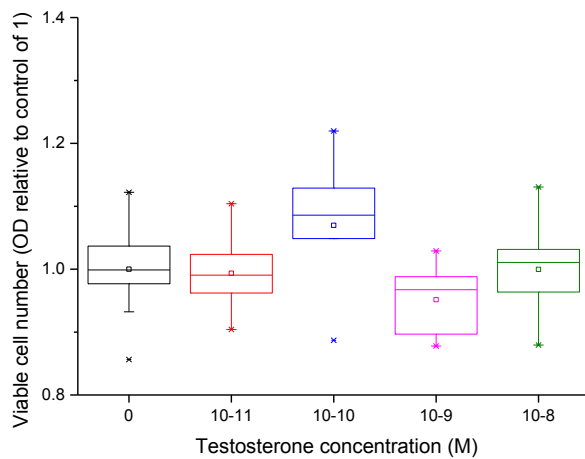
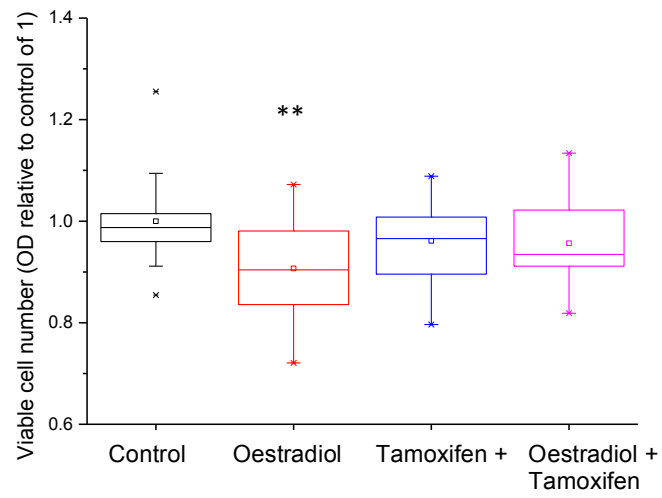


Figure 30. Effect of increasing concentrations of (A) oestradiol, (B) DHEA and (C) testosterone on MLO-Y4 cell cell number (**p<0.01 ***p<0.001 vs. control) Each experiment was performed three times (n=36 in each treatment group). Band = median optical density (OD) relative to control of 1 (box = first and third quartiles, whiskers = top and bottom values).

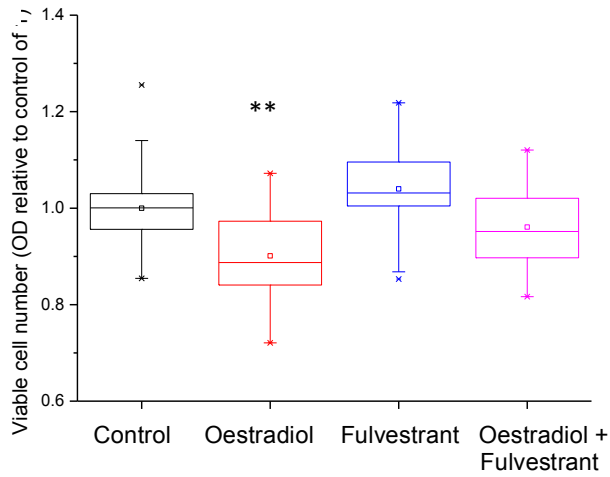
7.4.ii Effect of the addition of ER modulators (tamoxifen and fulvestrant) or aromatase inhibitor (anastrozole).

In the presence of either tamoxifen (10^{-7} M) or fulvestrant (10^{-7} M) the reduction in cell number caused by oestradiol was abolished (Figure 31A and B). The reduction in cell number caused by DHEA (10^{-8} M) was also prevented by the addition of the aromatase inhibitor anastrozole (Figure 31C).

A



B



C

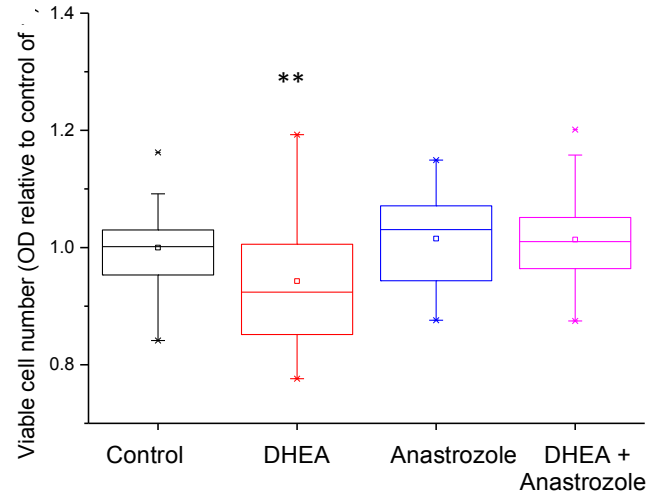


Figure 31. Effects of oestradiol (10^{-8} M) in the presence or absence of ER antagonists (A) tamoxifen and (B) fulvestrant (both 10^{-7} M) on MLO-Y4 cell number. (C) Effects of DHEA (10^{-8} M) in the presence or absence of the aromatase inhibitor anastrozole (10^{-7} M) on MLO-Y4 cell number. (**, $p < 0.01$ vs. control). Each experiment was performed three times.

7.4.iii Effect of oestradiol, testosterone and DHEA on VEGF₁₆₄ secretion by MLO-Y4 cells

Oestradiol (10^{-9} – 10^{-11} M) significantly increased VEGF₁₆₄ concentration (23.5-34.3% increase, $p=0.04$ - <0.001) (Figure 32A). DHEA also increased VEGF₁₆₄ secretion, although this was only significant at 10^{-10} M (19.2% increase, $p<0.05$) and 10^{-8} M (20.4% increase, $p<0.05$) (Figure 32B). Testosterone had no significant effect (Figure 32C).

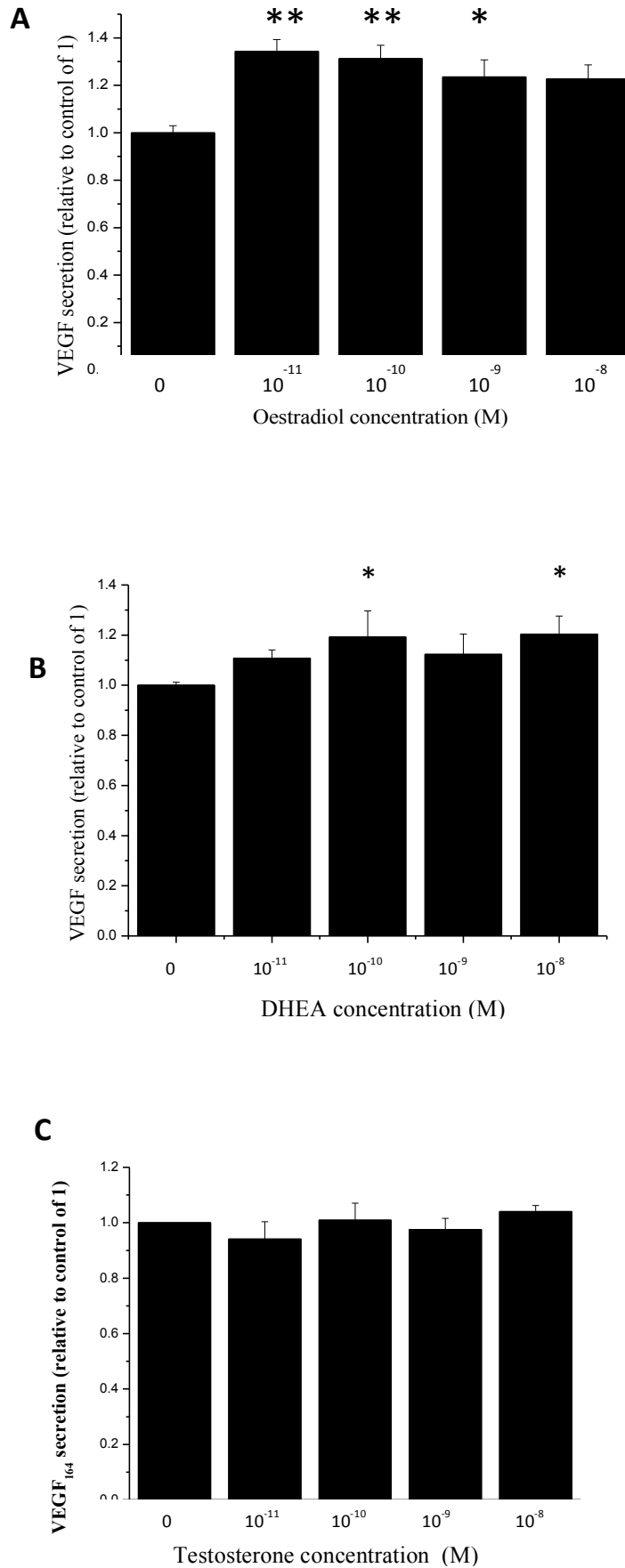


Figure 32. Effects of increasing concentrations of (A) oestradiol, (B) DHEA and (C) testosterone on VEGF₁₆₄ secretion by MLO-Y4 cells (*p<0.05, **p<0.01 and ***p<0.001 compared to control). Each experiment was

conducted in triplicate (n=3) for each treatment group. (A) Oestradiol, (B) DHEA and (C) testosterone.

7.4.iv Effects of the addition of oestrogen receptor modulators (tamoxifen and fulvestrant) or aromatase inhibitor (anastrozole).

Neither pre-treatment for 1 hour with ER antagonists or addition at the same time as oestradiol could prevent the increase in VEGF₁₆₄ secretion measured (Figure 33 A&B).

Similarly, anastrozole treatment did not prevent the increase in VEGF₁₆₄ secretion caused by DHEA (Figure 33C).

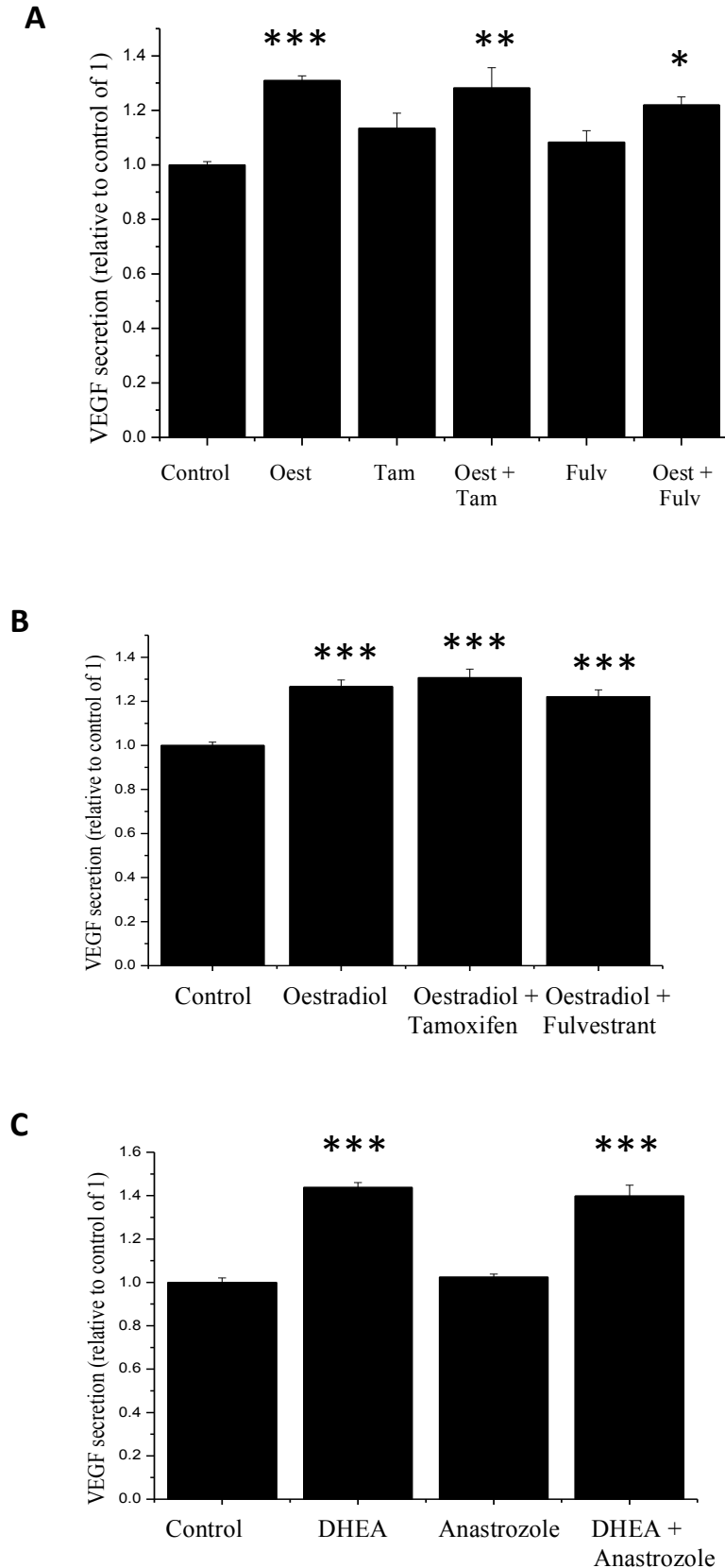


Figure 33. Effects of oestradiol (10^{-8} M) in the presence or absence of ER antagonists tamoxifen and fulvestrant (both 10^{-7} M) either (A) added at the same time as oestradiol or (B) pre-treated for 1 hour on VEGF₁₆₄ secretion by MLO-Y4 cells. (C) Effects of DHEA in the presence or absence of the aromatase inhibitor anastrozole on MLO-Y4 VEGF₁₆₄ secretion. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control) Each experiment was performed three times ($n=9$ in each treatment group).. Data representative of mean VEGF secretion (\pm SEM) relative to a control of 1.

7.4.v Effects of oestradiol in the presence or absence of dexamethasone on MLO-Y4 VEGF₁₆₄ secretion

As demonstrated previously in chapter 3, dexamethasone (10^{-7} M) significantly decreased VEGF₁₆₄ secretion (20.5% reduction, $p < 0.05$). Oestradiol treatment resulted in a 28.4% (10^{-10} M) and 41.2% (10^{-8} M) increase in VEGF₁₆₄ secretion. When dexamethasone (10^{-7} M) and oestradiol (10^{-10} M and 10^{-8} M) were added in combination, the increase in VEGF₁₆₄ secretion caused by oestradiol was abolished by dexamethasone and VEGF₁₆₄ levels were similar to control values (Figure 34).

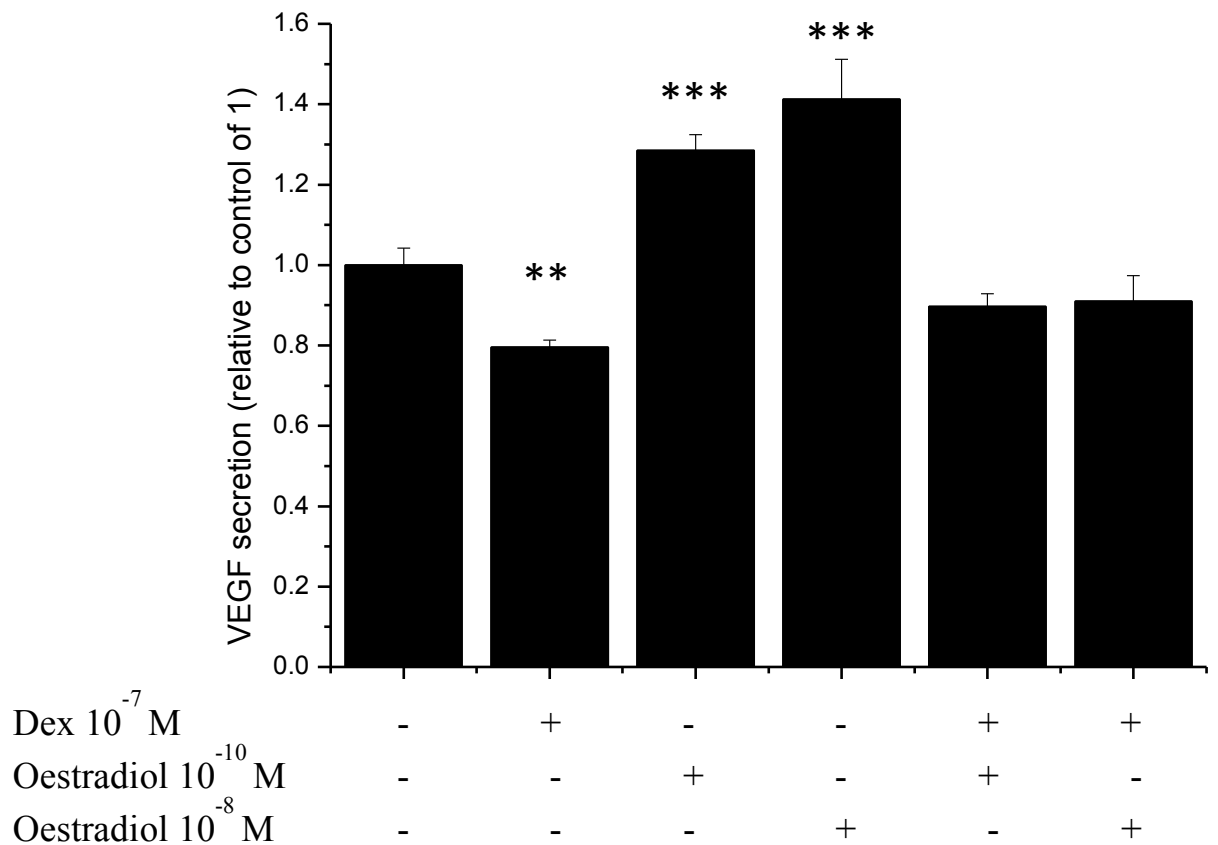


Figure 34. Effect of oestradiol (10^{-10} and 10^{-8} M) in the presence or absence of dexamethasone (10^{-7} M) on VEGF₁₆₄ secretion by MLO-Y4 cells. (** $p < 0.01$, *** $p < 0.001$ compared to control). Each experiment was performed three times ($n = 9$ in each treatment group). Data representative of mean VEGF secretion (\pm SEM) relative to a control of 1.

7.4.vi Effect of dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$ on ER expression by MLO-Y4 cells.

Both dexamethasone (10^{-7} M) and $1\alpha 25(\text{OH})_2\text{D}_3$ (10^{-8} M) significantly decreased ER α expression by MLO-Y4 cells. (51.9% $p=0.002$ and 37.6% $p<0.05$ reduction respectively).

When added in combination there still seemed to be a reduction (31.2%) but this did not reach statistical significance ($p=0.07$) (Figure 35A).

Although a 54.4 % reduction in ER β expression was seen with $1\alpha 25(\text{OH})_2\text{D}_3$ treatment this did not reach statistical significance. No significant effect of dexamethasone on ER β expression was demonstrated (Figure 35B). No significant effect of either $1\alpha 25(\text{OH})_2\text{D}_3$ or dexamethasone on GPR30 gene expression was seen (Figure 35C).

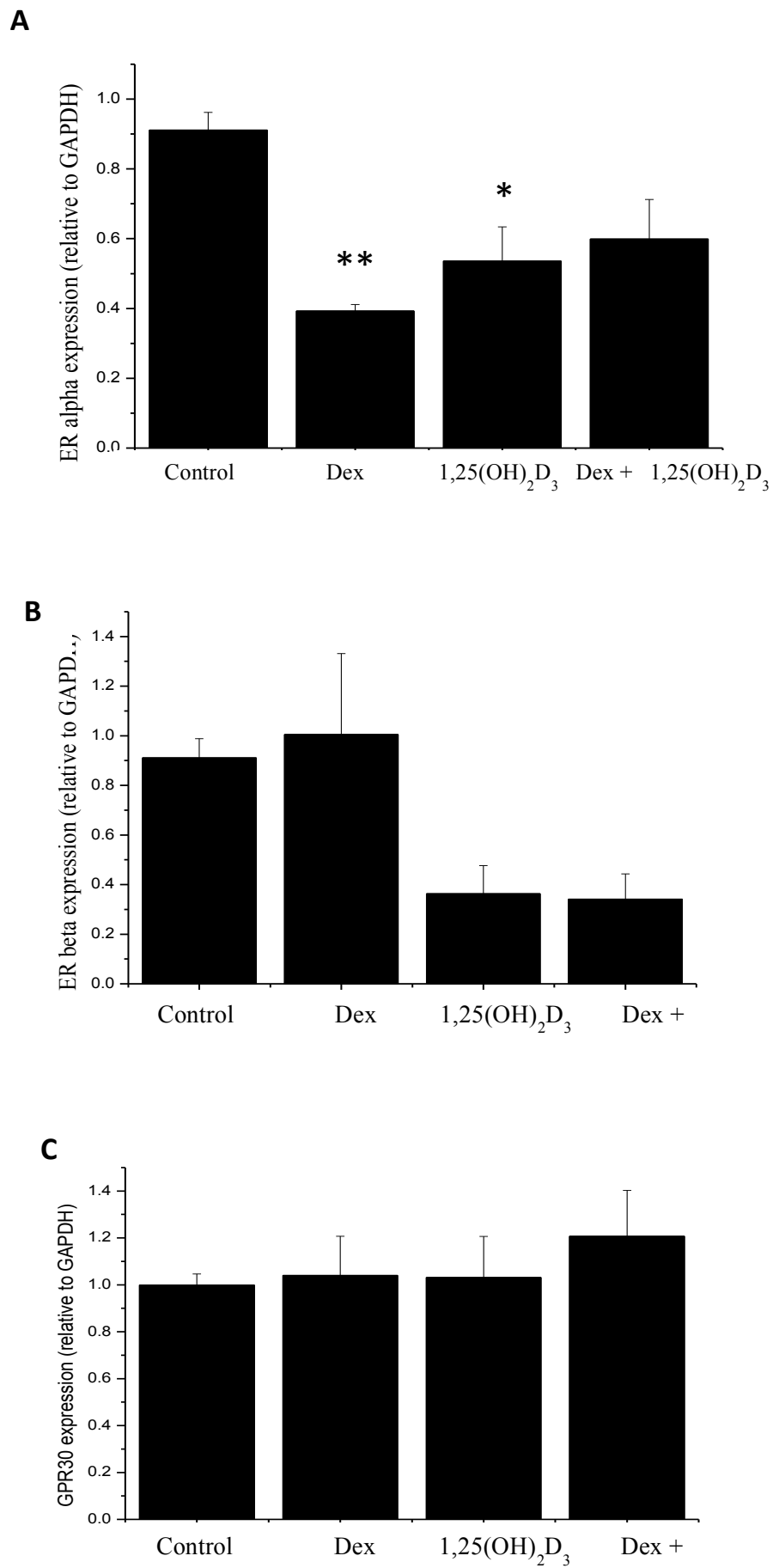


Figure 35. Effects of dexamethasone (10^{-7} M), $1\alpha 25(\text{OH})_2\text{D}_3$ (10^{-8} M) alone or in combination on (A) ER α , (B) ER β and

7.5 Discussion

Both oestradiol and DHEA significantly reduced MLO-Y4 cell cell number. In this particular assay the 10^{-8} M concentration of DHEA did not reach statistical significance whereas all of the lower concentrations tested did. The reason for this is not clear but is likely to be technical as results of further assays carried out as part of the testing of the mechanism of action of DHEA, demonstrated that the 10^{-8} M concentration did cause a statistically significant reduction (as demonstrated in Figure 31).

The MTS assay is unable to determine the mechanism by which this reduction occurred but morphologically cells did not appear to have died and oestradiol has been shown in previous studies to protect osteocytes from apoptosis induced by etoposide or serum starvation (Marathe et al., 2012, Wiren et al., 2006). Indeed, withdrawal of oestradiol after pre-treatment for 14 days induced apoptosis in both MLO-Y4 and MC3T3 osteoblast cells (Brennan et al., 2014b). Withdrawal of oestrogen also resulted in apoptosis in human bone (Tomkinson et al., 1997). Given this evidence, it is likely that the reduction in cell number seen is due to a decrease in proliferation. This raises the question whether these two agents promote differentiation of osteocytes and it would be interesting to investigate this using the IDG-SW3 cell line which has been developed specifically as an *in vitro* model of osteoblast to mature osteocyte differentiation (Woo et al., 2011). Interestingly in osteoblasts, oestradiol has been shown to promote differentiation but only when administered intermittently rather than continuously (Rao et al., 2003).

The above experiments were unable to demonstrate any effect of testosterone on osteocyte cell number. Although there is very little published work on this area, one study has shown that dihydro-testosterone (DHT) induces apoptosis in osteocyte-like cells (Wiren et al., 2006).

The reduction in cell number caused by oestradiol, was blocked by the addition of fulvestrant and tamoxifen suggesting that oestradiol is acting via the classical ER α/β pathway. A similar study looking at oestradiol-induced Cx43 expression in MLO-Y4 cells also demonstrated blockage of oestradiol effects by tamoxifen and fulvestrant. Treatment of cells with a GPR30 agonist had no significant effect. This study concluded that gap junction intercellular communication occurs mainly via the ER pathway, and sensitises osteocytes to mechanical loading (Ren et al., 2013). Further evidence for the importance of the ER pathway in osteocytes has been demonstrated by the fact that ER α in osteocytes is important for trabecular bone formation in mice (Borjesson et al., 2013, Windahl et al., 2013) and the less well-studied ER β appears to facilitate effects of strain including activation of extracellular signal-regulated kinase and down regulation of SOST in well-differentiated cells of the osteoblast lineage including osteocytes (Galea et al., 2013b).

Antagonism of the conversion of DHEA to oestrogens using the aromatase inhibitor anastrozole blocked the reduction in cell number caused by DHEA. This suggests that the action of DHEA on cell number occurs indirectly as a result of its conversion to oestradiol. Androgens, including DHEA are well known to have a profound effect on the physiology of bone and muscle particularly in women. By acting both directly and via transformation into oestrogens they have been shown to modulate the bone-remodelling cycle (Notelovitz, 2002). DHEA exerts direct activity by binding to the AR which is expressed by MLO-Y4 cells as well as embedded osteocytes in human pathological samples (Abu et al., 1997). AR expression is up regulated by GCs including dexamethasone as well as oestradiol and vitamin D (Kasperk et al., 1997, Notelovitz, 2002). A specific receptor for DHEA has also been identified in osteoblast cells (Notelovitz, 2002) as well as murine T-cells (Meikle et al., 1992) but the exact role of this receptor as well as its presence or absence in osteocyte cells is yet to be established.

Sex steroids acting on bone cells can either be derived from the circulation or can be metabolised locally by a process known as 'intracrinology' as described by Labrie et al (Labrie et al., 2000). A number of enzymes are involved in the conversion of sulphated precursors into the active metabolites 17 β -oestradiol, testosterone, DHT and DHEA. Osteoblasts (van der Eerden et al., 2004) as well as rat tibial growth plates (Van Der Eerden et al., 2002) have been shown to express a number of these enzymes including steroid sulfatase, type I and II 17 β -hydroxysteroid dehydrogenase, aromatase and 5 α reductase. The presence or absence of these enzymes in osteocytes however has yet to be demonstrated.

Both oestradiol and DHEA resulted in a significant increase in osteocyte VEGF secretion. This suggests that these agents may play a role in regulating bone angiogenesis, a finding supported by the fact that oestradiol treatment promotes endothelial cell differentiation and migration in rat bone marrow-derived endothelial progenitor cells (Zhang and Lu, 2013) as well as increasing VEGF production in osteoblasts both *in vitro* and *in vivo* (Pufe et al., 2007, Clarkin and Gerstenfeld, 2013). This is the first study to demonstrate such effects in osteocyte-like cells. A study investigating a potential steroid-sparing effect of DHEA in HCC1 osteoprogenitor cells demonstrated no effect of DHEA on VEGF gene expression (Harding et al., 2006) but there do not appear to be any other studies into the relationship between DHEA and VEGF in bone.

Contrary to the results on MLO-Y4 cell number, the increase in VEGF secretion caused by oestradiol could not be blocked by tamoxifen or fulvestrant despite pre-treatment with these agents for 1 hour before addition of oestradiol. This suggests that oestradiol exerts its effects on VEGF activity not via the classical ER pathway but by a non-genomic pathway such as the GPR30 receptor. The GPR30 receptor was first identified to be expressed by all three major bone cell types by Heino et al in 2008 (Heino et al., 2008). This study also

demonstrated that osteocytes more frequently expressed GPR30 than osteoblasts and that GPR30 positivity declined during pubertal development in osteocytes. In MLO-Y4 cells GPR30 is known to be mainly confined to the plasma membrane and has been shown to activate calcium release by these cells (Ren and Wu, 2012). Oestradiol has also been demonstrated to induce osteoblast proliferation via the GPR30 receptor (Noda-Seino et al., 2013). Despite these findings, the role of GPR30 in bone remains relatively poorly understood. Similarly very little is known about any potential role of GPR30 in angiogenesis, although one study has demonstrated that the GPR30 agonist G-1 blocks endothelial cell proliferation and angiogenesis *in vitro* (Holm et al., 2012). Another study of endometrial carcinoma cells showed increased levels of VEGF in cells with high levels of GPR30 expression (Smith et al., 2013).

Anastrozole was also unable to block the increase in VEGF secretion caused by DHEA, suggesting that DHEA is exerting direct activity via an AR rather than by conversion to oestrogens.

When cells were co-treated with dexamethasone and oestradiol, oestradiol prevented the reduction in VEGF secretion caused by dexamethasone. This appears to be the first study to show a relationship between GCs, oestradiol and VEGF in any tissue. In terms of ON it may be that dexamethasone interferes with oestrogen –induced VEGF signalling in osteocytes and thus partly explains why the adolescent female population are at greatest risk. A study investigating the role of ACTH in ON has demonstrated that ACTH increases VEGF production by osteoblast cells and infers that the down-regulation of ACTH by GC treatment thus impairs bone angiogenesis and results in ON (Blair et al., 2011), however this study does not address the female preponderance to ON. A further explanation for this may be due to down regulation of ER α by dexamethasone as demonstrated by qRT-PCR in the above results section. ER α is the predominant receptor for mediation of oestradiol

effects in osteocytes and the above results have demonstrated a reduction in its expression following treatment with dexamethasone. A similar result has been previously shown in a study of MC3T3 osteoblasts (Park, 2012) as well as breast cancer cells (Karmakar et al., 2013). No effects of dexamethasone was demonstrated on ER β expression and a literature search found no other studies investigating any relationship between GCs and ER β in bone. In rat hypothalamus where ER β is the predominant ER, dexamethasone treatment up-regulated its expression (Suzuki and Handa, 2004). Although results did not achieve statistical significance, 1 α ,25(OH) $_2$ D $_3$ did appear to reduce ER β expression, a finding which is believed to be novel. The significance of this finding remains unclear as the role of ER β in bone remains poorly understood and no other studies of vitamin D and ER β expression in any tissue could be found.

Chapter 8

Discussion

8.1 Introduction

This Chapter reflects on the implications of the overall findings reported in this thesis within the context of the current literature, and their clinical relevance to the pathogenesis of GC-induced ON. Limitations of the work as well as recommendations for future studies are also discussed.

8.2 Novel findings of this work

Studies outlined in this thesis have identified a number of novel findings that contribute to the overall understanding of the pathogenesis of GC-induced ON. Firstly, the effect of dexamethasone on the expression of VEGF isoforms within osteocytes supports previous studies of the role of VEGF and angiogenesis in the bone healing process. The modulating effects of vitamin D on the reduction in VEGF expression caused by dexamethasone is also previously unpublished in osteocytes and lends support for a therapeutic role for vitamin D in the management of ON as well as osteoporosis. The findings that sex steroids also influence osteocyte angiogenesis, and that dexamethasone interferes with this effect, is also novel. To date there is very little published data on sex steroids and ON and results of the *in vitro* experiments in this thesis have identified a number of important interactions that provide a baseline for future *in vivo* work. Adolescent females are well known to be at greatest risk for the development of ON and it is likely that one reason for this is the interruption of oestradiol-driven angiogenic pathways by GC agents.

8.3 Pathogenesis of GC-induced ON

8.3.i Effects of dexamethasone on osteocyte number

The results outlined in this thesis add support to previous literature demonstrating a potent effect of dexamethasone on osteocyte biology. Pathological studies of GC-induced-femoral head ON show that osteocyte apoptosis is a major contributory mechanism (Weinstein et al., 2000, Kabata et al., 2000, Eberhardt et al., 2001, Weinstein, 2012a). This is pathologically distinct from other causes of ON such as alcohol and trauma where apoptotic osteocytes are virtually absent (Weinstein et al., 2000), suggesting that although these conditions are grouped under the “umbrella term” of ON, that they may be in fact separate conditions. One limitation of this study is that the mechanism by which dexamethasone reduced osteocyte cell number *in vitro* was not analysed, but previous studies have shown several mechanisms of cell death following GC-treatment of osteocytes including interruption of FAK-kinase signalling as well as autophagy, suggesting that the mechanism is not as simple as “osteocyte apoptosis” alone (Plotkin et al., 2007, Jia et al., 2011). The complexity of the actions of GCs on bone is highlighted in a recent study that shows that although GCs induce autophagy in osteocytes, suppression of autophagy in osteocytes does not accentuate the negative effects of GCs on the skeleton (Piemontese, 2013).

A second limitation was the fact that cells were cultured in monolayer, which is very different from the 3D orientation of osteocytes in bone. When the work described in this thesis began, the differentiation of osteoblasts to osteocytes in 3D gels was still in the early stages of development (Atkins et al., 2009, Boukhechba et al., 2009) and other models were being developed (Uchihashi et al, 2013). Interestingly when MLO-Y4 cells were cultured in 3D collagen gels, the reduction in cell number and viability caused by dexamethasone was not demonstrated, suggesting that either the collagen gel matrix

somehow protected the osteocytes from dexamethasone exposure and/or that cells in 3D are more robust to the effects of GCs. Clearly some dexamethasone was able to penetrate the collagen as there was a reduction in VEGF secretion, as seen in monolayer cultures. These results highlight the importance of using the 3D model to assess osteocyte biology *in vitro* as clearly different results can be obtained in 3D compared to monolayer. There are on-going studies to further refine and develop the 3D model using both MLO-Y4 cells, IDG-SW3 cells and human osteocytes (Elford et al., 2012, Scully et al., 2013).

A further important limitation of this study is the use of murine cell lines rather than primary mouse or human osteocytes. Due to the fact that osteocytes are embedded within the mineralised bone matrix, they have proved very difficult to isolate, culture and maintain. The murine cell lines MLO-Y4 and more recently IDG-SW3 are still widely used as templates for osteocyte biology *in vitro* (Artsi et al., 2014, Ueda et al., 2014, Woo et al., 2011, St John et al., 2014). There are however recent examples of isolation of primary mouse osteocytes, for example using retinoic acid (Mattinzoli et al., 2014) or a process of extended collagenase digestions combined with EDTA-based decalcification (Stern et al., 2012) and on-going studies to isolate and culture human osteocytes (Elford et al., 2012) may have important implications for future research studies.

8.3.ii Effect of dexamethasone on osteocyte angiogenesis

Angiogenesis or new blood vessel formation is vital not only for embryonic bone development (Gerber et al., 1999) but also for fracture healing (Dong et al., 2014) as well as bone repair in other conditions including osteoporosis and ON (Saran et al., 2014). VEGF has historically been described to be the single most important molecule in this process where it acts as a coupling factor between angiogenesis and osteogenesis (Gerber et al.,

1999). The hypoxic environment of injured bone causes release of a number of agents, of which VEGF is the most studied. The resulting endothelial cell proliferation and migration results in revascularisation and bone repair (Saran et al., 2014, Schindeler et al., 2008).

Until recently it was thought that osteoblasts were the main cell responsible for the release of VEGF and other angiogenic factors that promote bone healing (Mayr-Wohlfart et al., 2002, Wang et al., 1996, Steinbrech et al., 2000, Spector et al., 2001), but it is becoming increasingly clear that osteocyte cells provide a vital contribution to this process. Apoptotic MLO-Y4 cells are known to produce VEGF and CM from these cells has been shown to promote endothelial cell proliferation (Cheung et al., 2011) as well as osteoclast precursor recruitment and differentiation (Al-Dujaili et al., 2011). Results in this thesis support the findings of previous research that osteocytes are a vital source of VEGF, but we also report the novel findings that $1\alpha 25(\text{OH})_2\text{D}_3$ treatment of osteocytes prevents the reduction in VEGF secretion caused by dexamethasone. A major limitation of this study is that despite the use of a 3D model which is closer to the natural environment of an osteocyte, all of the experiments have taken place *in vitro*. The results however are promising for a potential therapeutic role for $1\alpha 25(\text{OH})_2\text{D}_3$ in the prevention and/or treatment of GC-induced ON and results in this thesis provide sufficient evidence for initiation of *in vivo* studies. There is a plethora of evidence for the use of vitamin D supplementation along with calcium (Reid et al., 2014) in both prevention and treatment of osteoporosis (Khan et al., 2013, Avenell et al., 2014) but no such studies exist for ON. Results in this thesis support the development of a randomised controlled trial of vitamin D supplementation versus placebo in patients taking high dose GCs, with incidence of ON as a primary outcome measure. A study showing a modest reduction in dexamethasone sensitivity of precursor B-cell ALL blasts treated with vitamin D (Antony et al., 2012) has raised sufficient concerns to prevent such a trial taking place in ALL patients at the present time. These concerns need to be addressed with further investigations before vitamin D could routinely be given to ALL patients. It is

likely therefore that any initial studies of vitamin D for GC-induced ON would take place in benign conditions, requiring high dose corticosteroid therapy.

A further novel finding of this thesis is the MLO-Y4 cell expression profile of genes involved in angiogenesis, and the modulation of 13 angiogenesis genes by dexamethasone. To date, studies of angiogenesis in osteocytes have mainly been restricted to the activity of VEGF. A number of growth factor agents (TGF β , PDGF and FGF) have been shown to be important in differentiation of bone marrow MSCs into adipogenic, chondrogenic, and osteogenic lineages (Ng et al., 2008) but their role in osteocyte biology remains unstudied. PDGF is known to be important in endothelial cell proliferation and migration (Kilian et al., 2004) and so is likely to be an important angiogenic factor in osteogenesis.

Subsequent to the findings of the gene array outlined in Chapter 7, all of the gene changes have been confirmed by continuing work in the laboratory (Adams et al., 2013). Three of the genes that were modulated by dexamethasone, have also been shown to be modulated by oestradiol treatment. These studies reveal exciting insights into the angiogenic profile of the osteocyte and the effects of GCs and oestradiol which open up new lines of investigation to further understand why adolescent females are at greatest risk of ON. This work remains in the early phase, but future studies are recommended using the gene array to investigate any modulatory effect of dexamethasone on the angiogenic pathways of oestradiol treated cells as well as any influence of 1 α 25(OH) $_2$ D $_3$ on dexamethasone treated cells.

8.3.iii Effect of dexamethasone on markers of bone remodelling

During the time that experiments in this thesis were carried out, studies within the osteocyte field have rapidly progressed. Knowledge of osteocyte biology is continuing to increase and there is now undisputed evidence that the osteocyte is the key cell responsible for controlling bone turnover and remodelling (Sapir-Koren and Livshits, 2014, Nakashima, 2013, Nakashima et al., 2011, O'Brien et al., 2012). It does this by communication (both direct and indirect) with other cell types including osteoblasts, osteoclasts and fibroblasts (Fujita et al., 2014, Sims and Vrahnas, 2014, Lloyd et al., 2014, Plotkin, 2014, Uzer et al., 2014).

The following table adapted from a recent paper by Bellido (Bellido, 2014), outlines what is currently understood regarding osteocyte gene expression.

Gene	Expressed in	Function
E11	Early, embedding osteocyte	Dendrite formation
CD44	Highly expressed in osteocytes compared to osteoblasts	Hyaluronic acid receptor associated with E11 and linked to cytoskeleton
Fimbrin	All osteocytes	Dendrite branching
MT1-MMP	Osteoblasts and osteocytes	Matrix degradation, canaliculi formation
Phex	Early and late osteocytes	Phosphate metabolism
MEPE	Late osteoblasts through osteocytes	Inhibitor of bone formation, phosphate metabolism
DMP1	Early and mature osteocytes	Phosphate metabolism, mineralisation
FGF-23	Mature osteocytes	Phosphate metabolism
Dkk1-related protein 1 (Dkk1)	Osteoblasts and osteocytes	Inhibitor of bone formation
SOST	Mature osteocytes	Inhibitor of bone formation
RANKL	Osteocytes and osteoblasts	Osteoclast differentiation and survival
OPG	Osteoblasts and osteocytes	Inhibitor of osteoclast differentiation
MCSF	Osteoblasts and osteocytes	Osteoclast proliferation and survival factor

Table 11. Summary of genes expressed by osteocytes and function. Boxes group four main categories: (1) genes related to dendritic morphology and canaliculi formation, (2) genes related to phosphate metabolism and matrix mineralisation, (3) genes that regulate bone formation, and (4) genes that regulate bone resorption. Adapted from (Bellido, 2014).

Osteocytes are known to express a number of genes involved in bone formation (including Dkk1 and SOST). SOST is known to antagonise several members of the BMP family and both Dkk1 and SOST (Bonewald, 2011, Bellido, 2006) bind to LRP5 and LRP6, preventing activation of Wnt signalling which is vital for osteoblastogenesis. It is now clear therefore that through the expression of Wnt and BMP antagonists, osteocytes have the potential to regulate the formation and activity of osteoblasts (Bellido, 2014).

The exact mechanism by which osteocytes control bone resorption is still not fully understood but it is thought that a major triggering factor is osteocyte apoptosis (Kennedy et al., 2014, Bellido, 2014). Apoptosis of osteocytes appears to release RANKL as well as promoting osteoblasts to secrete RANKL (albeit in lower concentrations than osteocytes) (Nakashima et al., 2011). In fact it has been shown that it is osteocyte-derived RANKL that drives bone resorption in cases of calcium deficiency (Xiong et al., 2014). There is also recent evidence to suggest that SOST under certain conditions also regulates differential RANKL and OPG production, and creates a dynamic RANKL/OPG ratio, leading either to bone formation or resorption (Sapir-Koren and Livshits, 2014). Furthermore osteocytes can negatively regulate osteoclastogenesis by secreting β -interferon (Hayashida et al., 2014).

It remains unknown, however, whether osteocytic membrane-bound or sRANKL is involved in osteocyte-driven bone resorption. Recent studies have suggested that the contribution of sRANKL to osteoclastogenesis is minimal and that instead, direct contact between membrane-bound RANKL expressed in the osteocytic dendrites and RANK expressed in osteoclast precursors appears to be required to initiate osteoclast development (Xiong et al., 2011, Aguirre et al., 2006, Honma et al., 2013).

Results in this thesis show that both membrane bound mRNA expression and sRANKL protein secretion in the CM of osteocytes are markedly increased when cells are treated with dexamethasone in combination with $1\alpha 25(\text{OH})_2\text{D}_3$, suggesting that these two agents act synergistically to aid osteoclastogenesis and drive bone remodelling. These results are in contrast to the antagonistic effect of $1\alpha 25(\text{OH})_2\text{D}_3$ on preventing the reduction in VEGF secretion caused by dexamethasone. These findings are believed to be novel and the results highlight the complexity of the bone remodelling process and further studies are required to fully understand the interactions between GCs and $1\alpha 25(\text{OH})_2\text{D}_3$ in osteocytes. Further *in vitro* studies are recommended to investigate the effect of CM (and therefore sRANKL) from dexamethasone and $1\alpha 25(\text{OH})_2\text{D}_3$ treated osteocytes directly on osteoclastic activity. An *in vivo* model is also recommended where animals (most likely mice) are treated with combinations of vitamin D and GCs and RANKL/OPG levels studied as well as looking for any features of GC induced ON and whether vitamin D alters this.

Another limitation of studies within this thesis is the fact that the majority of the experiments were carried out on a single cell line (MLO-Y4 cells). The MLO-Y4 cell represents an early osteocyte, and exhibit high expression of OCN, Cx43 and E11 and low expression of ALP but they do not express the mature osteocyte markers including SOST (Rosser, 2011). PTH treatment of MLO-Y4 cells had very little effect on VEGF and IL-6 secretion as well as expression of the VEGF, RANKL and OPG genes. This is despite the increasingly documented evidence that PTH receptor signalling in osteocytes from transgenic mice plays an important role in inducing bone resorption by direct regulation of the RANKL gene (Ben-Awadh et al., 2014) as well as increasing expression of FGF-23 (Rhee et al., 2011b). The activity of the PTH used was tested with a positive control and so the lack of effect particularly on RANKL expression/secretion in this thesis (evidence from the literature would suggest it should increase (Ben-Awadh et al., 2014, Delgado-Calle, 2013) remains unexplained. One potential explanation for the lack of PTH effect seen comes from

a study of osteosarcoma cells that can differentiate into mature osteocytes. This study demonstrated that PTH increased RANKL expression only at very early stages of osteocyte differentiation (Prideaux et al., 2014). Verbal communication with this research group (M Prideaux, personal communication) revealed little effect of PTH on the MLO-Y4 cell line. The IDG-SW3 cell line only became available towards the end of this project. In view of the well documented effects of PTH on osteocyte biology, future studies will reassess the effects of PTH on osteocyte angiogenesis (including VEGF expression) using a different cell line such as the IDG-SW3 cells.

8.3.iv Effect of sex steroids on osteocyte biology (and their interaction with dexamethasone)

The results of this study have identified novel information regarding the effects of oestradiol and DHEA on osteocyte number and VEGF secretion. The fact that both oestradiol and DHEA independently up-regulate osteocyte VEGF secretion has not been previously reported and represents exciting new information which may be relevant to the pathogenesis of GC-induced ON. The protective effect of oestradiol in preventing osteocyte apoptosis (including that caused by GCs) is well documented (Brennan et al., 2014a, Marathe et al., 2012, Gu et al., 2005) and lends support to the role of hormone replacement therapy in preventing osteoporosis and fractures in post-menopausal women. This is the first study however to document oestradiol effects on osteocyte VEGF and modulation of the reduction in VEGF secretion by dexamethasone. Since this project was completed further studies within this research group have demonstrated oestradiol to modulate a number of other angiogenesis genes (Adams M. et al., 2013) and work is ongoing in the laboratory to investigate this further. We have also demonstrated a down-regulation of ER α expression in osteocytes treated with dexamethasone, a further novel

finding which may contribute to the pathogenesis of GC-induced ON. ER α is known to be an important receptor for signalling molecules promoting bone growth (Clegg and Palmer, 2013) during puberty and studies have demonstrated that polymorphisms of ER α can lead to constitutional delay in growth and puberty (Kang et al., 2013). Adolescents treated for ALL are at approximately 10-fold greater risk of developing ON than young children. It may be that dexamethasone treatment down-regulates ER α expression in the osteocytes of these patients, impairing normal bone growth and turnover during puberty and this combined with impaired angiogenesis predisposes the cells to apoptosis and therefore the development of GC-induced ON. The findings of this research warrant further studies using pathological specimens from ALL patients undergoing surgery for ON. Specimens could be analysed for VEGF expression as well as ER α expression which would be expected to be lower than in control samples. Discussions regarding studies of this nature are on-going within the UKALL 2011 ON working group.

8.4 Potential treatment strategies for GC-induced ON

As previously discussed the mainstay of current ON management is surgical, including core decompression with arthroscopic debridement, with or without bone grafting in early stage disease and total joint (usually hip) replacement in more advanced cases where there is evidence of subchondral fracture or acetabular involvement (Issa et al., 2013). Core decompression is only successful in approximately 60% of cases meaning that the remaining 40% will also need joint replacement (Hungerford, 1983). Evidence for conservative management such as not weight-bearing, bed rest or hyperbaric oxygen therapy has shown very limited effects (Reis et al., 2003, Okazaki et al., 2012).

This section uses the findings of the work from this thesis as well as other published literature to outline potential agents which may have a role in preventing or treating GC-induced ON. As there are two main mechanisms (which the findings of this work supports) thought to be responsible for GC-induced ON namely i) osteocyte apoptosis and ii) interruption to VEGF signalling and angiogenesis, it may be that targeting key molecules within osteoblasts and osteocytes in order to promote osteogenesis and angiogenesis may prove to be beneficial.

8.4.i Stem cell therapies

Once established ON rarely spontaneously regresses with the natural history of the disease being progression to total hip replacement (Flouzat-Lachaniette et al., 2012, Hernigou et al., 2010, Hernigou et al., 2006). It is thought that in patients with ON, inadequate supply of progenitor cells to remodel the area of necrosis (Hernigou et al., 2009). Previous animal studies have suggested using intra-bone marrow injection of autologous bone marrow cells to increase osteoblast numbers. This appears to be an effective therapy in a rabbit model of GC-induced ON (Asada et al., 2008) and another study reports tissue recovery following transplantation of MSCs into a sheep model of ON (Feitosa et al., 2010). Early human studies of injection of autologous MSCs combined with core decompression into an area of necrosis showed good results in preventing need for joint replacement. The results were particularly effective in those with early stage disease and showed a dose-dependent effect of MSC dose injected. The study also showed that certain risk factors including GC treatment as the cause of ON to correlate with increased risk of treatment failure (Hernigou and Beaujean, 2002). Since this original study a number of larger prospective studies have shown similar results with autologous injection of MSCs being associated with better outcome and less risk of progression to joint replacement than core decompression alone, the results of which are summarised in a review by Houdek et al (Houdek et al.,

2014). This review makes the following suggestions for patients suitable to undergo stem cell therapy.

Indications	Contraindications
Symptomatic stage 1 or 2 ON of the femoral head	Stage 3 or higher ON of the femoral head
Bilateral ON where one hip symptomatic and the other stage 0 and asymptomatic	Rapidly progressive ON
Large (30%) asymptomatic lesions identified on MRI	Active or chronic infection

Table 12. Indications and contraindications for stem cell therapy in ON of the hip (Houdek et al., 2014)

Despite these recommendations from this North American study, routine practice in the UK at present does not include stem cell therapy. This may be for a number of reasons; firstly autologous stem cell collection is a relatively invasive procedure requiring either bone marrow harvest or peripheral leucaphoresis and secondly harvesting, processing and storage of MSCs is expensive and so may not be cost effective although there does not appear to be any evidence that the cost effectiveness of stem cell therapy vs. joint replacement has been analysed and certainly there is no NICE guideline for the management of ON. In ALL patients the timing of autologous stem cell harvest would be crucial as it must be at a time point when the bone marrow is completely clear of disease, and many clinicians may feel uncomfortable injecting autologous bone marrow cells back in to a patient with a history of ALL.

8.4.ii PTH

The rationale for using recombinant PTH (1-34) (Teriparatide) in ON stems from the fact that PTH is known to induce osteogenesis through promotion of osteoblastogenesis and reducing osteoblast apoptosis (Chen et al., 2002, Jilka et al., 1999) as well as preventing osteocyte apoptosis (Weinstein et al., 2010a). The mechanism by which PTH is thought to work is by suppression of SOST expression in osteocytes and subsequent increase of Wnt/ β -catenin signalling and bone formation (Bellido et al., 2005, Rhee et al., 2013). Several studies have shown a benefit of Teriparatide in the treatment of bisphosphonate-induced ON of the jaw (Kim et al., 2014b, Yoshiga et al., 2013, Cheung and Seeman, 2010). Bisphosphonates are anti-resorptive agents used to preserve or increase BMD in patients with osteoporosis. One of the side effects of bisphosphonates is ON of the jaw, the mechanism for which is not fully understood. The mechanism by which Teriparatide works in ON of the jaw is thought to be initial stimulation of bone formation by osteoblasts and subsequent bone resorption by osteoclasts and thus can reactivate of suppressed bone remodelling (Kim et al., 2014b). There have been no studies of Teriparatide in GC-induced or any other types of ON.

8.4.iii Bisphosphonates

Somewhat counterintuitively, given that ON of the jaw is a well-recognised side effect of bisphosphonate therapy, there have been reports of the use of bisphosphonates in a randomised clinical study of ON (Lai et al., 2005) with initial promising results and reports of improved pain scores in children with ON treated for ALL (Kotecha et al., 2010). However, longer term follow up of the randomised study cohort above revealed no benefit of bisphosphonates over placebo in terms of radiological appearance or need for joint replacement surgery (Chen et al., 2012a). Other properties that may lend to a potential therapeutic role of bisphosphonates have been demonstrated within our laboratory.

Bisphosphonates have been shown to increase osteocyte cell number *in vitro* (personal communication) and other studies have shown an anti-apoptotic effect on both osteoblasts and osteocytes *in vivo* (Plotkin et al., 2008). On the other hand, bisphosphonates are potent inhibitors of angiogenesis and have been demonstrated to reduce VEGF concentrations in a number of studies (Tang et al., 2010, Wood et al., 2002, Hanai et al., 2006). In view of the results of this thesis and other published evidence that GC-induced impairment of VEGF secretion is a major contributory role in the development of ON, at the present time there is insufficient evidence to warrant treatment of patients with bisphosphonates outside of the research setting.

8.4.iv Nitric Oxide

There is evidence that nitric oxide (NO) mediates the prosurvival effects of oestrogen on osteocytes (Marathe et al., 2012) There is also evidence that nitrates reduce bone resorption in post-menopausal women (Jamal et al., 2004, Jamal et al., 2011). Genetic polymorphisms of the enzyme that synthesises NO, endothelial NO synthase (eNOS) have also been reported in patients with ON of the femoral head (Gagala et al., 2013, Kim et al., 2013, Koo et al., 2006). Interestingly there has been a study of the use of transdermal nitrate patches which prevented GC-induced ON in a rabbit model (Drescher et al., 2011a). To date there are no clinical studies of the use of nitrates to prevent GC-induced ON in humans. It would be interesting to assess whether NO treatment of osteocytes *in vitro* modulates the reduction in VEGF secretion caused by dexamethasone.

8.4.v VEGF

Both the results of this study and those of others (Street et al., 2002, Fessel, 2013) suggest that VEGF therapy (either systemically or local injection into the affected joint) may be an effective treatment for ON. Other studies however have expressed concern that VEGF accelerates bone turnover, resulting in a mechanically fragile ossification that is prone to fracture and collapse (Boss et al., 2004). The main concern however regarding use of VEGF in malignant conditions is the concern that VEGF may promote tumour growth and metastasis or in the case of ALL, progression of disease and resistance to treatment. The relationship between serum VEGF levels and prognosis in childhood ALL remains controversial with a wide variation in results (probably due to small sample sizes) demonstrated (Kalra et al., 2013, Leblebisatan et al., 2012, Yang et al., 2013, Todorovic et al., 2012). While there remains any concern that VEGF therapy may impair response to treatment or adversely affect prognosis in ALL patients, it would not be advisable to subject these patients to such therapy. Despite these concerns, impaired bone angiogenesis does appear a significant mechanism in the development of GC-induced ON and therefore trials of VEGF therapy in non-malignant conditions may prove to be an effective therapy.

8.4.vi Other potential therapies

A number of medical therapies have also been undertaken with variable and inconsistent results including the use of calcium channel blockers (nifedipine), prostaglandin infusions, low molecular weight heparin, and statins (Laroche et al., 1990, Li et al., 2003, Jager et al., 2009). These pharmacologic agents predominantly ameliorate the regulation of blood supply targeting local ischemia or lipocyte proliferation (Mostoufi-Moab and Halton, 2014).

Anti-SOST antibodies have also recently become available (Li et al., 2009, Padhi et al., 2011) and have been shown in clinical trials to be well tolerated and treatment leads to an increase in markers of bone formation and BMD (Padhi et al., 2011) A recent animal study has shown anti-SOST antibodies to be superior to PTH therapy in increasing femoral cortical and vertebral strength in metaphyseal bone healing (Agholme et al., 2014).

Extracorporeal shock wave therapy (ESWT) is a further treatment that has been investigated in the management of ON. ESWT has been shown to improve bone angiogenesis and increase serum NO concentrations as well as increasing a number of osteogenic factors within femoral head ON (Lin et al., 2006, Wang et al., 2012, Wang et al., 2005). A recent systematic review of ESWT in ON of the femoral head did suggest therapeutic potential but highlighted the need for randomised controlled studies (Alves et al., 2009).

8.5 Final conclusions

With increasing numbers of long-term survivors of childhood ALL (and other malignancies requiring treatment with high dose steroid therapy) the prevalence of ON will continue to increase. This has implications firstly and most importantly for the patients, for whom quality of life can be severely impaired and secondly, significant cost implications for the NHS (the average cost of a single hip replacement is approximately £5000) (Fordham et al, 2012).

Curing patients of their ALL remains the number one priority of treatment and GCs are a vital part of therapy. Although the UKALL 2011 trial is aiming to reduce the cumulative dose of GCs and consequently the incidence of ON, it is inevitable that this disabling disease will continue to affect certain cohorts of patients, particularly teenagers and young adults. The

development of ON in adolescent patients often results in early termination of GC therapy which can have adverse effects on prognosis, which is of particular significance as survival rates in this age group are already inferior to those achieved in younger patients.

All of the above highlights the importance of establishing new strategies to both prevent the development of ON and to treat established disease. Current therapies are mainly surgical and outcomes are limited by the fact that significant joint damage has already occurred at the time of intervention. In order for new, definitive therapies to be developed the pathogenesis of GC-induced ON needs to be better understood. It is vital therefore that the results of this thesis and other published literature are developed further in order to improve knowledge of this disabling condition. The current focus of many new cancer therapies is the development of molecular targeted agents against certain pathways leading to tumour growth and survival. In the future it is likely that side effects such as ON will also be managed with targeted therapies and therefore it is vital that work like this continues to identify molecules and pathways to target.

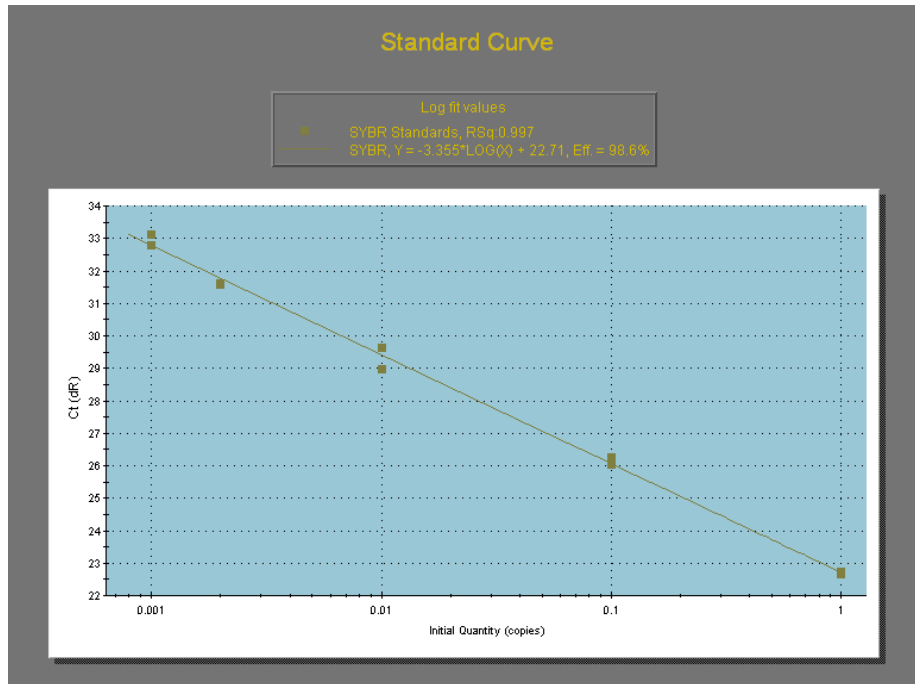
As previously stated, the main limitations of this study are its' *in vitro* nature and the use of osteocyte cell lines rather than for example, osteocytes directly obtained from pathological specimens from patients with GC-induced ON. Patients with ALL (and some forms of lymphoma) seem to be at particular risk of ON compared with patients treated with high dose steroids for other conditions such as inflammatory bowel disease. The reasons for this are unclear but are likely to be related to the direct effects of the bone marrow disease on the surrounding bone architecture as well as circulating levels of bone remodelling factors including vitamin D and PTH. RANKL and PTHrP for example has been shown to be elevated in some forms of adult leukaemia and is associated with hypercalcaemia (Gao *et al*, 20015). There have been no such studies in childhood ALL. Despite these limitations the experiments outlined in this thesis do identify important effects of GCs on a number of

factors (vitamin D, VEGF and RANKL) all of which could be used as potential management agents in either prevention or treatment of ON in clinical practice. Further *in vitro* experiments using pathological specimens from patients with ALL and *in vivo* animal models of GC-induced ON are required to verify these findings and results from this thesis are being used to inform on-going investigations (for example a study of SNPs of a number of genes including VEGF) by the UKALL 2011 osteonecrosis working group.

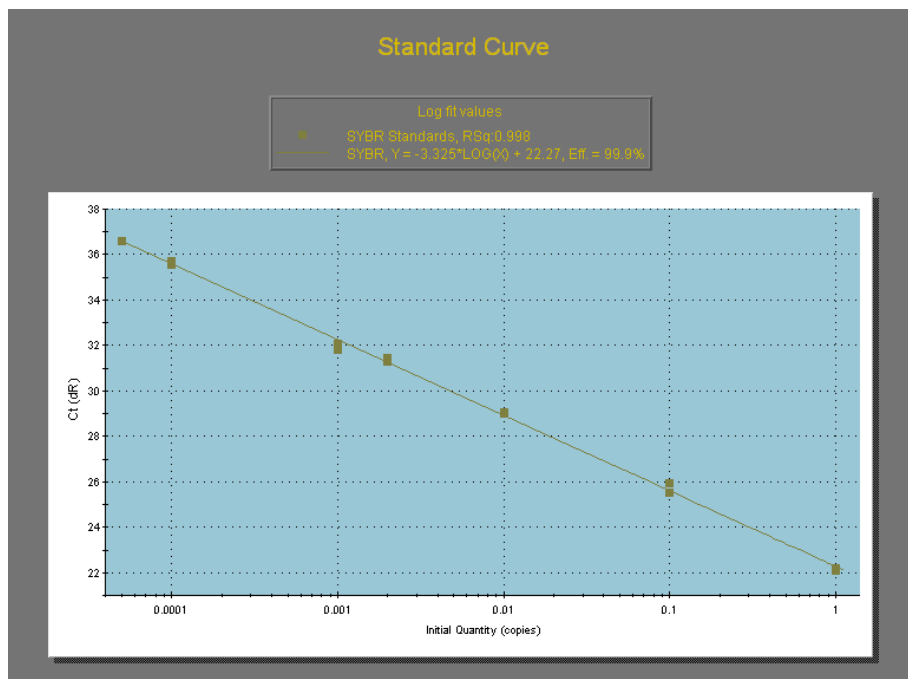
APPENDIX 1

Examples of standard curves for primers used in this thesis

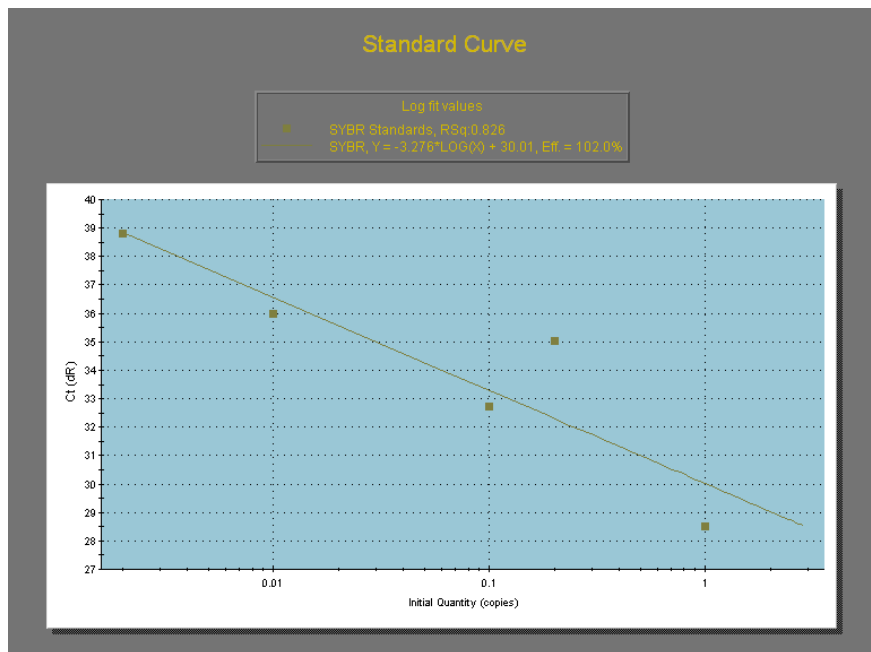
1. VEGF₁₆₄



2. VEGF₁₂₀



3. RANKL



APPENDIX 2

Angiogenesis gene expression profile of the MLO-Y4 cell using the Qiagen® RT² Profiler mouse angiogenesis PCR array. Positive expression defined as C_T value of <35. (Genes not expressed C_T value highlighted in red)

Symbol	Description	Ct value
Akt1	Thymoma viral proto-oncogene 1	24.74
Ang	Angiogenin, ribonuclease, RNase A family, 5	32.57
Angpt1	Angiopoietin 1	25.85
Angpt2	Angiopoietin 2	31.58
Anpep	Alanyl (membrane) aminopeptidase	25.03
Bai1	Brain-specific angiogenesis inhibitor 1	Undetermined
Ccl11	Chemokine (C-C motif) ligand 11	34.38
Ccl2	Chemokine (C-C motif) ligand 2	26.88
Cdh5	Cadherin 5	39.36
Col18a1	Collagen, type XVIII, alpha 1	27.19
Col4a3	Collagen, type IV, alpha 3	33.91
Csf3	Colony stimulating factor 3 (granulocyte)	35.05
Ctgf	Connective tissue growth factor	21
Cxcl1	Chemokine (C-X-C motif) ligand 1	28.35
Cxcl2	Chemokine (C-X-C motif) ligand 2	32.82
Cxcl5	Chemokine (C-X-C motif) ligand 5	25.36
Edn1	Endothelin 1	35.22
Efna1	Ephrin A1	28.64
Efnb2	Ephrin B2	32.21
Egf	Epidermal growth factor	32.91
Eng	Endoglin	25.46
Epas1	Endothelial PAS domain protein 1	27.37
Ephb4	Eph receptor B4	27.9
ErbB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	28.12
F2	Coagulation factor II	37.48
F3	Coagulation factor III	29.33
Fgf1	Fibroblast growth factor 1	Undetermined
Fgf2	Fibroblast growth factor 2	33.46
Fgf6	Fibroblast growth factor 6	Undetermined
Fgfr3	Fibroblast growth factor receptor 3	26.69
Figf	C-fos induced growth factor	26.68
Flt1	FMS-like tyrosine kinase 1	37.37
Fn1	Fibronectin 1	19.44
Hgf	Hepatocyte growth factor	25.08
Hif1a	Hypoxia inducible factor 1, alpha subunit	25.23
Ifng	Interferon gamma	Undetermined

Igf1	Insulin-like growth factor 1	35.75
Il1b	Interleukin 1 beta	37.21
Il6	Interleukin 6	27.77
Itgav	Integrin alpha V	25.04
Itgb3	Integrin beta 3	28.58
Jag1	Jagged 1	28.83
Kdr	Kinase insert domain protein receptor	36.62
Lect1	Leukocyte cell derived chemotaxin 1	36.29
Lep	Leptin	38.55
Mapk14	Mitogen-activated protein kinase 14	26.62
Mdk	Midkine	33.91
Mmp14	Matrix metalloproteinase 14 (membrane-inserted)	25.57
Mmp19	Matrix metalloproteinase 19	27.22
Mmp2	Matrix metalloproteinase 2	21.23
Mmp9	Matrix metalloproteinase 9	33.98
Nos3	Nitric oxide synthase 3, endothelial cell	35.95
Nrp1	Neuropilin 1	25.97
Nrp2	Neuropilin 2	24.55
Pdgfa	Platelet derived growth factor, alpha	27.14
Pecam1	Platelet/endothelial cell adhesion molecule 1	36.86
Pgf	Placental growth factor	28.35
Plau	Plasminogen activator, urokinase	25.56
Plg	Plasminogen	Undetermined
Ptgs1	Prostaglandin-endoperoxide synthase 1	25.37
Ptk2	PTK2 protein tyrosine kinase 2	26.3
S1pr1	Sphingosine-1-phosphate receptor 1	26.83
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	29.99
Serpinf1	Serine (or cysteine) peptidase inhibitor, clade F, member 1	25.22
Smad5	MAD homolog 5 (Drosophila)	26.76
Sphk1	Sphingosine kinase 1	27.98
Tbx1	T-box 1	38.08
Tek	Endothelial-specific receptor tyrosine kinase	34.93
Tgfa	Transforming growth factor alpha	Undetermined
Tgfb1	Transforming growth factor, beta 1	24.7

Tgfb2	Transforming growth factor, beta 2	28.38
Tgfb3	Transforming growth factor, beta 3	28.31
Tgfbr1	Transforming growth factor, beta receptor I	27.93
Thbs1	Thrombospondin 1	25.99
Thbs2	Thrombospondin 2	20.56
Tie1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	37.73
Timp1	Tissue inhibitor of metalloproteinase 1	25.78
Timp2	Tissue inhibitor of metalloproteinase 2	22.42
Tnf	Tumor necrosis factor	31.35
Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	27.85
Tymp	Thymidine phosphorylase	34.57
Vegfa	Vascular endothelial growth factor A	25.53
Vegfb	Vascular endothelial growth factor B	26.69
Vegfc	Vascular endothelial growth factor C	38.55
Actb	Actin, beta	20.48
B2m	Beta-2 microglobulin	19.74
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	21.72
Gusb	Glucuronidase, beta	26.16
Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	22.24

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