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Responses of bone cells to microinjury

Lauren Elizabeth Mulcahy,
BSc, MSc

A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the degree of

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Department of Mechanical and Manufacturing Engineering
Trinity College Dublin
&
Department of Anatomy
Royal College of Surgeons in Ireland

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Supervised by Prof. David Taylor, Dr. Garry Duffy & Prof. Clive Lee

Internal Examiner: Dr. Bruce Murphy
External Examiner Prof. Mitchell Schaffler

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Summary

Microdamage in bone has been implicated as a principal stimulator of bone remodeling through the release of numerous biochemical factors. Such factors initiate the bone resorbing and bone forming potential of the constitutive cells of the Basic Multicellular Unit (BMU). Further to this, an accumulation of microdamage has been implicated in potentiating the risk of fracture in osteoporotic patients, and current treatments for osteoporosis have been thought to exacerbate the risk of fracture through suppression of the normal microdamage mediated response in bone. The overall goal of this study was to determine the effects of microdamage, in terms of microinjury, most notably the impact of injury size, on the biochemical pathways involved in stimulating bone remodeling, and subsequently, the cellular response to microinjury. Additionally, the effects of bisphosphonates, which are commonly used therapeutics in the treatment of osteoporosis, on bone remodeling activities, were also determined.

Using a 3D *in vitro* model of microdamage in bone, termed microinjury, whereby osteocytes were cultured in a matrix to facilitate cell process interactions, linear microinjury, of constant length and width with varying thickness (160-800 μm) was applied. This is the first time such work has been completed, and it is significant to note that the work was performed in the absence of any mechanical stimulus, cyclic loading, or fluid flow. Initially the responses of the two principal cytokines in bone remodeling, RANKL and OPG were determined, and an inverse relationship in their activity was observed- i.e. an increase in microinjury size caused an increase in RANKL activity and decrease in OPG activity. Further to this, conditioned media from these experiments was applied to preosteoclasts and their osteoclastogenic response determined. Increased damage was shown to increase preosteoclast migration, maturation and osteoclastogenesis, in terms of TRAP activity within cells. Additionally, conditioned media from microinjury induced osteoclastogenesis was applied to preosteoblastic cells, and the osteogenic potential of this was determined by analysis of calcium and phosphate mineralisation. It was found that microinjury induced osteoclastogenesis caused an

increase in the migration and mineralisation of preosteoblasts, in an injury size dependent manner, in the absence of osteogenic media.

Using the same model of microinjury in bone, a number of biochemical pathways were investigated using a proteomic approach, to determine whether microinjury induced bone remodeling was exclusively regulated by RANKL and OPG activity. Again, work such as there, whereby an array of proteins has been determined from an *in vitro* simulation of microinjury in osteocytes. It was found that microinjury to an osteocytic network stimulates the action of Interleukin, TNFα and Wnt/ β-catenin pathways, which have been implicated in stimulating bone remodeling, yet their relationship between bone remodeling and microdamage had been shown to be unfounded. The role of two commonly used bisphosphonates, alendronate and zolendronate, on the biochemical and cellular activities involved in the BMU, in response to microinjury, was also determined. It was found that bisphosphonate treatment worked synergistically with microinjury, through the suppression of RANKL and OPG activity, yet a degree of osteoclastogenesis and osteogenesis was maintained, which depended on the magnitude of the microinjury applied. Furthermore, using proteomics, it was found that bisphosphonates treatment suppresses many of the pathways involved in stimulating bone remodeling, yet still maintained a degree of interleukin and Wnt/ β- catenin pathway activity, in addition to BMP-2 activity, which may still be capable of initiating a remodeling response.

This thesis demonstrated the significance of microinjury size in initiating a bone remodeling response. The studies described in this thesis have shown that RANKL and OPG activity, and subsequent biochemical activity, which may act independently of these two cytokines, is strongly affected by the presence of damage and is dependent on damage size. Further to this, the role of damage size on osteoclastogenesis and osteogenesis was determined and again, found to be governed by the degree of damage size. Finally, the role which bisphosphonates play in an environment with elevated microinjury, demonstrated that in the absence of RANKL and OPG, again, a remodeling response can ensue. In conclusion, this study has demonstrated, overall, that microinjury size is a critical factor in mediating a remodeling response in bone.

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Nomenclature

%	Percentage
°	Degrees
°C	Degrees Celsius
l	Litre
ml	Millilitre ($x10^{-3}$)
µl	Microlitre ($x10^{-6}$)
µm	Micrometre ($x10^{-6}$)
cm	Centimetre ($x10^{-2}$)
m	Metre
nm	Nanometre ($x10^{-9}$)
mm	Millimetre ($x10^{-3}$)
µε	Microstrains
g	Grams
mg	Milligrams ($x10^{-3}$)
ng	Nanograms ($x10^{-9}$)
pg	Picograms ($x10^{-12}$)
µg	Micrograms ($x10^{-6}$)

M	Molar
mM	Milimolar ($x10^{-3}$)
nM	Nanomolar ($x10^{-9}$)
2D	2 Dimensional
3D	3 Dimensional
α MEM	Alpha Modified Eagles Medium
ANOVA	Analysis of Variance
ALP	Alkaline Phosphatase
AP-1	Activator protein 1
BMD	Bone Mineral Density
BMP	Bone Morphogenic Protein
BMU	Basic Multicellular Unit
cAMP	Cyclic Adenosine Monophosphate
CATK	Cathepsin K
Cbfa1	Core-Binding Factor Alpha 1
CD	Cluster of Differentiation
CO_2	Carbon Dioxide
COLIA	Collagen Type 1 Alpha 1
CS	Calf Serum
CSF-1	Colony Stimulating Factor 1
Cx	Connexin
DAPI	4',6-diamidino-2-phenylindole
DEXA	Dual Energy X-ray Absorptiometry
DKK	Dikkopf Factor
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid

dPBS	Dulbecco's Phosphate Buffered Saline
DPX	Di-N-Butyl Phthalate in Xylene
E-coli	Escherichia Coli
ELISA	Enzyme Linked Immunosorbent Assay
EP	Prostaglandin Receptor
ERK	Extracellular Signal Receptor Kinase
ERK	Extracellular signal-regulated kinase
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
Fig	Figure
FPPS	Farnesyl Diphosphate Synthase
GAB2	GRB2-Associated-Binding Protein 2
GFP	Green Fluorescent Protein
GTP	Guanosine 5'-Triphosphate
H_2O	Water
HCl	Hydrochloric acid
IFN	Interferon
IKK	Inhibitor of Kappa B Kinase
IL	Interleukin
ILGF	Insulin Like Growth Factor
JAK	Janus Kinase
JNK	Jun N-terminal Kinase
K+	Potassium
kDa	Kilodalton
LB	Luria Bertani
LRP	Low Density Lipoprotein Receptor-Related Protein

Ltd	Limited
MAPK	Mitogen Activated Protein Kinase
MEKK	MAPK/ERK kinase
MITF	Micropthalmia Associated Transcription Factor
MMP	Matrix Metalloprotease
N	Normality
NF κ B	Nuclear Factor Kappa B
NFAT	Nuclear Factor of Activated T Cell
NFATC1	Nuclear Factor of Activated T-cells, Cytoplasmic 1
NO	Nitric Oxide
OPG	Osteoprotegrin
OSM	Oncostatin M
Osx	Osterix
PBS	Phosphate Buffered Saline
P-C-P	Phosphate-Carbon-Phosphate
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
pDNA	Plasmid Deoxyribonucleic Acid
PGE_2	Prostaglandin E2
PnPP	P-nitrophenyl Phosphate
PO_3	Phosphonate
PtH	Parathyroid Hormone
RANK	Receptor Activator of Nuclear Factor Kappa B
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
rpm	Revolutions per minute
Runx2	Runt Related Transcription Factor
SERMS	Selective Estrogen Receptor Modulators

SFRP	Secreted Frizzled-Related Proteins
STAT	Signal Transducer and Activator of Transcription
TACE	Tumour Necrosis Factor-Alpha Converting Enzyme
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TNFRSF11A	Tumour Necrosis Factor Receptor Superfamily 11A
TNFRSF11B	Tumour Necrosis Factor Receptor Superfamily 11B
TRAF	Tumour Necrosis Factor Receptor-Associated Factor
TRAP	Tartrate Resistant Acid Phosphatase
WHO	World Health Organisation
WIF	Wnt inhibitory factors
X	Magnification

Publications, Presentations and Prizes

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Papers:

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Mulcahy, L.E., Lee, T.C., Taylor, D., Duffy, G.P. (2011). **Bisphosphonates change the normal response to microdamage but maintain osteogenesis and osteogenesis (In preparation)**

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Taylor, D., Mulcahy, L.E., Tisbo, P., Dooley, C., Duffy, G., Geraghty, D., Lee, T.C. (2010). **The ``Scissors'' Model of Microcrack Detection in Bone: Effects of Extreme Loading and Disease.** ***Biological Materials and Structures in Physiologically Extreme Conditions and Disease.*** *San Francisco, April 5th- 9th 2010*

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Mulcahy, L.E., Taylor, D., Lee, T.C., Duffy, G.P. (2011) **Osteoclastogenesis and Osteogenesis is stimulated by cell process rupture in an injury-size dependant manner using an *in vitro* simulation of microdamage in bone.** *17th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland*

Mulcahy, L.E., Taylor, D., Lee, T.C., Duffy, G.P. (2011). **The effects of bisphosphonate treatment on osteoclastogenesis and osteogenesis in an *in vitro* simulation of microdamage in bone.** *RCSI research day, April 2011.*

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Chapter 1

Microdamage in bone and repair mechanisms

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1.1 Introduction

The human skeleton comprises of 206 bones and functions primarily in providing strength, protection and support for the soft tissues of the human body and its organs, in addition to the production of red and white blood cells in the red and yellow bone marrow of the medullary cavities. Bone is a dynamic organ, and consists of an anisotropic, nanocomposite material composed of osseous tissue, which predominantly presents an inorganic component comprising of calcium phosphate, or in the chemical form, hydroxyapatite, which allows for mechanical strength and contributes to 75% of bone weight. The organic constituent includes Collagen Type I, which contributes up to 90% of the organic material, along with other non-collagenous proteins, or ground substance, which include osteocalcin, glycoproteins and proteoglycans, which provide durability. Collagen fibres provide a framework for the deposition of hydroxyapatite during mineralisation, and act as a template for the formation of lamellae, in addition to contributing to the mechanical properties of bone (Reid, 1986).

Bone homeostasis is controlled by the coordination of various cell types, which provide approximately 2% of bone mass, and include osteocytes which are included in the permanent architecture of bone, osteoclasts, which remove bone tissue, and osteoblasts, which replace bone tissue. These cells allow for maintenance of calcium and phosphorous levels in bone, thus maintaining the structure and function of bone. Following resorption of bone by osteoclasts, numerous products are released which stimulate the differentiation and proliferation of osteoblasts, which, in turn, form new matrix which becomes mineralised, forming new bone. This process is known as bone remodeling.

Bone remodeling is an intricate process encompassing numerous paracrine and autocrine biochemical pathways and mechanical mechanisms. It is responsible for maintaining bone homeostasis, ultimately leading to preservation of structural integrity and function. Central to the bone remodeling process is the Basic Multicellular Unit (BMU), which controls bone resorption coupled with bone formation, by means of osteoclasts and osteoblasts, respectively. Osteoclasts function in the resorption of bone and are coordinated by the action of Receptor Activator of Nuclear Factor Kappa B ligand (RANKL) binding to Receptor Activator of Nuclear Factor Kappa B (RANK), which is counterbalanced by its decoy receptor Osteoprotegrin (OPG), which

subsequently stimulates osteoblast activity, and thus the production of new bone (Lacey *et al,* 1998, Hayden *et al,* 1995).

The RANKL-RANK-OPG cytokine system has been identified as the principal mediator of maintenance of bone cell function, and deviations from this system have been implicated in the pathogenesis of metabolic bone disease. Theories surrounding the initiation and control of bone remodeling postulate a role for various factors, including mechanical loading, fluid flow and microdamage. Microdamage is a naturally occurring phenomenon in bone and occurs as a result of daily loading, such as walking, running, and any mechanical impact. It manifests itself as a series of cracks, ranging from 2-400 μm in length. Microdamage in bone has been considered to be one of the principal mechanisms influencing RANKL and OPG signaling and subsequent bone remodeling. Concentrations of RANKL and OPG ultimately determine the fate of bone homeostasis, and deviations from normal levels occur in individuals with metabolic bone diseases, such as osteoporosis, leading to an increased incidence of fracture.

1.2 Categories of bone

The bones of the human skeleton are classified into six types, and these may present themselves as individual or fused, or partially fused bones, some of which are detailed in **Fig. 1.1.** These are: long (a), short (b), flat (c), irregular, sutral and sesamoid bones. Long bones, such as the femur and humerus, exhibit a long diaphysis and tend to consist of compact bone with little cancellous bone. Short bones, as the name suggests, are shorter and are cuboidal in shape, and constitute the bones of the wrist and ankle. These two categories provide a mechanical role, facilitating movement of an organism in tandem with muscle attachment. Flat bones are generally fused and are found in the skull and sternum. These provide a protective role and serve to shield organs such as the brain, heart and lungs, from injury. The latter 3 types of bone have a more complex structure and provide numerous functions. Sutral bones are found in the skull, and as implicated, assist in joining the flat bones of the skull. Irregular bones, as the name suggests, do not conform to a specific structure like long and short bone, and these include the vertebrae. Finally, sesamoid bones are located in tendons and function in providing distance between the joint and the tendon, and an example of this is the patella.

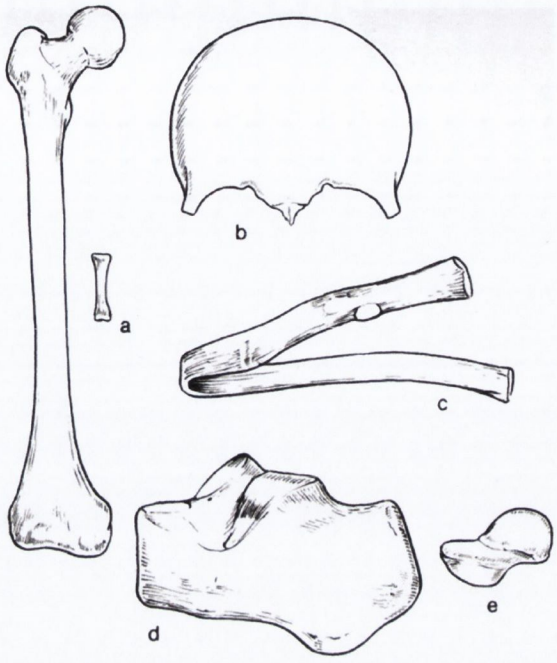


Fig. 1.1 Types of bone found in the human body (Dandy *et al*, 2003)

1.3 Bone structure and functional properties

1.2.1 Types of Bone

In human bone, two types of bone tissue are observed- cortical and trabecular, which form the extracellular matrix. Cortical bone, which is highly calcified, is present on the outer layers of the diaphyses of bones, and provides mechanical strength and constitutes 80% of bone weight. Cortical bone is characterised physically by a series of lamellae surrounding blood vessels. Trabecular or cancellous bone is spongy and more durable, characterised by a parallel lamellar system, more metabolically active and forms the majority of bone surface tissue and the ends of long bones (Adler, *et al*, 2000, Ashman *et al,* 1988). The bone is covered by a fibrous layer called the periosteum, which is composed of flattened osteoprogenitor cells, which coats all of the bone except the articular cartilage on the joint surfaces. Bone is infiltrated with blood vessels via the Volkmanns canals, which penetrate the periosteum. Below the surface of the periosteum is the endosteum, also consisting of osteoprogenitor cells. Osteocytes inhabit the

lacunae, which are cavities found mainly in osteonal tissue. The structure of bone, as a whole, tends to vary for each respective bone shape, and also its function, however, the majority of bones consist of trabecular and/or cortical bone. Long bones are what most frequently experience mechanical stress and tend to be load bearing bones. Long bones consist of a diaphysis, which is a long, tubular shaft, on the end of which is an epiphysis, connected via a metaphysis. The wall of the diaphysis consists of layers of compact bone surrounding the medullary cavity, which is rich in bone marrow. The epiphysis consists broadly of trabecular bone covered by a thin layer of cortical bone.

1.3.2 Trabecular bone

Trabecular bone is spongy and porous, and occupies the epiphyses of all long bones and can predominate in short and irregular bones, within the epiphyses, in addition to being found within the sternum and pelvis. It is comprised of numerous microstructural struts, called trabeculae, which form a 3 dimensional open porous space filled with bone marrow. The trabecular microstructure is orientated in a direction which provides structural strength and stiffness, distributing the force and impact received during normal movement, maintaining the anisotropic nature of bone.

1.3.3 Cortical bone

Cortical, or compact bone, is generally found on the surfaces of bone, and comprises 80% of bone- the remainder being trabecular bone. It is composed of inorganic salts and ground substance, giving rise to tiny spaces maintaining osteocytes, and is more isotropic than trabecular bone, allowing mechanical load to become impacted from any direction. Compact bone is comprised of tightly packed osteons and consists of various canals, known as Haversian canals. Running between the lacunae and Haversian canal are canaliculi. These organelles function in allowing for fluid flow, provision of nutrients, and are often a product of the removal and replacement of bone (Buckwalter *et al*, 1987, Hilka, 2003). Compact bone is lamellar in structure, giving a layered appearance, and is infiltrated by vascular canals, known as Haversian canals. These create a vascular network for providing nutrients for osteocytes. Surrounding the Haversian canals are circular structures known as osteons, consisting of numerous concentric

layers, which are formed during bone remodeling, where compromised bone is removed by osteoclasts and replaced by osteoblasts. This will be discussed in greater detail later in this thesis.

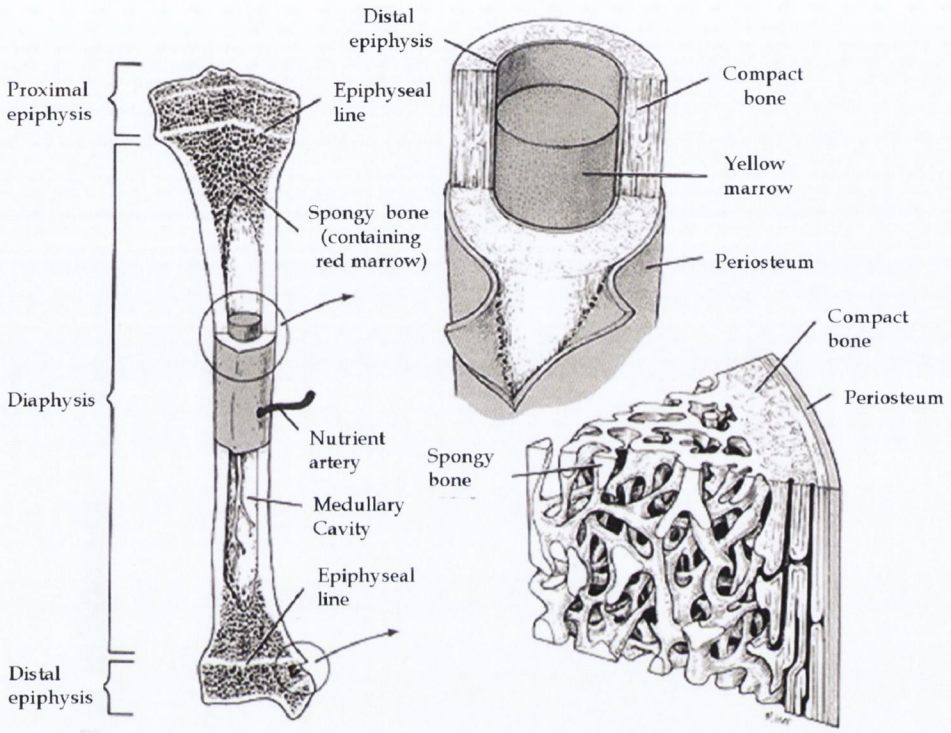


Fig. 1.2 Bone structure, trabecular and cancellous bone (Basic Medical Anatomy, by Alexander Spence (Benjamin/Cummings 1990)

1.4 Formation of Bone

Primary bone is formed, *de novo*, through a process known as osteogenesis, which occurs during embryonic development, or following a mechanical stimulus, resulting in damage to an osteocytic network, or from use of the bone, and may form from soft tissue or in cartilaginous tissue. This is followed by a period of mineralization, where the new bone becomes hardened. The process of osteogenesis can be divided into 2 primary forms of bone formation: intramembranous ossification or endochondral ossification. Secondary bone is formed during bone remodeling, which will be discussed in greater detail below.

1.4.1 Intramembranous ossification

Intramembranous ossification occurs during embryogenesis and is characterised by the formation of a primary ossification centre, whereby mesenchymal stem cells differentiate to form osteoblasts, which secrete osteoid, which subsequently becomes mineralized by hydroxyapatite salts. This is followed by the formation of spicules, which are small struts progressing from the ossification centre. During this process, osteoblasts become trapped in the matrix and furthermore differentiate to form osteocytes. The spicules subsequently fuse, and trap blood vessels infiltrating the new bone material. The newly formed bone is spongy in structure; however, remodeling of this bone surrounding the blood vessels facilitates the formation of osteons, which characterise compact bone. Further to this, connective tissue surrounding the bone becomes mineralized, forming a fibrous layer, the periosteum. Subsequent remodeling of the newly formed bone allows the bone to become hardened and resistant, whilst the organic content allows to bone to remain durable. This form of bone formation produces flat bones, such as the skull, and sutral bones. This process is illustrated in **Fig. 1.3.**

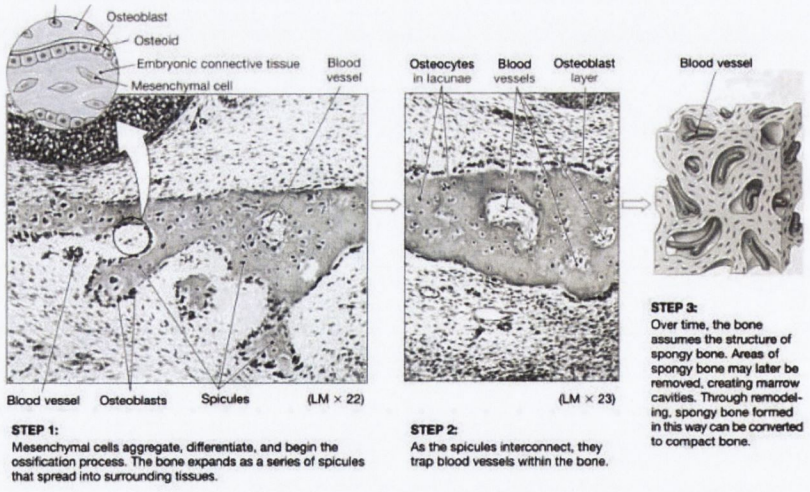


Fig. 1.3 The process of intramembranous ossification (Fundamentals of Anatomy and Physiology, Martini)

1.4.2 Endochondral ossification

Endochondral ossification is the second type of ossification, and, as the name suggests, is characterised by the formation of bone from hyaline cartilage, and is responsible for limb development. A series of interstitial growth, where the cartilage expands, precedes appositional growth, whereby new cartilage is produced on the surface. Within cartilage, chondrocytes expand and increase in size (in a process known as hypertrophy) and number and following this enlargement, the lacunae expand, forming struts which can become calcified by hydroxyapatite crystals. The enlarged chondrocytes become essentially starved as they are void of a nutrient supply, and die. Blood vessels infiltrate the new tissue, the perichondrium, in which, cells differentiate to form osteoblasts. These again, start to form new bone, and the perichondrium progresses to what is essentially the periosteum. During this period, blood vessels furthermore infiltrate the tissue and circulating fibroblasts differentiate to form osteoblasts, which lay down more tissue. Bone development now begins at what is known as the primary ossification centre. This spreads towards both ends of the cartilaginous model, forming a spongy bone like tissue. The bone enlarges, and at this point, osteoclasts appear and erode the trabeculae at the centre of the newly formed diaphysis, followed by a phase of appositional growth. A secondary ossification centre subsequently forms, following calcification of the epiphysis. These become followed with spongy bone. A cap of cartilage remains on the ends of the new bone and is maintained as the articular cartilage. Subsequent remodeling of the bone facilitates the hardening of the new bone, again, to maintain strength and rigidity of the bone. This process is illustrated in **Fig. 1.4.**

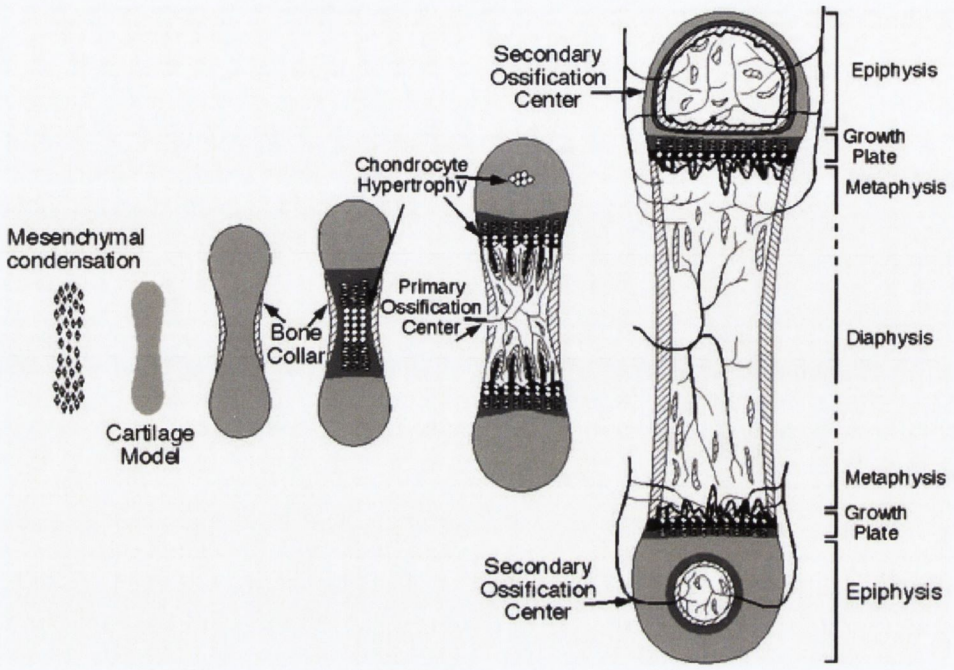


Fig. 1.4 The process of endochondral ossification (Baron *et al,* 2008)

Mineralisation is the stage whereby osteoid, which is secreted by osteoblasts, becomes mineralized by peripheral hydroxyapatite crystals. There are 2 distinct phases of mineralization- primary and secondary. Primary ossification occurs during bone remodeling where hydroxyapatite crystals are deposited within the newly formed matrix. This only takes a few days to complete. Secondary mineralization occurs over a period of up to 6 months, and is dependent on the rate of bone remodeling.

1.4.3 Formation of secondary bone

During normal bone remodeling, compromised bone is removed by osteoclasts and new bone is formed by osteoblasts, through the working of the BMU. This process is responsible for the formation of secondary bone. Secondary bone is characterised by the presence of a highly organised network of lamellar bone, presenting a layered appearance comprised of collagen and mineral matrix. Within the collagen framework, fibres run in opposite helical directions to that of adjacent lamellae. Three forms of lamellar bone exist: osteonal, circumferential, and interstitial,

which function in surrounding Haversian canals, Volkmanns canals, and the marrow cavity, respectively.

1.5 Bone Cells

There are four main types of cells present in bony tissue which are: osteoblasts, osteoclasts, osteocytes, and bone lining cells, as shown in **Fig. 1.5.** These act in a highly synchronised fashion within the BMU, and act under constant biochemical control, to maintain homeostasis within bone. The function of bone cells is, as an entire entity, to maintain bone mass and a balance between osteoclastic bone resorption and osteoblastic bone formation is required. Osteoblasts mediate bone turnover, effectively renewing bone tissue, whilst bone resorption is carried out by osteoclasts (Kane, 2007).

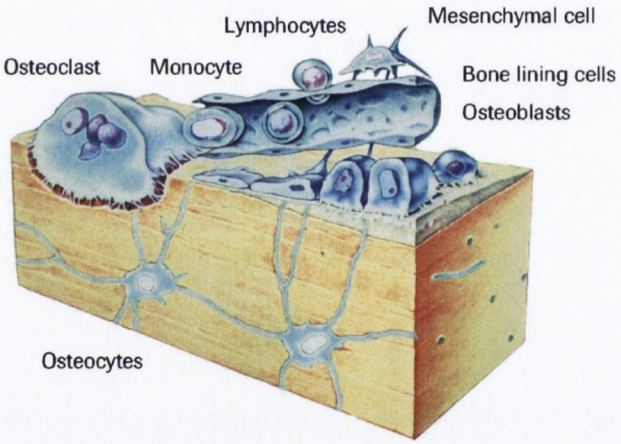


Fig. 1.5 Bone cells : demonstration of the principal cell types constituting bone : osteocytes, osteoclasts, osteoblasts, progenitor cells and bone lining cells (www.roche.com)

1.5.1 Osteocytes

Osteocytes are terminally differentiated osteoblasts that function regulating bone remodeling. Osteocytes are present in quantities of typically 25,000 cells per mm^3, and originate from mesenchymal cells, and are the most abundant cell in bone, constituting 90-95% of bone cells (Vaananen *et al*, 2000, Kato *et al,* 1997, Bonewald, 2007). Osteocytes are characterised by the presence of a network of between 50 and 100 dendritic cell processes which result from osteoblast differentiation, which allow cell-cell contact between osteocytes and osteoblasts and

bone lining cells. These processes also penetrate the canaliculi, facilitating osteoclast-osteocyte communication, and have been shown to infiltrate bone marrow (Kamioka, *et al*, 2001).

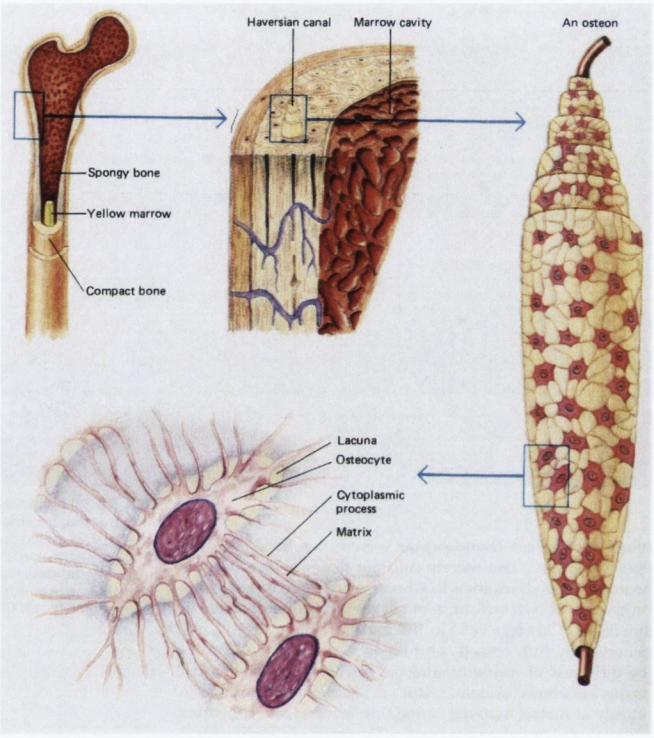


Fig. 1.6 Osteocytes and canaliculi (Villee *et al*, 1989)

Osteocytes are considered to be sensor cells; for example mechanical impact promotes them to produce nitric acid and prostaglandins (Klein-Nulend, *et al*, 1995). The expression of gap junctions has facilitated their role as mechanosensory cells; osteocytes form networks via cell processes joined by gap junctions, and these allow one cell to detect a mechanical stimulus, and transmit a signal to surrounding cells (Turner *et al*, 1994,). A further attribute to the osteocyte is its intricate cytoskeleton, which is arranged in bundles of microfilaments running parallel to the cell body, filling the cell processes. Further to maintaining the stellate morphology of osteocytes, these function in the transport of nutrients and also may play a mechanosensory role (Tanaka, *et al*, 1998). The mechanosensory role of osteocytes will be discussed in greater detail later on in this introduction.

1.5.2 Osteoclasts

Osteoclasts are terminally differentiated, multinucleated bone cells, originating from a monocyte-macrophage lineage, responsible for the dissolution of mineralised bone matrix following injury, disease or during bone remodeling (Takahashi *et al*, 2007). Osteoclasts are characterised phenotypically by being "giant" cells, expressing many nuclei, and may have a "foamy" cytoplasm (Akatsu *et al*, 1992, Wezeman *et al,* 1979) These specialised cells function by secreting lytic enzymes capable of dissolving extracellular matrices in response to RANKL, a cytokine responsible for osteoclastogenesis (Boyle, *et al,* 2003). This occurs following RANKL-RANK binding, whereby lysosomal enzymes are released from transport vesicles within the cell. Osteoclasts resorb bone in three distinct phases: (1) through polarisation to bone, and so (2) creating a ruffled membrane, composed of vinculin and actinin, surrounded by F-actin, through which lytic enzymes are released, including Vacuolar H^+- ATPase and cathepsin K, (3) resorbing bone (Katagiri *et al,* 2002). Attachment to the bone cell is via the vitronectin receptor, collagen receptor, and through extracellular proteins (Vaananen *et al,* 2000).

1.5.3 Osteoblasts

Osteoblasts are functional cells which originate from a mesenchymal lineage, and are responsible for the formation of new bone. They secrete osteoid, an organic extracellular matrix component comprised predominantly from collagen type I, which becomes mineralised by calcium salts (Aubin *et al*, 2002). This occurs under the control of the Cbfa1/Runx2 pathway. Osteoblasts are characterised by their mononucleate morphology, in addition to expressing high levels of bone sialoprotein and osteocalcin. Additionally, they express numerous gap juntions between adjacent osteoblasts, which function in a paracrine role (Cheng *et al*, 2004). Furthermore, osteoblasts are characterised by their ability to secrete vast amounts of alkaline phosphatase (ALP), in addition to the expression of parathyroid hormone, estrogen, androgens and vitamin D3 reseptors (Harada *et al*, 2003, Zanello *et al,* 2004). Osteoblasts contain a few cytoplasmic processes which become extended into the osteoid matrix, whereby they contact the osteocyte processes within the canaliculi (Bianco *et al,* 1983, Ferretti *et al,* 2002).

Osteoblasts become terminally differentiated into osteocytes, which, as described, play a mechanosensory role in bone. Also, like osteocytes, they express RANKL and its decoy receptor,

OPG (Takahashi *et al*, 1999, Hofbauer *et al,* 2004). Osteoblasts are principally controlled by BMPs, of which several types exist. BMPs are responsible for the phosphorylation of Smad proteins, the action of which instigates osteoblastic differentiation (Massague *et al*, 2000). Osteoblast differentiation is controlled moreover by the transcription factor Osterix (Osx), which encourages cellular differentiation, as well as Runx2. This can furthermore be phosphorylated into mitogen activated protein kinase (MAPK) and this, in turn, elevates levels of BMPs and facilitates the production of extracellular matrix (Nakashima *et al,* 2002, Otto *et al,* 1997). The formation of osteoid occurs in a sequence of events orchestrated by the presence of ALP. Acquisition of ALP is followed by increase in collagen type I synthesis. This is followed by an upregulation in the expression of osteocalcin, osteopontin and bone sialoprotein, which become depositied within the matrix, and subsequently mineralised (Aubin *at al,* 1996). Following secretion of osteoid, which become subsequently mineralised, osteoblasts differentiate furthermore to form osteocytes or bone lining cells (Nijweide *et al*, 1988).

1.5.4 Bone lining cells

Bone lining cells, or periosteal cells, as the name suggests, are flat elongated cells that are responsible for lining over 80% of endosteal bone surfaces of bone and are characterised by flat, ovoid shaped cells joined by junctions similar to those of gap junctions (Dierkes *et al,* 2009, Miller *et al,* 1980). Bone lining cells function in providing regulation of exchange of substrates between bone and its fluid environment and as a mechanical sensor in the osteocytic network, and in doing so, assist in the bone remodeling process (Dierkes, *et al*, 2009). It has been postulated that bone lining cells are derivatives of osteoblasts, and act in a syncytium with activated osteoblasts (Martin, 2000, Doty *at al,* 1981).

1.6 Bone diseases

It is necessary to maintain correct hormonal and chemical levels to allow instigation of the BMU, thus ensuring normal functioning of the bone remodeling process. Accumulation of damage and increased incidence of stress fractures may be indicative of a malfunctioning BMU, which could suggest poor hormonal function and regulation, and is symptomatic of bone disease.

Diseases of the bone result due to incorrect hormonal functioning, such as osteogenesis imperfecta, Paget's disease and osteopenia (Rodan *et al*, 2000, Thiebaud *et al,* 1994). Conditions affecting hormonal functioning have also been implicated in the pathogenesis of this disease, including menopause, Turners syndrome, Kallman syndrome and hypogonadism, which lead to a decrease in estrogen, creating symptoms similar to that of menopause (Harper, *et al*, 1998, Shaw *et al,* 2003). The most noteworthy of these conditions is the marked reduction in estrogen at women following menopause, leading to osteoporosis, which will be discussed in greater detail within this introductory chapter.

1.7 Bone remodeling

Similarly to bone formation, bone turnover and remodeling is controlled through a series of cells, cell signaling, and by the BMU. This occurs in cycles of bone resorption and remodeling, into which osteoclasts and osteoblasts are organised (**Fig. 1.7**). On average, the BMU progresses longitudinally at a rate of 25 μm per day, for a period of 6-9 months, and approximately 10% of bone is replaced per year. Bone resorption and formation are coupled synchronously by RANKL and OPG activity, and under normal circumstances, when one mechanism increases, the other generally decreases.

1.7.1 Bone resorption

Bone resorption by osteoclasts generally occurs over a period of 2-3 weeks, whereas bone formation takes several months. The rate of BMU function depends on numerous factors, including the lifespan of the BMU, rate of BMU origination, efficiency of cell signaling, and rate of bone turnover at a given location (Jaworski *et al,* 1992, Hernandez *et al,* 2002). It has been found that normal functioning of the BMU decreases with age, facilitating a decrease in trabecular and cortical bone volume, leading to osteoporosis. Bone remodeling and the BMU are essential for maintaining skeletal integrity and for the reparation of microcracks and injury sustained from daily wear and tear.

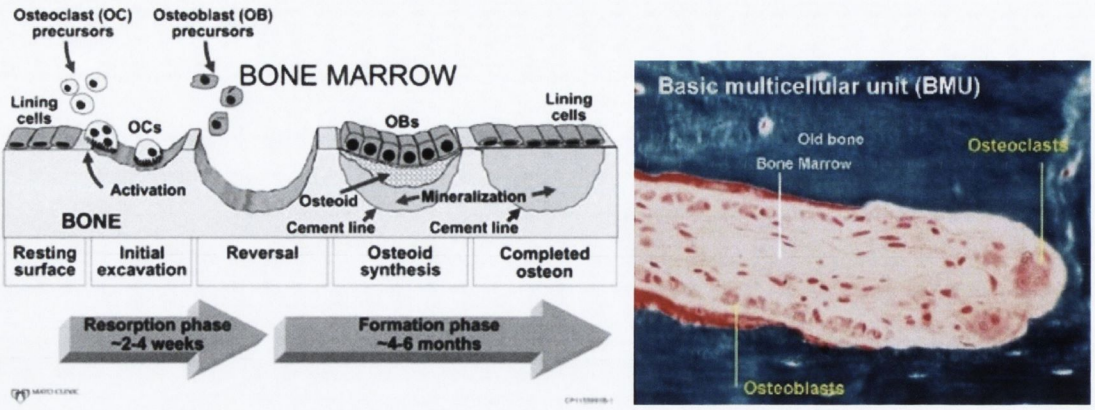


Fig. 1.7 The process of bone remodeling. A The process of bone remodeling as performed by the Basic Multicellular Unit (Riggs *et al,* 2004). Fig. 1.7 B Histological image of the arrangement of osteoclasts and osteoclasts in a BMU (http://www.talosproject.nl)

A quiescent period is experienced in bone following the removal of tissue by osteoclasts. It is then subjected to infiltration of mononuclear cells and removal of the remaining debris left from the breakdown and absorption of bone (Mulari *et al*, 2004). The BMU is three dimensional in structure, 200 μm in diameter, and comprises of a band of osteoclasts capable of breaking down bone. A major marker of bone resorption is Tartrate Resistant Acid Phosphatase (TRAP), which is a lysosomal enzyme involved in the breakdown of bone, and will be used throughout this study to determine the rate of osteoclast activity (Eyre *et al*, 1987). TRAP is a hydrolase enzyme which has a molecular weight of 32 kDa and exists as a phosphatase in its active form (Nenonen *et al*, 2005). Activity of TRAP is regulated by trypsin and cathepsin induced cleavage. Two forms exist in blood serum, where it is found in a complex with α-2 macroglobulin: TRAP 5A, a sialic acid containing isoform, which also exists in a non - sialylated form, TRAP 5B, both of which are differentially glycosylated (Schaffer-Brehme *et al*, 1999, Nenonen *et al*, 2005). It has been found that TRAP 5B is secreted from osteoclasts, however, the origin of TRAP 5A is still unknown (Halleen *et al*, 2000). TRAP functions in removing the by-products of bone degradation, which are produced from the functional secretory domain of osteoclasts, through the action of reactive oxygen species present in transcytotic vesicles on osteocytes (Salo *et al*, 1997).

1.7.2 Bone Formation

The process of bone resorption is followed by the infiltration of a group of mononuclear cells which mediate the reversal stage of bone remodeling. Following this group of cells are the osteoblasts, which function in the deposition of osteoid, or new bone matrix (Eriksen *et al*, 1995). On cessation of BMU movement, the formation of a Haversian canal is evident through the mineralisation of a groove or tube in the bone (Robling *et al*, 2006). **Fig. 1.7A** demonstrates the process of bone remodeling. The formation of bone is marked predominantly by ALP, which is found in the plasma membrane of osteoblasts, and has been shown to bud from this membrane during bone development. ALP is a hydrolase, forming a large family of dimeric enzymes, characterised by their ability to release phosphate on catalysis of phosphomonoesters (Mornet *et al*, 2001). The main function of ALP is the regulation of osteoblast homeostasis in its carboxylated form, remineralisation of bone tissue, and it accounts for up to 20% of non collagenous proteins in bone (Szulc *et al*, 1996, Lee *et al*, 2004). Furthermore, it has been hypothesised that ALP is responsible for the breakdown of pyrophosphate, and in doing so, assists in the maintenance of inorganic bone (Risteli *et al*, 1993).

The presence of BMPs and TGF β which are released from bone resorption stimulate osteoblastic activity and the secretion of bone matrix proteins, which includes osteoid and collagen Type I, in addition to non collagenous proteins, including proteoglycans, which encompass decorin and byglycan and glycoproteins, such as osteonectin and fibronectin, which function in bone cell adhesion through production of osteopontin and bone sialoprotein, as well as stimulating and regulating the migration and functioning of the cells of the BMU (Shi *et al*, 2002. Mackie *et al*, 1990). Osteoblasts furthermore regulate the mineralisation of osteoid through the aforementioned release of ALP It has been found that the birth rate and longevity of the BMU is directly proportional to the frequency and intensity of microcracks and that mechanical stimulus is a major contributing factor to the dedifferentiation process of osteocytes into osteoblasts (Robling *et al*, 2006, Tuner *et al*, 1998).

1.7.3 Cell signaling within the BMU- The RANKL-RANK-OPG signaling pathway

As described, bone remodeling is under constant biochemical and physiological control. Central to this process is the RANKL-RANK-OPG signaling pathway (**Fig. 1.9**). RANKL is released from both osteocytes and osteoblasts as a result of membrane perturbation, following mechanical stimulation or damage, and it binds to its receptor RANK, on osteoclasts. Following bone resorption, OPG is released, which acts to bind to and sequester RANKL, ceasing its activity and stimulating bone formation by osteoblasts.

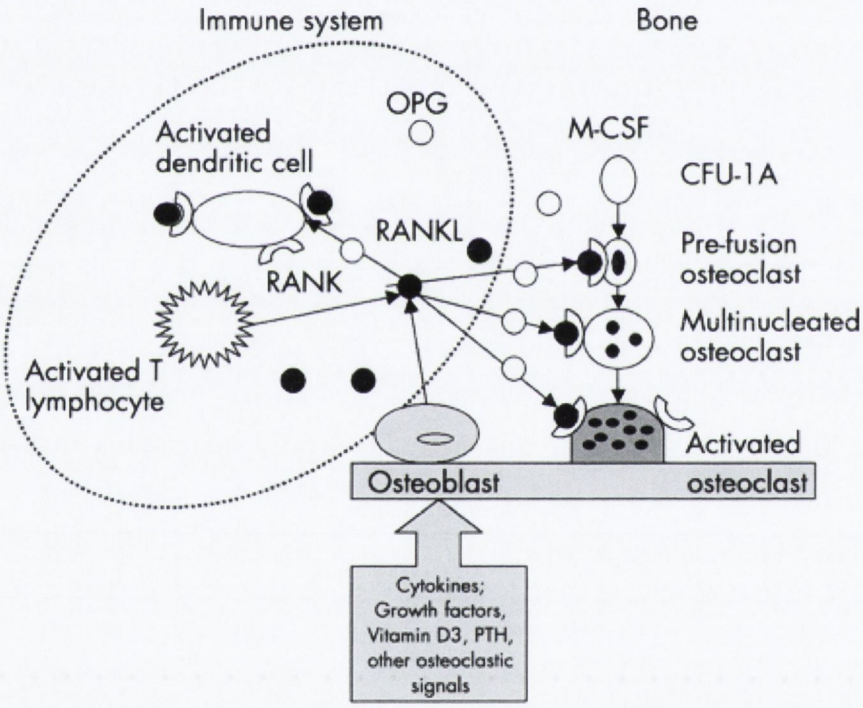


Fig. 1.8 The RANKL-RANK-OPG signaling pathway and related cellular crosstalk (Livshits *et al*, 2004)

1.7.4 RANKL

RANKL (TNFRSF11A) is a type II transmembrane protein tumour necrosis factor homologue, released by the metalloprotesase, disintegrin TNFα (TACE) on the surface of osteoblasts, preosteoclasts and dendritic cells. It is the primary mediator in the activation and differentiation of preosteoclasts into osteoclasts, and so functions in stimulating and sustaining bone remodeling (Lum, *et al*, 1999, Yasuda *et al*, 1998). Human and murine RANKL exist as

316 and 317 amino acid residues, respectively, and are released from osteoblasts on receiving paracrine or endocrine chemical messages, including parathyroid hormone (PtH), tumour necrosis factor (TNF) and Interleukins (IL). The cytokine then binds to the RANK receptor on the cell surface of preosteoclasts and mature osteoclasts, in a membrane bound or soluble form, and this has been shown to increase the absorptive function of mature osteoclasts 7 fold (Lacey *et al*, 1998, Boyle *et al*, 2003, Burgess *et al*, 1999). RANKL has been shown to be induced by numerous factors, including parathyroid hormone tumour necrosis factor alpha (TNF), TGF α, Oncostatin M(OSM), prostaglandin E_2 (PGE_2), interleukin 1 (IL-1), interleukin 6 (IL-6) and interleukin 11 (IL-11) (Oyajobi *et al*, 2001, Walton *et al*, 2002). RANKL has also been shown to be upregulated by the presence of calcitropic elements such as 1, 25 dihydroxyvitamin D3, ionomycin, cyclopiazonic acid and thapsigargin (Takami *et al*, 1997). These factors act alone or in tandem with other cytokines, creating a signaling pathway.

Osteoclast activation is a result of polarisation by RANKL binding to the cytoplasmic tail of RANK (Darnay *et al*, 1998). This results in a massive influx of NF κ B cytokines, and is the first major step in the upstream activation of the osteoclastogenic signaling cascade. On stimulation of RANK by RANKL, trimerization of the receptor ensues, followed by a massive influx of tumour necrosis factor associated receptor factors (TRAFs), 1, 2, 3 and 5, which bind to the carboxyl end of the RANK dimer (Palmqvist *et al*, 2004). This elicits a downstream cascade resulting in TRAF dependant activation of NFκ B, mitogen activated protein kinases (MAPK), activation of fc-Jun N terminal kinase (JNK), and p38, an inhibitor of NF κ B kinase. This followed by protein tyrosine kinase- c-Src mediated activation of phosphatidylinositol 3-kinase/Akt pathway, inducing an anti-apoptotic effect on osteoclasts (Boyle *et al*, 2003). This causes alterations of the actin in the cytoskeleton of the osteoclast and the formation of tight junctions in the basement membrane in preparation for the degradation of bone. This, along with polypeptide growth factor 1 (CSF-1) , leads to the upregulation of the genes encoding TRAP, the calcitonin receptor, integrins and pro - cathepsin k (CATK), which are all necessary mediators in osteoclastogenesis (Boyle *et al*, 2003).

1.7.5 OPG

RANKL is antagonised by Osteoprotegrin (TNFRSF11B), a member or the tumour necrosis factor (TNF) family, which functions by binding to and sequestering RANKL, acting as a decoy receptor, impeding the resorption of bone. This inhibits osteoclastogenesis and prohibits the functioning of osteoclasts (Bekker *et al*, 2001, Lacey *et al*, 1998). This RANKL-RANK-OPG pathway is controlled by a series of cytokines and chemokines. OPG is released from the osteoblast lineage of cells on receipt of signals such as estrogen, bone morphogenic proteins and transforming growth factor beta (Boyle *et al*, 2003). OPG, following stimulation by RANKL-RANK interaction, affects osteoblastic density and is the major factor contributing to the inhibition or activation of bone resorption, respectively (Wan *et al*, 2001).

Osteoblasts initially produce OPG as a monomer, but it only becomes functionally active as a dimer, and it is in this configuration that allows binding to RANKL (Simonet *et al*, 1997). The production of a small mimetic by OPG, OP3-4, binds to trimeric RANKL, and blocks receptor function through alteration of orientation and by blocking cytoplasmic interactions and NFκ B activation (Cheng *et al*, 2004). Despite its action as an anti-RANKL cytokine, OPG is controlled by a similar series of metabolic cytokines and mediators including TNFs, TGFs, cholcalciferol and prostaglandin E2. Murine OPG is a 401 amino acid glycoprotein exhibiting a 21 amino acid residue which is subjected to cleavage, leaving a 380 amino acid protein. It has been found that OPG is secreted by osteoblasts and may exist as a 60kDa monomer or a 120 kDa disulphide linked homodimer (Simonet *et al*, 1997, Yamaguchi *et al*, 1999). Release of OPG often occurs inadvertently due to stimulation by the biproducts of bone resorption and bone remodeling. Initially, RANKL is released, as described, and along with its release, PGE_2 and PHT are released, via NFκB activation, in addition to TGF-β stimulation, which functions in stimulating OPG (Hofbauer *et al*, 2004). OPG expression is also regulated by the Wnt/β-catenin pathway, as will be described later on in this chapter (Glass *et al*, 2005).

1.7.6 Other biochemical factors in bone remodeling

The bone remodeling cycle is, as described, principally under the control of the RANKL-RANK-OPG signaling pathway, however, within this pathway, there are numerous other factors contributing to both the release and activation of these cytokines, and also may be responsible for stimulating a bone remodeling response on their own. During bone resorption, for example, numerous bone formation stimulatory factors are released, including TGF β, PTH and ILGF have been thought to play a role in stimulating osteoblast function.

The combination of macrophage colony stimulating factor (M-CSF), which acts as a stimulator of RANKL, and RANKL has shown to upregulate BMP-2 mediated differentiation of osteoclasts. BMPs, however, are not fundamentally involved in RANKL-RANK- OPG crosstalk due to being predominantly under the control of Smad signaling pathways, and so, BMPs are somewhat excluded from the crosstalk involved in osteoclastogenesis (Itoh *et al*, 2001). Smads are serine/threonine kinases, of which 8 forms exist, and these are furthermore subdivided into 3 groups: receptor regulated Smads, common partner Smads, and inhibitory Smads (Fujii *et al*, 1999). Smads function through other BMPs, in addition to being mediated by activin A and TGFs, and are involved in osteoblasts specific gene expression (Lee *et al*, 2000). On the other hand, BMPs do stimulate the upregulation of numerous transcription factors, including core bonding factor (CBFa 1), and osteoblast specific factor (OSF) which in turn upregulate osteoblastic genes including osteocalcin, osteopontin, and bone sialoprotein, and so potentiate osteoblast differentiation, releasing OPG (Gao, *et al*, 1998). There has been no further study to elucidate the relationship between RANKL, its receptors, and BMPs; however, one could conclude that BMPs act indirectly in the process of osteoclastogenesis.

As described previously, RANKL is stimulated by the presence of PGE2, IL-1 and TNF-α and membrane bound RANKL is released and solubilised by the presence of matrix metalloproteases (MMPs) (Klier *et al*, 2001). RANKL functions following release of M-CSF, which in turn triggers the transcription of extracellular signal-related kinase (ERK). This is the first step in the regulation of osteoclastogenesis. M-CSF is released by bone marrow stromal cells, in response to IL-1 (Kimble *et al*, 1996). IL-1, has also been shown to, in conjunction with TNF, increase the formation of TRAP positive osteoblasts in culture through upregulation of

RANTES (Cho *et al,* 2006). M-CSF attaches to the c-Fms receptor on osteoclastic cells and this ultimately determines the fate of these cells, and allows differentiation into osteoclasts (Udagawa *et al*, 1990). The c-Fms receptor is a transmembrane receptor kinase, and on binding of M-CSF to the receptor, dimerisation occurs followed by phosphorylation of tyrosine residues (Takeshit *et al*, 2007).

A major molecular participant in the signaling process is TRAF6, another member of the tumour necrosis factor superfamily which permits binding to RANK. It has been shown that TRAF6 knockout mice tend to express osteopetrosis and overgrowth of teeth (Lomaga *et al*, 1999). TRAF6 maintains the pathways of activator proteins and NF κ B proteins, and facilitates binding of RANKL and RANK (Boyle *et al*, 2003). Furthermore, TRAF6 interacts, under the direction of NF κ B, with c-Fos, the latter of which is produced by RANKL stimulation by interferon β, facilitating osteoclastogenesis (Takayanagi *et al*, 2002, Wang *et al*, 1992). The mechanisms of this cascade are mediated by RANK binding to RANKL. This allows for the recruitment of growth factor receptor bound protein 2 associated binding protein 2 (GAB2), one of the principal activators of Phosphatidylinositol 3-kinase, P13. GAB2 is a potent inhibitor of osteoclastogenesis, and so these cytokines work in a negative feedback loop (Itoh *et al*, 2001). This leads to C-fos activation, which, in turn stimulates NFAT, which has been described as the major factor contributing to terminal differentiation of osteocytes, and so is one of the final aspects in the signaling cascade in osteogenesis. These cytokines ultimately combine to initiate the transcription of osteoclastic genes downstream of RANKL, and maintain integrity and longevity of osteoclasts (Boyce and Xing, 2007, Schett *et al*, 2005). Further to this, activation of the JAK/STAT and MAPK pathway has shown increase leukemia inhibitory factor mediated RANKL activation, thus stimulating bone resorption (O'Brien *et al*, 1999, Palmqvist *et al*, 2004).

Ephrin is a major molecular participant in functionalising the BMU and initiating the bone remodeling process, requiring cell to cell contact between osteoblasts and osteoclasts. Two classes of Ephrins exist, Class I (Ephrin B1-B3) are 26k Da proteins and Class II (Ephrin A1-A5), which are 37 kDa in size. These are ligands for tyrosine kinase receptors Eph B1-B6, and GPI anchored EphA receptors, (A1-10), respectively (Mundy *et al*, 2006). There are 14 Ephrin receptors and 8 ligands in existence. Ephrins, namely the Ephrin B2 ligand on osteoclasts and the EphB4 receptor on osteoblasts, are responsible for the bidirectional signaling during contact

between cell types, mediating cellular activation and inactivation. Osteoclasts lack receptors for ephrin, yet express Ephrin B1 and B2, whereas osteoblasts express both ligands and receptors. Interaction between Ephrin 2 and EphB4 prevents c-fos activation of NFATC1, thus inhibiting osteoclastogenesis. The process of EphB receptor activation is considered to be "foreword signaling", and ephrin ligand activation is known as "reverse signaling". Ephrin B2 and Eph B4 crosstalk effectively acts to turn on bone resorption via osteoclasts. Once sufficient resorption has occurred, ligand mediated Ephrin B2 signaling switches off osteoclasts activity through reverse signaling. This occurs in tandem with TGF β mediated attenuation of osteoclast activity. This then results in activation of osteoblasts and the laying down of bone tissue, acting in a continuous cycle until the process is complete (Zhao *et al*, 2006).

It was suggested by Zhao *et al*, that forward signaling from osteoclasts is dependent on RhoA, a member of the Ras superfamily of GTPases, through the exchange factors intersectin and kalirin. This signaling, like RANKL, also is enhanced by PTHrP (Parathyroid hormone related protein) (Edwards *et al*, 2008, Irie *et al*, 2002, Hill *et al*, 1995). Other Ras family members, including H-Ras and R-Ras, are regulated by eph receptors, in turn, activating the MAPK pathway (Miao *et al*, 2001). Ephrins also signal, to a lesser extent, through the Janus kinase/signal transducers and activators of transcription (Jak/Stat) and focal adhesion kinase (FAK) pathways. These pathways facilitate the transduction of cytokine and protein signals within the bone cell. It has been proposed by Pierroz *et al* (2008), that regulation is furthermore controlled by beta arrestins. Beta arrestins are proteins capable of being activated and phosphorylated by G protein coupled receptors, and function in initiating MAPK pathways, then preventing G protein coupled signaling, and PTH stimulated intracellular signaling. Further to this, bone formation is potentiated by the activation of the canonical pathway, which is focused on the wnt signaling cascade. Wnt proteins encompass numerous biochemical factors including bind to frizzled and low density lipoprotein receptor related protein receptors (LRP) has shown to regulate the expression of OPG by increasing the OPG-RANKL ratio, thus inducing bone formation (Glass *et al*, 2005). The canonical pathway is antagonised by the presence of frizzled related proteins and Dikkopf proteins (DKK), which effectively act by binding to frizzled and LRP receptors, thus interfering with and restoring the RANKL/OPG ratio, subsequently stimulating bone resorption (Qiang *et al*, 2005, Diarra *et al*, 2007).

1.8 Microdamage

It has long been thought that microdamage in bone is one of the principal mediators of bone remodeling and of the initiation of the BMU, which then functions in removing damaged or compromised bone surrounding microcracks. It was first proposed by Frost (1960), that repair of bone following micro-injury is fundamental in maintaining skeletal integrity. Frost proposed the concept that cells are essentially mechanostats, and act as an integral part of establishing bone strength, based around cellular detection of mechanical signals to trigger a response. The mechanosensory mechanism, as described by Frost, details the stimulation of the cell through conversion of mechanical signals to biochemical signals (Klein-Nulend *et al*, 1995). This then signals for the commencement of the BMU or to osteoblasts to deposit collagen matrix.

Like any weight bearing structure, bone is subject to fatigue induced damage however, unlike engineered materials, it has the ability of self reparation. Carter and Hayes, 1977, described how fatigue results in the deterioration in mechanical properties such as strength and material durability, and how repeated stresses can cause mechanical failure due to a build up of damage (Fazzalari *et al*, 1998). Microcracks accumulate naturally as a result of cyclic loading, day to day stress and strain and repetitive insult to the area under stress, and the amount of microdamage observed in an individual has shown to be dependent on the mass of the individual (Brianza *et al*, 2011). It has been observed in compact bone both *in vivo* and *ex vivo* (Carter *et al*, 1977, Frost 1960). It has also been found that microdamage collects at points of diminished osteocyte integrity (Qiu *et al*, 1997, Vashishth, *et al*, 2000). The production of microcracks allows bone to take in energy without breaking (Schaffler *et al*, 1994).

Microdamage manifests itself as a series of cracks ranging up to 400 μm (**Fig. 1.10**), depending on the nature or the crack and exceeding these values, the microcrack becomes a potential stress fracture. Two patterns of microcracks, linear and cross-hatched, have been observed. Linear microcracks were observed in both the central portion and near surfaces of trabeculae (Wenzel *et al*, 1994). Larger, linear cracks result from compression, and tend to be observed 30-40° from the bones axis. These cracks tend to be more related to the bone's microstructure and form around the bone's cement lines and inter-lamellar boundaries (Ebacher *et al*, 2007, Boyce *et al*, 2008, Carter and Hayes, 1976, Schaffler *et al*, 1989). Groups of smaller

cracks, from 2-10 μm long, known as diffuse damage, result from tensile strain, and run parallel to the loading direction (Martin and Burr, 1985, Fazzalari *et al*, 1998, Zioupus *et al*, 1994). Histological staining has shown that cracks extend further in the longitudinal direction than in the transverse direction, thus presenting an elliptical shape (O'Brien *et al*, 2003). A build up of microcracks in bone is a factor in reduced strength and resistance to fracture in bone (Schaffler *et al*, 1996).

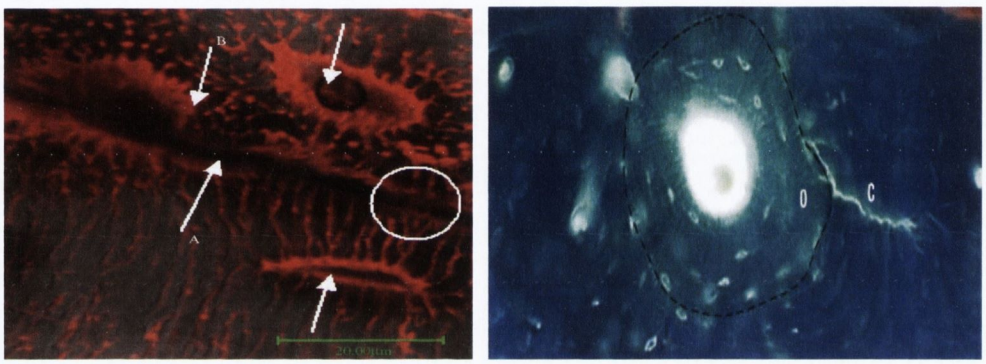


Fig. 1.9 Histological Images of microcracks in bone (Taylor *et al*, 2003). A: This image demonstrates broken cell processes on crack interface; B This image demonstrates the crack progressing through an osteocyte lacuna. Within the circle, intact processes can be viewed spanning the crack interface. The microcrack is found to be encountering and being deflected by an osteon (O'Brien *et al*, 2003).

Cracks can be observed mainly in the interstitial bone between osteons, on channels and lacunae, for example, it was recently demonstrated that increased lacunar density elicits a greater propensity for bone to accumulate microdamage (Soicher *et al*, 2011). Microcracks have been shown to be prevented from progressing and are often stopped in their tracks by collision with osteonal cement line (Boyde *et al*, 2003, Martin, 2003). This has predominantly been observed in linear microcracks, for example, in a study using an ovine radial fracture model, short microcracks were stopped at the osteonal cement line, and medium sized cracks were deflected by osteons, whereas large linear cracks had a tendency to penetrate osteonal barriers (Reilly *et al*, 1999, O'Brien *et al*, 2005). The microstructure of bone functions therefore in facilitating crack propagation in addition to acting as a barrier preventing progression of microcracks, most notably at microstructural interfaces (Diab *et al*, 2007, Chapurlat *et al*, 2008). Furthermore, it was hypothesised by Martin and Burr (1982) that the Haversian canal adjacent to the crack will produce a new osteon, and essentially traps the microcracks within the lamellae of the osteon

(Martin and Burr *et al*, 1982). Interaction of microdamage with cells can increase mechanical strain, and in doing so, triggers bone remodeling. Mechanisms of controlling such an action were considered by Parfitt, 1984, and included the incidents of fatigue damage, changes in cell membranes, hydrostatic pressure on cell membranes thanks to alterations in fluid flow, and stress potentials. In addition to this, microcracks have been associated with increased intracortical resorption and in compact bone in overuse fractures in dogs (Burr *et al*, 1985, Mori *et al*, 1993).

Human bone can tolerate strain levels of up to 2000 με in quantities from 4-10 million cycles prior to failure, and the greater the strain encountered by the bone, the more frequent the microdamage and the greater the loss of stiffness is experienced (Pattin *et al*, 1996, Schaffler *et al*, 1989). If strain exceeds 7000 με, it will be irreparably damaged, and bone will die (Burr *et al*, 1985). It has been theorized that microdamage in bone is one of the principal mechanisms involved in the initiation of apoptosis, which is, however, confined to areas surrounding the microcrack. This function coincides with bone resorption by the BMU and the instigation of the bone remodeling process, whereby apoptotic regions have been observed, in addition to resorption sites being noted in close proximity to microdamage (Verborgt *et al*, 2000). The events occurring at the site of the cracks have been of great interest and the source of considerable hypothesising as to whether cells are necrotic or apoptotic. To consider this question, it was demonstrated in a study by Noble *et al*, 1998, which described an increase in osteocyte apoptosis following deformation of bone, preceding high rates of bone resorption. More recently, Cardoso *et al*, 2009, described how osteocyte apoptosis, found local to an area of fatigue induced microdamage, is necessary to initiate intracortical remodeling, and that apoptosis may be necessary to stimulate bone remodeling, and that bone remodeling may occur in a dose response relationship with regard to the magnitude of microdamage. This also provided a platform to consider microcracks as a modulator of bone remodeling. Klein-Nulend suggested that levels of nitric oxide (NO) produced following microdamage are sufficient to induce apoptosis (Klein-Nulend *et al,* 2005). Turner *et al*, 2002, proposed that cells directly at the site of injury signal through a series of gap junctions within osteocytic processes, forming a syncytium, allowing cell-cell signaling.

1.8.1 Microdamage and bone remodeling

There are numerous theories surrounding the initiation of bone remodeling, including mechanical force and loading, fluid flow and microdamage in bone. The main issue is that these factors often occur concurrently, and one must examine each aspect alone or together to illustrate the mechanisms by which bone repairs itself. Galileo first noted the relationship between bone morphology and the amount of load applied to bone. This was furthermore expanded upon by Julius Wolff, who considered that bone mass is an adapted 3D structure in conjunction with load, to obtain a higher efficiency (Wolff 1982). As previously described, Frost (1960) proposed that microdamage is necessary to maintain skeletal strength, yet he also considered the concept that mediators of bone remodeling may not be mutually inclusive. Cell process rupture in canaliculi and alterations in fluid flow then gave rise to the mechanostat theory of bone remodeling (Frost, 1960). These theories are conceptually viable, however, the precise mechanisms by which the basic multicellular unit is directed to an area of compromised tissue has yet to be founded.

Mechanical loading and fluid flow often go hand in hand - application of load to bone directly influences fluid flow, and one could connect this to Wolff's Law. Cell membrane perturbation is due to alterations in fluid flow resulting from mechanical loading, producing fluid shear stress. This is detected by mechanoreceptors in the canaliculi of osteons (Klein-Nulend *et al*, 1995). This kick starts a chain of events with the release of prostaglandins, which are principal mediators of bone remodeling, an influx of calcium ions, cAMP, and increased alkaline phosphatase activity, ultimately leading to stimulation of osteoblasts (Frangos *et al*, 1985, Lane *et al*, 1987). There is solid evidence that fluid flow affects cytokine and prostaglandin release. Similarly Lozupone (1996) demonstrated how osteocyte integrity is increased following mechanical loading. Takai (2004) furthermore demonstrated how hydrostatic pressure increased osteoblast function, and Bakker *et al*, 2004 demonstrated how mechanical loading preceding fluid flow is a vital component in ensuring osteocyte survival. Burger *et al*, 2003 showed that fluid flow as a result of strain acts to prevent NO production, and in doing so, cause a chain of events including osteocyte apoptosis, recruitment of osteoclasts followed by products of resorption stimulating the action of osteoblasts, thus replacing the compromised bone.

1.8.2 Microdamage, bone remodeling and "the Scissors Model"

The exact mechanism whereby bone remodeling is stimulated has been postulated to be several different mechanisms, all resulting from a repetitive, cyclic pattern of activity. Fluid flow, mechanical force and microdamage have all been implicated as stimulators of osteoclasts, and in turn, osteoblast activity. The principal mediator of bone remodeling is the osteocyte, which, as described, may play a mechanostimulatory role in bone remodeling, ie: it receives a stimulus and elicits a remodeling response. Osteocytes possess a multitude of cell processes which radiate from the cell body and infiltrate the canaliculi of bone, where there connect to the cell processes of other osteocytes. As described above, Frost first described the concept that cracking of the bone following mechanical impact may rupture cell processes, thus providing a stimulus for bone remodeling (Frost, 1960). Ehrlich *et al*, 2002, described how osteocytes respond to strain as a population of cells as opposed to individual cells, and so this "networking" of cells is a vital component in initiating a remodeling response. Further to this concept, a hypothesis within our group is based on the concept that compressive loading causes cell processes to be cut in a shear like mechanism, similar to that of being cut by a pair of scissors, essentially rupturing cell processes, and stimulating a response (Taylor *et al*, 2003, Taylor *et al*, 2002, Hazenberg et al, 2009). This so called "Scissors Model" has become central to the hypotheses of this group. Cracks in bone are often elliptical in shape and occur along the direction of the materials anisotropy, and cell process spanning the crack are subject to shear displacements, and these displacements, as thought within our group, cut the cell processes, as observed microscopically, like one would with a pair of scissors (Hazenberg *et al*, 2006). This action allows osteocytes to function in their mechanosensory role, estimating the size of the crack, and releasing RANKL and OPG, and stimulating subsequent bone remodeling (Hazenberg *et al* 2006, Cowin *et al*, 2002, Klein Nulend *et al*, 1995).

The mechanosensory and mechanostimulatory role of osteocytes has additionally been well characterised by determining the cellular response to fluid flow. Turner *et al*, 1994, suggested that osteocytes regulate targeted bone remodeling in response to interstitial fluid flow. Fluid flow can occur via load induced means, by fluid pressure and through shear stress (Cowin *et al*, 1991, Turner *et al*, 1994). The effects of fluid flow on osteocytes can cause an influx of calcium ions and ATP, the latter of which bind to ionotropic and metabotropic purinergic

receptors. This binding allows for the release of PG_2, which in turn binds to its EP receptors, facilitating increased bone formation. Pressure in the marrow cavity has been shown to stimulate the production of nitric oxide synthase, and so the release of nitric oxide, a known inhibitor of RANKL. This action increases OPG secretion and so functions in the accumulation of bone matrix (Robling *et al*, 2006, Klein-Nulend *et al*, 2005). The emission of nitric oxide has also been shown to potentially direct the BMU along the areas which have experienced most stress (Klein-Nulend *et al*, 2005).

As previously discussed, microdamage has also been implicated in initiating the bone remodeling process (Martin, 2001). Similarly to fluid flow, microdamage is a natural occurrence linked to mechanical loading and accumulates following day to day wear and tear, yet levels of microdamage increase proportionally to age, hormonal status, and degree of loading on a bone (Verborgt *et al*, 2000, Mori *et al*, 1993). Considering that remodeling is initiated in close proximity to microdamage, one could consider this to be a mechanism utilised by bone to repair itself (Martin 2001). Burr demonstrated that the prevalence of BMUs was 4-6 times greater following fatigue induced microdamage than non induced bone (Burr *et al*, 1993). Moreover, Bentolila (1998) demonstrated that in rats, which do not experience as great a frequency of bone remodeling, BMU activity increased significantly following fatigue induced microdamage. A decline in elastic modulus of bone, which results following fatigue loading has been associated with an elevation in microdamage accumulation (Prendergast and Taylor, 1994, Pattin *et al*, 1996, Schaffler *et al*, 1989). In addition to this, Lee *et al*, 2002, found consistency in the presence of secondary osteons and resorption cavities in regions where microdamage has occurred. Martin (2007) and Klein Nulend *et al* (2005) have considered that the presence of microcracks effectively directs BMUs to the point of insult, and initiate remodeling from that area in bone matrix. However, at present, there is little experimental data to prove the biochemical events involved in this hypothesis.

1.9 Osteoporosis and microdamage

Osteoporosis gains its name from the appearance it produces within bone, and originates from the Greek terms, osteo and poroso, for bone and porous, respectively. It is the most prevalent of bone diseases, is characterised by the reduction in bone density, and alteration of the

osseous microstructure, resulting in a low mineral density and deterioration of the structural microarchitecture of bone. This leads to an increase in the risk of fractures, and over the age of 60, occurs at a frequency of 1 in 3 women and 1 in 12 men (WHO, Kong *et al*, 2000). Given that human ages are increasing, in addition to increasing obesity and tendency for one to enjoy a sedentary lifestyle, the impact of osteoporosis is ever increasing (International osteoporosis foundation).

The international osteoporosis study group (2002), states that osteoporosis cost the economy just over 17 billion annually (United States), and this figure is constantly growing. In Ireland, the cost of treating osteoporosis sits at 10 million euro per annum. The physical manifestations of osteoporosis include pain in the lower back and presentation of a "dowagers hump", due to compression of the vertebrae (Lindsay *et al*, 2001). Furthermore, osteoporotic fractures are common, and even minor trauma can result in fracture, most commonly in the wrist and hip. The world health organisation has provided a physiological outline by which osteoporosis can be defined: Patients with osteoporosis present a bone mineral density (BMD) >2.5 standard deviations below the mean value of young, healthy individuals, which is usually defined as 833 mg/cm^2. This can be determined with DEXA or bone densitometry.

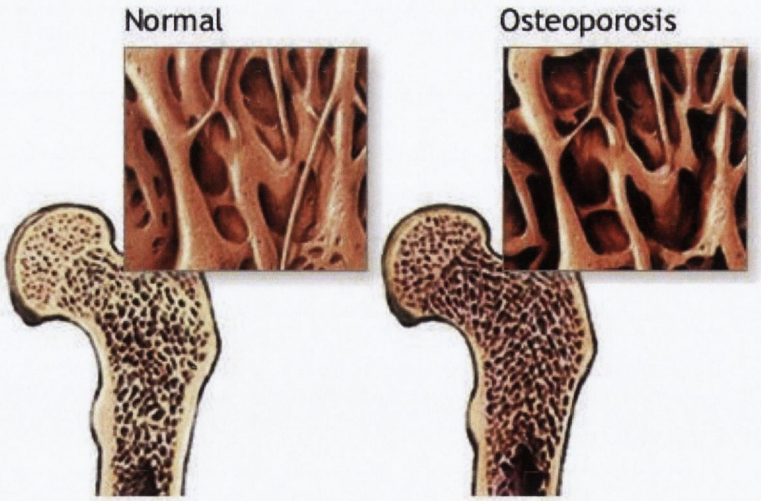


Fig. 1.10 Comparison between healthy bone and osteoporotic bone (http://www.medhelp.org).

1.9.1 Types and causes of Osteoporosis

Osteoporosis may be classified into two distinct forms, primary and secondary osteoporosis, based on whether they result from a physiological imbalance or due to medication, respectively. Primary osteoporosis can be further subdivided into Type I and II. Following menopause, estrogen levels in women decrease significantly, causing depletion of calcium levels from the skeleton, making the bone fragile due to loss of mineral content. This is Type I primary osteoporosis and results in fractures of the wrist and forearm, in addition to the hip and vertebrae. Type II osteoporosis is a result of the imbalance between bone resorption and formation, due to impaired cell signaling pathways, and is primarily characterised by increased femoral and spinal fractures. Secondary osteoporosis results from medications or a medical condition, such as steroids and diuretics, kidney disease, hyperthyroidism, coeliac disease, gastrointestinal disease, hypogonadism, and also alcoholism.

Osteoporosis occurs when there is a marked lack of homeostasis between the resorption of bone and formation of bone, where bone resorption by osteoclasts exceeds bone formation by osteoblasts. This is due to a lack of biochemical equilibrium, most principally in the RANKL-RANK-OPG signaling pathway. This loss of balance in cell signaling has been linked to a depletion or ablation of estrogen levels, most notably following surgical or spontaneous menopause. The presence of estrogen has been associated with providing longevity in the viability of osteocytes (Westbroek at al, 1998). Loss of estrogen increases the risk of osteocyte apoptosis, and affects osteocyte viability (Tomkinson *et al*, 1997, Tompkinson *et al*, 1998) thus contributing to fragility in bone (Qie *et al*, 1997). The region of apoptosis has been found to be localised around regions of microdamage, however, in certain instances, this can be considered a positive feature, facilitating bone remodeling (Verborgt *et al*, 2000). Loss of estrogen has been implicated in stimulating excessive bone loss. Kamada *et al*, 1997, demonstrated that during menopause, osteoclast activity increased. Estrogen functions in increasing OPG secretion, and thus, a lack of estrogen means a subsequent loss of OPG activity. Osteoblasts express α and β estrogen receptors and so failure to receive estrogen is implied in malfunctioning bone remodeling (Hofbauer *et al*, 2004). As a result of this, RANKL-RANK binding and subsequent osteoclastogenesis, can prevail, and OPG remains unable to bind to RANKL within the bone itself, thus promoting osteoclastogenesis and preventing any RANKL-OPG mediated bone

formation (Bucay *et al*, 1998). It was also suggested by Arko *et al,* 2002, that polymorphisms in the gene encoding OPG promoter region also contribute to osteoporosis.

The loss of balance in the regulatory feedback loop maintains osteoclast activity and allows subsequent bone resorption to prevail, resulting in bone being devoid of remineralisation, displaying a marked reduction in density and strength. Further to this, estrogen deficiency has been shown, by Turner *et al*, 1994, to increase the resorption capacity and potential of osteoclasts, facilitating the formation of excessively deep resorption pits, which, if osteoblast activity does occur, they may not be able to infiltrate the pits, and these remain unfilled, contributing to skeletal fragility. Osteoporosis has also been shown to display polymorphisms in the genes encoding collagen type I synthesis: COLIA 1 and COLIA 2, as well as mutations in the vitamin D receptor gene (Grant *et al*, 1996), which will ultimately affect the structural component of bone.

Lack of or reduced mobility, such as following illness, or in elderly individuals has been implicated in increasing osteoporosis. As previously described, bone is a highly dynamic, living material, capable of self renewal and remodeling, to remove compromised bone. This however, is a result of stimuli, such as microdamage, which result from normal movement, so a lack of activity reduced the incidences of damage, and thus, increases the risk of fracture. On the contrary, microdamage accumulation is one of the major contributors to fracture and breakages in osteoporosis due to impaired repair mechanism (Burr *et al*, 1998). Bone, following estrogen withdrawal, can become more sensitive to fracture (Bakker *et al*, 2005).

1.9.2 Treatment of Osteoporosis

The treatment of osteoporosis has shown to be widely successful, in spite of some controversy surrounding it. It has been shown that the chemical treatment of disease affecting bone remodeling may potentiate the pathogenesis of this disease. Treatments include hormone replacement therapy, selective estrogen receptor modulators, hormone treatment, "dietary supplements" and chemical analogues. The most commonly used treatment for both osteoporosis is bisphosphonates, which bind to the mineral surface of bone, normalise alkaline phosphate levels, and inhibit osteoclast function and, in doing so, inhibit bone resorption (Gonzalez *et al*, 1999). These will be discussed in greater detail in this section. Initially, the greatest way of

treating osteoporosis is to avoid acquiring the disease from the first instance, having a diet rich in calcium and vitamin D, and maintaining an exercise regime. Calcium is one of the principal mineral components in bone, and thus a diet rich in calcium is essential for preventing fracture and also in skeletal maintenance. It was reported by Bendich *et al,* 1999, that the supplementation of postmenopausal women's diets with calcium could reduce fracture risk by up to 50%. In addition to this, vitamin D has shown to increase calcium and phosphorous absorption by the body, and is paramount in maintaining mineral homeostasis in bone (Regnistar *et al*, 1995).

At onset of menopause, estrogen, among other hormone levels, is depleted, and this leads to a bone density loss of up to 30%. Treatment with hormone replacement therapy had become commonplace, to increase the presence of circulating estrogens, thus increasing the release of OPG, and therefore assisting in the cessation of excessive bone resorption. However, this treatment has become less popular due to an associated increase in the risk of cancer. Treatment with parathyroid hormone and its analogues, such as teripatatide, was also used as a common treatment for osteoporosis, due to its ability to regulate calcium and phosphorous homeostasis, in addition to increasing the body's potential to absorb vitamin D, thus maintaining a healthy skeleton. Teriparatide is a synthetic analogue of PTH and has been shown to increase bone mineral density and reduce the risk of fracture. Further to this, new generation estrogen like drugs, such as Tamoxifen, have become available on the market, termed, SERMs, or selective estrogen receptor modulators. These function in mimicking natural estrogens, and thus stimulate OPG release, thus sequestering RANKL and preventing the over-resorption of bone by osteoclasts. More recently, a treatment with a human monoclonal antibody, Denosumab, has shown to bind to and inhibit RANKL activity, thus preventing excessive bone resorption and osteoclastogenesis.

A common treatment of osteoporosis is bisphosphonate therapy, of which numerous types exist. Two principal forms of bisphosphonate exist, nitrogenous and non nitrogenous, however, and these drugs are synthetic analogues of inorganic pyrophosphate. All bisphosphonates share a common phosphate-carbon-phosphate (P-C-P) backbone. The phosphate-carbon-phosphate backbone of the bisphosphonate is responsible for facilitating the avid binding to hydroxyapatite crystals on bone surfaces, which has been observed principally at

the sites of bone remodeling (Rogers *et al*, 2003). In addition to this P-C-P backbone, two PO_3 side chains exist, which provide the characteristic name for the bisphosphonates, and a side chain, termed R2, which may constitute a hydroxyl group, and determines the chemical properties, mode of action and potency of the drug. Another side chain, termed R1, is responsible for the pharmacokinetic properties of the drug. The mode of action of bisphosphonates is based on reducing osteoclast activity and increasing the capacity of bone to become mineralised. This is based on the concept that osteoclasts resorb bisphosphonates, and this is followed by interference with farnesyl pyrophosphate synthase (FPPS), which is a key enzyme in the 3-hydroxy-3 methylglutaryl co enzyme A reductase pathway. This leads to reduced osteoclastic activity, increased osteoclast apoptosis, and reduced capacity to resorb bone, by disrupting the melavonic pathway (Russel *et al,* 2008, Gallacher *et al*, 1991). The reduced resorptive ability of osteoclasts is due again to inhibition of GTPases, at the ruffled border of osteoclasts, preventing attachment to bone (Sato *et al*, 1996). Regarding mineralisation of bone, it has been demonstrated, *in vitro*, that bisphosphonate treatment increases the rate of osteoblast differentiation, and has been shown to inhibit osteoclastogenesis (Reinholtz *et al,* 2000) As a result of this, bone becomes stronger and more resistant to fracture (Black *et al,* 1996, Resrud *et al,* 1997, Fleish *et al,* 1987, Harris *et al,* 1999, Boivin *et al,* 2002). The effects of bisphosphonate treatment on the cellular and biochemical events in bone remodeling is an aim of this thesis, and thus, further mechanisms of action of bisphosphonates will be described in more detail in chapter 4.

1.10 Stress Fractures and Fragility Fractures

Poorly functioning BMUs and an accumulation of microdamage has been shown to increase stress and fragility fractures, especially in runners, army recruits, and subjects with diseases such as osteoporosis, due to increased bone resorption. In normal, healthy individuals, as described above, bone can tolerate high levels of strain prior to failure (Pattin *et al*, 1996), however, in bone where there is an accumulation of microdamage, i.e. following constant mechanical impact, or where there is diminished structural integrity, stress and fragility fractures can occur. Homeostasis between the rate of microcrack propagation and microdamage repair is fundamental in maintaining structural integrity in bone (Taylor *et al*, 1997). In conditions whereby the structure of bone is compromised, and stress levels below the yield strength for a

static load for normal bone are present, mechanical failure of bone tissue can ensue. This allows for the formation and accumulation of microcracks, reducing elastic modulus, which poses serious consequences in terms of skeletal strength (Burr *et al*, 1998). An accumulation rate of cracks exceeding the rate of repair, such as what is observed in osteoporosis, can be categorised as a fragility fracture (Schaffler *et al,* 1995, Ziopus *et al,* 2001, Major *et al*, 1997). Stress fractures, on the other hand, are what occur with an accumulation of microdamage, such as what is observed in military recruits and athletes, whereby bone has not adapted to increased stress (Johnson *et al,* 1994, Milgrom *et al*, 1985, Daffner *et al*, 1992). Regardless of the rate of bone turnover, no difference has been observed in the rates of stress fractures in both men and women (Bennell *et al,* 1998). Both of these manifestations are marked by the increase in microcrack size to a more critically sized macrocrack, which ultimately leads to mechanical failure and fracture.

1.11 Aims of this thesis

It is already known that compressive loading, such as results from running, walking, and daily activities, causes microdamage to occur in bone. Such damage, as described in the theories developed by our research group, may disrupt cell processes in a way similar to that of cutting them like a pair of scissors, hence, the coining of the term "the Scissors Model". Rupture of the processes in such a manner may release a multitude of biochemical factors, which may be responsible for stimulating the BMU to initiate bone remodelling. This has allowed us to pose two simple questions; (i) How does bone detect microcracks? And (ii) on detection of these microcracks, how does it elicit a repair process? A large number of hypotheses have been posed to determine the route by which bone remodeling occurs. These include the aforementioned stress mechanical stimulus, fluid flow and microdamage. However, no substantial evidence exists to support any of these ideas. This brings us to the principal hypothesis of this study: that linear microinjury of a given size will stimulate the biochemical and cellular events surrounding the bone remodeling system. This study is focusing on microinjury as a principal stimulator of bone remodeling, and granted this; we put forth the following set of aims;

1. To characterise the biochemical signaling pathways involved in the detection of microcracks as a result of damage. Binding of RANKL to RANK elicits an osteoclastogenic response, thus facilitating the resorption of bone. Products of bone resorption then triggers OPG activity, the decoy receptor of RANKL, and binding of OPG to RANKL allows the generation of osteoblasts and the laying down of bone. Further to this, it has been thought that small cracks, in the order of a few microns, tend to go undetected and are overlooked by the BMU, until they increase and become of critical size. We aim to disclose whether this is a biochemical or physiological phenomenon, using damage applied to 3D osteocyte cell process networks by means of micro-needles, to determine RANKL and OPG activity, in addition to the effects of damage to cell viability and apoptosis. In addition to this, other researchers within our group are working on a theoretical model to determine RANKL and OPG activity as a function of damage size, in addition to the number of cell processes ruptured and their relationship to crack size in *ex vivo* samples.

2. A secondary aim of this thesis is to determine the cellular response to microinjury and microinjury induced osteoclastogenesis using our *in vitro* model of microdamage in bone, using the constitutive cells of the BMU. Given our hypothesis, that microinjury of a certain size may the various stages in the bone remodeling process, which include migration of osteoclasts towards damage; osteoclastogenesis; osteoblastogenesis and migration, and subsequently, mineralization of newly created bone, we aim to determine if there is a relationship between crack size and the rate and magnitude at which these stages occur. Conditioned media from microinjury to an osteocytic network, and also, microinjury induced osteoclastogenesis will be applied to stimulate bone remodeling activities of preosteoclasts and preosteoblasts, respectively, to provide further clarification of the role of microdamage size in bone remodeling.

3. The third aim of this thesis is to determine the biochemical and cellular events which occur in an *in vitro* model of microdamage in bone, to bisphosphonate treatment. As described, bisphosphonates are a common treatment of osteoporosis, and it has been established that osteoporotic bone exhibits a higher propensity to experience microcracks than healthy bone. Using our model, this chapter aims to determine the biochemical response, focused on RANKL and OPG signaling, following treatment with two commonly used bisphosphonates, alendronate and zolendronate. In addition to this, we aim to establish a cellular response, using the aforementioned constitutive cells of the BMU, and to illustrate how bisphosphonates determine the fate of osteoclast and osteoblast activity, in relation to microdamage, *in vitro*.

4. The final aim of this thesis is to establish if there is an alternative mechanism of stimulating bone remodeling, through assessment of numerous cytokine, chemokines, growth factors and soluble factors. It has been suggested, that following microdamage, there is a marked increase in factors such as IL-1, TNF-α, and NFκB, which are capable of stimulating osteoclastogenesis and osteoclastogenesis. Using the model of microinjury established within this thesis, the aim of this chapter is to elucidate the other biochemical factors involved on comparing microinjury to an osteocytic network to undamaged samples.

Chapter 2

RANKL and OPG activity is regulated by microinjury size in networks of osteocyte-like cells

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2.1 Introduction

As described in the introductory chapter, bone is a dynamic material which is continuously being remodelled through the action of the BMU, in which compromised bone is removed by osteoclasts and replaced with osteoblasts. It is thought that this is regulated following perturbation of osteocytes. Furthermore, a series of cellular crosstalk mechanisms and cell signaling cascades function in order to maintain bone homeostasis (Lacey *et al,* 1998, Simonet *et al,* 1997). RANKL, and its antagonist, OPG, are the principal biochemical factors involved in this process and are the central stimulators of differentiation, proliferation and inhibition of osteoclasts, and are pivotal in the bone remodeling process (Hayden *et al,* 1995, Zhao *et al,* 2006). RANKL is a type II transmembrane protein tumour necrosis factor homologue, released by the metalloprotesase, disintegrin TNFα converting enzyme (TACE) on the surface of osteoblasts, preosteoclasts and dendritic cells. It is the primary mediator in the activation and differentiation of preosteoclasts into osteoclasts, and so functions in stimulating and sustaining bone remodeling (Lum, *et al*, 1999, Yasuda *et al*, 1998). As described in chapter one, RANKL is released from osteocytes and binds to its receptor, RANK, on preosteoclasts, allowing them to differentiate into mature, fully functioning osteoclasts, and thus initiates a resorptive response. In turn, OPG is released from osteocytic cells on receipt of signals such as oestrogen, bone morphogenic proteins and transforming growth factor beta, which result from RANKL-RANK binding and subsequent bone resorption (Boyle *et al*, 2003). OPG binds to and sequesters RANKL, facilitating recruitment and activation of osteoblasts, and in doing so, stimulate the secretion of osteoid by osteoblasts, which subsequently becomes mineralised, forming new bone. This was discussed in greater detail in chapter 1.

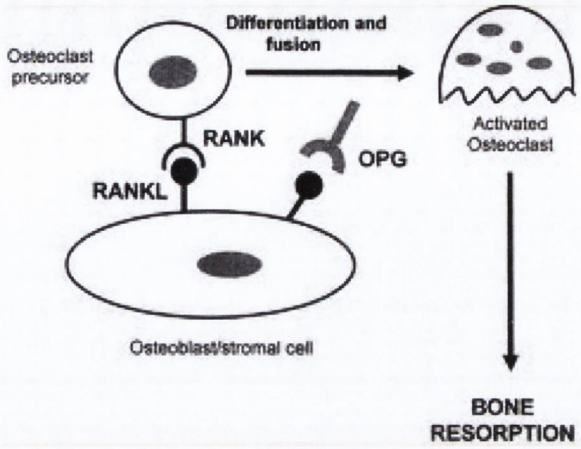


Fig. 2.1 The RANKL-RANK-OPG signaling pathway (Coetzee *et al,* 2004). On receipt of a stimulus, RANKL is released which binds to RANK, initiating an osteoclastogenic response. In turn, OPG is released, which acts are a decoy receptor for RANKL, stopping its activity and stimulating subsequent osteoblast activity.

Microdamage occurs naturally in bone as a result of fatigue and daily wear and tear, and an accumulation of damage can cause fragility fractures due to diminished structural integrity (Lacey *et al,* 1998, Simonet *et al,* 1997). Microdamage manifests itself as a series of cracks ranging from 100-400 μm in length in the case of linear microcracks, or can be a collection of small cracks known as diffuse microdamage. Linear microcracks tend to be ellipsoidal in morphology (Hayden *et al,* 1995, Zhao *et al,* 2006). Cracks originate mostly in the interstitial bone between osteons, on channels and lacunae, and their progression into fractures is frequently halted through the crack's collision with osteonal cement lines (Martin, 2003).

Based on this perception, it has been suggested that microdamage in bone is one of the principal mediators of bone remodeling and involved in the initiation of the BMU, which then functions in removing damaged or compromised bone surrounding the crack (Burr *et al,* 1985, Lee *et al,* 2002, Robling *et al,* 2006). This concept, first proposed by Frost (1960), was based on the idea that repair of bone following microdamage is fundamental in maintaining skeletal integrity. Frost proposed that cells, namely osteocytes, act essentially as mechanostats, and are an integral part of establishing bone strength, based around cellular detection of mechanical signals to trigger a response. The mechanosensory mechanism acts via stimulation of the cell through conversion of mechanical signals to biochemical signals (Klein-Nulend *et*

al, 1999). This then initiates bone remodeling through RANKL mediated activation of osteoclasts through the process of osteoclastogenesis, which in turn initiates a further cascade of events, resulting in OPG sequestration of RANKL, stimulating osteoblast activity and deposition of osteoid, which becomes mineralised, forming new bone.

The relationship between microdamage and RANKL-OPG activity has long been considered, however, the relationship between crack size and bone remodeling has yet to be determined. Following a stimulus, which may be fluid flow, cyclic compression or microdamage, a chain of events is initiated, starting with the release of prostaglandins, which mediate bone remodeling, an influx of calcium ions, cAMP, and increases RANKL activity. In turn, OPG release occurs inadvertently following microdamage, due to stimulation by the biproducts of bone resorption and bone remodeling (Hofbauer *et al,* 2004). Additionally, there is strong evidence to suggest that remodeling is targeted to regions containing microcracks implying that the osteocyte network is capable of detecting them (Burr *et al,* 1985). Furthermore, it has been found that the birth rate and longevity of the BMU is directly proportional to the frequency and intensity of microcracks and that mechanical stimulus is a major contributing factor to the dedifferentiation process of osteocytes into osteoblasts (Robling *et al,* 2006). As yet, the mechanism of detection is unclear, though some workers have suggested that this could be performed via the cells' mechanotransduction potential (Turner *et al,* 1994). An alternative mechanism has been proposed within this research group, by which cellular processes spanning the crack could be ruptured by a shearing mechanism similar to the action of a pair of scissors, which has been named "the Scissors Model", thus potentially releasing RANKL and initiating a cascade of events (Taylor *et al,* 2003, Hazenberg *et al,* 2006, Hazenberg *et al,* 2009). In this model, it has been thought that compressive loads, such as what would result from normal day to day activities such as walking and running, would cause cell processes to become subject to shear displacements, cutting processes, and releasing bone remodeling cytokines (Hazenberg *et al* 2005).

2.2 Aims of this study

The principal objective of this study was to determine the effect of microinjury on RANKL and OPG activity and to quantify this effect as a function of the severity of the damage applied. To understand the bone remodeling response to microdamage, a microenvironment using an osteocyte cell line (MLO-Y4) seeded in collagen-matrigel constructs will be developed. Planar defects applied to cellular constructs will facilitate the quantification of biochemical response by ELISA and luciferase assay. Additionally, it has been considered by Nobel *et al*, 2003, that microdamage may cause apoptosis of osteocytes, and more recently, it has been shown that microdamage causes an apoptotic response *in vivo* (Cardoso et al, 2009), thus, the effects of microinjury on osteocyte apoptosis within this model was also assessed

Considering this, the specific aims of this particular study were as follows:

1. To develop a 3D *in vitro* model to study the response of the osteocyte like cells, MLO-Y4, to microinjury of varying size.

2. To elucidate the signaling pathways between osteocytes following microinjury.

3. To assess the effect of microinjury on cell viability and apoptosis by means of caspase 3 & 7 activity.

2.3 Methods

2.3.1 Materials

Cell culture materials including α-modified essential medium (α MEM) , foetal bovine serum (FBS), calf serum (CS), penicillin/streptomycin, Dulbeccos phosphate buffered saline (1X), Trypsin/EDTA, Dulbeccos modified eagles medium (DMEM), 0.1 N Sodium Hydroxide solution, Trypan Blue stain, TRIS/EDTA, Neomycin, Kanamycin, Amplicllin, 1α,25-Dihydroxycholecalciferol (Vitamin D 3) and B-D Precisionglide 21 and 26 gauge syringe needles were all purchased from Sigma Aldrich Ireland Ltd, Airton Rd, Tallaght, Dublin 24. RANKL and OPG ELISA were purchased from R&D systems. Dual-Luciferase® Reporter (DLR™) Assay System and ApoTox-Glo™ Triplex Assay were purchased from Promega. OptiMEM, DAPI, Lipofectamine 2000, and phalloidin 488 were purchased from Invitrogen. Matrigel basement membrane matrix and rat tail collagen were purchased from BD Biosciences. RNeasy Mini prep and Maxi prep kits were purchased from Qiagen. Classic Plus acupuncture needles (160, 300, 400 and 800 μm diameters) were purchased from Harmony Medical Distributions Ltd, Leytonstone, London, United Kingdom. Cell culture plastic-ware was purchased from Sarstedt Ltd, Sinnottstown, Wexford, Ireland. MLO-Y4 cells were provided by Professor Lynda Bonewald, University of Missouri, Kansas, U.S.

2.3.2 MLO-Y4 cell culture

In this study, MLO-Y4 cells were used as an osteocyte cell line, which are a transgenic murine cells, (murine long bone osteocytes Y4), developed by Kato in 1997, by means of a SV-40 oncogene under the control of an osteocalcin promoter driving the large T antigen (Bonewald, 1999). Phenotypically similar to osteocytes in their stellate morphology, MLO-Y4 cells also produce large amounts of osteocalcin, and low amounts of alkaline phosphatase and type 1 collagen. On the other hand, MLO-Y4 cells lack mRNA for osteoblast specific factor 2 (perostin), and also express a K+ current, which has yet to be observed in osteocytes (Gu, 2001). Cells exhibit dendritic processes, positive for osteopontin, T- antigen, CD 44 and a major gap junction, connexin 43 (Kato *et al*, 1997). An attribute of MLO-Y4 cells is their ability to differentiate into osteocytes in the absence of vitamin D and other osteotropic factors, and express exceptionally high levels of osteocyte specific antigen, E11,

which localises only to osteocytes (Zhao, *et al*, 2002, Ahuja *et al*, 2003). Osteocyte like cells also bear a capability to establish numerous gap junctions composed of Cx43 when exposed to fluid flow, and show a massive increase in prostaglandin production, when compared to their osteocytic counterparts (Bonewald, 1999, Alford *et al*, 2003). MLO-Y4 cells were cultured on collagen coated flasks (0.15 mg/ml rat tail collagen type 1) in culture medium of α MEM supplemented with 5% FBS, 5% CS and 1% penicillin/ streptomycin. Cells were incubated at 37 °C at 5% CO_2. To observe cell morphology in 3D, and to characterise microinjury and broken processes, cells were stained with F actin specific phalloidin, to stain the cellular cytoskeleton, and 4',6-diamidino-2-phenylindole (DAPI), which binds avidly to the A-T rich regions to DNA, thus staining the nucleus. These fluorescent dyes produce green and blue fluorescence, respectively. Gels were fixed in formalin for 1 hour and stored in rinsed x3 with dPBS. Cells were incubated for 20 minutes in a 1:1000 solution of DAPI in dPBS followed by 3 further rinses with dPBS. This was followed by staining cells in a 1:800 solution of phalloidin for 20 minutes, followed by 3 x rinses with dPBS. Cells were imaged with and without microinjury using the Leica DM IL florescent imaging system.



Fig. 2.2. 20 X Image of MLO-Y4 cells in 2D culture. Note the stellate morphology of the cells, characteristic of osteocytes.

2.3.3 *In vitro* simulation of microinjury

2.3.3.1 Construction of hydrogels seeded with MLO-Y4 cells

In order to simulate a 3D *in vitro* bone micro-environment, MLO-Y4 cells were embedded in a rat tail collagen type I /Matrigel construct. Matrigel is an extract from a kidney carcinoma that facilitates formation of vessel like structures, in addition to maintaining the formation of cellular processes. Constructs were prepared by the addition of 1 volume of Matrigel basement membrane matrix to 1 volume of a collagen solution comprised of 58% Collagen, 26% 5x DMEM, 2.5 % FBS, 2.5% CS, 5% Sodium Hydroxide and 5% cell suspension. Such 3D culture ensures a greater propensity for cell process formation in osteocytic networks. The collagen / Matrigel constructs containing 1 x 10^6 cells/ml of gel were cultured in 24 well plates and incubated for 1 hour prior to addition of normal growth medium. They were cultured for 5 days prior to experimentation to facilitate cell process network formation.

2.3.3.2 Application of microinjury

Microdamage manifests itself as a series of cracks in bone and tend to be, on average, about 100 μm long, and may be up to 400 μm in length- cracks exceeding this size in transverse length are considered to be macro cracks. *In vivo*, it is postulated that small microcracks tend to go unrepaired until they grow to a critical size where it is postulated that they initiate a repair response. In order to apply microinjury to the gel embedded MLO-Y4 cells, gels were subjected to microinjury of a set length and width and variable thickness using acupuncture needles of four different diameters: 160, 300, 400 and 800 μm. A single planar defect was created in each culture well by inserting a needle vertically into the centre of the hydrogel (see **Fig.2.3**) and drawing it through at a right angle, creating a defect with dimensions of approximately 7 mm in length x 6 mm in depth. It is significant to note that the length and depth of the defect remained constant in these experiments, and the diameter of the needle was the only variable to consider. Negative controls were uninjured cells and hydrogels excluding cells.

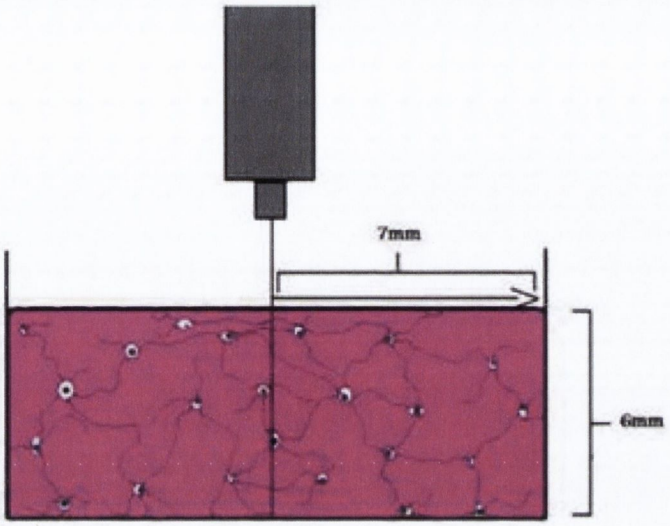


Fig. 2.3 Schematic of application of microinjury to gel embedded MLO-Y4 cells. Needles were inserted into the centre of the gel and a planar defect created by drawing the needle through at a right angle to the point of insertion.

2.3.3.3 Theoretical analysis of the injury model

Based on the above model, passage of the needle through the 3D cellular construct affects the cellular processes reflective of damage size i.e. the thickness of the needle, which is the main variant within these experiments. The cell density remains consistent throughout experiments, and is based on the volume of cells being 1 x 10^6 cells per ml, which results in 1000 cells per cubic mm of gel. This gives, in a homogenous distribution of cells, a spacing of approximately 100 µm between the centre of each cell. Thus, it may be assumed that injury sizes of less than 400 µm, would tend to rupture more cell processes, whereas a greater needle diameter would facilitate the movement of cells following passage of the needle through the construct. Each injury applied to the model maintains an approximate depth of 6mm and approximate length of 7mm, whereas width varies from 160-800 µm, which encompasses what would be small and standard sized micro crack *in vivo*, and also what may be considered failure. Considering this, damage applied would increase based on the volume of gel embedded osteocyte like cells within the construct, and the smaller the damage applied, fewer

cells would be affected and fewer cells may be "moved" from their *in situ* environment, whereas, at an injury size of 300 and 400 μm, which would be a more typical crack size observed *in vivo*, passage of the needle through the construct may affect a greater number of cells. Taking the variable values (injury size (needle width)), and the constant values (injury length and width of 7mm and 6mm, respectively), the total percentage of cells affected by the damage size can be calculated. Considering this is a volume model, the gel volume (mm^3) may be calculated by width (X) x height (h) x length (l), and from this, the number, and thus the percentage of cells affected, may be calculated. Taking the smallest injury size of 160 μm, which would be the lower limit of the experiments in this study, the total gel volume affected would be 6.72 mm^3 (0.16 mm x 7mm x 6 mm), and thus affecting 6720 cells (taking that there are 1000 cells per cubic mm of gel), and thus 0.672% of cells were affected. Using this calculation, at 300 and 400 μm, the percentage number of cells affected would equate to 1.26% and 1.68 % of cells, respectively, and thus a greater response would be expected to be observed.

When considering an exceptionally large damage size, what may be thought of as being "failure", an 800 μm injury to the gel embedded MLO-Y4 cells, the needle may ablate or displace any cells and gels as it moves through the passage, affecting 3.36% of the total cells. However, at this damage size, cells within the matrix may experience a different response due to cells potentially being displaced from their in situ environment. This may affect the biochemical and cellular processes which wouldn't normally ensue following microdamage and thus an alternative mechanism of response may be observed.

2.3.4 Effects of microinjury on RANKL and OPG secretion

Gels were incubated using OptiMEM serum free media. Samples were taken at 24 hour intervals over a 72 hour time period. RANKL and OPG were quantified by means of single site specific ELISA (Mouse RANKL and OPG ELISA) under manufacturers' instructions. 24 hour serum free media samples were removed for a total of 72 hours. Samples were centrifuged to remove particulates and used immediately or frozen at -20° C. Kit controls were reconstituted with 1.0 ml deionised water. A wash buffer was prepared by the addition of 25 ml wash buffer concentrate to 625 ml deionised water. Initially a standard curve was

constructed by reconstituting the mouse RANKL and OPG Standard with 2.0 ml of Calibrator Diluent RD5-3, producing a stock solution of 2000 pg/ml. 200 µl of calibrator diluent was added to an eppendorf tube and the stock solution used to create a 2-fold dilution series to create a range of concentrations from 31.2 pg/ml – 2000 pg/ml. Calibrator Diluent RD5-3 served as the zero standard (0 pg/ml). 50µl of calibrator diluent was added to each well of the ELISA plate, followed by 50 µl of standard or sample. The plate was tapped gently and incubated for 2 hours. Following this, each well was aspirated and rinsed 5 times with 400 µl of wash buffer. 100 µl of appropriate conjugate was added to each well and incubated for a further 2 hours. Following incubation, wells were rinsed as before. Equal volumes of colour reagent A and colour reagent B were mixed to form the substrate solution and 100 µl of this was added to the wells and incubated in darkness for 30 minutes. 100 µl of stop solution was then added to the substrate solution. The absorbance was read at 450nm and 570 nm using the Victor 3 Wallac 1420 multilabel centre and graphed accordingly. This was repeated using varying sized microinjury (160, 300, 400 and 800 µm). Gels were fixed in formalin for 1 hour and stored in dPBS for microscopic analysis. n=3 cultures were assayed at each microinjury level, in addition to non-injured controls.

2.3.5 Transfection of MLO-Y4 cells with RANKL promoter plasmid

A RANKL promoter reporter construct (clone name NR00int, 7244 base pair) was obtained from Dr Charles O' Brien, which contains a luciferase gene reporter for the activity of RANKL downstream of the RANKL promoter, as well as a neomycin resistance gene. The concept behind the use of a promoter construct is to perform a luciferase assay following application of microinjury to 3D cell culture. Expression of luciferase downstream of the RANKL promoter indicates that RANKL has also been produced in the cells. This then allows for quantification of RANKL promoter activity which correlates to RANKL production. The RANKL – luciferase plasmid was prepared in 50µl TRIS/EDTA. One Shot chemically competent Escherichia *coli* (E-*coli*) cells were transformed with 5 µl RANKL ligation reaction and grown overnight in 2ml Luria Bertani (LB) Agar containing 100 mg/ml kanamycin. Plasmid cultures were isolated and grown overnight in LB broth preceding plasmid purification using Qiagen Miniprep kit (under manufacturers instructions). A 250ml overnight culture of transformed cells was cultured under shaking conditions (225 rpm) and the plasmid

purified by means of Qiagen Maxiprep kit (under manufacturers instruction). Plasmid DNA was quantified by mean of a Mason Nanodrop 1000, and stored at – 80 °C.

2.3.6 Optimisation of transfection conditions with GFP plasmid

MLO-Y4 cells were grown at a density of 1 x 10^5 cells in a 24 well plate 24 hours prior to experimentation. Transfection was optimised by means of a green fluorescent protein (GFP) positive plasmid (see **Fig. 2.4**), and was conducted using a transfection agent, Lipofectamine 2000). 3 ratios of ug/ml plasmid: lipofectamine were used to establish the best means of transfection efficiency; 1:1, 1:2 and 2:1. Plasmid DNA (pDNA) and lipofectamine was diluted accordingly with an appropriate quantity of optiMEM (50 µl: 50 µl lipofectamine complex: plasmid complex) and applied to cells. Following 6 hours of incubation, the plasmid-lipofectamine solution was replaced with MLO-Y4 culture medium and incubated for 48 hours. Cells were analysed by means of fluorescent microscopy to establish the optimum transfection conditions and lipid/pDNA transfection ratios showing the greatest quantity of GFP positive cells.

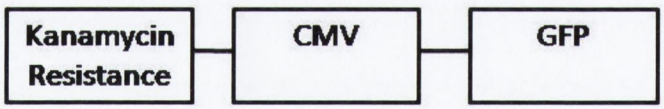


Fig 2.4. GFP plasmid map

2.3.7 Transfection of MLO-Y4 cells with RANKL promoter reporter plasmid

MLO-Y4 cells were grown at a density of 1 x 10^5 cells in a 24 well plate 24 hours prior to experimentation, and cells were transfected with a plasmid containing the reporter gene luciferase under the control of the RANKL promoter (see **Fig. 2.5**). Transfections were conducted using a 2:1 ratio RANKL promoter + luciferase plasmid and lipofectamine at a concentration of 1µg/µl plasmid. Plasmid DNA and lipofectamine was diluted accordingly with an appropriate quantity of optiMEM (50 µl: 50 µl lipofectamine complex: plasmid complex) and applied to cells. Following a 6 hour incubation, the plasmid-lipofectamine solution was replaced with MLO-Y4 culture medium and incubated for 48 hours. Luciferase

activities were measured using a luciferase reporter assay kit followed by quantification using spectroscopy. Culture medium was removed and cells were rinsed with dPBS. 400 µl lysis reagent was added to cells followed by a 15 minute incubation. The lysis buffer was placed in a tube and incubated briefly. 20 µl of cell lysate was then added to 100 µl luciferase assay reagent and the luminescence measured with the Victor 3 Wallac 1420 multilabel centre.



Fig 2.4. RANKL promoter reporter plasmid map

2.3.8 Effects of microinjury on RANKL activity

Collagen-Matrigel constructs were set up and microinjury applied as previously described. Constructs contained MLO-Y4 cells transfected with the plasmid containing the reporter gene luciferase under the control of the RANKL promoter. Negative controls were uninjured cells and hydrogels excluding cells. Aliquots were removed at 24 hour time points over a 72 hour period. A luciferase assay was performed under manufacturer's instructions (Dual-Luciferase® Reporter (DLR™) Assay System). Briefly, reagents were prepared as follows: 1 volume of 5X Passive Lysis Buffer (PLB) was added to 4 volumes of distilled water and mixed well. To prepare the LAR ll, the lyophilized luciferase assay substrate was resuspended in luciferase assay buffer ll. The stop and glo reagent was prepared by adding 2.1ml of 50X Stop & Glo® Substrate to 105ml of Stop & Glo® Buffer. Cells were lysed through removal of media from hydrogels and rinsing with 1x PBS. An appropriate quantity of passive lysis buffer was added to each well followed by a 15 minute incubation. The cell lysate was then placed into a tube. 20 µl of cell lysate was added to 100 µl LAR ll followed by 100 µl Stop & Glo reagent, and luminescence measured by spectroscopy to quantify release of RANKL with the Victor 3 Wallac 1420 multilabel centre to quantify release of RANKL. n=3 cultures were assayed at each microinjury level, in addition to non-injured controls.

2.3.9 Effects of microinjury on entire cell population

To determine the effect of microinjury on undamaged cells, conditioned media was added to intact gel embedded MLO-Y4 cells. The principal behind this experiment was to determine whether injured cells are responsible for emitting a signal to stimulate bone remodeling or whether the cells surrounding the area of compromised cell viability are capable of mediating a response. Collagen-Matrigel constructs were set up and microinjury applied as previously described. Constructs contained MLO-Y4 cells transfected with the plasmid containing the reporter gene luciferase under the control of the RANKL promoter. Conditioned media from previous experiments on non-transfected cells having received 400 μm microinjury at 48 (A) and 72 (B) hours was placed on undamaged matrices for 72 hours and the production of RANKL quantified by means of luciferase activity (determined by luciferase assay). n=3 cultures were assayed in each case.

2.3.10 Effects of microinjury on cell viability and apoptosis

To determine the effect of microinjury on cell viability and caspase 3 and 7 activity in our *in vitro* model, 100 μl of collagen/matrigel construct containing 1 x 10^6 cells/ml gels was cultured in a 96 well plate under conditions previously described. Microinjury was applied following a culture period of 72 hours, apoptosis and cell viability was determined using an ApoTox-Glo™ Assay, under manufacturers' instructions. This kit measures 2 independent biomarkers simultaneously to determine the effect of varying parameters on cell viability through measuring live/dead cell protease activity, in addition to caspase 3 and 7 activity for apoptosis. Briefly, media was removed from gels and incubated for 30 minutes in the viability/toxicity reagent at 37 °C. Fluorescence was measured at $400_{EX}/505_{EM}$ to determine cell viability. 100 μl of Caspase-Glo Reagent was then added to wells, and luminescence measured following a 30 minute incubation to determine the degree of caspase 3 and 7 activity, following application of microinjury.

2.3.11 Statistics

All data was analysed for significance ($p \leq 0.05$) using two way ANOVA, as a function of microinjury size and time, post hoc Tukey test, to compare means.

2.4 Results

2.4.1 Imaging of 3D MLO-Y4 cell process networks and the application of microinjury

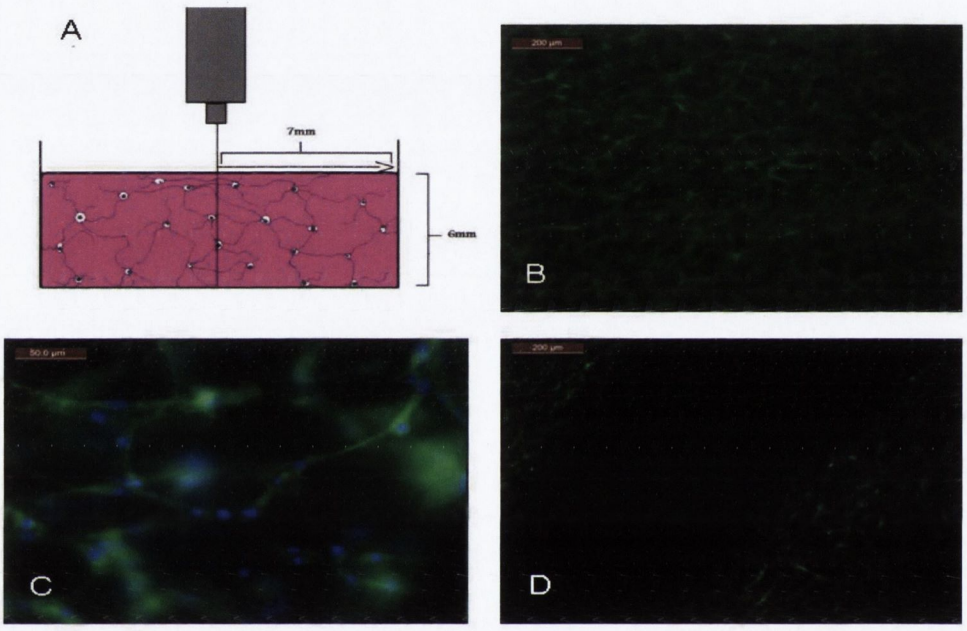


Fig. 2.6 Application of microinjury and staining of cell process networks of fluorescently labelled MLO-Y4 cells. A: Schematic diagram of application of microinjury **B:** Phalloidin stained gel embedded MLO-Y4 cells. Phalloidin is F actin specific, allowing imaging of the cytoskeleton of the cell. **C:** DAPI and phalloidin staining of gel embedded MLO-Y4 cells. DAPI stains the nuclei of the cells blue. **D:** Image of microinjury applied to phalloidin stained gel embedded MLO-Y4 cells

2.4.2 Microinjury affects RANKL and OPG activity in an injury size dependant manner

The effect of defect thickness on RANKL release was measured using ELISA. There was no significant difference in RANKL release between negative control samples with cells and 160 μm microinjury samples over the 72 hours; however, a significant increase is seen in the 300 and 400 μm microinjury samples ($p \leq 0.05$) as seen in **Fig. 2.7**. For the 800 μm damage samples, in contrast, a decrease in RANKL activity was observed compared to the 300 and 400 μm samples.

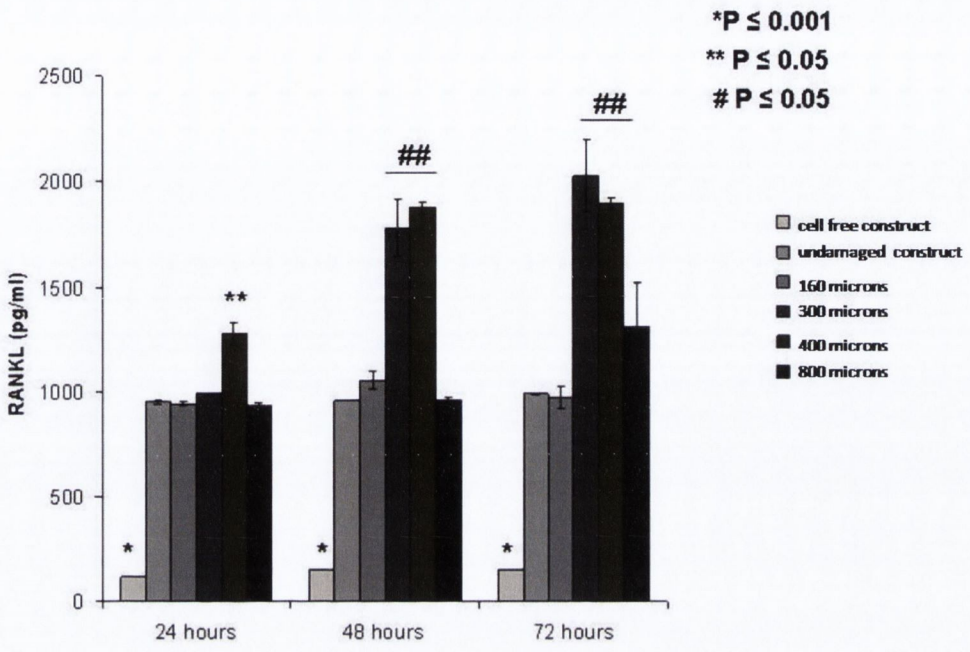


Fig. 2.7 Microinjury affects RANKL release in a size dependent manner. Results show RANKL release as quantified by ELISA. A significant increase ($P \leq 0.05$) in RANKL release was seen in the 400 μm samples when compared to controls and other microinjury samples at 24 hours. In the 48 and 72 hour groups, both 300 and 400 μm samples showed a significant increase in RANKL production when compared to other samples within the group ($P \leq 0.05$). * indicates $P \leq 0.001$ when comparing 24 negative control data to other data within the experimental group. **denotes $p \leq 0.05$ significance of 400 μm data to other samples at 24 hours, # denotes significant of 0.05 when comparing 300 and 400 μm samples to other groups at 48 and 72 hour time points. Error bars are indicative of standard deviation, n = 9.

Furthermore, the effects of defect thickness on OPG release again quantified by ELISA. OPG release found to be least apparent in 400 μm samples at all time points when compared to other defect sizes as seen in **Fig. 2.8**. OPG release then peaked at 48 hours for all samples, however, again for the 400 μm microinjury size, release was significantly lower. A significant decrease in OPG release was subsequently seen at 72 hours in 400 μm samples. For 800 μm, release was comparable to smaller sized injuries, and was significantly less than 400 μm injuries ($p \leq 0.05$).

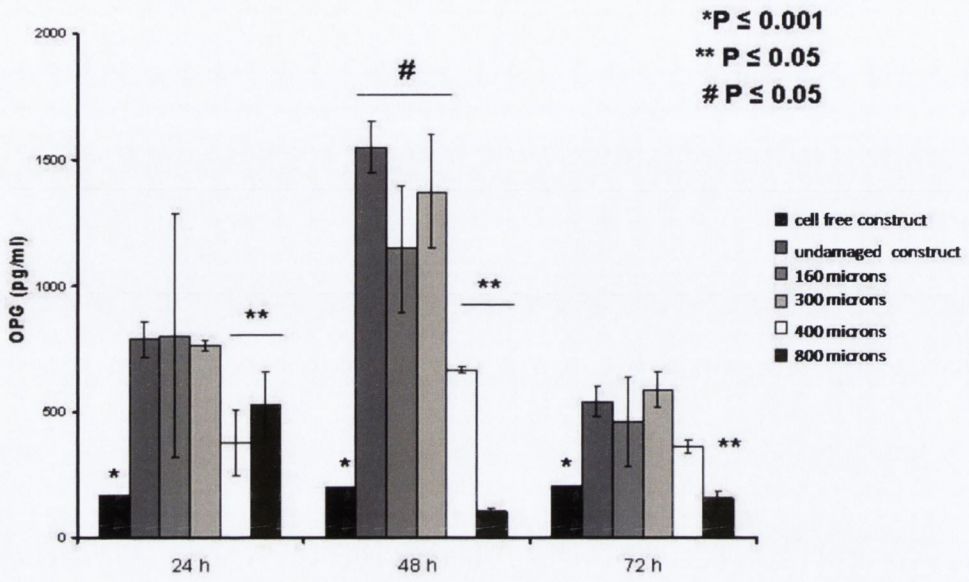


Fig. 2.8 Microinjury affects OPG release in a size dependent manner. Results show OPG release as quantified by ELISA. A significant increase ($P \leq 0.05$) in OPG release was initially seen in both control and injured sample. 48 hours post microinjury, a statistically significant increase in OPG is noted in all samples when compared to 24 hours, however, this decreases significantly in all samples at 72 hours ($P \leq 0.05$). * indicates $P \leq 0.001$ when comparing negative control data to all other data, ** denotes $p \leq 0.05$ when comparing significance within a group at a specific time point. # denotes significance to same microinjury size compared to 24 hours and 72 hours. Error bars are indicative of standard deviation, n = 9.

2.4.3 Optimisation of cell line expressing RANKL promoter plasmid

2.4.3.1 GFP plasmid- determination of optimum transfection conditions

A GFP reporter plasmid was used to optimise the transfection efficiency of MLO-Y4 cells. Cells exhibiting the greatest number of GFP positive cells following a 48 hour incubation were chosen and the ratio of plasmid to lipofectamine used for the RANKL promoter construct. This was found to be a 2:1 ratio (**Fig. 2.9**). In order to assess the effect of microinjury on the upregulation of RANKL activity in MLO-Y4 cells, 3D gel embedded cells were transfected as described. Samples were taken and analysed using a luciferase assay, in which a substrate, luciferin, is bound by luciferase, emitting luminescence which was measured using the Victor Wallac.

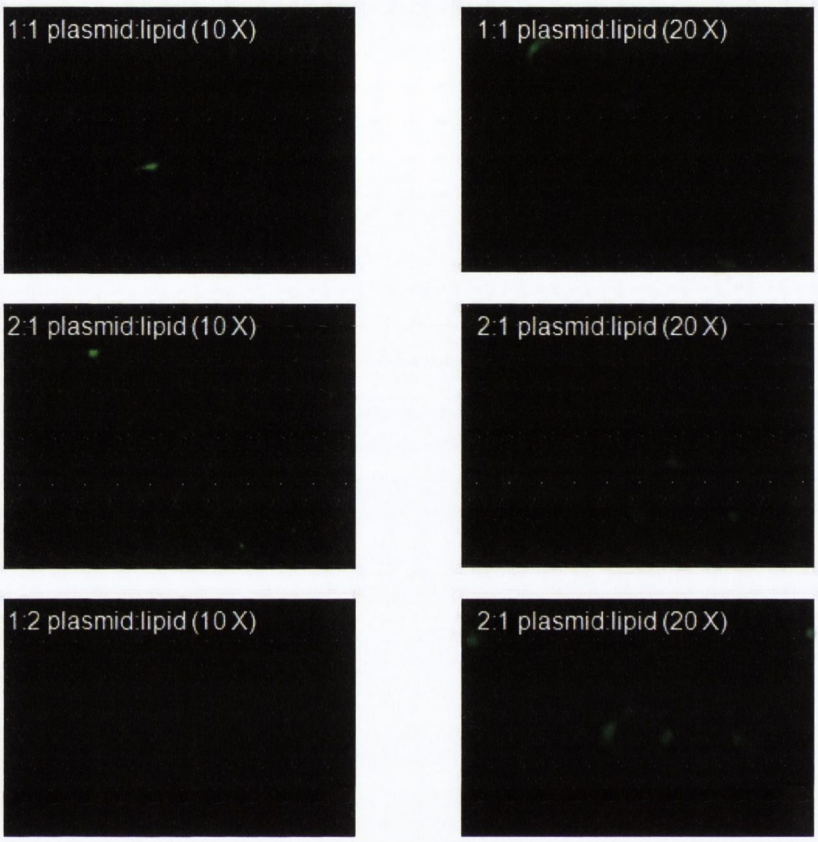


Fig. 2.9 Optimum transfection conditions for GFP plasmid in MLO-Y4 cells. These images demonstrate the ratios of GFP plasmid:lipofectamine required to optimise transfection efficiency. Images were taken 72 hours after transfection at 10X and 20X magnification. Samples exhibiting the greatest number of green fluorescent cells were chosen to be cells incubated under the greatest transfection efficiency, which was found to be a 2:1 ratio of plasmid: lipofectamine.

2.4.3.2 RANKL promoter activity increases 7 days following transfection

A RANKL promoter plasmid, NR00int, with the luciferase gene downstream of the RANKL promoter was transfected into MLO-Y4 cells using Lipofectamine 2000. **Fig. 2.10** depicts the release of RANKL from promoter cells when compared with the control, 7 days after transfection, and also the comparison between cells transiently expressing RANKL (after 48 hours) and cells expressing RANKL promoter in the genome. A marked increase in promoter activity was noted in both samples when compared to the control ($P \leq 0.05$).

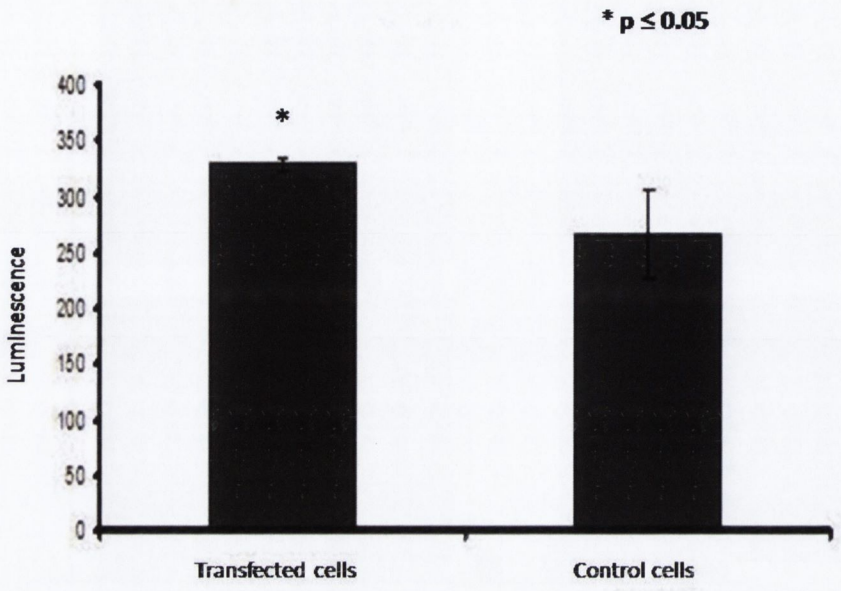


Fig. 2.10 RANKL promoter activity is increased 7 days after transfection. This graph demonstrates the results of a luciferase assay of 2D MLO-Y4 cells transfected with the RANKL promoter, 7 days following transfection and neomycin selection. Cells were transfected and incubated in neomycin enriched media, 48 hours following transfection. A significant increase in luciferase activity was noted after 7 days when compared to parental cells, indicating that an increase in reporter activity can be concluded. * denotes $p \leq 0.05$ when comparing transfected to parental cells. Error bars are indicative of standard deviation, n = 9.

2.4.4 An increase in microinjury size causes an increase in RANKL promoter activity

At 24 hours, promoter activity was seen to be greatest in 300 and 400 μm samples when compared to control 160 and 800 μm microinjury samples, with a significant increase being seen in the 400 μm sample as seen in **Fig. 2.11**. At 48 hours, a similar situation was noted, with the largest increase seen in 400 μm samples. At 72 hours, a highly significant increase in RANKL promoter activity occurred in both 300 and 400 μm samples. Again, the 800 μm microinjury size showed a decrease in RANKL production which is consistent with RANKL release. These results are broadly consistent with the ELISA results shown in **Fig. 2.7**, with regard to an increase in RANKL activity being most noteworthy at 400 μm and reduced at 160, 300 and 800 μm, however, a more stepwise increase in RANKL production was noted in the results of the luciferase assay, at the same time points, whereas RANKL release was more profound in the ELISA study. Overall, however, there results indicate the significant effects of microinjury on RANKL production and release.

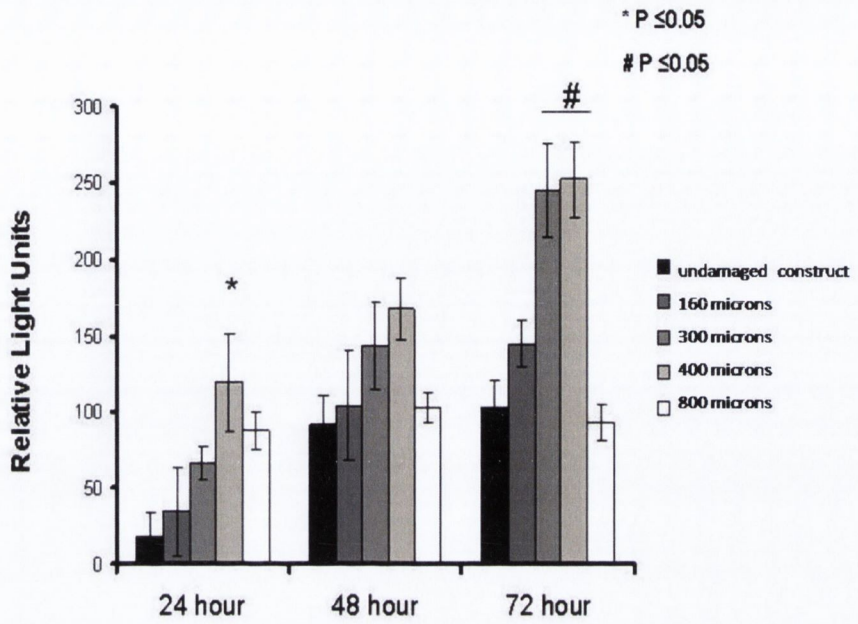


Fig. 2.11 Microinjury increase RANKL production in a size dependant manner. Results show the effect of varying size microinjury on RANKL production in gel embedded MLO-Y4 cells. Quantification of luciferase assay showed increase in RANKL activity over time in microinjury samples. Production of RANKL was found to be significantly highest in samples with greater damage (300 and 400 μm) at 72 hours ($P \leq 0.05$). * denotes $p \leq 0.05$ showing significance in increase in luciferase activity at 400 μm at 24 hours. # indicates $p \leq 0.05$, showing 300 and 400 μm at 72 hours being significantly greater than the same microinjury size at 24 and 48 hours. Error bars are indicative of standard deviation, n = 9.

2.4.5 Microinjury stimulates RANKL release in undamaged cells

To determine the effects of microinjury on undamaged cells, conditioned media from damaged cells was applied to transfected MLO-Y4 cells and the response quantified by luciferase assay. **Fig. 2.12** shows luciferase activity in populations of undamaged cell networks after application of conditioned media, i.e. media taken from injured networks in which the 400 μm defect had been created. The results indicted that after 72 hours, non injured cells are capable of emitting a signaling response to other cells to stimulate osteoclast recruitment, 72 hours following microinjury.

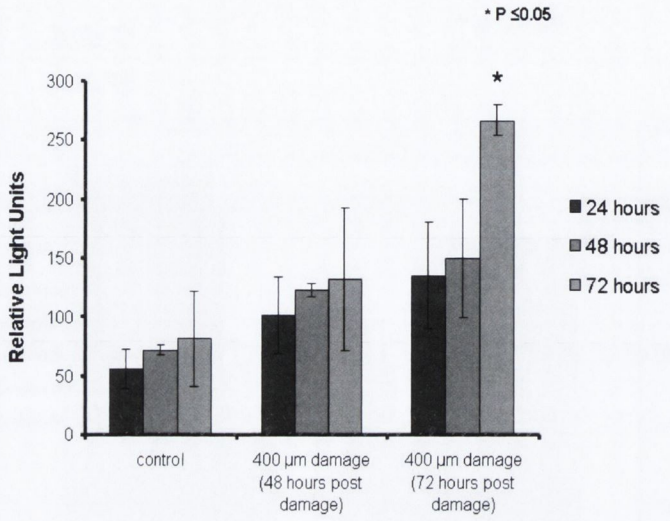


Fig. 2.12 Microinjury stimulates RANKL production in undamaged cells. Luciferase activity was used to quantify RANKL promoter activity 72 hours after microinjury on cells surrounding microinjury. Results showed that at 72 hours, a 2 fold increase was noted when compared to all other samples. * denotes $p \leq 0.05$ when comparing a significant increase in luciferase activity at 72 hours following microinjury and 72 hours after exposure to conditioned media.

2.4.6 Microinjury decreases cell viability and increases cellular apoptosis in an injury size dependent manner

The effect of microinjury on cell viability and caspase 3 and 7 activity is illustrated in **Fig. 2.13.** A significant decrease ($p \leq 0.01$) in cell viability was noted in 300 and 400 µm samples when compared to the control, 160 and 800 µm damaged samples. Caspase 3 and 7 activity was measured to determine whether cells were apoptotic following microinjury (**Fig. 2.14**). No significant difference was observed between control, 160, 300 and 800 µm samples; however, a significant increase in caspase activity was seen in 400 µm samples.

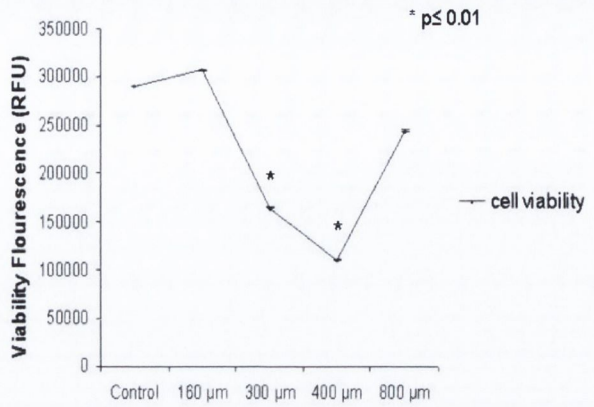


Fig. 2.13 Microinjury affects cells viability in a size dependent manner. A significant decrease ($p \leq 0.01$) in cell viability was noted in 300 and 400 µm samples when compared to the control, 160 and 800 µm damaged samples. No significant difference was observed in cytotoxicity in control, 160, 300 and 400 µm samples; however, a significant decrease (* $p \leq 0.05$) in viability was noted in 800 µm samples.

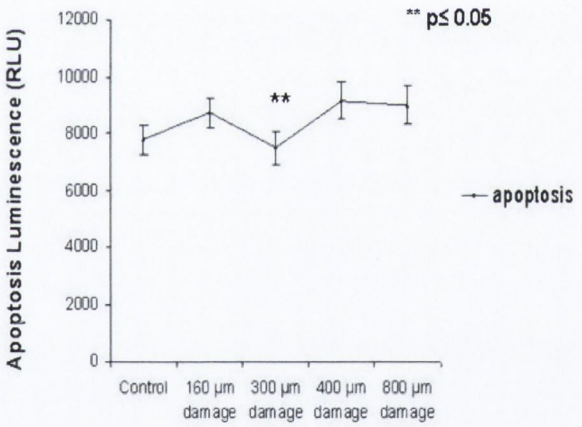


Fig. 2.14 Microinjury increases cellular apoptosis accordingly with increased damage size. No significant difference was observed between control, 160, 300 and 800 µm samples. A significant increase in caspase activity was seen in 400 µm samples when compared to the control, 160 and 300 µm samples. ** denotes $p \leq 0.01$ when comparing 400 µm sample to other samples.

2.5 Discussion

Microdamage, i.e. the accumulation of microcracks, has long been implicated as being one of the principal factors initiating the bone remodeling process (Burr *et al,* 1985). Microdamage is a natural occurrence which accumulates following day to day wear and tear, yet levels of microdamage increase proportionally with age, hormonal status, and degree of loading on a bone (Verborgt *et al,* 2000, Mori *et al,* 1993). Bone remodeling is initiated in close proximity to microdamage, and has been seen to be activated by microdamage, so it may be considered to be an important mechanism by which bone maintains its mechanical structure through an intricate repair process by the BMU (Martin *et al,* 2001). This particular study aimed to compare and contrast the effect of planar crack like defects, of specific but varying size, on the production of the osteoclastogenic cytokine, RANKL, and its antagonist, OPG.

It is significant to note that that in the microinjury experiments in this study; no other mechanical stimulus was used beyond a single microdamage event. Therefore, microinjury was the only moiety involved as a single stimulator of bone remodeling, as opposed to a cyclic application of the stimulus such as matrix strain or fluid flow, which occurs under normal physiological circumstances such as walking (Turner *et al,* 1994, Burger *et al,* 2003). However, it is important to note that fluid flow and cyclic compression can occur via load induced means, by fluid pressure and through shear stress (Turner *et al*, 1994). Additionally, the process of fluid flow and compression are as a result of similar stimuli, whereby the application of a load to a bone causes a change in the flow of fluid, which results from mechanical loading, thus this is detected by osteocytes (Klein Nulend *et al*, 1995). As described in chapter 1, it has been demonstrated that fluid flow, in particular, affects the release of numerous cytokines and growth factor necessary to stimulate a remodeling response osteoblasts (Lozupone *et al,* 1996, Takai *et al,* 2004). Furthermore, as discussed, microdamage has shown to effect fluid flow throughout bone by impairing the normal route through which fluid flows in bone (Tami *et al*, 2002). These three postulated mechanisms which are considered to the principal mediators of bone remodeling are found to be linked, and produce similar responses in terms of a biochemical response, however, in spite of the evidence in favour of each stimulus, the aim of this thesis is to determine the role of microinjury in the remodeling response in an *in vitro* simulation of microdamage in bone.

A microcrack may remain in the bone, and as previously described, can be repaired or may encounter an osteon which impedes its progression in the matrix. If left unrepaired, it may grow over time, so its effective repair requires that it continue to be detected by the surrounding cells so long as the defect remains. This refers us back to the "Scissors Model" for the detection of microdamage in bone. As described, compressive loads may cause' cell processes to rupture in a shear like mechanism, initiating the release of cytokines such as RANKL, to stimulate bone resorption via microdamage mediated osteoclastogenesis. This then stimulates the release of OPG, ceasing osteoclastogenesis and allowing for the maturation and functioning of osteoblasts and subsequent bone formation. Given this, it may be assumed that there is a need for, at certain critical defect sizes, a mechanism for the detection of a crack, to thus allow for a stimulus to initiate the repair of the crack.

In this study, cytokine release was found to be proportional to microinjury size, and an inverse relationship in cytokine release was observed (more RANKL and less OPG), the exception of this being the 800 μm microinjury, which showed relatively little effect. The effect of microinjury on RANKL and OPG was assessed by ELISA. Over a 72 hour time period, RANKL release remained relatively stable in control samples and 160 μm damage samples. However, at 300 μm, a two fold release of RANKL was observed from 48 hours. These results suggest that the larger the damage, the greater the release of RANKL, with a plateau effect being noted between 48 and 72 hours. Additionally, a damage of 800 μm shows a RANKL release comparable to that of 160 μm, suggesting 300-400 μm defects to be a critical microinjury size to release bone remodeling cytokines, and thus determine the rate of bone remodeling. Furthermore, the anomalous effect of the 800 μm damage may be indicative that either at this defect size, cells are being ablated from the hydrogels and thus cannot elicit a response, or may be involved in a different signaling pathway, considering the greater number of cells damaged, and thus, the signaling mechanism may be considered unnecessary, as the damage level is too high. This however is speculative, and therefore requires a greater detail of investigation. High levels of RANKL are indicative of bone resorption and microinjury at early stages, and increased OPG secretion is associated with bone deposition and maintenance of bone structure *in vivo* (Robling *et al,* 2006). The increasing effect is most likely due to the increased numbers of cells being affected - the cells were approximately 100 μm apart so the thicker needles would have encountered more cells and more processes. It has to be noted,

however, again that the damage caused in this experiment differs somewhat to what is speculated to be observed *in vivo*; our group hypothesises that cell processes are cut in a shearing mechanism, as discussed above, however, in this model we are somewhat more crudely disrupting the cells and respective network of cell processes. However, as will be discussed later on, we are observing an apoptotic response at various damage sizes, which has been observed in *ex vivo* samples of bone, where osteocytes around a region of microinjury have been shown to be apoptotic (Cardoso *et al*, 2009), thus the "end result" of our damage, compared to the *in vivo* scenario, is the same,

OPG release generally remains consistent in control samples and in samples below 300 μm. At 400 μm, release of OPG is significantly lower than what is seen in small sized injuries. This correlates with RANKL release being highest at 400 μm at all time points. Moreover, this may be suggestive of the binding and sequestering of RANKL by OPG and a lack of sufficient signals being released to trigger OPG production. This may be indicative of the greater amount of RANKL being released, the lower the amount of OPG released. Moreover, this may be suggestive of the binding and sequestering of RANKL by OPG and a lack of sufficient signals being released to trigger OPG production. Considering this, microinjury of less than 160 μm *in vivo* may cause the release of OPG to stop the action of RANKL, and to "lay down" and "repair" the microinjury (through osteoblast secretion of osteoid), as opposed to releasing RANKL prior to OPG to remove compromised tissue. However, it may be hypothesised that more OPG is noted in 160 μm samples, due to a larger amount of RANKL being released at 400 μm, thus increasing the propensity for OPG to fulfil its role and become bound to RANKL, and thus, less free OPG may be available for detection by ELISA. Recently, Kennedy *et al,* 2012 demonstrated that in ex vivo samples, unfatigued bone exhibited a greater expression of OPG, and increasing levels of fatigue resulted in a decrease in OPG activity, which is reflective of this result. A similar response was observed by Hazenberg, *et al*, 2009, whereby in osteocytic networks subjected to cyclic damage, in samples exposed to less damage, more OPG was released.

A different biochemical response was observed in 800 μm samples, and the release pattern of OPG differed in that less OPG was released. Based on a speculative assumption, this may be due to again, a different signaling pathway being initiated, or perhaps due to less

RANKL being released, which may interfere with subsequent OPG release. Furthermore, the reduction in OPG activity may also be due to the amount of cells damaged being reflective of what would be indicative of mechanical failure, and so, there would effectively be no need for the release of OPG to sequester RANKL or initiate an osteogenic response. As a whole, however, on observation of the results of this section of the study it may be reasonable to conclude that release of RANKL peaks at microinjury size between 300 and 400 μm with an inverse relationship being demonstrated with OPG, thus reinforcing the concept that microinjury size is critical in the initiating of bone remodeling. These results suggest that RANKL production from cells is determined by the size of microinjury. This result is consistent with the findings of Taylor *et al* 2002, which demonstrated that crack size is critical for repair and remodeling in bone. Taylor *et al,* 2003 described that cracks with a length of less than 30 μm were too small to elicit a biochemical response; however, larger cracks of up to 300 μm in length may also be overlooked by the BMU. However, it is noteworthy to establish that these studies were focused on altering crack length as opposed to variations in defect thickness, and the use of this model has found to be comparable to what occurs *in vivo*, in terms of magnitude of damage and the fate of cellular viability, as will be discussed further on in this chapter.

Additionally, examination of the effect of microinjury on a non-injured cell population has allowed for clarification as to whether the cells, having undergone process rupture as a result of microinjury, stimulate a signaling response to damage from surrounding cells. Treatment of cells with conditioned media, which contains RANKL, among other biochemical factors, which may include NFκB, IL-1, IL-6 and PGE_2, in addition to matrix metalloproteases capable of initiating RANKL cleavage, harbours the potential for undamaged cells to release greater quantities of RANKL than what normally would be release at a baseline level. This may have shown that, in spite of an injured cells' impaired viability, microinjury may still be able to be repaired by a BMU through the recruitment of a neighbouring cell response. This result has also provided a background for a future study in this thesis, whereby the biochemical factors present in conditioned media will be elucidated. Furthermore, assessment of cell viability and apoptosis within our model has provided data to further elucidate some of the cellular responses to microinjury. The results have shown that cell viability remains unaffected in control and small defect sizes; however, a significant decrease in cell viability occurs at 300

and 400 μm defect sizes, only to then return to normal levels at 800 μm. Regarding apoptosis levels, Caspase 3 and 7 activities remained consistent in control and 160 μm defects, which then significantly increases at 400 μm. These suggests that perhaps, at 160 μm, cells remain unaffected by the damage applied, or perhaps are undergoing delayed apoptosis, and may require a greater amount of time for cells to become apoptotic, whereas at 300 and 400 μm, damage is sufficient enough to instigate a more instantaneous apoptotic response. At 800 μm, however, results were comparable to that of 400 μm, suggesting that cells are indeed undergoing apoptosis; however, the magnitude of the reaction is not affected by a vast increase in damage size. This may be due to ablation of cells following the passing of the needle through the osteocytic network, thus providing no variation in an increase of decrease of osteocyte apoptosis. These results are consistent with the increased release of RANKL and decrease in OPG release at 72 hours in 300 and 400 μm, and furthermore demonstrate the similar behaviour of cells to large and small damage size (160 & 800 μm). Furthermore, the consistency of the rate of cellular apoptosis between 400 and 800 μm is suggestive that the damage caused in both instances influence the fate of cell viability, i.e. cells are apoptotic, however, it may indicate that, indeed, a difference signaling response is initiated at both damage sizes. Additionally, these results are reflective of the findings of Cardoso *et al*, 2009 and Noble *et al,* 2002, whereby microdamage has shown to increase incidences of apoptosis, and subsequent bone remodeling. The cumulative results of the apoptosis study and also considering that microinjury affects uninjured cells, eliciting a response, further verifies the findings of Verborgt et al, 2000, that in *ex vivo* samples, microdamage stimulates apoptosis at the site and also in cells within 100 μm of the site of the microcrack. This finding further illustrates the fine line between microdamage and what may be considered failure, in an *in vitro* model of microdamage to an osteocytic network.

This work is consistent with many previous findings which have linked microdamage to remodeling (Burr *et al,* 1985, Robling *et al,* 2006) and which have suggested that individual microcracks can be detected by the surrounding cells which can signal to initiate the repair response, more so than mechanical stimulation and fluid flow (Martin *et al,* 1992, Taylor *et al,* 1995). The results obtained in this study are indicative that microdamage alone, in the absence of cyclic strain or fluid flow, can play a role in the release of RANKL and OPG, thus are

important factors in the repair of microcracks in bone. Considering the quantitative results of both the ELISA and luciferase assay as a whole, whereby RANKL release and production were assessed, respectively, it is evident that RANKL activity is governed by damage size, however, release is more instantaneous and more so determined by damage size –i.e. at 400 μm, release was greatest at all time points, whereas RANKL production occurred at a more steady state, albeit again, production was found to occur as a function of damage size. Membrane bound RANKL is released from following cleavage via disintegrin, a metalloprotease, on the cell surface, and so any stimulation or alteration in the cell's environment may be responsible for inducing the cleavage and subsequent release of soluble RANKL (Lum *et al,* 1999). As previously described, OPG release is preceded by RANKL, and so, the release of RANKL is usually followed by OPG, which acts as a decoy receptor for RANKL, blocking its function. These are the basis for the bone remodeling system. However, the use of a promoter construct within the genome of the cells allows for quantification of RANKL production in the cell. These results correlate with the concepts of Hazenberg *et al* 2006, among previous studies, which suggested that RANKL and OPG release, and ultimately, bone remodeling, was microdamage size dependant (Hazenberg *et al,* 2006, Kurata *et al,* 2006), and indeed that microdamage manifests its effects through cellular disruption (Hazenberg *et al,* 2006).

It may also prove necessary to consider that the findings of this chapter, in terms of the inverse relationship between RANKL and OPG, and the levels of apoptosis, may be reflective of the studies of Cardoso *et al,* 2009, whereby osteocyte apoptosis is a stimulator of bone remodeling. Further to this, it has been found that RANKL levels tend to be lower in regions of apoptotic cells, yet tend to be released from cells surrounding apoptotic regions (Kennedy *et al,* 2011). Hence, the results of this chapter, as a whole, demonstrate that, our model bears a similarity to those of other research groups, whereby *ex vivo* samples are utilised to determine the relationship between microdamage and biochemical responses, cellular apoptosis and subsequent bone remodeling, also cell signaling from un-injured cells. This chapter may effectively mimic the *in vivo* scenario and provide data supporting these other findings. The net results of these studies, including those of this chapter, pose the question as to whether it is cell process rupture that causes apoptosis, or whether cellular disruption causes release of RANKL and subsequent bone remodeling. The results in this

study have shown that microinjury causes a greater release of RANKL than a combination of factors, and so, it may be reasonable to consider that microdamage plays a greater role in the repair of microcracks.

The principal limitation to this study is that in our model crack size is significantly larger than what would be observed *in vivo*, despite a similarity in shape, and so it is reasonable to assume that a greater number of cell processes and cells as a whole were damaged. However, to achieve a greater understanding, it is necessary to consider that there are some differences in morphology between the MLO-Y4 cells in our experiments and real osteocytes in bone. We measured an average of 13 processes per cell, whereas there are typically 50 processes per osteocyte *in vivo*. Furthermore, in this experiment, the average distance between cells, with 1 x 10^6 cells per ml gel, is 100 μm. *In vivo*, the density of osteocytes is 12,000-20,000 per mm^2 (Martin *et al* 1989), giving a spacing of 37 μm between cells. One can estimate the number of processes which will cross a crack of given size, both in our experiment and in bone. This calculation shows that number of processes spanning the crack in our experiment, 10,833, would be the same as the number spanning a crack in bone of transverse length 500 μm. This would be at the upper end of the lengths of cracks which actually exist: a more typical *in vivo* crack length is 100 μm. It is also significant to note, as described above, that the width and length of the defect in this model remains constant, and the diameter of the needle, and thus the defect thickness is the only variant, and thus would affect the amount of cells being disrupted in the matrix, which may be reflective of observing varying crack sizes *in vivo*. This, of course, would bear an impact on the number of cells being disrupted by the passing of the needle throughout the network, however, following the calculations, which consider defect length, depth and thickness, it can be deduced that although the crack-like damage in the experiment is physically much larger, the actual number of cells and processes being affected is quite similar to that for microcracks in bone *in vivo*.

2.6 Conclusion

In conclusion, this study has demonstrated that microinjury size may be critical in affecting the instigation of bone remodeling through release of biochemical factors and that the subtle difference between damage and failure may have implications in bone repair. This chapter has shown that RANKL activity and OPG secretion is dependent on crack size and the greater the size, the more RANKL produced and the smaller the microinjury, the greater amount of OPG is produced. However, a different pattern is observed in excess of 400 μm, whereby RANKL and OPG activity is markedly reduced. This is suggestive of the initiation of a different signaling pathway, perhaps indicative of what occurs when the number of cells damaged correlates with what may be observed when bone reaches mechanical failure, *in vivo.* Further to these findings, this study has amply demonstrated that microinjury to an osteocytic network is capable of instigating a systemic response, allowing other, undamaged cells to release RANKL and stimulate a remodeling response. Additionally, it has been confirmed that cell viability is compromised in accordance with microinjury size, in that cell viability is reduced in accordance with an increase in damage size. Additionally, cellular apoptosis, which has been shown to be initiated *in vivo* following microdamage, has shown to increase with damage size, in terms of caspase 3 and 7 activities. In spite of the fact that the damage caused using this model is not entirely analogous to what is observed *in vivo* (i.e. cell networks were disrupted more so than ruptured by a shearing mechanism, this study has demonstrated that damage size to an osteocytic network, similar to what is observed *in vivo,* may be critical in initiating a remodeling response. From this chapter, a platform for the future study to investigate the effect of damage size to an osteocytic network on the bone remodeling activities of constitutive cells of the BMU, has been created. In summary, this study shows that microinjury may be a principal mediator of bone remodeling through its effect on RANKL release and may confirm the reason as to why small cracks are left unrepaired in bone *in vivo.*

Chapter 3

Osteoclastogenesis and Osteogenesis is stimulated by cell process rupture in a microinjury-size dependant manner using an *in vitro* simulation of microdamage in bone

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3.1 Introduction

As described in previous chapters, microcracks can occur in bone following daily wear and tear, leading to fatigue damage. An accumulation of damage can cause fragility fractures due to diminished structural integrity (Frost, 1960, Burr, 1997). It has been suggested that microdamage, among other factors such a fluid flow and mechanical force, is responsible for stimulating the bone remodeling phenomenon, and in the previous chapter, it was demonstrated that microdamage, of certain size, is capable of initiating the release of bone remodeling cytokines in a manner which coincides with microdamage size (Martin and Burr, 1982, Prendergast and Taylor, 1994, Burr *et al,* 1985, Lee *et al,* 2002).

Bone turnover and remodeling is controlled through a series of cells, cell signaling, and by the BMU, as illustrated in **Fig.1.7**, chapter 1. This occurs in cycles of bone resorption and remodeling, into which osteoclasts and osteoblasts are organised. Following RANKL ligation to RANK, osteoclast precursors fuse to become large, multinucleated bone resorbing cells, attaching to bone via a β3 integrin receptor, which creates a microcompartment between the ruffled membrane of the cell and the bone surface. These specialised cells function by secreting lytic enzymes and H+ ions capable of dissolving extracellular matrices, followed by protease degradation of the organic matrix, in response to RANKL, a cytokine responsible for osteoclastogenesis (Boyle, *et al,* 2003). The resulting cavity remaining after osteoclast mediated bone resorption is the Howship's lacuna, which can be observed histologically in samples demonstrating a BMU. Osteoclasts and osteoclast activity is marked principally by the presence of Tartrate Resistant Acid Phosphatase (TRAP), which is a lysosomal enzyme involved in the breakdown of bone, as described in chapter 1 (Eyre *et al,* 1987).

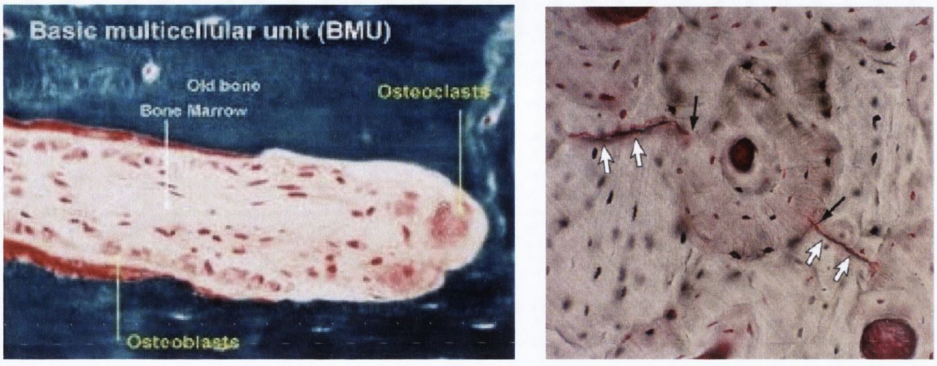


Fig 3.1. A: Image of a Basic Multicellular Unit (BMU) in action. The BMU is characterised by a band of large, multinucleated osteoclasts preceding a group of bone forming osteoblasts. B: Fuschin stained microcrack in bone, microcracks are maintained within the interstitial bone, and the crack is impeded from progression by the osteonal cement line (Martin, 2000).

Following this group of cells are the osteoblasts, which are preceded by a band of evolving mononuclear cells and precursor cells, and function in the deposition of osteoid, or new bone matrix (Eriksen *et al,* 1995). Osteoblasts, along with other mitogenic factors, are responsible for the production of new bone tissue though the setting down of unmineralised matrix in response to the RANKL-RANK-OPG signaling cascade. The expression of transcription factors such as runt related transcription factor 2 (Runx2), distal-less homobox, and msh homobox homologue 2, are responsible for mediating the differentiation of osteoblasts from a mesenchymal lineage (Ducy *et al,* 1997). At this stage, high levels of collagen type 1, and bone sialoprotein are expressed by immature osteoblasts. Osteoblasts are principally controlled by BMPs, of which several types exist. BMPs are responsible for the phosphorylation of Smad proteins, the action of which instigates osteoblastic differentiation (Massague *et al,* 2000). Osteoblast differentiation is controlled moreover by the transcription factor Osterix (Osx), which encourages cellular differentiation, as well as Runx2. This can furthermore be phosphorylated into mitogen activated protein kinase (MAPK) and elevates levels of BMPs and facilitates the production of extracellular matrix (Nakashima *et al,* 2002, Otto *et al,* 1997).

An increased frequency in cellular apoptosis has been observed in instances relating to increased microdamage (Brennan *et al,* 2011). Following microdamage, as observed in *ex*

vivo samples, cell processes become ruptured, and osteocytic cells undergo apoptosis, and this has been shown to be vital in instigating a remodeling response (Verborgt *et al,* 2000, Cardoso *et al,* 2009). Initially, MCS-F is released, most principally from endothelial cells, and its expressing may be mediated by TNF α, and thereby it functions in the p13/Akt pathway, and MAPK/ERK pathway, to assist in the maintenance of cell viability and to stimulate proliferation. RANKL is subsequently released via the metalloproteinase, disintegrin, on the cell surface membrane of osteocytes, under the regulatory control of TGF-β1 and this binds to the RANK receptor on preosteoclasts, promoting osteoclastogenesis and bone resorption (Yan *et al,* 2001). A signaling cascade ensues, which recruits a myriad of key osteoclastogenic proteins, including TRAFs, MEKK 1 and 2, which is activated upstream of RANKL, by TRAF2, which in turn, activates JNK and MEK1 mediated sSCC and ERK activity. This, in turn, upregulates TRAF6, thus stimulating NFκ B activation (Darnay *et al,* 1998, Wong *et al,* 1998). This, requires the expression of various genes, including AP-1 member, c-fos, micropthamia-associated transcription factor (MITF), nuclear factor of activate T cells, calcineurin dependant 1 (Matsuo *et al,* 2004, Wang *et al,* 1992, Partington *et al,* 2004). These facilitate the differentiation and maturation of preosteoclasts, and RANKL regulates the longevity of their survival. In addition to this, osteoclastogenesis has been shown to be promoted by TNF α, and this occurs via a Type 1, or p55 receptor, and can act synergistically with RANKL, with each complementing the stimulatory potential of the other (Abu-Amer *et al*, 2000). The reaction stimulates the release of OPG, which binds to and sequesters RANKL, stimulating osteoblast activation and mineralization of the tissue.

3.2 Aims of this study

Much debate exists regarding the role which microdamage plays in bone remodeling, and so, it could be reasonable to suggest the hypothesis that bone remodeling, as a separate entity, is regulated by microdamage size. Microdamage, as described in the introductory chapter, has been shown to be a principal stimulator of bone remodeling (Burr *et al,* 1985). However, whether microdamage itself causes osteoclastogenesis, and in turn, osteoblast activity, is not clear. It was previously demonstrated in Chapter 2 that cell process damage to the osteocyte like cell line, MLO-Y4, releases RANKL and OPG in an microinjury size dependent manner, suggesting that the degree of bone remodeling is also microinjury size dependent (Mulcahy *et al,* 2011), yet, its effect on the constitutive cells of the BMU has yet to be elucidated. Based on the finding that RANKL and OPG activity is microinjury size dependant, it could be reasonable to suggest the hypothesis that the principal steps in bone remodeling, in terms of cellular activity, are regulated by microinjury size.

Considering this, the aims of the present study were:

1: To determine the rate of progenitor cell infiltration towards a stimulus i.e. microinjury of varying size and respective microinjury induced osteoclastogenesis.

2: To develop an *in vitro* experiment determining the cellular events surrounding bone remodeling using the constitutive cells of the BMU and relate the levels of osteoclastogenesis and osteoblast activity to microinjury size.

3: To determine how linear microinjury of a varying size, stimulates osteoclastogenesis; osteoblastogenesis and migration, and subsequently, mineralization, using conditioned media, i.e. spent media from microinjury to an osteocyte cell process network and microinjury induced osteoclastogenesis, which contains cytokines and growth factors which is hypothesised to elicit a cellular response.

3.3 Methods

3.3.1 Materials

Cell culture materials including α-modified essential medium (α MEM) , foetal bovine serum (FBS), calf serum (CS), penicillin/streptomycin, Dulbeccos phosphate buffered saline (1X), Trypsin/EDTA, Dulbeccos modified eagles medium (DMEM), 0.1 N Sodium Hydroxide solution, Trypan Blue stain, β-glycerophosphate, ascorbic acid, dexamethasone, Cetylpyridium Chloride, Leukocyte acid phosphatase kit, Alizarin Red staining, Silver Nitrate, and B-D Precisionglide 21 and 26 gauge syringe needles were all purchased from Sigma Aldrich Ireland Ltd, Airton Rd, Tallaght, Dublin 24. OptiMEM reduced serum media was purchased from Gibco. Matrigel basement membrane matrix and rat tail collagen were purchased from BD Biosciences. Classic Plus acupuncture needles (160, 300 and 400μm diameters) were purchased from Harmony Medical Distributions Ltd, Leytonstone, London, United Kingdom. Cell culture plastic-ware was purchased from Sarstedt Ltd, Sinnottstown, Wexford, Ireland. MLO-Y4 cells were provided by Professor Lynda Bonewald, University of Missouri, Kansas, U.S. Raw 264.7 cells and MC3T3 cells were purchased from ATCC. Recombinant RANKL was purchased from R&D systems. 8-μm-pore hanging cell culture inserts were purchased from Millipore, Little Island, Cork, Ireland. PnPP and OptiMEM were purchased from Invitrogen. Calcium Liquicolour Kit was purchased from StanBio Laboratories.

3.3.2 Cell culture - Raw 267.7 cells and MC3T3-E1 cells

MLO-Y4 cells were maintained in α Modified Eagles medium (Biosera) supplemented with 5% foetal bovine serum, 5% iron supplemented calf serum and 1% antibiotics (penicillin/ streptomycin). Cells were cultured in collagen coated flasks (0.15 mg/mL rat tail collagen type 1) at 37 °C at 5% CO_2. Preosteoclasts (Raw 264.7 cells) were cultured in high glucose Dulbecco's Modified Eagles Medium supplemented with 10% heat inactivated foetal bovine serum and 1% antibiotics (penicillin/ streptomycin). Raw 264.7 cells

are monocytic cells of a hematopoetic lineage. Cells were established from tumour ascites induced in a male mouse by induced pluripotent injection of Abselon Leukemia Virus. Raw 264.7 cells can become readily differentiated into osteoclastic cells, exhibiting a multinucleated morphology and high TRAP activity, following stimulation with RANKL in the absence of M-CSF. Preosteoblasts (MC3T3-E1 cells) were maintained in α Modified Eagles medium supplemented with 10% foetal bovine serum 1% L-glutamine, and 2% antibiotics (penicillin/ streptomycin). MC3T3-E1 cells are clonal, non transformed, preosteoblastic cells of a mesenchymal linage, which were isolated from calvariae of late stage mouse embryos. These cells exhibit a high Alkaline Phosphatase activity, among other characteristic properties of osteoprogenitor cells (Yamaguchi *et al,* 2000), and they display a potential to become mineralised in the presence of osteogenic media, which contains acid, β-glycerophosphate, and dexamethasone, which stimulates osteoblastic cells to produce calcium and phosphate, mimicking intramembranous ossification (Sudo *et al,* 1983).

3.3.3 Generation of conditioned media - microinjury to 3D MLO-Y4 cell cultures

Conditioned media samples were obtained following application of microinjury to gel embedded MLO-Y4 cells, as previously described (Section 2.3, **Fig. 2.3**) (Mulcahy *et al,* 2011). 1 ml conditioned media was collected 72 hours after microinjury, from several constructs, and pooled accordingly.

3.3.4 Cell migration of preosteoclasts (Raw 264.7) to conditioned media from osteocytes following microinjury

In order to assess whether microinjury stimulates the migration of preosteoclast cells towards a site of microinjury, a cell migration assay was performed as previously described (Duffy *et al,* 2009). This was performed to determine whether microinjury initiated the BMU by stimulating an influx of cells. The assay was based on the concept that following a period of serum starvation, cells will migrate through a porous membrane towards a stimulus and the number of cells counted on the membrane is indicative cell migration. Under serum free conditions, 1 x 10^4 preosteoclasts were cultured on the upper side of 8-μm pore hanging cell

culture inserts. Following a 2 hour incubation, serum free media was replaced with conditioned media from microinjury experiments (undamaged, 160, 300 and 400 μm), as illustrated in **Fig.3.2**. Controls were serum free media and standard cell culture media. Cells were incubated for 18 hours, following which; cells were fixed in 4% formalin for 10 minutes, and stained with hematoxylin for 10 minutes. The membrane was then removed and mounted on a glass slide, bottom side down. Cell migration was quantified by counting the cells on the bottom side of the membrane in 5 random fields. n = 3 cultures were assayed in each case.

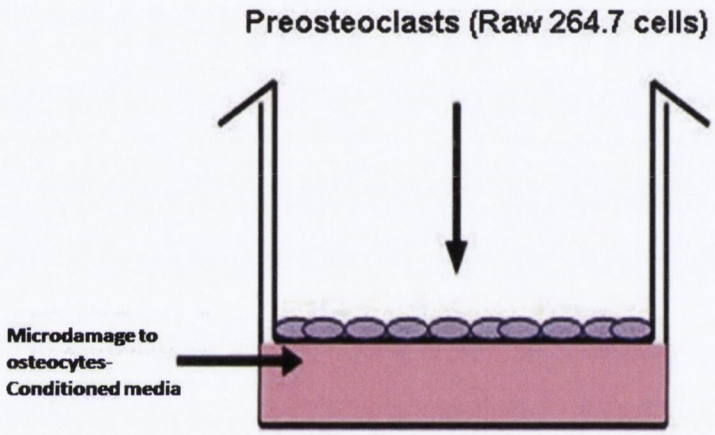


Fig.3.2 Modified Boyden chamber seeded with preosteoclastic cells. Cellular migration was stimulated with conditioned media from varying sized microinjury and the number of cells which migrated to the bottom side of the membrane was found to be relative to the number of cells migrated towards the stimulus.

3.3.5 Verifying the efficacy of Raw 264.7 cells for use as a preosteoclastic cell line

Murine preosteoclasts were seeded at a density of 4 x 10^4 cells/ well in a 12 well plate and cultured for 24 hours prior to experimentation. Additionally, cells were seeded on poly-d-lysine (1M) coated coverslides to determine TRAP activity. Recombinant RANKL was added to the culture medium at concentrations of 0-100 ng/ml. This was performed, to firstly determine the efficacy of this cell line to undergo RANKL mediated osteoclastogenesis, and also to justify the use of this cell line as a preosteoclastic cell line. Cells were cultured for 21 days and at 7 day intervals, TRAP staining and TRAP quantification was performed on preosteoclasts to determine osteoclastogenesis. n = 3 cultures were assayed in each case.

3.3.6 TRAP quantification

Preosteoclasts were cultured for 21 days, and at 7 days intervals, TRAP activity was quantified as follows. The presence of TRAP catalyses the conversion of pNPP to PNP, which is characterised by a maximal absorbance at 405 nm, which provides a relative reading of TRAP activity in the osteoclasts. Culture media was removed from preosteoclasts, followed by an incubation of 1 hour in 1mM citrate solution at 37°C. 50µl of a solution containing 1 mol PnPP, 200 mmol sodium citrate and 80 mmol sodium tartrate per litre was added to a 96 well plate. 20 µl of the culture medium was assayed and the solution incubated for 30 minutes at 37°C. 20 µl of 1M NaOH was added to stop the reaction and absorbance was read at 405 nm by spectroscopy. This provides a relative reading to TRAP activity in a sample. TRAP activity was furthermore determined by TRAP staining using a leukocyte acid phosphatase kit under manufacturer's instructions and number of TRAP positive multinucleated cells was observed. n = 3 cultures were assayed in each case.

3.3.7 TRAP staining of Raw 264.7

TRAP activity was determined by TRAP staining using a leukocyte acid phosphatase kit, under manufacturer's instructions and the number of TRAP positive multinucleated cells was observed. Briefly, 4 x 10^4 Raw 264.7 cells were seeded on a 1x poly d lysine coated coverslip and 24 hours prior to treatment. Following treatment, cells were stained for TRAP activity. Cells were rinsed with dPBS and fixed for 5 minutes with 1x citrate/acetone solution. Cells were rinsed x 3 with dPBS and allowed to air dry for 10 minutes. A staining solution, composed of 46 ml warm (37°C) H2O, 2 ml naphthol, 1 fast garnet capsule, 2 ml sodium tartrate, filtered, was added to the slides and incubated for 1 hour at 37°C. Cells were rinsed x 3 with dPBS and counterstained for 10 minutes with Acid Hematoxylin. This was followed by 3 rinses with dPBS. Slides were allowed to air dry and mounted cell side down on a microscope slide with 200 µl DPX mountant, and allowed to dry before viewing microscopically.

3.3.8 Effect of microinjury on osteoclastogenesis

3.3.8.1 TRAP quantification in Raw 264.7 cells

Preosteoclasts were seeded at a density of 4 x 10^4 cells/ well in a 12 well adherent plates (Cruinn diagnostics). Cells were also seeded on poly d lysine (1M) coated coverslides to determine TRAP activity. Conditioned media samples were collected from gel embedded MLO-Y4 cells 72 hours following the application of microinjury (As per **Fig. 3.3**). 24 hours after seeding, RAW 264.7 cell culture media was replaced with conditioned media from undamaged, 160, 300 and 400 µm MLO-Y4 samples. Conditioned media was changed every 3 days. TRAP activity was quantified as previously described and TRAP staining performed to determine osteoclastogenesis, at 7 day intervals over a 21 day time period. Furthermore, the number of TRAP positive multinucleated cells were quantified, from 3 random fields of view, at 20 X magnification, and their frequency expressed as a percentage of cells within the field of view n = 3 cultures were assayed in each case.

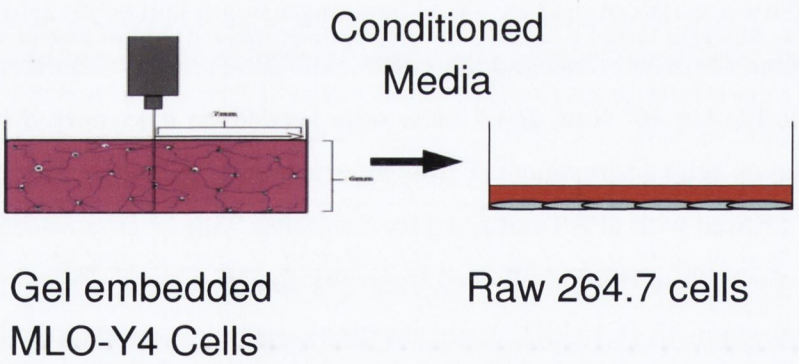


Fig 3.3 Conditioned media treatment of Raw 264.7 cells following microinjury. Following a 5 day incubation, gel embedded MLO-Y4 cells were subjected to microinjury through insertion of varying diameter microneedles into the centre of the gel. A planar defect was created through pulling the needle through the gel and a right angle to the point of insertion. 72 hours after the microinjury, conditioned media was placed on the preosteoclastic cell line, RAW 264.7, to stimulate osteoclastogenesis.

3.3.9 Effect of microinjury induced osteoclastogenesis on MC3T3 migration

In order to assess whether microinjury induced osteoclastogenesis stimulates the migration of osteoblast precursors towards a site of microinjury, a cell migration assay was performed as described above. This was performed to determine whether microinjury induced osteoclastogenesis initiated cell migration towards an osteoclastogenic stimulus, as illustrated in **Fig.3.4.** Again, cell migration was quantified by counting the cells on the bottom side of the membrane. n = 3 cultures were assayed in each case.

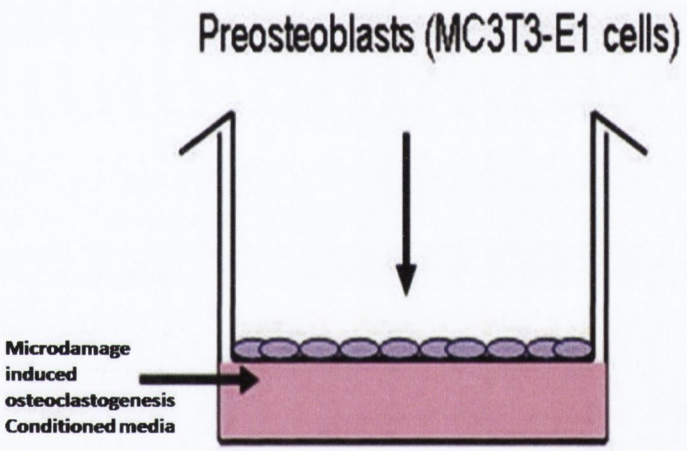


Fig 3.4 Modified Boyden chamber seeded with preosteoblastic cells. Cellular migration was stimulated with conditioned media from microinjury induced osteoclastogenesis and the number of cells which migrated to the bottom side of the membrane was found to be relative to the number of cells migrated towards the stimulus.

3.3.10 Effect of microinjury induced osteoclastogenesis on osteogenesis in MC3T3 cells

Preosteoblasts were grown at a density of 4 x 10^4 cells in 6 well plates. Culture media was replaced after a 24 hour incubation and replaced with conditioned media from microinjury induced osteoclastogenesis experiments combined in a 1:1 ratio with cell culture media and osteogenic media (see **Fig.3.5**). Osteogenic media was prepared by the addition of ascorbic acid (50 μg/ml), β-glycerophosphate (10mM) and dexamethasone (100nM) was added to

standard α-MEM supplemented with 10% FBS, 1% L-glutamine and 2% penicillin/streptomycin. Cells were cultured for 21 days under normal conditions and mineralisation assays performed at 7 day intervals to determine osteogenesis.

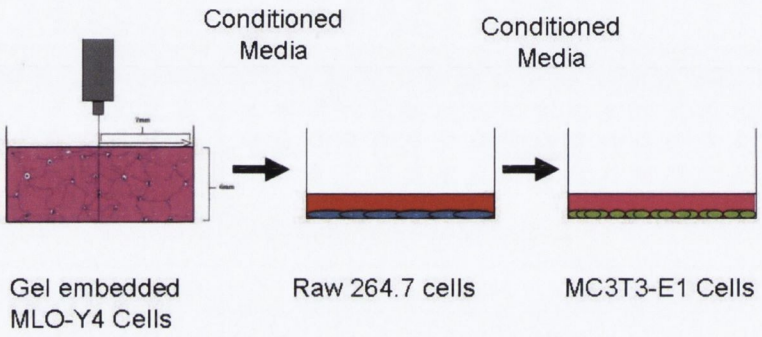


Fig. 3.5 Conditioned media treatment of RAW 264.7 cells from microinjury experiments and conditioned media treatment of MC3T3-E1 cells from microinjury induced osteoclastogenesis experiments. Following 21 days of osteoclastogenic stimulation, conditioned media was mixed with normal or osteogenic media in a 1:1 ratio and applied to the preosteoblastic cell line, MC3T3-E1, to stimulate osteogenesis.

3.3.11 Calcium quantification

To determine the effect of microinjury induced osteoclastogenesis on calcification in preosteoblasts, incubated with conditioned media, in the presence and absence of osteogenic media. Osteogenic media has shown to stimulate the production of calcium in MC3T3-E1 cells, and additionally, we hypothesise that cytokines and growth and stimulatory factors produced from microinjury induced osteoclastogenesis will stimulate osteogenesis *in vitro*. Calcium production was assessed using a StanBio Calcium Liquicolour Kit under manufacturer's instructions. Briefly, culture media was removed from cells after 7 days in culture, followed by rinsing with PBS. 0.5 ml of 1M hydrochloric acid was added to wells and the cells scraped and transferred into a tube. This was repeated twice. Samples were incubated overnight in the refrigerator. 10 µl of sample was added to a well of 96 well plate followed by 200 µl working solution of 1:1 of binding reagent and working dye. Calcium concentration was determined by spectroscopy at 595 nM. n = 3 cultures were assayed in each case.

3.3.12 Alizarin Red staining

Alizarin red staining was used to determine calcium mineralization in preosteoblasts following the addition of conditioned media, as previously described. Alizarin red staining is characterized by staining calcium bright red. After 7 days in culture, cells were rinsed and fixed in 4% formalin for 10 minutes. Cells were rinsed and stained with 2% Alizarin red solution for 5 minutes. This was followed by 3 rinses with PBS. Calcification was observed histologically using a light microscope.

3.3.13 Alizarin Red Quantification

At 7 day intervals, cells were rinsed with dPBS, and stained with alizarin red, as described. A 10% solution of Cetylpyridium Chloride was dissolved in distilled water, whilst stirring over heat. 1mL of Cetylpyridinium Chloride was added to each well, and after a 5 minute incubation, the solution was mixed thoroughly by pipetting. 100µl of the solution was pipetted into a 96 well plate, and the absorbance read at 540nm.

3.3.14 Von Kossa staining

Von Kossa staining was used to determine phosphate mineralization in preosteoblasts following the addition of conditioned media, as previously described. Von Kossa staining is characterized by staining phosphates a brown/black colour. After 7 days in culture, over a 21 day time period, cells were rinsed and fixed in 4% formalin for 10 minutes. A 3% silver nitrate solution was prepared by adding 300mg silver nitrate to 10 mL water. 1ml of this solution was added each well followed by a 10 minute incubation in the dark. This was followed by 3 rinses with PBS, reserving the final rinse. Plates were exposed to warm bright light for 15 minutes. Phosphate mineralization was observed histologically using a light microscope.

3.3.15 Statistics

All data was analysed for significance ($p \leq 0.05$) using two way ANOVA, as a function of microinjury size and time, post hoc Tukey test, to compare means.

3.4 Results

3.4.1 An increase in microinjury increases preosteoclast migration

The quantity of cells which passed through the membrane of the cell insert in response to stimuli in conditioned media from varying injury size to an osteocytic network is illustrated in **Fig.3.6**. A significant increase in cell migration was observed in all samples, 72 hours after microinjury, which occurred in accordance with increase in microinjury size, i.e. migration at 400 μm was significantly greater than at 300 μm, and migration at 300 μm was greater that what was observed at 160 μm. No significant difference was seen between undamaged and 160 μm samples. Also, a significant increase was noted in the control sample with complete cell culture media, when compared to other control groups.

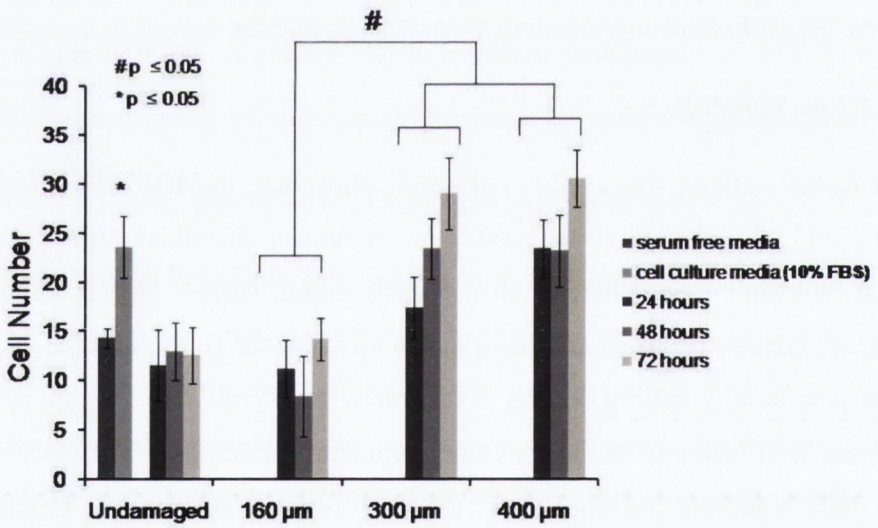


Fig. 3.6 Increase in microinjury increases preosteoclast migration. A significant increase in cell migration was noted in all microinjury samples at all time points when compared to conditioned media from undamaged cells. Also, a significant increase was noted in the control sample with complete culture media, when compared to other control groups. * denotes $p \leq 0.05$ when comparing between control groups. # denotes $p \leq 0.05$ when comparing between varying microinjury sizes (160, 300 and 400 μm microinjury size) between groups. Error bars are indicative of standard deviation, n = 3.

3.4.2 The efficacy of Raw 264.7 cells for use as a preosteoclastic cell line

3.4.2.1 RANKL stimulates osteoclastogenesis and demonstrates that RAW 264.7 cells are a viable cell line for studying osteoclastogenesis

Cells were given varying concentrations of RANKL to promote osteoclastogenesis (**Fig.3.7**). At Day 7, a difference in cell morphology was noted in samples with 50 ng/ml or more of RANKL, which was also observed at day 14 and 21. Additionally, the greatest numbers of large, multinucleated cells were observed in samples treated with 50 and 75 ng/ml of RANKL, which is indicative that Raw 264.7 cells are capable of undergoing RANKL mediated osteoclastogenesis and are suitable cell line for studying osteoclastogenesis.

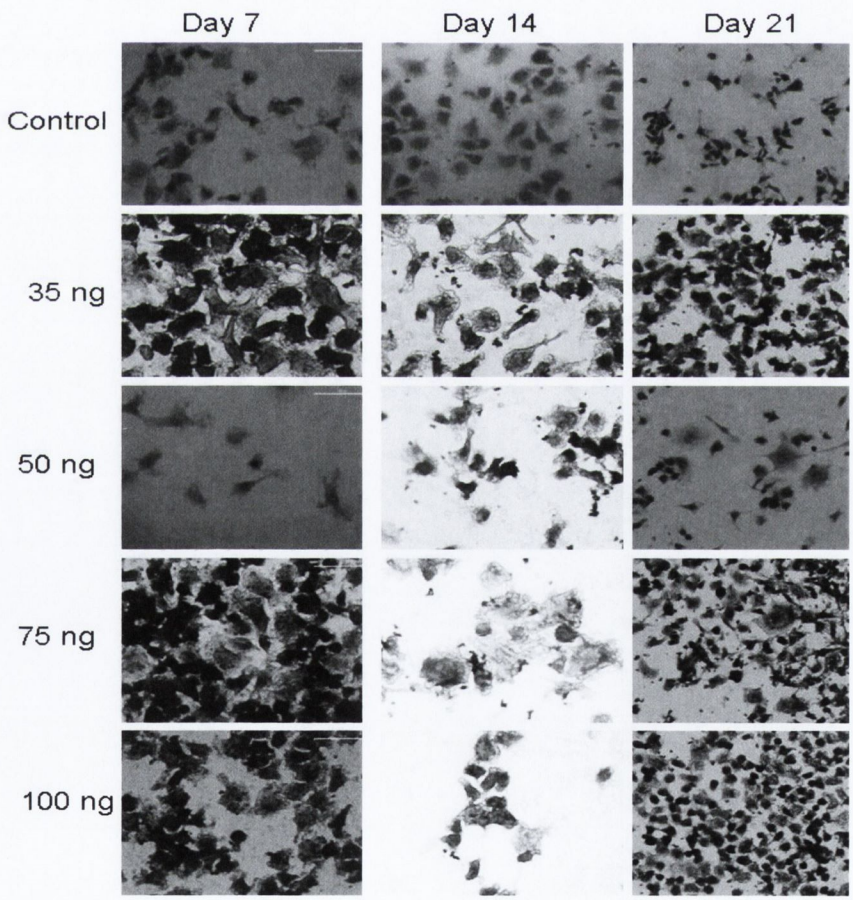


Fig 3.7 RANKL stimulates osteoclastogenesis and demonstrates that RAW 264.7 cells are a viable cell line for studying osteoclastogenesis. An increased number or large, multinucleated TRAP + cells was observed at 50 and 75 ng/ml RANKL, demonstrating that Raw 264.7 cells make a valid cell line for studying osteoclastogenesis.

3.4.2.2 50 ng/ml is the optimum concentration to stimulate osteoclastogenesis, thus Raw 264.7 cells are a viable cell line for studying osteoclastogenesis

This assay was previously performed to determine the differentiation potential of Raw 264.7 cells in the presence of cytokines. Determining the osteoclastic response allows us to measure the amount of osteoclastogenic activity in preosteoclasts from other stimuli. TRAP is a substance, as previously described, released from mature osteoclasts. This assay was performed to furthermore clarify an osteoclastogenic response from preosteoclasts. On addition of 50 ng/ml RANKL, there was a significant increase in TRAP activity over a 21 day time period, as shown in **Fig. 3.8**. This indicated that treatment of preosteoclasts with RANKL stimulated TRAP activity and thus cells could undergo RANKL mediated osteoclastogenesis.

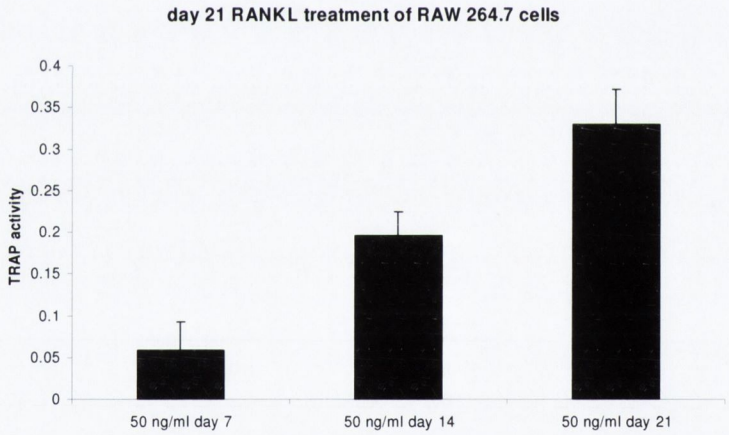


Fig 3.8 50 ng/ml is the optimum concentration to stimulate osteoclastogenesis, thus Raw 264.7 cells are a viable cell line for studying osteoclastogenesis. 50 ng/ml was the optimal dose of RANKL to differentiate pre-osteoclast to mature osteoclasts, so was used to determine the osteoclastogenic potential of Raw 264.7 cells. Graph depicts the increase in TRAP activity at 7 day intervals over a 21 day time period, thus verifying them as a suitable cell line for studying osteoclastogenesis.

3.4.3 The effect of microinjury on osteoclastogenesis

3.4.3.1 Microinjury increases the amount of TRAP positive cells in a damage size dependent manner

The effect of microinjury was determined by TRAP staining of preosteoclasts at 7 day intervals over a 21 day period following treatment with conditioned media. **Fig.3.9** shows TRAP staining results at 10 X magnification. Cells were given conditioned media from control and injured gel embedded MLO-Y4 cells to promote osteoclastogenesis. The presence of large multinucleated cells was greatest in 300 and 400 μm samples when compared to 160 μm and control samples at all time points.

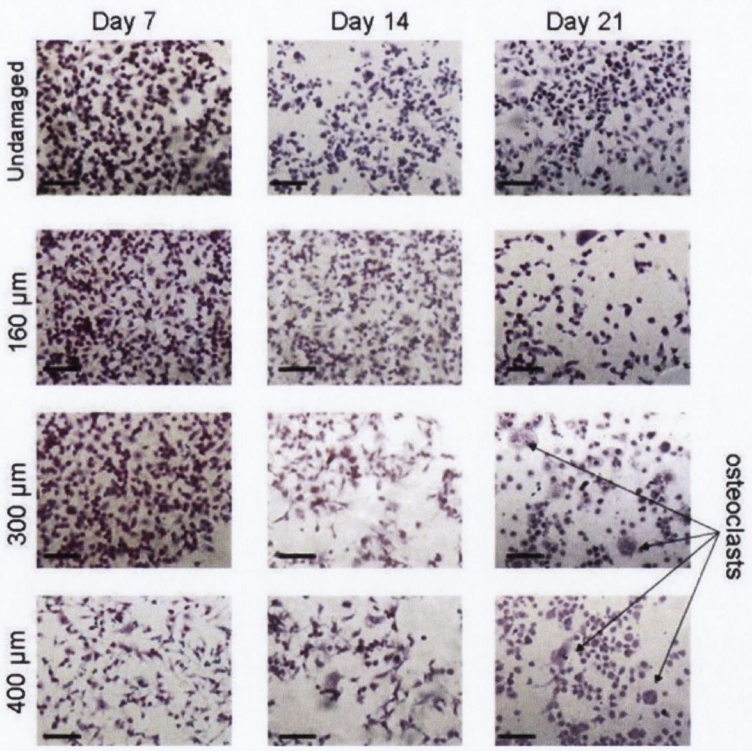


Fig 3.9 Microinjury increases the amount of TRAP positive cells in a damage size dependent manner. Cells were treated with conditioned media from previous microinjury experiments. The presence of large multinucleated cells was greatest in 300 and 400 μm samples at all time points when compared to other samples.

Additionally, the number of TRAP positive cells was quantified and expressed as a percentage of the total cell number within the field of view. Again, a significant increase in

large, TRAP positive, multinucleated cells was observed in the large microinjury sizes; however, all microinjury sizes were all significantly greater than the undamaged control. A six fold increase in the number of TRAP positive cells was observed in 400 μm samples when compared to its undamaged control at 21 days, as can be observed in **Fig. 3.10**.

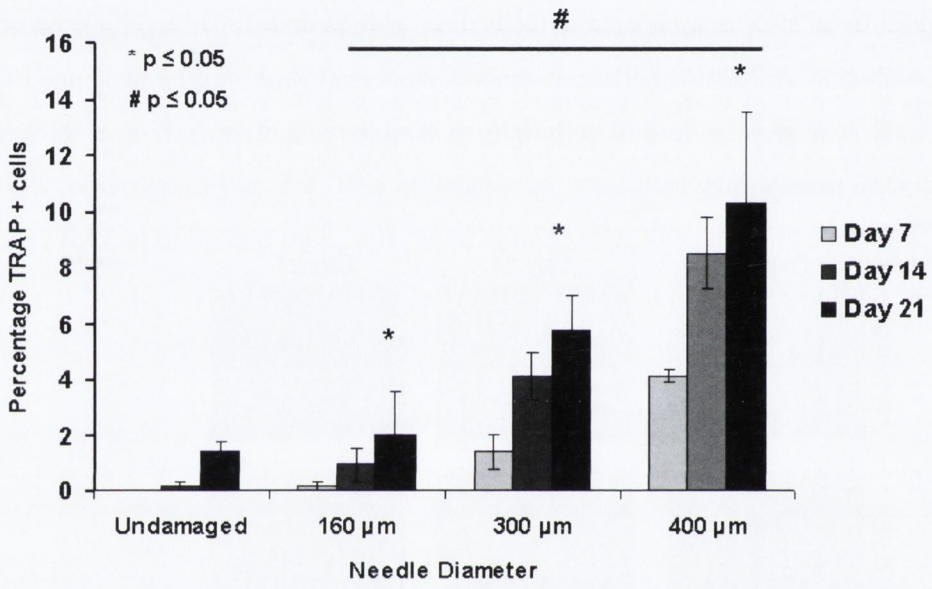


Fig 3.10 Microinjury increases the number of TRAP+ cells according to damage size. Quantification of TRAP positive cells in a preosteoclast population treated with conditioned media from varying microinjury sizes to gel embedded MLO-Y4 cells. A significant increase in TRAP positive cells was observed in 160 μm and 300 μm at 7 days and 400 μm samples at 14 and 21 days. Again, it must be noted that TRAP activity increased significantly accordingly with increase in damage size. * denotes $p \leq 0.05$ when comparing varying damage sizes within a group. # denotes $p \leq 0.05$ when comparing varying damage sizes between groups at any time point.

3.4.3.2 Microinjury increases TRAP activity according to damage size

TRAP activity was quantified in preosteoclasts treated with conditioned media from control and injured gel embedded MLO-Y4 cells. TRAP activity significantly increased in 160 and 300 μm samples at 72 hours and 400 μm samples at 48 and 72 hours, when compared to undamaged samples and other time points, as can be observed in **Fig. 3.11**. It must be noted that TRAP activity increased significantly accordingly with increase in microinjury size.

TRAP activity was significantly less in undamaged samples when compared to all other samples.

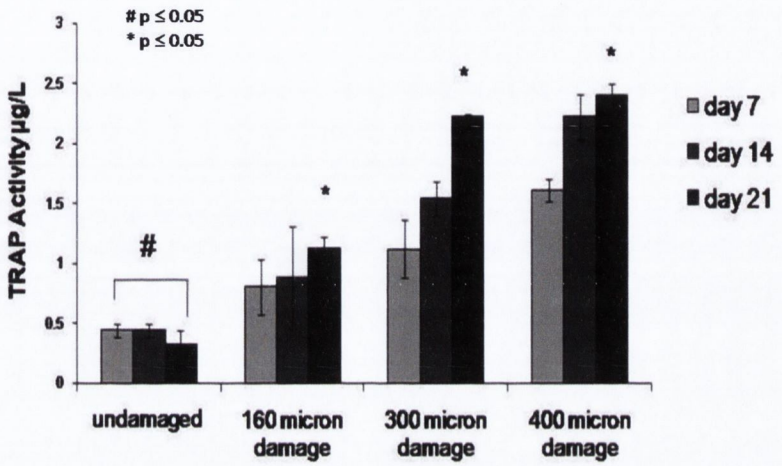


Fig 3.11 Microinjury increases TRAP activity in preosteoclastic cells according to damage size. Quantification of TRAP activity in preosteoclasts treated with conditioned media from control and injured gel embedded MLO-Y4 cells. A significant increase in TRAP activity was noted in 160 µm and 300 at 72 hours and 400 µm samples at 48 and 72 hours. However, it must be noted that TRAP activity increased significantly accordingly with increase in microinjury size. * denotes $p \leq 0.05$ when comparing varying microinjury sizes within a group. # denotes $p \leq 0.05$ when comparing varying microinjury sizes between groups. Error bars are indicative of standard deviation, n = 3.

3.4.4 Microinjury induced osteoclastogenesis increases preosteoblast migration depending on damage size

The quantity of pre-osteoblastic cells which passed through the membrane of the cell insert in response to stimuli in conditioned media from microinjury induced osteoclastogenesis is illustrated in **Fig. 3.12**. A significant increase in cell migration was observed in all samples, 72 hours after microinjury, which occurred in accordance with increase in microinjury size, i.e. migration at 400 µm was significantly greater than at 300 µm, and migration at 300 µm was greater that what was observed at 160 µm. No significant difference was seen between undamaged at 160 µm samples. Also, a significant increase was noted in the control sample with complete cell culture media, when compared to other control groups.

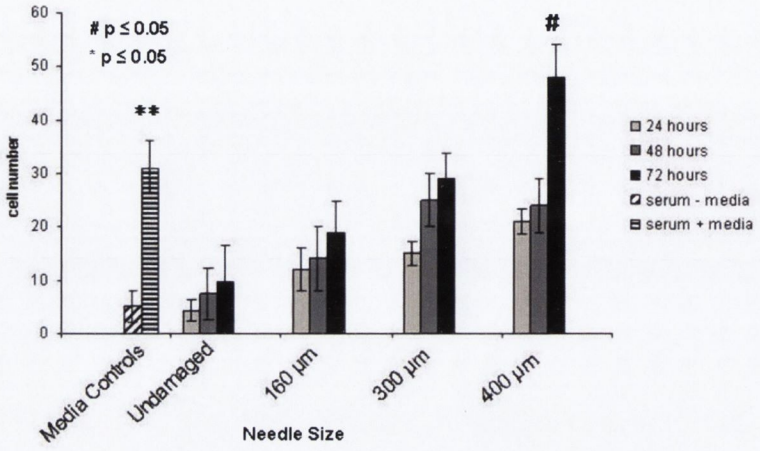


Fig 3.12 Microinjury induced osteoclastogenesis increases preosteoblast migration depending on damage size. A modified Boyden chamber was used to determine the migration of preosteoblasts towards conditioned media from microinjury induced osteoclastogenesis. A significant increase in cell migration was noted in all damage samples at all time points when compared to conditioned media from undamaged cells. Also, a significant increase was noted in the control sample with complete culture media, when compared to other control groups. # denotes $p \leq 0.05$ when comparing between control groups. * denotes $p \leq 0.05$ when comparing between varying damage sizes (160, 300 and 400 µm damage size) between groups. Error bars are indicative of standard deviation, n = 3.

3.4.5 Microinjury induced osteoclastogenesis increases mineralisation of preosteoblasts in a size dependent manner

This assay demonstrated the potential of preosteoblasts to undergo calcification following the addition of conditioned media from microinjury experiments and microinjury induced osteoclastogenesis experiments, with an aim to A: determine the osteogenic response to varying microinjury size, and B: to determine whether an osteogenic response results from microinjury induced osteoclastogenesis. **Fig. 3.13** shows a calcium assay of preosteoblasts with conditioned media from microinjury induced osteoclastogenesis over a 21 day time period. There was significantly more calcium present in MC3T3-E1 cells cultured in osteogenic media when compared with their counterparts cultured in standard cell media with conditioned media from microinjury induced osteoclastogenesis at day 21. However, there was significantly more calcification noted in 400 µm samples without osteogenic media, when

compared to other damage sizes without osteogenic media. These results show that even in the absence of osteogenic media, microinjury size is critical in initiating an osteogenic response (ie: at 400 µm, there is a significantly greater degree of calcium mineralisation when compared to other damage sizes within the group).

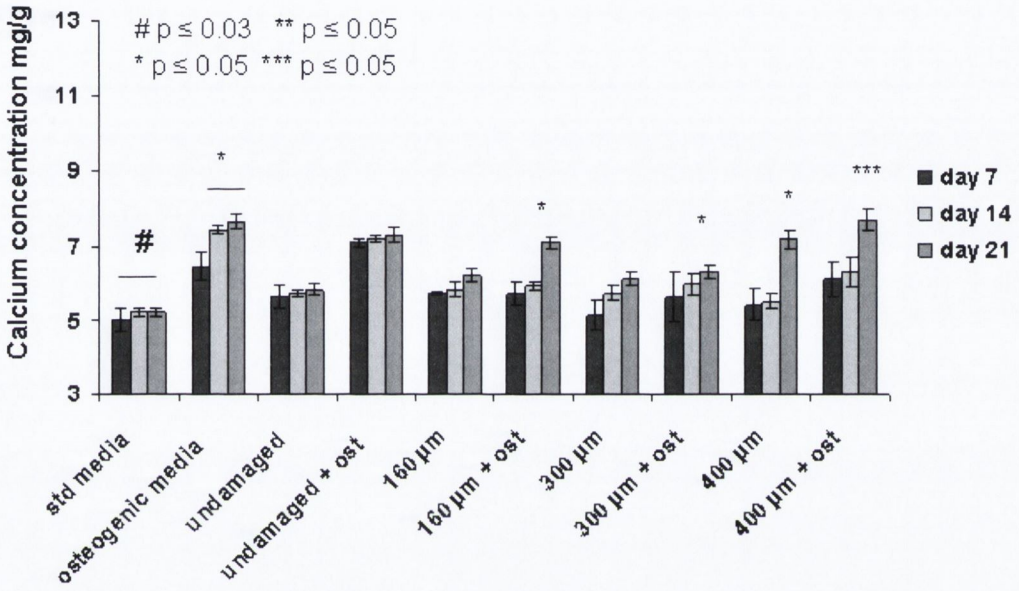


Fig.3.13 Microinjury induced osteoclastogenesis increases mineralisation of preosteoblasts in a size dependent manner. Microinjury induced osteoclastogenesis produced a reduced level of calcium mineralisation when compared to preosteoblasts treated with conditioned media from microinjury experiments. A similar response was observed however, with a significant increase was seen in all samples with osteogenic media at day 21 and in 400 µm samples without osteogenic media. * denotes $p \leq 0.05$ when comparing significance between damage size with and without osteogenic media. ** denotes $p \leq 0.05$ when comparing significance within control groups. *** denotes $p \leq 0.05$ when comparing 400 µm sample with ost with all other samples. # denotes $p \leq 0.05$ when comparing between all groups. Error bars are indicative of standard deviation, n = 3.

3.4.6 Alizarin Red staining demonstrates the effects of microinjury induced osteoclastogenesis on calcium mineralisation

Alizarin Red S staining of preosteoblasts at 10 X magnification demonstrated the potential of preosteoblasts to undergo matrix deposition and mineralisation following the addition of conditioned media from microinjury induced osteoclastogenesis, with and without the presence of osteogenic media. Alizarin Red stains calcium bright red. As **Fig. 3.14** shows,

there was mineralisation observed at 7 days only in 400 µm samples with osteogenic media. However, at day 14, mineralisation was observed in all samples, with the greatest quantity of mineralisation being observed in 300 and 400 µm samples in the presence and absence of osteogenic media. At day 21, extensive mineralisation was observed in all samples and again, was most abundant in 300 and 400 µm damage samples. These results are depicted graphically, in **Fig. 3.15**, which details quantification of alizarin red. Again, these results show that the quantity of alizarin red was significantly greater in 400 µm samples, both with and without osteogenic media, most notably at day 21. Furthermore, these results are reflective of the calcium assay, again, with significantly more calcification noted in 400 µm samples without osteogenic media, when compared to other samples without osteogenic media.

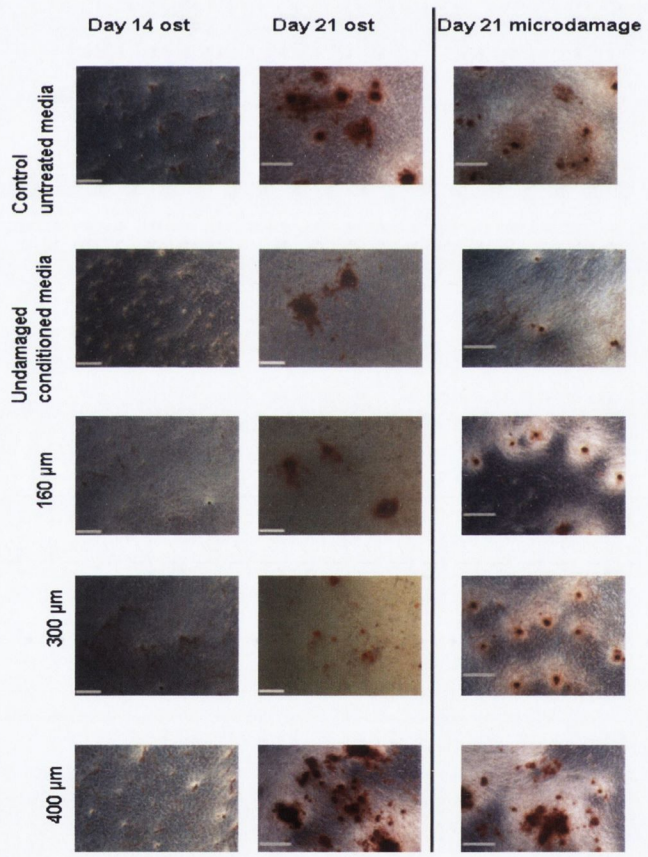


Fig. 3.14 Alizarin Red staining demonstrates the effects of microinjury induced osteoclastogenesis on calcium mineralisation At days 14 and 21, both calcium mineralisation was observed in all samples with and without osteogenic media, with the greatest quantity of mineralisation being observed in 300 and 400 µm samples with osteogenic media. Calcium and phosphate mineralisation was most abundant in 300 and 400 µm samples with and without osteogenic media. Scale bar shown in images = 200 µm.

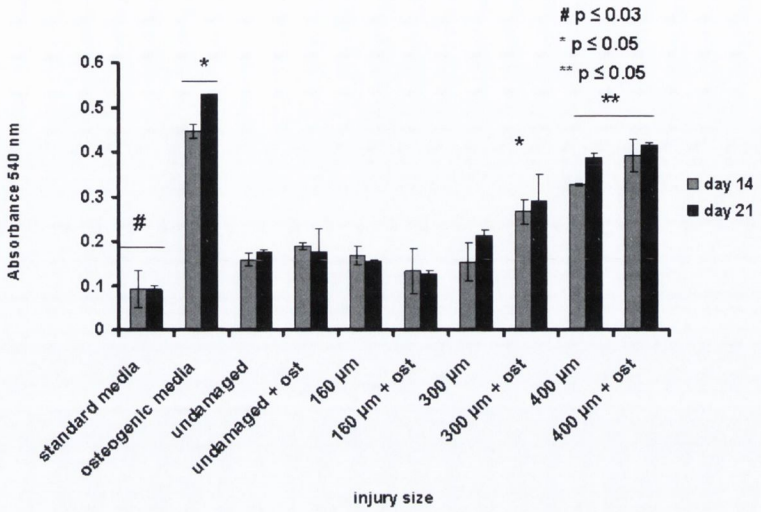


Fig. 3.15 Quantification of alizarin red staining demonstrates the effects of microinjury induced osteoclastogenesis on calcium mineralisation. Results show that again, the quantity of alizarin red was significantly greater in 300 and 400 µm samples, both with and without osteogenic media, most notably at day 21. Most notably, significantly more calcification noted in 400 µm samples without osteogenic media, when compared to other samples without osteogenic media. * denotes $p \leq 0.05$ when comparing significance between various samples ** denotes $p \leq 0.05$ when comparing 400 µm samples to other damaged and undamaged samples, both with and without osteogenic media. # denotes $p \leq 0.05$ when comparing standard media with all other samples. Error bars are indicative of standard deviation, n = 3.

3.4.7 Von Kossa staining demonstrates the effects of microinjury induced osteoclastogenesis on phosphate mineralisation

Von Kossa staining of preosteoblasts at 10x magnification determined the effect of microinjury and microinjury induced osteoclastogenesis on phosphate mineralisation, again, in the presence and absence of osteogenic media. Von Kossa characterises silver nitrate binding to phosphates, producing a brown/black stain. As **Fig. 3.16** shows, phosphate mineralisation was observed at 7 days only in all samples. Again at day 14, mineralisation was observed in all samples with the greatest quantity of phosphate mineralisation being observed in 300 and 400 µm samples both with and without osteogenic media. At day 21, extensive mineralisation was observed in all samples and was greatest in 160, 300 and 400 µm samples

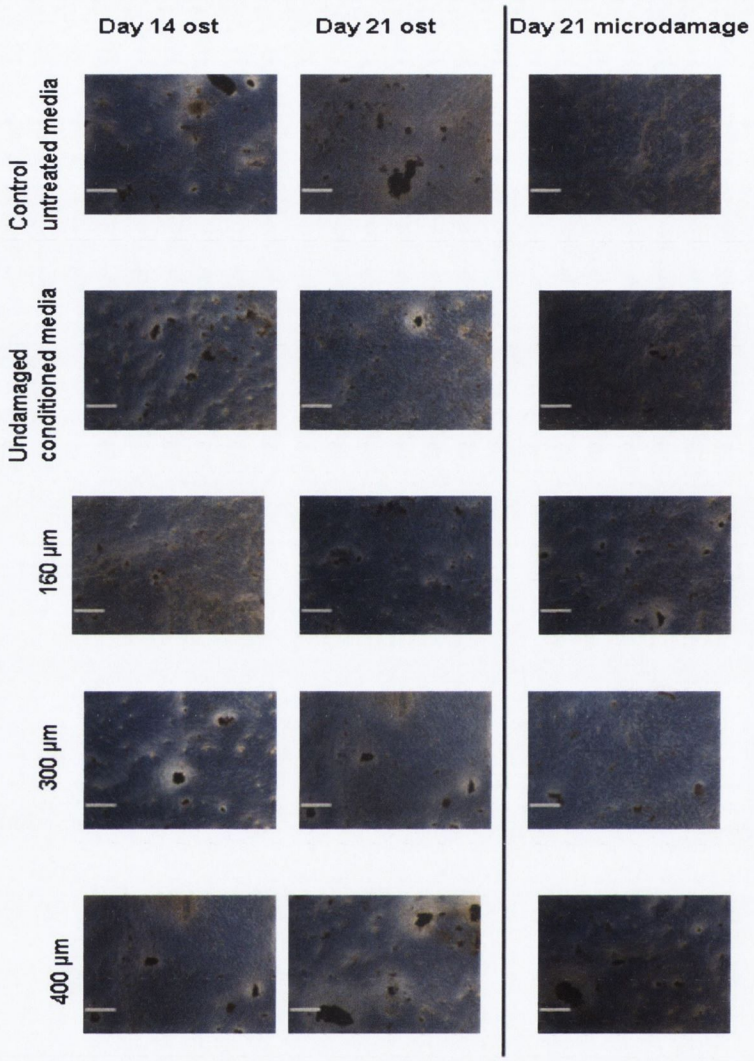


Fig. 3.16 Von Kossa staining demonstrates the effects of microinjury induced osteoclastogenesis on phosphate mineralisation. Phosphate mineralisation was observed at 7 days only in samples with 400 µm conditioned media from microinjury induced osteoclastogenesis with osteogenic media (ost), by the presence of brown/black staining of the cells, as shown by Von Kossa staining. Again, at day 14, both calcium mineralisation was observed in all samples with and without osteogenic media, with the greatest quantity of mineralisation being observed in 300 and 400 µm samples with osteogenic media. Calcium and phosphate mineralisation was most abundant in 300 and 400 µm samples with and without osteogenic media. Scale bar shown in images = 200 µm.

3.5 Discussion

Skeletal strength and durability are maintained by the removal of compromised tissue as a result of microcracks, an accumulation of which can potentiate the risk of stress fractures. This is performed by the actions of the BMU through the process of bone remodeling (Burr *et al,* 1997). The role of microdamage in bone has long been considered, and it has been shown that microdamage stimulates the release of bone remodeling cytokines. This particular study investigated, for the first time, the effect of varying sized microinjury, which resulted from the application of planar defects on 3D osteocyte cultures, on osteoclastogenesis and osteogenesis, *in vitro.* These results should ultimately reflect the process of bone remodeling through the activation of the BMU, with regard to progenitor cell infiltration towards a specific stimulus (i.e. microinjury and microinjury induced osteoclastogenesis, osteoclastogenesis and osteogenesis. In the previous chapter, we discussed how activity of RANKL and OPG, the principal cytokines involved in the stimulation of bone remodeling, increased and decreased according to microinjury size, respectively (Mulcahy *et al,* 2011). However, the effect of microinjury on the various stages of bone remodeling in the constitutive cells of the BMU has yet to be established. As discussed in the previous chapter, it was found that an increase in microinjury size was related to an increase in RANKL activity and decrease in OPG activity

The hypothesis of this study was that variations in damage size would present variations in the degree of precursor cell migration, osteoclastogenesis and osteogenesis, in the constitutive cells of the BMU. This hypothesis provided the principal aim of this study, which was to determine the cellular behaviour of osteoclasts and osteoblasts to varying microinjury size. It has been reported that damage to an osteocytic network following microdamage stimulates osteoclastogenesis, and thus would be a principal mediator of bone remodeling (Verborgt *et al,* 2000, Frost *et al,* 1973, Parfitt *et al,* 1983). However the relationship between severity of damage and rate of remodeling has yet to be determined. Conditioned media from 3D gel embedded MLO-Y4 cells, having received controlled, planar defects of set microinjury size, may possess soluble RANKL, in addition to other factors, elevated to a concentration which corresponds to damage size, so it is significant to note that conditioned media samples were obtained from both varying sized microinjury and microinjury induced

osteoclastogenesis experiments. Considering this, it may be assumed that the degree of mineralization and osteogenesis would increase in correlation with osteoclastogenesis induced by microinjury.

The initial portion of this study was conducted to examine the migration of preosteoclasts towards a stimulus, i.e. soluble factors released from osteocytes following microdamage, at varying time points and damage sizes, thus demonstrating the "activation" stage of bone remodeling. This was performed to determine whether the influx of preosteoclasts, associated with the initiation of a BMU depended on microdamage, and if so, whether the size of microdamage was significant in stimulating osteoclast precursor migration. It is thought that following microdamage, preosteoclasts infiltrate the tissue and eventually progress to the site of damage, whereby they resorb compromised bone (Mulari *et al,* 2004). Activation of the BMU is wholly dependent on RANKL and OPG ratios (Hofbauer *et al,* 2000), the rate of which was found to be dependent on damage size, as detailed in chapter 2. It was found that there was no significant difference in cell migration between undamaged controls and 160 μm conditioned media samples. However, a two fold increase in preosteoclast migration was noted in 300 and 400 μm samples, 72 hours following microinjury. This is indicative that initiation of bone remodeling depends on microinjury size, and that smaller cracks may be left unrepaired *in vivo*. It has been considered that cracks exceeding a critical size of 300 μm, and thus rupturing a certain number of osteocyte cell processes, relative to what we have observed in our model, can send out a signaling response to activate a BMU (ie- cell process rupture at specific microdamage sizes releases RANKL to recruit osteoclasts) (Mori *et al,* 1993), and so, these results may confirm this concept. A similar response was observed in the treatment of Raw 264.7 cells with conditioned media from apoptotic MLO-Y4 cells, where an increase in preosteoclast recruitment was observed, however this is the first time a relationship between damage size and cell migration has been observed, thus reinforcing the concept that damage size is critical in bone remodeling. Additionally, it was demonstrated that resorption spaces were evident as little as 8 days after the application of fatigue induced microdamage (Mori *et al,* 1993), which is reflective of our results, however, we have shown that microinjury size may be critical in determining the rate of bone remodeling based on the differentiation response of the constitutive cells of the BMU

Our finding, within this "stage" of bone remodeling, is that preosteoclast migration is stimulated by damage and increases with damage size, may be indicative that initiation of bone remodeling depends on the severity of the damage.

The next aim of this study was to determine the effect of microinjury, of varying size, on osteoclastogenesis. Conditioned media from gel embedded MLO-Y4 cells was added to preosteoclasts and cultured for 21 days, with TRAP activity and TRAP staining performed at 7 day intervals. This study showed a trend in an increased osteoclastogenic response with increased microinjury size. TRAP, a major marker of osteoclast activity, was quantified over at 7 day intervals over a 21 day time period. A significant increase in TRAP activity was noted in all injured samples, however, in 300 μm samples at 72 hours after microinjury, and 400 μm at 48 and 72 hours after microinjury, a fivefold increase in TRAP activity was observed when compared to undamaged control samples. To complement these findings, a TRAP stain was performed to determine the frequency of large, multinucleated cells, i.e., osteoclasts. Again, a higher frequency of osteoclasts were observed in 300 and 400 μm samples, 21 days after exposure to microinjury. Furthermore, quantification of the number of large, multinucleated, osteoclastic cells, expressed as a percentage of total cell number, showed an increase in the quantity of osteoclasts over time, and also a marked increase occurred along with greater microinjury sizes (ie: at 400 μm). These results show a similar pattern to what was observed in a study performed by Hazenberg et al, 2009, whereby damage was applied to collagen embedded MLO-Y4 cells and an osteoclastic response observed in bone marrow cells. However, this is the first time a relationship between damage size and the rate of preosteoblast migration and osteoclastogenesis has been observed. Thus, this is suggestive that osteoclastogenesis is effected by microdamage size.

The final step of this study was to determine the effect of microinjury alone and microinjury induced osteoclastogenesis on osteogenesis. Preosteoblastic MC3T3-E1 cells were used to determine osteoblast migration and *in vitro* mineralization following microinjury induced osteoclastogenesis, and thus the reversal phase of bone remodeling. Similarly to the activation stage of bone remodeling, the reversal phase requires an influx of osteoblast precursor cells. *In vivo*, preosteoblasts infiltrate the BMU in order to secrete osteoid, and form new bone. Once osteoclasts have removed a sufficient amount of compromised bone, a

reversal phase is noted, whereby bone resorption switches to bone formation. This process occurs in tandem with RANKL sequestration by OPG. Osteoclasts undergo apoptosis, releasing osteoblast recruitment factors, including BMPs and TGF β. Similarly to osteoclast migration, osteoblast migration was quantified after an 18 hour incubation on a porous membrane exposed to a stimulus- i.e. conditioned media. It was found that there was no significant difference in cell migration between undamaged controls and 160 μm conditioned media samples. However, a two fold increase in preosteoclast migration was noted in 300 and 400 μm samples, 72 hours following microinjury. As before, these results are indicative that microinjury size is pivotal in instigating a remodeling response with regard to influx of osteoblast precursor cells, to mediate the formation of new bone.

Once osteoblasts have been recruited to repair the Howslips lacuna left following osteoclast activity, it is necessary for osteoblasts to secrete osteoid, which will become mineralised with calcium and phosphate, forming an osteon, and essentially, new bone (Eriksen *et al,* 1995). It proved viable to determine the osteogenic potential of microinjury induced osteoclastogenesis on bone formation. To complete the analysis into the effect of microinjury on BMU activity, preosteoblastic MC3T3-E1 cells, were used to determine calcium mineralization. Assays were performed in the presence and absence of osteogenic media. In a functioning BMU, a band of osteoclasts precedes preosteoblasts which differentiate into osteoblasts and lay down new matrix (Miyazaki *et al,* 2004). 7 day analytes of preosteoblasts were assayed for mineralization via a quantitative calcium assay over a 21 day time period. This is indicative of osteoblast differentiation and mineralization, thus bone formation. Calcium concentration was found to be significantly greatest in 400 μm samples at day 21 with osteogenic media. Furthermore, a significant increase in calcium concentration was observed in 400 μm samples without osteogenic media when compared to other microinjury sizes at the same time points. Further to this, these results may imply that crack size may be crucial in initiating a remodeling response, that is, to release a sufficient signal to activate the constitutive cells of BMU.

To further reinforce these findings, alizarin red staining was performed on cells under the same parameters, to determine calcium mineralization, characterised by bright red staining of cells. Alizarin red staining showed, histologically, that the greatest amount of calcium

mineralization in 300 and 400 μm microinjury samples at days 14 and 21 when compared to 160 μm and undamaged samples. Quantification of alizarin red staining again reflected this, and demonstrated again that the greatest amount of calcium mineralization in 300 and 400 μm microinjury samples at days 14 and 21 when compared to 160 μm and undamaged samples. Furthermore, Von Kossa was employed to determine phosphate mineralisation, as shown by brown/black staining. Again, histological results confirmed that the greatest amount of both calcium and phosphate mineralization was in the 300 and 400 μm microinjury samples at days 14 and 21 when compared to 160 μm and undamaged samples. The principal finding in is that osteoclastogenesis and mineralization through osteoblast activity, is stimulated by microinjury in an microinjury size dependent manner, i.e.: the greater the microinjury size, the more likely it is that osteoclasts will infiltrate the compromised tissue, remove bone, recruit osteoblasts and lay down new tissue, again and it is noteworthy that this occurs in the absence of osteogenic media. Indeed, it can be argued that, considering in our previous chapter, we observed at an increase in damage size, i.e. at 400 μm, a reduction in OPG was observed, and thus, one might expect a reduction in the level of osteogenesis at this damage size, however, it has been shown that osteoblast mediated mineralisation can occur in the absence of OPG. As described in the introductory chapter, crosstalk between Ephrin B2 and Eph B4 functions to stop RANKL activity, which stops bone resorption and stimulates bone formation. In addition to this, it has been recently shown that osteocytes may stimulate osteogenesis directly through the secretion of bone forming cytokines, which include ILGF-1, TGF-β, and BMPs, and also Wnt10a and sphingosine-1-phosphate, the latter two of which have also been implicated in osteogenesis following osteoclastogenesis (Pederson *et al,* 2008). This may be suggestive that, even in the absence of a strong OPG signal to stimulate osteogenesis, the level of osteoclastogenesis may be sufficient to stimulate an osteogenic response.

Considering the osteoclastogenic and osteogenic results in tandem, it is evident that microdamage bears a significant effect on stimulating the activity of the constitutive cells of the BMU. Furthermore, it has been suggested that microdamage size may be critical in the degree of bone remodeling which occurs, which has been demonstrated within this chapter. These findings are consistent with previous findings and hypotheses. It has long been known that interference with a cells normal environment, be it through mechanical stimulation, fluid

flow and microdamage, instigates the release of RANKL, which initiates a chain of events leading to the release of OPG (Wyzga *et al,* 2004, Suda *et al,* 1999). This would bear an effect on activation of the BMU. Noble *et al,* 2003, stated that microdamage is capable of initiating an osteoclastogenic response, and it was furthermore demonstrated that osteocytes act as a regulatory system in bone, determining rate of bone resorption by osteoclasts (Mori *et al,* 1993, Tompkinson *et al,* 1997, Noble *et al,* 2003). The results demonstrate that bone remodeling may result from damage directly to osteocyte cell processes It can, based on these findings, be thought that a bone remodeling response in the constitutive cells of the BMU is stimulated by the damaging of cell process networks, which further reinforces the bone remodeling theories of Hazenberg *et al,* 2006, which stated that bone remodeling was damage size dependant, and furthermore demonstrates the concept of targeted bone remodeling, whereby, not only is the BMU activated, but steered towards a site of damage, following cellular apoptosis (Burr *et al,* 1997, Martin *et al,* 2006 Verborgt *et al,* 2000). It is also reflective of our previous study, where an increase in osteocyte apoptosis occurred following an increase in microinjury size, which furthermore complements the work of Verborgt *et al,* 2006, which stated that osteocyte apoptosis initiates bone remodeling following microdamage (Mulcahy *et al,* 2011). Correlations have been noted between the effects of osteocyte damage and apoptosis on osteoclastogenesis in our model and previous studies (Hazenberg *et al,* 2009), however, no work to date has been conducted to determine the effects of microinjury induced osteoclastogenesis on osteogenesis, thus the latter portion of this study present a very novel finding. Considering this, the principal finding in this particular study is that osteoclastogenesis and mineralization through osteoblast activity is stimulated by microinjury in a size dependent manner, thus fulfilling the hypothesis of this study. Furthermore, this study has shown the potential of osteocytes as a principal stimulator of bone remodeling activity, thus linking into previous studies, by which any disruption to osteocytes, resulting in osteocyte apoptosis and RANKL release from cells, stimulates BMU activity *in vivo* (Cardoso *et al,* 2009, Burger *et al,* 2003, Marotti *et al,* 1992, Sobelman et al, 2004).

A limiting aspect to this study is that, in our previous study, we determined the biochemical response to microinjury, and clarified the relationship between damage size and RANKL/OPG activity, and thus, it could be argued that the results of this chapter could allow

one to predict the cellular response to microdamage. While this is, indeed, true, other factors are involved other than RANKL and OPG in stimulating the cellular responses to microinjury, and so this chapter serves to reinforce the findings of the previous chapter and also to provide a platform for the next chapter, whereby the other biochemical factors involved in bone remodeling are elucidated. Further to this, the results of the previous chapter may indicate that OPG may have been blocked by endogenous RANKL and thus unable to be detected by ELISA, however, was still able to fulfil its function, as previously observed by Hazenberg et al, 2009. Additionally, whilst this study has shown the relationship between severity of microinjury and the functioning of the constitutive cells of the BMU, it does not illustrate the effects of microinjury size on the BMU as an entire entity. Also, it is known that RANKL is a stimulator of osteoclastogenesis and inadvertently, osteogenesis, and we have established that there is a relationship between microinjury size and cytokine release.

3.6 Conclusion

To conclude, this study has demonstrated, in a set of novel experiments, the potential of microdamage size to affect the principal steps in bone remodeling, using an *in vitro* simulation of this phenomenon, using the constitutive cells of the BMU. The study shows that microinjury is capable of stimulating the migration of osteoprogenitor cells to a site of cellular damage, and subsequent osteoclastogenesis and osteogenesis *in vitro*, the degree of which is also microinjury size dependant. These results furthermore show that microinjury may be the principal mediator of BMU activation, and reiterate the concept that microinjury size is critical in the instigation of bone remodeling.

Chapter 4

Bisphosphonates change the normal response to microinjury but maintain osteoclastogenesis and osteogenesis

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4.1 Introduction

Osteoporosis is a disease of the bone, characterized by a reduction in bone density and low bone mass, resulting the deterioration of the structural microarchitecture of bone, thus increasing the risk of fracture, and is one of the most common of all diseases in bone. Osteoporosis occurs in approximately 8 million women and 2 million men in the United States (WHO, Kong *et al*, 2000). According to the World Heath Organization, osteoporosis can be defined as a pelvic or spinal bone mineral density (BMD), 2.5 standard deviations below the average BMD for young, healthy individuals. Osteoporosis, as described in the introductory chapter, can be divided into 2 separate groups, primary and secondary osteoporosis, based on an age related decline in hormonal release, such as menopause, or a reduction of bone quality as a result of other diseases or medication, respectively. Patients presenting with osteoporosis tend to demonstrate an elevated incidence of microdamage due to the reduction in structural integrity resulting from loss of bone mass, and impaired bone remodeling. Further to this, it has long been suggested that an accumulation of microdamage, such as in osteoporosis, can lead to mechanical failure of bone (Schaffler *et al*, 1989).

Osteoporosis progresses following estrogen depletion, interrupting the normal RANKL-OPG homeostasis in bone, as described in chapter 1. RANKL is released from osteocytes, which binds to RANK on the surface of preosteoclasts, promoting their differentiation, activity and survival (Boyce *et al,* 2008, Kearns *et al*, 2008). OPG, in turn, is regulated by the degree of RANKL-RANK interaction, in addition to estrogen, bone morphogenic proteins and transforming growth factor beta (Boyle *et al*, 2003). Following OPG release, the catabolic effects of RANKL are inhibited, thus stimulating osteoblast infiltration. The function of these cytokines is under biochemical and hormonal control, and homeostasis within these is paramount to maintain balanced bone remodeling (ie: bone is resorbed and formed equally). This, in turn, maintains the mechanical strength and functional capacity of bone. In osteoporotic bone, there is a marked imbalance between bone resorption and bone formation, and excessive bone resorption is observed. Following menopause, or disease and drug treatment (characteristic of primary and secondary osteoporosis, respectively) estrogen levels are reduced or depleted and this can have a

remarkable effect on RANKL-OPG activity. As described above, a reduction in estrogen levels results in reduced OPG, thus RANKL-RANK binding occurs avidly, with no cessation, thus bone resorption exceeds bone formation to a detrimental degree, leading to the presence of fine, fragile struts in cancellous bone, and increased porosity in cortical bone, and a greater potential for bone to acquire fractures.

For the last 40 years, osteoporosis treatment has been focused on bisphosphonates therapy, which has demonstrated great promise regarding their mechanism of action. Bisphosphonates function by inhibiting bone resorption through reducing osteoclastogenesis, decreasing bone turnover, and have also been shown to increase bone mass by increasing the mineral content of bone. Bisphosphonates can be administered orally or intravenously and their binding efficiency to bone depends on the compound itself. Bisphosphonates are synthetic analogues of inorganic pyrophosphate, and consist of two phosphonic acids joined to a carbon (P-C-P), inclusive of two side chains, which may constitute a hydroxyl group, (R2), or another functional group responsible for the pharmacokinetic properties of the bisphosphonate. The phosphate-carbon-phosphate backbone of the bisphosphonate is responsible for facilitating the avid binding to hydroxyapatite crystals on bone surfaces, principally at the sites of bone remodeling (Jin *et al*, 2000, Rogers *et al*, 2003). Following binding to hydroxyapatite, via the P-C-P backbone of the drug, which essentially "hooks" onto hydroxyapatite in bone, nitrogenous bisphosphonates are released from bone, and are phagocytosed by osteoclasts. Bisphosphonates additionally become embedded in bone, following binding of the P-C-P backbone to hydroxyapatite, for a long period of time, prior to release into lacunae generated following osteoclastic bone resorption. As a consequence of this, bisphosphonates can prevail in bone for up to 6 months, and still can be available to inhibit further osteoclast activity (Thompson *et al*, 2006).

The inhibition of bone resorption is pivotal in their function to preserve structural integrity of bone; however this action has been shown to disturb the normal bone remodeling process, resulting in a further accumulation of microdamage and a lack of repair of bone, exacerbating the clinical symptoms of osteoporosis. *In vitro*, it has been demonstrated that osteoblasts differentiate and become mineralized in the presence of

nitrogen containing bisphosphonates, and additionally, it has been shown that conditioned media from osteoblasts cultured in the presence of bisphosphonates has had an inhibitory effect on osteoclastogenesis (Reinholtz *et al*, 2002, Frediani *et al*, 2004, Sahni *et al*, 1993). The result of these actions is that bone becomes stronger and more resistant to fracture (Black *et al*, 1996, Resrud *et al*, 1997, Fleish *et al*, 1987, Harris *et al*, 1999, Boivin *et al*, 2002). This can prove to be a further factor influencing fracture following the treatment of bisphosphonates. However, it has been furthermore suggested, by Mashiba *et al*, 2000, that the increase in microdamage which is observed in bone following treatment with osteoporotic drugs does not affect the mechanical properties of bone, due to the ability of bisphosphonates to increase mechanical strength in bone (Burr *et al*, 1997). Mashiba *et al*, 2001, further demonstrated that, in spite of the increase in microdamage, there was no decrease in mechanical strength in vertebrae following bisphosphonate treatment.

From a biochemical perspective, bisphosphonates have been shown to affect the RANKL-RANK-OPG signaling pathway (Engel *et al*, 2003). Bisphosphonates have been shown to function by increasing upregulation of OPG *in vivo* (Viereck *et al*, 2002), which would decrease RANKL-RANK binding, and thus, osteoclastogenesis, effectively restoring equilibrium within the BMU. Additionally, it has been demonstrated that bisphosphonates may decrease RANKL levels as a result of increased RANKL cleavage by upregulation of tumour necrosis factor-α-converting enzyme (TACE), which is a known RANKL sheddase, among other metalloproteases responsible for RANKL cleavage (Lum *et al*, 1999, Pan *et al*, 2004). The net result of interaction with the RANKL-OPG signaling pathway, in the reduction of RANKL and promotion of OPG, would be a reduced number of osteoclasts and thus, bone resorption, and an increase in the amount of osteoblasts, and thus, bone formation. In bone, osteocytes function as the primary mechanosensory and mechanostimulatory moiety, facilitating signal transduction and controlling biochemical events following a mechanical stimulus. Bisphosphonates have been shown to prevent osteocyte apoptosis though the activation of extracellular signal-related kinases through the opening of connexin 43 channels (Plotkin *et al*, 2001). It was demonstrated by Brennan *et al*, 2011, and Follet *et al*, 2007, that zolendronate and alendronate can prevent osteocyte apoptosis in both ovine and rodent models of osteoporosis.

There is a great amount of contradictory data existing regarding the mechanistic functioning of bisphosphonates, to determine their role both biochemically and physiologically. In this particular chapter, the action of 2 commonly used nitrogenous bisphosphonates, alendronate and zolendronate, was evaluated. Alendronate, or as it is chemically known, 4-amino-1-hydroxybutlidene bisphosphonic acid monosodium (see **Fig. 4.1 A**), is available commercially as Fosamax, and is a nitrogenous bisphosphonate shown to reduce fracture risk and is used to treat the low mineral density associated with osteoporosis. Alendronate has been shown to be 1000 times more effective in reducing fracture risk than other early generation bisphosphonates such as etidronate and ibandronate (Black *et al*, 1996). It has also been shown to reduce the amount of resorption pits at sites of bone remodeling, thus contributing to improved microarchitecture in bone (Seeman *et al*, 1999, Liberman *et al*, 1995, Seeman *et al*, 2007) In addition to this, alendronate functions by inhibiting osteoclastic bone resorption, through the mechanisms described above (Bergstrom *et al*, 2002). Structurally, alendronate contains a four carbon side chain which displays a very high binding affinity to hydroxyapatite, consequently inhibiting bone resorption, following uptake by osteoclasts, and has also been shown to increase bone mass (Schenk *et al*, 1986). Additionally, alendronate has been shown to inhibit the mevalonate pathway, and inhibit prenylation and subsequent Ras, Rho, and Ras activity, thus increasing osteoclast apoptosis and reducing their capacity to resorb bone (Russel *et al*, 2008, Bergstrom *et al*, 2002 Gallacher *et al*, 1989). The reduced resorptive ability of osteoclasts is due again to inhibition of GTPases, at the ruffled border of osteoclasts, preventing attachment to bone (Sato *et al*, 1991).

Zolendronate, or 1-Hydroxy-2-(1H-imidazol-1-yl)-ethylidene bisphosphonic acid, as it is chemically known, is available commercially as Reclast or Zometa; it is another nitrogenous bisphosphonate responsible for reducing bone resorption through inhibition of osteoclasts and by increasing bone mineral density. Zolendronate, like all bisphosphonates, contains a P-C-P backbone which binds to hydroxyapatite on the bone surface; however, its action to do so is more potent than that of alendronate (Fig. 4.1 B). Additionally, its ability to inhibit FPPS is greater than that of alendronate, due to its heterocyclic side chain, which exhibits a greater potency than the presence of an alkyl side chain exhibited by alendronate

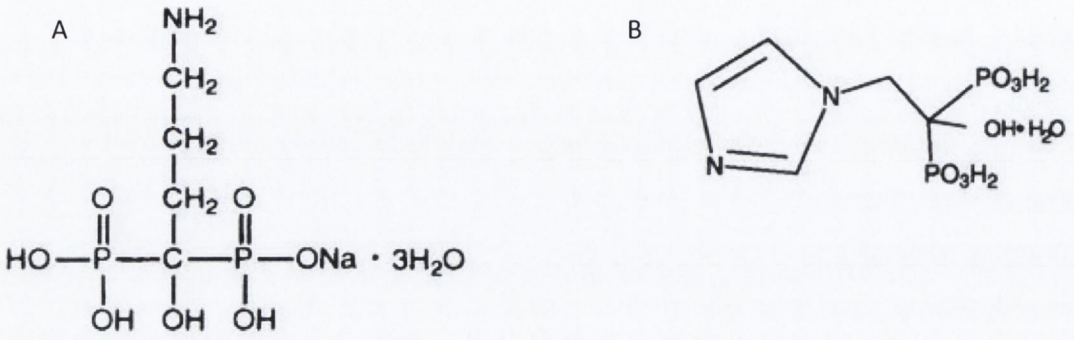


Fig. 4.1 A: The chemical structure of alendronate. Fig. 4.1 B: The chemical structure of zolendronate.

Suppression of the bone remodeling phenomenon, as described, can lead to the accumulation of microdamage in bone. This can prove to be a further factor influencing fracture following the treatment with bisphosphonates. It has been suggested, by Mashiba *et al*, 2000, that an increase in microdamage is observed in bone following treatment with osteoporotic drugs, however, this does not affect the mechanical properties of bone, due to the ability of bisphosphonates to increase mechanical strength in bone (Burr *et al*, 1997). Mashiba *et al*, 2001, further demonstrated that, in spite of the increase in microdamage, there was no decrease in mechanical strength in vertebrae following bisphosphonate treatment. In previous chapters, we have demonstrated the relationship between microinjury size and the rate of RANKL and OPG release, and also in the bone remodeling functions of the constitutive cells of the BMU. These results have been reflective of previous studies by Martin and Burr, and Schaffler, who have demonstrated the role of microdamage in stimulating bone remodeling, *in vivo*. During osteoporosis, a frequent diagnostic symptom is an increase in the presentation of fracture, due to diminished structural architecture of trabecular bone. This is, as described, a result of increased levels of microdamage due to diminished homeostasis in bone remodeling. Considering that microdamage has shown to be fundamental in stimulating the bone remodeling process, it is thus instrumental in maintaining the structural integrity of bone through the remodeling process (Lee *et al*, 2002, Prendergast *et al*, 1994). Therefore, interference with the normal

bone remodeling function, during the treatment with bisphosphonates, would pose a knock on effect in maintaining bone structure. We have demonstrated in chapter 2 and 3 that in our *in vitro* model of microinjury in bone, osteocyte cell apoptosis increases in accordance with damage size, and releases pro-resorptive cytokines including RANKL and OPG (Verborgt *et al*, 2000, Mulcahy *et al*, 2011). The anti-apoptotic effect of bisphosphonates on osteocytes may initially interfere with the release of such biochemical factors. This then may interfere with the stimulation of the BMU, thus demonstrating a knock on effect in the initiation and longevity of bone remodeling.

4.2 Aims of this study

Elevation of microdamage, implicated in osteoporosis, would suggest elevated levels of bone remodeling, however, in osteoporotic bone, this is not so. It has been suggested, as described above, that bisphosphonate treatment reduces RANKL activity, and subsequent osteoclast activity, as a principal function, and it has been shown to increase osteoblast function and proliferation. Given this, we hypothesize that treatment with bisphosphonates may affect the resorptive capacity of osteoclasts following microinjury, in addition to the subsequent osteogenic potential of microdamage in bone. Considering this, the aims and objectives of this study are as follows:

1. To determine the optimal dose of alendronate and zolendronate to maintain osteocyte viability.

2. To determine the microinjury induced cellular response to bisphosphonates, including quantification of RANKL and OPG, using an *in vitro* simulation of microdamage in bone.

3. To determine where in our hypothesized bone remodeling paradigm does the drug induce its action; during microinjury, following microinjury induced osteoclastogenesis, or during the osteogenesis phase of the bone remodeling activities of the constitutive cells of the BMU.

4.3 Methods

4.3.1 Cell Culture

Cell culture materials including α-modified essential medium (α MEM), foetal bovine serum (FBS), calf serum (CS), penicillin/streptomycin, Dulbeccos phosphate buffered saline (1X), Trypsin/EDTA, Dulbeccos modified eagles medium (DMEM), 0.1 N Sodium Hydroxide solution, Trypan Blue stain, Leukocyte acid phosphatase staining kit, sodium citrate, PnPP, sodium chloride, sodium tartarate, Alendronate and B-D Precisionglide 21 and 26 gauge syringe needles were all purchased from Sigma Aldrich Ireland Ltd, Airton Rd, Tallaght, Dublin 24. OptiMEM reduced serum media was purchased from Gibco. Zolendronate was purchased from Alphatech technologies, Blessington, Co. Wicklow, Ireland. Hematoxylin, Eosin and Nuclear Fast Red were purchased from Lennox Laboratory Supplies, Naas rd, Dublin. Matrigel basement membrane matrix and rat tail collagen were purchased from BD Biosciences. Classic Plus acupuncture needles (160 μm, and 400 μm) were purchased from Harmony Medical Distributions Ltd, Leytonstone, London, United Kingdom. Cell culture plastic-ware was purchased from Sarstedt Ltd, Sinnottstown, Wexford, Ireland. MLO-Y4 cells were provided by the Department of Anatomy, Royal College of Surgeons in Ireland, 123 St Stephens Green, Dublin 2.

4.3.2 Cell culture

As described in previous chapters, MLO-Y4 cells were maintained in α Modified Eagles medium (Biosera) supplemented with 5% foetal bovine serum, 5% iron supplemented calf serum and 1% antibiotics (penicillin/ streptomycin). Cells were cultured in collagen coated flasks (0.15 mg/mL rat tail collagen type 1) at 37 °C at 5% CO_2. Preosteoclasts (RAW 264.7 cells, ATCC) were cultured in high glucose Dulbecco's Modified Eagles Medium supplemented with 10% heat inactivated foetal bovine serum and 1% antibiotics (penicillin/ streptomycin). Preosteoblasts (MC3T3-E1 cells, ATCC) were maintained in α Modified Eagles medium supplemented with 10% foetal bovine serum 1% L-glutamine, and 2% antibiotics (penicillin/ streptomycin).

4.3.3 Determining the effect of alendronate and zolendronate treatment on cell viability

In order to study the pharmacological effects of bisphosphonates *in vitro*, it was necessary to determine their effects on cell viability. As osteocytes, in our model, are the intrinsic stimulators of bone remodeling, the effect of both Alendronate and Zolendronate on cell viability on the osteocyte like cell line, MLO-Y4, was determined. Cells were seeded at a density of 2 x 10^4 cells in an adherent 6 well plate, 24 hours prior to experimentation. Alendronate and Zolendronate were dissolved in culture media at concentrations of 10^{-5}M, 10^{-4}M, 10^{-3}M, 10^{-2}M and 10^{-1}M (Naidu *et al*, 2008), at 37 °C, to determine the cellular response to varying concentrations of the respective bisphosphonate, and to establish the optimum dose for maintaining cell viability. After 24 hours, 72 hours, and 7 days in culture, cells were trypsinised and stained with trypan blue, to distinguish between viable and dead cells, and the number of viable cells was counted using a hemocytometer. The dose of respective bisphosphonate to cause the least depletion in cell viability was used as the optimum dose for experimentation. This was used to determine the effects of bisphosphonates on the biochemical and physiological events in the bone remodeling activities, in subsequent experiments, using the constitutive cells of bone and the basic multicellular unit.

4.3.4 Construction of hydrogels seeded with MLO-Y4 cells

As described in chapter 2 (section 2.3.2), MLO-Y4 cells were embedded in a rat tail collagen type I /Matrigel construct (BD Biosciences), to simulate an *in vivo* environment, facilitating cell process network formation. Constructs were prepared as described, and incubated for 1 hour prior to addition of culture media, supplemented with 10^{-5} M treatment of both Alendronate and Zolendronate. They were cultured for 5 days prior to experimentation to facilitate cell process network formation.

4.3.5 Application of microinjury of varying sizes

In order to apply microinjury to the gel embedded MLO-Y4 cells, gels were subjected to microinjury of a set length and width, and diameter of 160 and 400 μm, as described in chapter 2. A single planar defect was created in each culture well by inserting

a needle vertically into the centre of the hydrogel (see **Fig. 2.3**, chapter 2) and drawing it through at a right angle, creating a defect with dimensions of 7 mm in length x 5 mm in depth.

4.3.6 Effects of bisphosphonates on RANKL and OPG activity following microinjury

Gels were incubated using OptiMEM (Invitrogen) serum free media, supplemented with 10^{-5} M concentration of both Alendronate and Zolendronate. Samples were taken at 24 hour intervals over a 72 hour time period. RANKL and OPG were quantified by means of single site specific ELISA (Mouse RANKL and OPG ELISA, R and D systems) under manufacturers' instructions, as described in chapter 2, section 2.3.4. n=3 cultures were assayed at each injury level, in addition to non-injured controls.

4.3.7 Generation of conditioned media from microinjury to an osteocytic network

Conditioned media samples were obtained from gel embedded MLO-Y4 cells treated with the respective bisphosphonates, as described in chapter 3, section 3.3.3. Conditioned media was collected 72 hours after injury, from 12 constructs, and pooled accordingly.

4.3.8 Cell migration of preosteoclasts (Raw 264.7) to conditioned media from osteocytes following microinjury treated with bisphosphonates

In order to assess whether alendronate and zolendronate stimulates preosteoclast migration towards conditioned media from microdamage to an osteocytic network, a cell migration assay was performed, as described in chapter 3, section 3.3.4. This was performed to determine whether the treatment of bisphosphonates, combined with microinjury, initiated the BMU by stimulating an influx of cells. Cell migration was quantified by counting the cells on the bottom side of the membrane in 5 random fields. n = 3 cultures were assayed in each case.

4.3.9 Quantification of TRAP activity in preosteoclasts cultured in conditioned media from bisphosphonate treated osteocytes following microinjury

To determine the effects of bisphosphonates on osteoclastogenesis, Raw 264.7 cells, which were used as a preosteoclastic cell line were seeded at a density of 4 x 10^4 cells/ well in 12 well adherent plates and cultured in standard cell culture media, 24 hours prior to experimentation, as described in chapter 3, section 3.3.6. Conditioned media samples were collected from gel embedded MLO-Y4 cells treated with bisphosphonates, 72 hours following the application of microinjury. Conditioned media was changed every 3 days. At 7 day intervals, TRAP activity was quantified as previously described, in chapter 3, section 3.3.6. n = 3 cultures were assayed in each case.

4.3.10 TRAP staining of preosteoclasts cultured in conditioned media from bisphosphonate treated osteocytes following microinjury

TRAP activity in preosteoclasts was furthermore determined by TRAP staining using a leukocyte acid phosphatase kit, under manufacturer's instructions and the number of TRAP positive multinucleated cells was observed, as described in chapter 3, section 3.3.7. Cells were mounted cell side down on a microscope slide with 200 µl DPX mountant, and allowed to dry before viewing microscopically. Furthermore, the number of TRAP positive multinucleated cells were quantified, from 3 random fields of view, at 20 X magnification, and their frequency expressed at a percentage of cells within the field of view n = 3 cultures were assayed in each case.

4.3.11 Effect of microinjury induced osteoclastogenesis on MC3T3 migration with conditioned media from bisphosphonate treated osteocytes following microinjury

In order to assess whether a stimulatory moiety is released to initiate the migration of osteoblast precursors towards a site of microinjury to osteocytes, treated with bisphosphonates, in the presence of bisphosphonates, a cell migration assay was performed as previously described in chapter 3, section 3.3.9. The aim of this experiment was to determine whether microinjury induced osteoclastogenesis initiated preosteoblast migration towards a chemical (bisphosphonate) and/or osteoclastogenic stimulus. The preosteoblastic cell line, MC3T3- E1 was used in these experiments, and cell migration was quantified by

counting the cells on the bottom side of the membrane. n = 3 cultures were assayed in each case.

4.3.12 Determining the effect of bisphosphonates and microinjury induced osteoclastogenesis on osteogenesis in MC3T3 cells

Preosteoblasts were grown at a density of 4 x 10^4 cells in 6 well plates. Culture media was replaced after a 24 hour incubation and replaced with conditioned media from microinjury induced osteoclastogenesis experiments, treated with 10^{-5}M concentration of the respective bisphosphonate, combined in a 1:1 ratio with cell culture media. Osteogenic media was prepared by the addition of ascorbic acid (50 μg/ml), β-glycerophosphate (10mM) and dexamethasone (100nM) (Sigma-Aldrich, Ireland) was added to standard α-MEM supplemented with 10% FBS, 1% L-glutamine and 2% penicillin/streptomycin, as described in chapter 3, section 3.3.10. Cells were cultured for 21 days under normal conditions and mineralization assays performed at 7 day intervals to determine osteogenesis.

4.3.13 Quantification of calcium activity in preosteoblasts following microinjury induced osteoclastogenesis treated with bisphosphonates

To determine the effect of bisphosphonates and microinjury induced osteoclastogenesis on calcification in preosteoblasts, calcium production was assessed using a StanBio Calcium Liquicolour Kit under manufacturer's instructions, as described in chapter 3, section 3.3.11. n = 3 cultures were assayed in each case.

4.3.14 Alizarin Red Staining in preosteoblasts following incubation with conditioned media from bisphosphonate treated osteocytes following microinjury

Alizarin red staining was used to determine calcium mineralization in preosteoblasts following the addition of conditioned media from bisphosphonate treated microinjury induced osteoclastogenesis experiments, as previously described in chapter 3, section 3.3.12. Calcification was observed histologically using a light microscope.

4.3.15 Alizarin Red Quantification in preosteoblasts following incubation with conditioned media from bisphosphonate treated osteocytes following microinjury

To further quantify the levels of calcium mineralization, alizarin red staining was quantified, as described in chapter 3, section 3.3.13. As before, 100µl of the solution was pipetted into a 96 well plate, and the absorbance read at 540nm.

4.3.16 Statistics

All data was analysed for significance ($p \leq 0.05$) as a function of injury size, drug treatment and time, using two way ANOVA, post hoc Tukey test, to compare means.

4.4 Results

4.4.1 Alendronate and Zolendronate treatment reduce cell viability in MLO-Y4 cells

As depicted in **Fig. 4.2 A & B**, in both treatments, after 7 days, there was a significant decrease in cell viability ($p \leq 0.04$ and $p \leq 0.007$, respectively); however, this occurred to the least extent in respective treatments of 10^{-4} and 10^{-5} M concentrations of bisphosphonate. As a result of this, a 10^{-5} concentration, which was consistent with concentrations suggested in the literature (Naidu *et al*, 2008). of each bisphosphonate was chosen to be the optimum dose to maintain osteocyte viability whilst still stimulating a temporal pharmacological response.

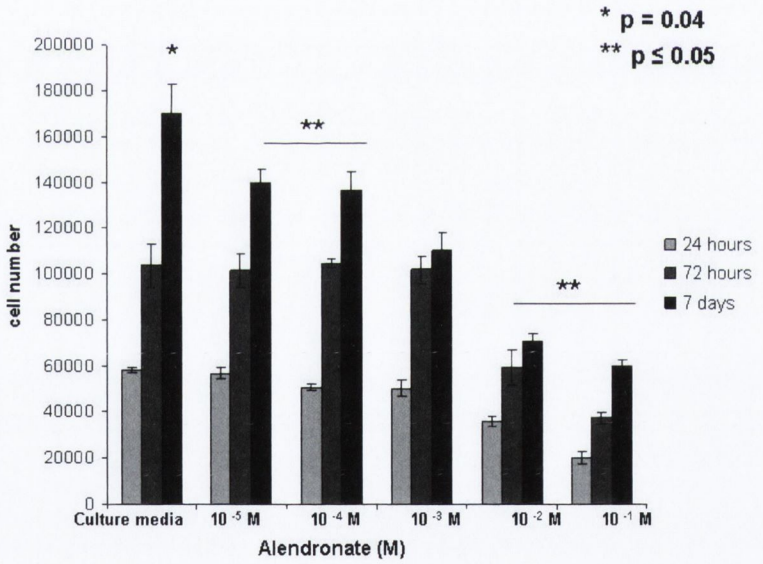


Fig. 4.2 A Alendronate affects cell viability of MLO-Y4 cells: Fig. 4.2A shows the effect of alendronate on cell viability. Following treatment with 10^{-4} and 10^{-5} M there were significantly more viable cells in culture when compared to higher concentrations, and so, a treatment of 10^{-5} M alendronate was chosen as an optimum dose. * is indicative of $p = 0.04$ when comparing non drug treated samples to other samples. ** indicates a significance of $p \leq 0.05$ when comparing between varying concentrations of bisphosphonate.

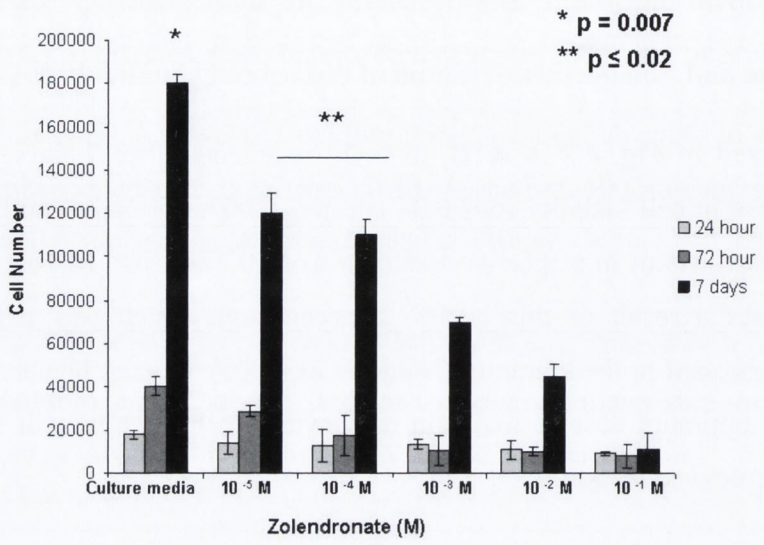


Fig. 4.2 B Zolendronate affects cell viability of MLO-Y4 cells. . At 7 days, there were significantly more at 10^{-4} and 10^{-5} M, when compared to higher concentrations of zolendronate, hence 10^{-5} zolendronate was chosen as the optimum dose. * is indicative of p = 0.007 when comparing non drug treated samples to other samples. ** indicates a significance of $p \leq 0.02$ when comparing between varying concentrations of bisphosphonate. Error bars are indicative of standard deviation, n = 3.

4.4.2 Alendronate (ALN) reduces RANKL and OPG activity, following microinjury to osteocytes.

The effect of osteocytes treated with alendronate following 160 and 400 μm damage, on RANKL and OPG release measured using ELISA is demonstrated in **Fig. 4.3 A& B** shows that, following treatment with alendronate, there was a significant decrease ($p \leq 0.009$) in the release of RANKL in the undamaged, 160 and 400 μm samples, when compared to the group cultured in standard growth media. However, no significant difference was observed between any of the microinjury groups treated with alendronate, regardless of injury size, indicting almost full suppression of RANKL activity following alendronate treatment. OPG release, as depicted in **Fig. 4.3 B**, showed again to be suppressed following alendronate treatment, with a significant decrease ($p \leq 0.001$) in activity noted in all samples when compared to samples cultured in standard growth media. OPG release was greatest in all non drug treated groups compared to 400 μm samples.

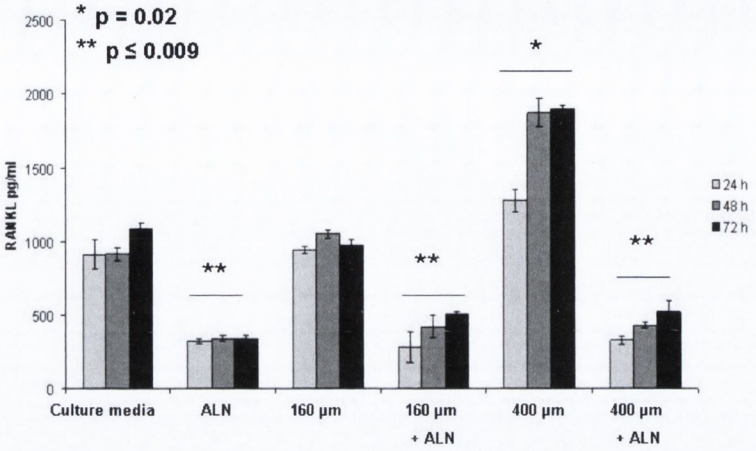


Fig 4.3 A Alendronate (ALN) reduces RANKL activity, following microdamage. The effects of alendronate treatment on RANKL release from 3D gel embedded MLO-Y4 cells, subjected to 160 and 400 µm microinjury were determined by ELISA, as depicted. In non drug treated samples, both damaged and undamaged, there was a significant increase in RANKL release at 400 µm, at all time points. Following treatment with alendronate, there was a significant decrease in RANKL activity in all samples when compared to their non drug treated counterparts. * is indicative of p = 0.02 when comparing 400 µm non drug treated samples to other non drug treated samples. ** indicates a significance of $p \leq 0.009$ when comparing all injury sizes treated with alendronate to non drug treated samples.

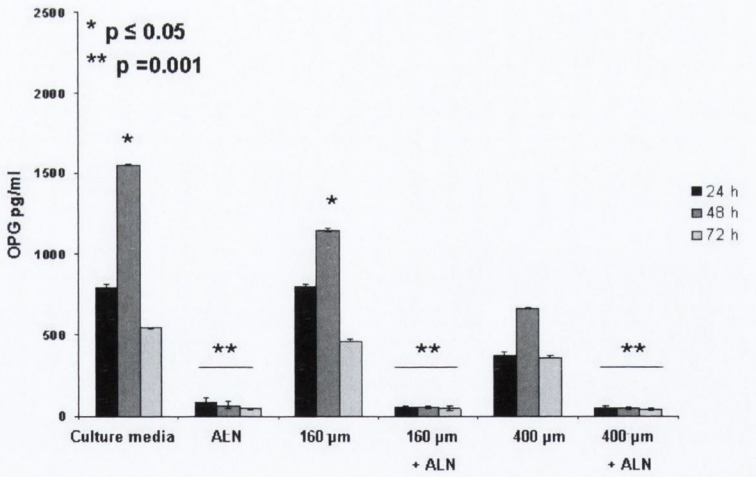


Fig. 4.3 B Alendronate (ALN) reduces OPG activity, following microinjury. Fig. 4.3 B shows the effect of alendronate on OPG activity. There was significantly less OPG released in all samples, both damaged and undamaged, following treatment with alendronate, versus the untreated controls. * is indicative of $p = \leq 0.05$ when comparing 400 µm non drug treated samples to other non drug treated samples. ** indicates a significance of $p \leq 0.001$ when comparing all injury sizes treated with alendronate to non drug treated samples. Error bars are indicative of standard deviation, n = 3.

4.4.3 Zolendronate (ZOL) reduces RANKL and OPG activity, following microinjury to osteocytes.

Treatment of gel embedded osteocytes with zolendronate and subsequent RANKL and OPG activity can be observed in **Fig. 4.4 A & B**. **Fig. 4.4** A demonstrates the effect of 160 and 400 μm microinjury on RANKL release as measured by ELISA. Results show, that in the zolendronate treated group, there was a significant decrease ($p \leq 0.05$) in the release of RANKL in the undamaged, 160 and 400 μm samples, when compared to the non drug treated group, again, with almost a full suppression of RANKL activity being observed, albeit to a lesser extent than what was observed following alendronate treatment. As depicted by **Fig. 4.4 B**, OPG release was greatest in all non drug treated groups compared to 400 μm samples, similar to that observed following alendronate treatment. In the bisphosphonate treated groups again, there was a significant depletion of OPG activity ($p \leq 0.008$), with no significant difference observed between the undamaged and damaged samples.

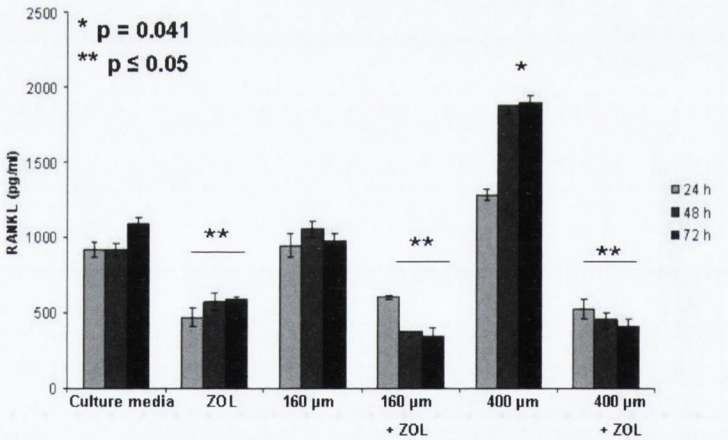


Fig. 4.4 A Zolendronate (ZOL) suppresses RANKL activity, following microinjury. The effects of zolendronate treatment on RANKL release from 3D gel embedded MLO-Y4 cells, subjected to 160 and 400 μm microinjury was determined by ELISA, as depicted. In non drug treated samples, both damaged and undamaged, there was a significant increase in RANKL release at 400 μm, at all time points. Following treatment with zolendronate, there was a significant decrease in RANKL activity in all samples when compared to their non drug treated counterparts. * is indicative of $p = 0.02$ when comparing 400 μm non drug treated samples to other non drug treated samples. ** indicates a significance of $p \leq 0.009$ when comparing all injury sizes treated with zolendronate to non drug treated samples.

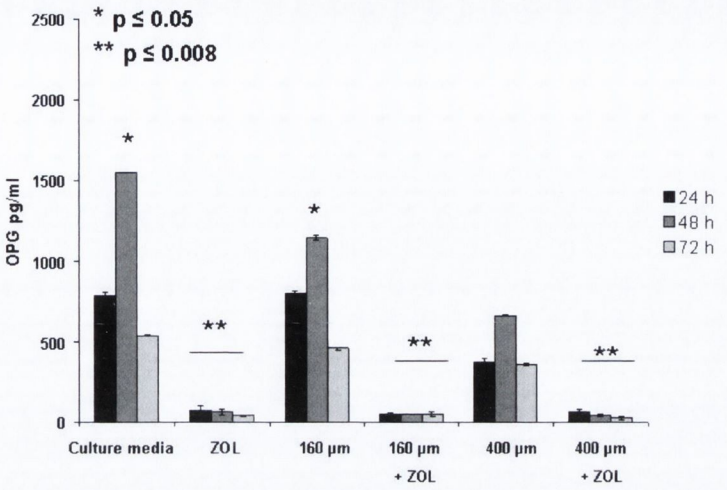


Fig 4.4 B: Zolendronate (ZOL) suppresses OPG activity, following microinjury. There was significantly more OPG released in undamaged and 160 µm samples without zolendronate, however, there was significantly less OPG released in all samples, both damaged and undamaged, following treatment with zolendronate. * is indicative of $p = \leq 0.05$ when comparing 400 µm non drug treated samples to other non drug treated samples. ** indicates a significance of $p \leq 0.001$ when comparing all injury sizes treated with zolendronate to non drug treated samples. Error bars are indicative of standard deviation, n = 3.

When considering each set of results from both alendronate and zolendronate, this section of the study has demonstrated that bisphosphonate treatment reverses the cytokine response of what is normally observed in an *in vitro* simulation of microinjury in bone, and shows that treatment with bisphosphonates affects the cells normal response to damage.

4.4.4 Alendronate and zolendronate reduce preosteoclast migration following microinjury to osteocytes.

To determine the effect of alendronate and zolendronate on preosteoclast migration towards a stimulus, i.e.; conditioned media from bisphosphonate treated microinjury to osteocytes; the number of cells which passed through the membrane of a modified Boyden chamber was quantified. As **Fig. 4.5** demonstrated, in the non drug treated groups (cells stimulated with culture media), a significant increase ($p \leq 0.005$) in cell migration was observed in all samples, 72 hours after damage, and this influx of cells was largely influenced by an increase in damage size, i.e. migration at 400 µm was

significantly greater than at 160 μm ($p \leq 0.05$). No significant difference ($p \leq 0.08$) was seen between undamaged or 160 μm samples. Following treatment with alendronate, there was a significant decrease ($p \leq 0.05$) in preosteoclast migration at 160 μm and the 400 μm groups. Zolendronate treatment suppressed preosteoclast migration; however, this was, although significant, less potent at suppressing preosteoclast migration when compared to alendronate treated cells. These results show that bisphosphonates reduce preosteoclast infiltration towards a microinjury induced stimulus.

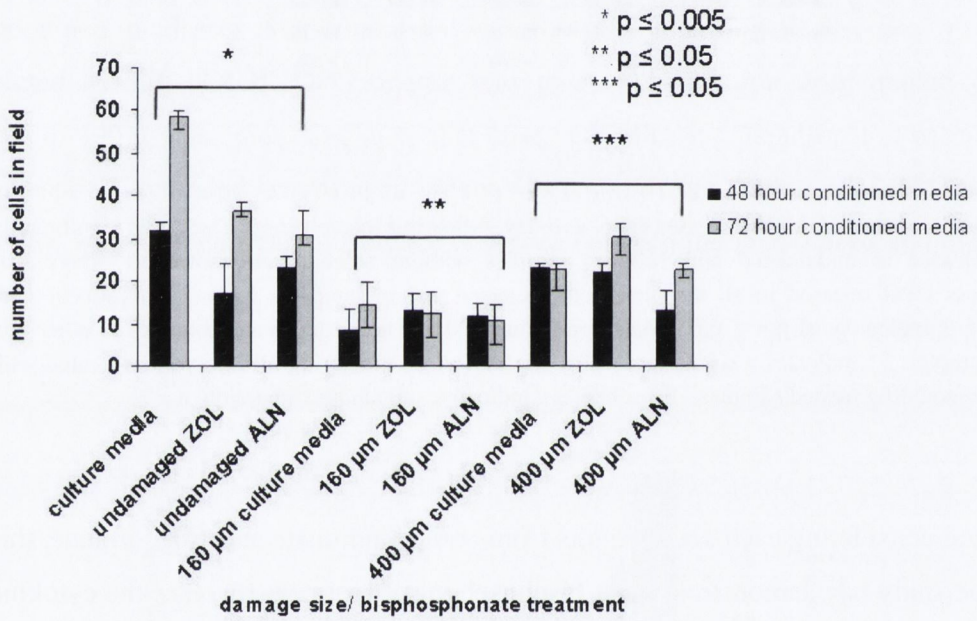


Figure 4.5 Alendronate and zolendronate reduce preosteoclast migration towards a microinjury induced stimulus. In standard culture media, there was a significant increase in preosteoclast migration when compared to culture media treated with alendronate and zolendronate. At 160 μm, there was no significant difference in the preosteoclast migration between non drug treated and bisphosphonates treated groups, however, at 400 μm, a significant increase was observed in all samples, however, this was suppressed by alendronate treatment, where a decrease in cell migration was observed. * is indicative of $p \leq 0.001$ when comparing undamaged samples to other non drug treated samples. ** indicates a significance of $p \leq 0.005$ when comparing samples treated with bisphosphonate to all other samples. *** indicates $p \leq 0.08$, when comparing all time points and treatments at 160 μm, which is significantly less than what is observed at 400 μm. Error bars are indicative of standard deviation, n = 3.

4.4.5 TRAP activity is suppressed in preosteoclasts treated with conditioned media from microinjury to gel embedded osteocytes treated with bisphosphonates

To determine the effect of alendronate and zolendronate following microinjury, TRAP activity was quantified in preosteoclasts as depicted in **fig .4.6**. 72 hours after the application of injury, conditioned media was added to preosteoclasts, and following a 21 day incubation, TRAP activity increased significantly ($p \leq 0.005$) in the damaged (400 μm) samples without drug treatment, when compared to the preosteoclast treated with conditioned media without damage. In the day 14 and day 21 samples, a significant increase in TRAP activity was observed in 400 μm samples. A significant decrease ($p \leq 0.01$) in TRAP activity was observed in the presence of both alendronate and zolendronate, regardless of the presence of microinjury, however, the decrease in TRAP activity was significantly more evident in undamaged samples, at day 14 and day 21. These results demonstrate the inhibitory effect of both alendronate and zolendronate on the now established osteoclastogenic potential of microinjury in bone.

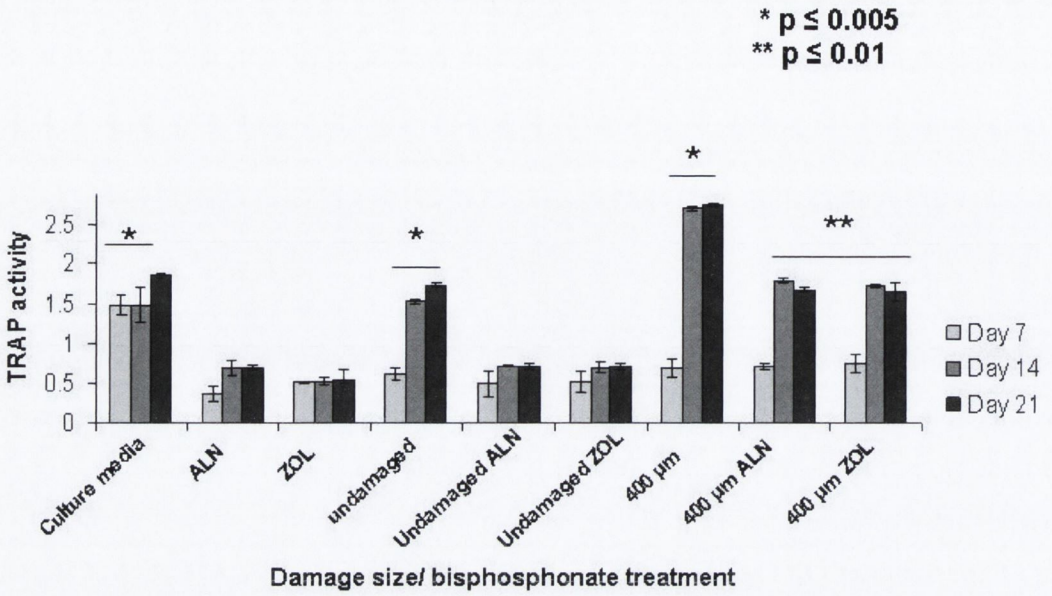


Fig. 4.6 TRAP activity is suppressed in preosteoclasts treated with conditioned media from microinjury to gel embedded MLO-Y4 cells. A significant increase in TRAP activity was noted in non drug treated 400 µm at 14 and 21 days when compared to undamaged samples at day 14 and 21. Release of TRAP was significantly less in samples treated with both alendronate and zolendronate at day 14 and 21 when compared to non drug treated samples. However, it must be noted that TRAP activity increased significantly in 400 µm even in the presence of microinjury, when compared to undamaged samples, however, this was still significantly less than non drug treated samples in this group. * denotes $p \leq 0.005$ when non drug treated to bisphosphonate treated groups. ** denotes $p \leq 0.01$ when comparing 400 µm bisphosphonate treated samples to other treated samples. Error bars are indicative of standard deviation, n = 3.

4.4.6 The number of TRAP positive cells is reduced by bisphosphonate treatment following microinjury to an osteocytic network

The effect of the osteoclastogenic potential of microinjury following bisphosphonate treatment was determined by TRAP staining of preosteoclasts at 7 day intervals over a 21 day period following treatment with conditioned media. **Fig. 4.7** shows TRAP staining results at 10 X magnification. Cells were cultured in conditioned media from undamaged and damaged gel embedded MLO-Y4 cells treated with alendronate and zolendronate, to determine their effects on the promotion of osteoclastogenesis at varying injury size. The morphology of the undamaged drug treated cells was markedly different to those of damaged cell populations, suggesting that what is released from the 400 µm

damage has an effect on osteoclast viability. The presence of large multinucleated cells, however, was greatest in 400 µm samples when compared to control samples at all time points without bisphosphonate treatment, however, following treatment with both alendronate and zolendronate, a marked decrease in the number of large, osteoclast like cells was observed at all time points, however, there were more osteoclastic cells at 400 µm treated with both alendronate and zolendronate when compared to smaller damage and undamaged samples. Also, it must be noted that after 21 days, there was a reduction in cell number in bisphosphonate treated samples, indicating increase in cellular apoptosis or necrosis following treatment.

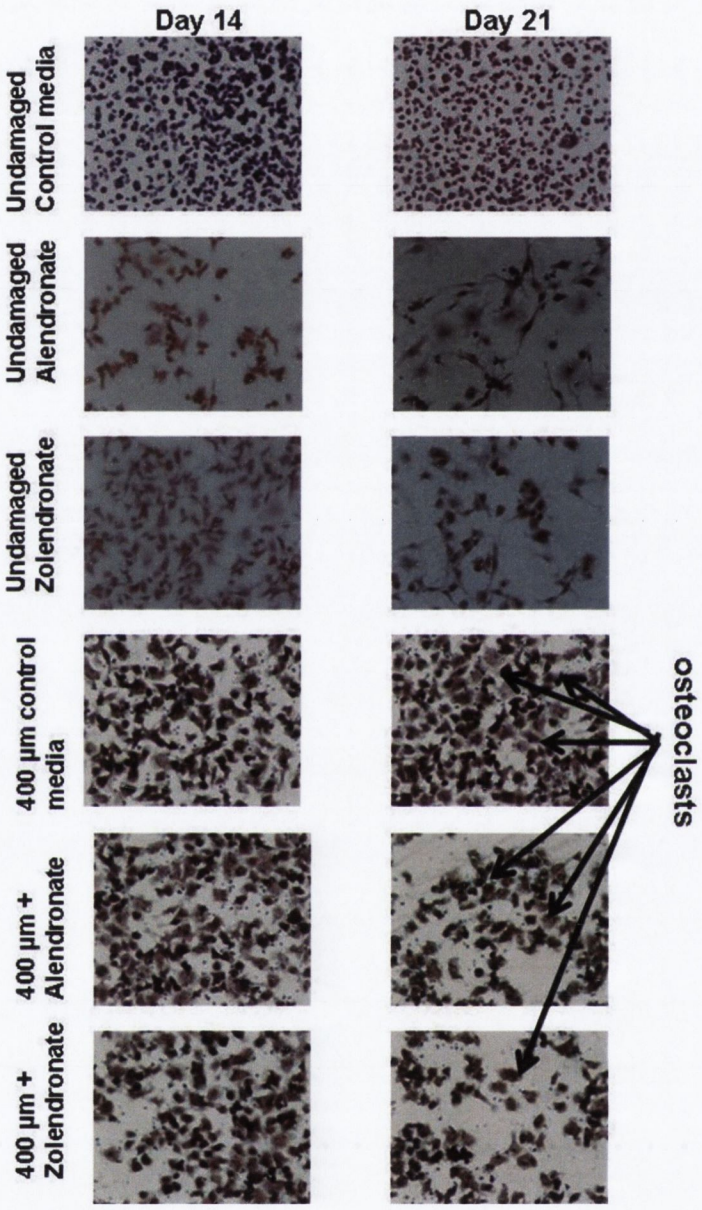


Fig. 4.7 The number of TRAP positive cells is reduced following treatment with conditioned media from microinjury to an osteocytic network with bisphosphonate treatment. This figure shows TRAP staining results at 10 X magnification. The presence of large multinucleated cells was greatest in 400 µm samples when compared to 160 µm and control samples at all time points without bisphosphonate treatment, however, following treatment with both alendronate and zolendronate, a marked decrease in the number of large, osteoclast like cells was observed at all time points, however, there were more osteoclastic cells at 400 µm treated with both alendronate and zolendronate when compared to smaller damage and undamaged samples.

Additionally, the number of TRAP positive cells was quantified and expressed as a percentage of the total cell number within the field of view. Again, a significant increase ($p \leq 0.01$) in large, TRAP positive, multinucleated cells was observed at 400 µm non drug treated samples compared to other non drug treated samples, however, treatment with alendronate and zolendronate caused a significant decrease in the number of TRAP positive cells, however, this reduction was observed to a lesser extent in 400 µm samples as can be observed in **Fig. 4.8**.

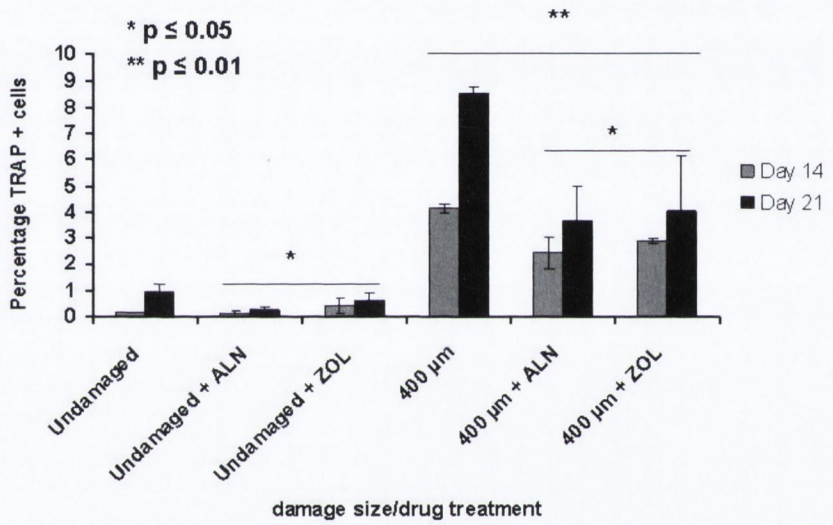


Fig 4.8 The number of TRAP positive cells following microinjury to an osteocytic network is reduced by bisphosphonate treatment. **Fig 4.8** shows the number of TRAP positive cells quantified, expressed as a percentage of the total cell number within the field of view. Treatment with bisphosphonate caused a significant decrease in the number of positive, multinucleated cells in undamaged and all damage sizes, however, this decline was significantly less in 400 µm samples treated with bisphosphonates, compared to other samples.* denotes $p \leq 0.05$ when comparing between bisphosphonate treatment and non drug treated samples at a given damage size. ** denotes $p \leq 0.05$ when comparing non drug treated 400 µm damage size to other undamaged samples. Error bars are indicative of standard deviation, n = 3.

4.4.7 Migration of preosteoblasts to conditioned media from microinjury induced osteoclastogenesis is increased after bisphosphonate treatment

Similarly to preosteoclast recruitment, preosteoblasts infiltrate tissue and constitute a functional unit of the BMU. As described above (section 4.3.11), preosteoblasts were seeded on membranes of a modified Boyden chamber and their

migration determined by quantifying the amount of cells which passed through the membrane towards conditioned media from microinjury induced osteoclastogenesis treated with alendronate and zolendronate. As shown in **Fig. 4.9**, the number of preosteoblasts having passed through the membrane increased according to injury size. In the absence of alendronate and zolendronate, a significant increase in cell migration was observed in osteoblasts stimulated with conditioned media from microinjury induced osteoclastogenesis when comparing undamaged and 400 μm samples. Treatment with alendronate significantly increased preosteoblast migration ($p \leq 0.05$), which was also observed following zolendronate treatment. However, in the 400 μm samples, a highly significant increase ($p \leq 0.01$) in preosteoblast migration was observed following treatment with zolendronate, however, this pattern was not consistent following treatment with alendronate where migration of cells was significantly less.

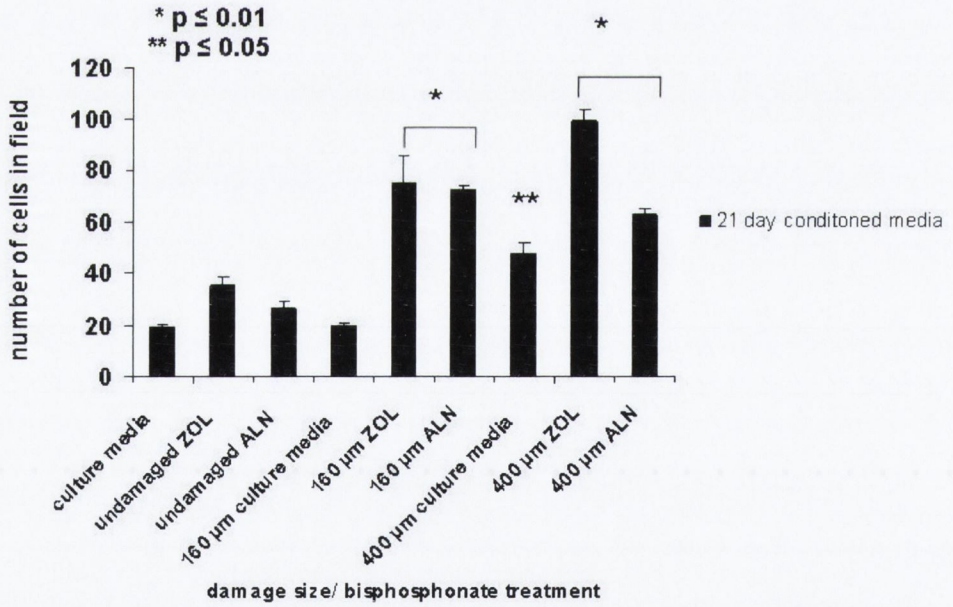


Fig. 4.9 Migration of preosteoblasts to conditioned media from microinjury induced osteoclastogenesis is increased after bisphosphonate treatment. A significant increase in cell migration was noted at 400 μm with and without bisphosphonate treatment, when compared to other samples, however, a significant increase in preosteoblast migration occurred in both 160 and 400 μm samples treated with both alendronate and zolendronate, when compared to non drug treated conditioned media from microinjury induced osteoclastogenesis experiments. * denotes $p \leq 0.01$ when comparing between bisphosphonate treatment and non drug treated samples at a given damage size. ** denotes $p \leq 0.05$ when comparing non drug treated 400 μm damage size to other non drug treated samples. Error bars are indicative of standard deviation, n = 3.

4.4.8 Calcium mineralisation in preosteoblastic cells treated with conditioned media from induced osteoclastogenesis increases following bisphosphonate treatment.

Alendronate and zolendronate have been shown to increase mineral mass in bone; however, the mechanisms dictating this action are currently unknown. Much controversy surrounds whether this is a cellular behaviour or due to a reduction in osteoclast mediated bone resorption. This assay aimed to demonstrate the osteogenic potential of alendronate and zolendronate on the maturation and differentiation of preosteoblasts into osteoblasts, and their subsequent mineralisation, following treatment with conditioned media from bisphosphonate treated microinjury induced osteoclastogenesis. This was to determine whether bisphosphonate treatment also affected the osteogenic response to microinjury. This was demonstrated by quantifying the presence of calcium over a 21 day time period in the preosteoblastic cell line, MC3T3-E1, as depicted in **Fig. 4.10**.

There was significantly more calcium present ($p \leq 0.05$) in preosteoblastic cells cultured in osteogenic media, and 10^{-5} M concentration alendronate and zolendronate, when compared with their counterparts cultured in standard cell media at all time points. However, there was significantly more calcification in all samples treated with both alendronate and zolendronate ($p \leq 0.01$), including those which were undamaged, with bisphosphonate treatment, when compared the undamaged samples without drug treatment, at day 21. A significant increase in calcium mineralisation was observed at 400 μm ($p \leq 0.05$), in both alendronate and zolendronate treated samples, suggesting that increased microinjury size, or elevated levels of microinjury, when combined with bisphosphonates, increases calcification in osteoblasts. These results demonstrate that alendronate and zolendronate, also when combined with microinjury, cause an increase in calcium mineralisation.

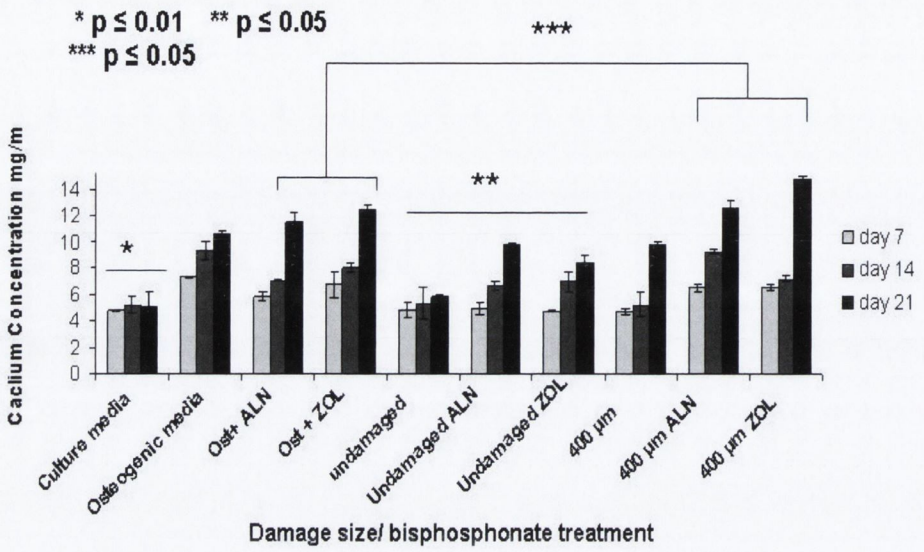


Fig. 4.10 Calcium mineralisation in preosteoblastic cells treated with conditioned media from induced osteoclastogenesis increases following bisphosphonate treatment. Calcium mineralisation was significantly less in MC3TC cells cultured in standard cell media when compared to both alendronate and zolendronate treated groups at day 14 and 21, the exception being cells cultured in osteogenic media. However a significant increase was in 400 µm samples without osteogenic media or bisphosphonate treatment at day 21, when compared to other non drug treated counterparts. Bisphosphonate treatment caused a significant increase in calcium mineralisation in 400 µm samples when compared to cells cultured in non drug treated conditioned media. * denotes $p \leq 0.05$ when comparing cells in standard culture media, without serum, and undamaged samples without bisphosphonates to other samples. ** denotes $p \leq 0.05$ when comparing significance between undamaged samples with bisphosphonate treatment to samples with osteogenic media and 400 µm *** denotes $p \leq 0.01$ when comparing significance between osteogenic media and bisphosphonate treated groups and 400 µm bisphosphonate treated groups to other samples. **** denotes $p \leq 0.05$, when comparing between 400 µm damage samples with bisphosphonates and osteogenic media samples with bisphosphonates. Error bars are indicative of standard deviation, n = 3.

The degree of calcium mineralisation was furthermore demonstrated through histological staining, as demonstrated by **Fig. 4.11 A** and **B**, and its related quantification of the staining, as depicted in **Fig. 4.11 C**. Alizarin red staining produces a bright red precipitate on cells, indicative of calcium mineralisation, and following staining, this precipitate can be removed and quantified through spectroscopy. Images show differentiated and mineralised preosteoblasts at 10 X magnification, which demonstrated the potential of alendronate (**Fig. 4.11 A**) and zolendronate (**Fig. 4.11 B**) to mineralise preosteoblasts the addition of conditioned media from microinjury induced osteoclastogenesis. As shown, at day 14, mineralisation was observed in all samples, with

the greatest quantity of calcium mineralisation being observed in 400 μm samples in the presence of alendronate. At day 21, extensive mineralisation was observed in all samples, and again, was most abundant in 400 μm inclusive of bisphosphonate treatment. Again, as depicted in the histological data, at day 14, mineralisation was observed in all samples, with higher calcium mineralisation being observed in 400 μm samples in the presence of zolendronate. At day 21, extensive mineralisation was observed in all samples, and again, was most abundant in 400 μm inclusive of bisphosphonate treatment.

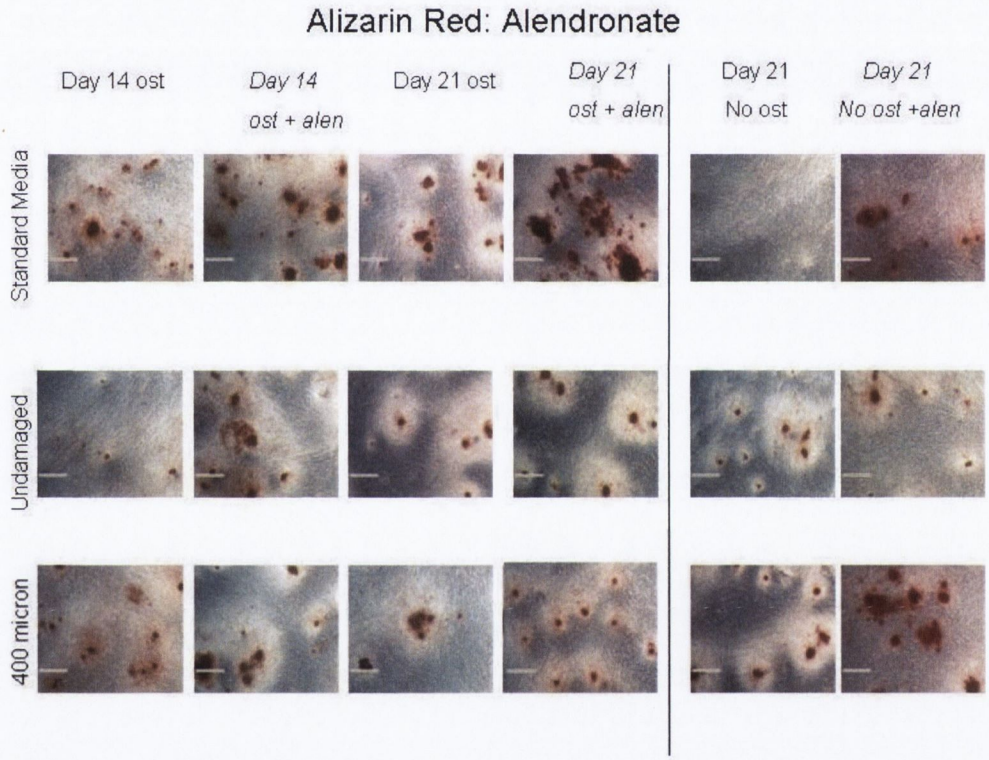


Fig. 4.11 A Calcium mineralisation was stimulated by alendronate in preosteoblasts treated with conditioned media from microinjury induced osteoclastogenesis. Calcium mineralisation was observed in all samples with and without osteogenic media (ost), with the greatest quantity of mineralisation being observed 400 μm samples across the board, also with and without bisphosphonate treatment. Calcium mineralisation was most abundant in 400 μm samples without osteogenic media at day 21 in the presence of alendronate. Scale bar shown in images = 200 μm.



Fig. 4.11 B Calcium mineralisation was stimulated by zolendronate in preosteoblasts treated with conditioned media from microinjury induced osteoclastogenesis. Calcium mineralisation was observed in all samples with and without osteogenic media (ost), with the greatest quantity of mineralisation being observed 400 µm samples across the board, also with and without bisphosphonate treatment. Calcium mineralisation was most abundant in 400 µm samples without osteogenic media at day 21 in the presence of zolendronate. Scale bar shown in images = 200 µm.

These results are depicted graphically, as can be observed in **Fig. 4.11 C** , where by quantification of alizarin red shows a similar trend to that of the calcium assay, whereby the presence of alendronate and zolendronate stimulated a significantly greater frequency of calcium mineralisation at 400 µm samples ($p \leq 0.05$) , again most significantly at day 21. No significant difference, however, was observed between alendronate and zolendronate samples at days 14 and 21 ($p \leq 0.07$).

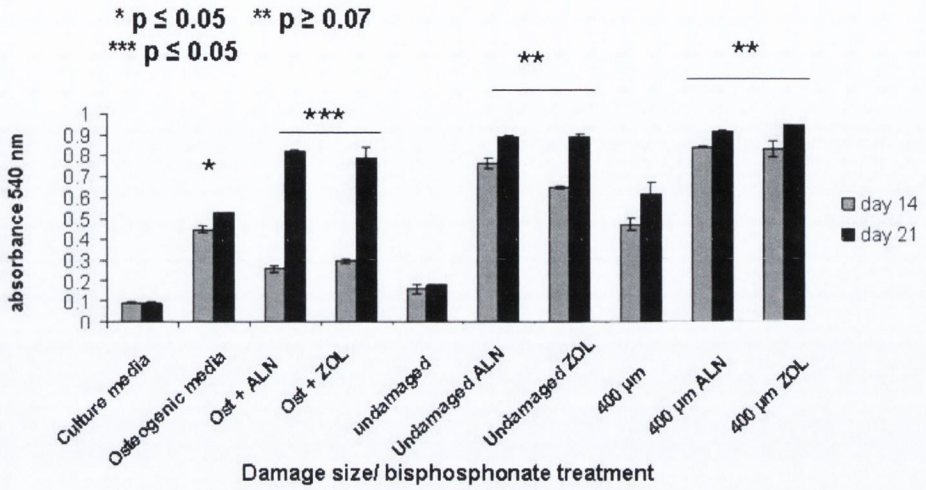


Fig. 4.11 C Calcium mineralisation was stimulated by bisphosphonates in preosteoblasts treated with conditioned media from microinjury induced osteoclastogenesis. This graph shows show that in the absence of a cellular stimulus, the quantity of alizarin red was significantly greater in all samples treated with both alendronate and zolendronate. No significance was noted, however between undamaged and 400 µm samples, following bisphosphonate treatment, at day 21 most. Additionally, a significant increase in calcification noted in non drug treated 400 µm samples without osteogenic media, when compared to other samples without osteogenic media. * indicates significance when comparing osteogenic media to standard culture media. ** denotes $p \leq 0.07$ when comparing drug treated samples to non drug treated samples in undamaged and 400 µm samples. *** denotes $p \leq 0.05$ when bisphosphonate treated controls to both osteogenic and standard culture media. Error bars are indicative of standard deviation, n = 3.

4.5 Discussion

For over forty years, bisphosphonates have been shown to assist in the treatment of bone diseases including osteoporosis, osteopenia, metastatic bone disease and Paget's disease through inhibition of osteoclast activity and inducing osteoclast apoptosis, in addition to increasing bone mass (Fleisch *et al*, 1968). In spite of the recognised attributes of bisphosphonates, the effects and mechanisms by which they function, is relatively unclear (Kimachi *et al*, 2011). Khosa, *et al*, 2007, initially suggested the mechanisms of action of bisphosphonates require greater investigation, to determine the exact cellular response, and this concept, coupled with the elevated incidences of microdamage in osteoporotic bone, was the backbone to this particular study. Bearing this in mind, this study aimed to investigate the stimulatory potential of two commonly used aminobisphosphonates, alendronate and zolendronate, on the biochemical factors involved in bone remodeling, such as RANKL and OPG activity, in addition to determining the cellular response to bisphosphonates in the constitutive cells of the BMU, in our previously established *in vitro* simulation of microdamage in bone.

In previous chapters, we reported that an inverse relationship between RANKL and OPG activity exists, and also that microinjury size has been implicated in determining the rate of osteoclastogenesis and osteogenesis. Results of this study have shown that microdamage size has an impact on the rate of osteoclastogenesis and osteogenesis in the respective cells of the BMU. An accumulation of microdamage has been implicated as one of the predisposing factors in increasing the risk of osteoporotic fractures (Arlot *et al*, 2008, Vashisth *et al*, 1997, Fazallari *et al*, 1998). These result from the reduced capacity of bone to initiate bone remodeling, or a lack of homeostasis in bone resorption and subsequent formation, resulting in fragile bone and diminished structural architecture (Burr *et al*, 1997). In turn, the greater propensity of bone to become predisposed to microdamage results from altered morphology and increased trabecular fragility, thus, the more brittle the bone, the more likely it is to experience microdamage. Considering the role of microdamage and the impact of microdamage of given size in osteoporosis, we developed the hypothesis of the present study. This was that the effect of microinjury, which has been shown to induce both osteoclastogenic and osteogenic responses, would be affected by the

presence of bisphosphonates. Additionally, bisphosphonate treatment of an *in vitro* model of microdamage may present variations in the degree of precursor cell migration, and may decrease osteoclastogenesis, and ultimately, increase the levels of osteogenesis, in the constitutive cells of the BMU, and may have an effect on the activity of RANKL and OPG. The biochemical effects of alendronate and zolendronate have been quite well documented *in vitro*, and it is known that they affect RANKL and OPG upregulation, release and activity. Furthermore, their effects on osteoclast activity have been determined, however, in a situation where microdamage is elevated, e.g., in osteoporotic bone, the effects of these drugs on biochemical and cellular activity have yet to be determined (Mackie *et al*, 2001, Glorieux *et al*, 1998).

In previous chapters, we investigated the role of a broader spectrum of injury sizes; however, in this study we used two needle sizes, 160 and 400 μm, the larger of which showed an overall significant difference regarding the impact of injury size on RANK/OPG activity, and the cellular responses to microinjury. At 24 hour intervals over a 72 hour incubation period, in the presence of both alendronate and zolendronate, cell culture supernatants were analysed for RANKL and OPG activity. As a control, non drug treated, damaged samples were used to compare to drug treatments. After 72 hours, there was a significant increase in RANKL activity, which also occurred in conjunction with an increase in damage size, and an inverse relationship was observed in the release of OPG, which is a behaviour which we have observed previously (Mulcahy *et al*, 2011). However, in both drug treated groups, most significantly alendronate, there was a two fold decrease in RANKL activity following treatment with alendronate in the undamaged and 160 μm samples, and a three fold decrease in the 400 μm groups.

A similar pattern was observed in zolendronate treatment; however, this occurred to a lesser extent. In both drug treatments, an almost entire elimination of OPG was observed, with up to a sixteen fold decrease in the undamaged group at 48 hours, which, under normal circumstances, would be the within the parameters optimising OPG release. This almost abolishment of OPG activity may be due to the marked reduction in RANKL activity- it is established that RANKL release closely precedes OPG release, and so, any disturbance in this signaling may produce a knock-on effect on any downstream

biochemical activity. Ablation of RANKL activity following alendronate and zolendronate treatment is consistent with previous studies, which have been shown, *in vitro*, that RANKL activity is substantially inhibited by treatment with aminobisphosphonates (Kimachi, *et al*, 2011, Giner *et al*, 2011), in osteoclasts and osteoblasts, respectively. However, Koch *et al*, 2010, demonstrated the stimulatory effect of alendronate and zolendronate on RANKL and OPG expression in osteoblasts. This study, however, demonstrated results via PCR and quantified gene expression, whereas in this model, we were describing RANKL and OPG protein release from cells. In this model of microinjury in bone, we assessed the effects of bisphosphonates following microinjury to a 3 dimensional osteocytic cell process network, and so, the results of this section of the study may be significant in demonstrating the fundamental biochemical activity (i.e.: RANKL and OPG activity), following treatment with bisphosphonate. From the perspective of OPG activity, very little work has been completed to date, however, previous studies using 2 dimensional osteoblast cultures have suggested OPG gene expression is not affected by bisphosphonate treatment (Koch *et al*, 2010, Enjuanes *et al*, 2009).

To determine where in the microinjury / bone remodeling paradigm bisphosphonates function in an *in vitro* model of microinjury in bone, it was necessary to determine the effects of alendronate and zolendronate on precursor cell recruitment, in addition to osteoclastogenesis and osteogenesis. Activation and recruitment refers to the stage by which an influx of osteoclast precursor cells infiltrate the area of osteocyte rupture and fuse to form giant, active osteoclasts (Mulari *et al*,2004, Parfitt *et al*, 1983). Treatment of osteoporosis with bisphosphonates, in particular alendronate and zolendronate, has been shown to pose an inhibitory effect on this stage of bone remodeling (Boonekamp *et al*, 1986). Additionally, it was recently demonstrated that zolendronate suppresses osteoclast migration through inhibition of the mevalonic pathway, which is responsible for maintaining osteoclast viability and activity through the presence of the key enzyme, farnesyl pyrophosphate synthase (FPPS) in the 3-hydroxy-3 methylglutaryl co enzyme A reductase pathway (Kimachi *et al*, 2011). In this study, preosteoclast migration towards a stimulus elicited by microinjury to osteoclasts treated with bisphosphonates was assessed. A reduction in quantity of preosteoclasts migrating was observed in all samples treated with each respective bisphosphonate;

however, this migration was significantly less in samples treated with zolendronate at 160 and 400 μm. This suggests that the presence of the bisphosphonate reduces the potential of preosteoclasts to migrate towards a stimulus, which is reflective of previous studies which demonstrated a similar response, albeit in the absence of microdamage (Kimachi *et al*, 2011, Stevenson *et al*, 1986, Li *et al*, 2011).

Recruitment of preosteoclasts precedes the fusion and maturation of precursor cells, to osteocytes, which break down bone (Katagiri *et al*, 2001). A significant decrease in TRAP activity was observed in all samples treated with bisphosphonate, albeit to a lesser extent in 400 μm samples, when compared to osteoclasts cultured in the absence of bisphosphonate. Additionally, histological results showed a decrease in the number of TRAP positive multinucleated osteoclastic cells, the number of which, when furthermore quantified, greatly reflected the results of TRAP quantification. This may be due to two reasons. Firstly, the reduced capacity of osteocytes to release RANKL, as stimulated by bisphosphonates, following microinjury, would certainly reduce the osteoclastic potential of microinjury. Secondly, it has been established that bisphosphonates interrupt the release of FPPS, a key enzyme in the mevalonic acid pathway (Hughes *et al*, 1995, Rogers *et al*, 2000, Caraglia *et al*, 2004).

The concept that TRAP activity, although suppressed, was still greater at 400 μm, suggests that the increased levels of RANKL, among other factors being released, such as TNF-α, which has been implicated as having an equally potent osteoclastic potential as RANKL are, indeed hampered by the effects of bisphosphonates, however, may still be great enough to elicit an osteoclastogenic response (Kimachi *et al*, 2011, Li *et al*, 2000). It has previously been demonstrated, *in vivo*, that alendronate is less potent at inhibiting osteoclast activity than zolendronate. The results of this study may suggest that, *in vitro*, bisphosphonate treatment affects the ability of preosteoclasts to migrate and differentiate. This potentially may occur through the inhibition of the mevalonate pathway, inducing cellular apoptosis, regardless of the reported potency of the bisphosphonate in question, and so, the use of only one dose of respective bisphosphonate may have an impact on these results (Watts *et al* 2010, Holen *et al*, 2010).

Preosteoblast recruitment and differentiation marks the final step in bone remodeling, whereby new bone is formed, following OPG binding to and sequestering RANKL. There showed to be no significant difference in cell migration between undamaged controls with and without bisphosphonate treatment, however, the presence of zolendronate caused a significant increase in preosteoblast migration when compared to non drug treated samples. Alendronate, however, increased the migration of preosteoblasts significantly, when compared to zolendronate and non drug treated groups, and both injury sizes. Little work has been performed regarding the effects of bisphosphonates on osteoblast recruitment, both *in vivo* and *in vitro*, so this shows, for the first time, the *in vitro* effects of alendronate and zolendronate on preosteoblast recruitment. However, it has been reported, by Garcia-Moreno *et al*, 1998, that the presence of alendronate does not affect the proliferative or functional capacity of human osteoblasts under normal culturing conditions, however, the use of an alternative cell type from a different species may pose an alteration on the mechanism of action of bisphosphonates.

The effects of bisphosphonates on the cellular capacity to mineralize *in vitro* was determined through performing a calcium assay, in addition to alizarin red staining and quantification, to furthermore determine the calcification potential of a preosteoblastic cell line, MC3T3-E1, treated with conditioned media from bisphosphonate treated microinjury induced osteoclastogenesis experiments. It has been shown that following bone resorption, numerous factors are released from osteoclasts to sustain bone formation, including IGF-I, IGF-II, FGF, TGFs, BMPs and PDGF (Boyle *et al*, 2003, Ducey *et al*, 2000). *In vivo*, zolendronate has been shown to increase mineral apposition rate following treatment (Recker *et al*, 2008), whereas it has been shown that other bisphosphonates, including alendronate fails to do this effectively, in spite of it stimulatory effect on osteoblastogenesis (Bravenboer *et al*, 1999, Duque *et al*, 2007). Furthermore, it has been suggested that, *in vivo*, bisphosphonates pose a stimulatory effect on osteoblastic activity (D'Aoust *et al*, 2000). Following treatment with both alendronate and zolendronate, there was a significant increase in calcium mineralization at day 21 in both undamaged and 400 μm samples. Further to this, there was a two fold increase in calcium mineralization in both alendronate and zolendronate treated samples at 400 μm when compared to undamaged constructs at 21 days. This result was also demonstrated with histological staining. As

shown in chapter 3, microinjury, in the absence of osteogenic media, demonstrated an ability to stimulate calcium mineralization in preosteoblastic cells. Further to this, bisphosphonates have been shown to increase mineralization and even restore the loss of mineral content associated with post menopausal bone loss (Brennan *et al*, 2011, Reinholtz *et al*, 2002, Boivin *et al*, 2002). Considering this, these results may be indicative of a relationship between bisphosphonates and microinjury to an osteocytic network within bone. The results of this section of the study imply that bisphosphonates, in the presence of biochemical factors released from microinjury and subsequent osteoclastogenesis, are capable of stimulating and enhancing *in vitro* osteogenesis in preosteoblasts, producing a cumulative effect, with microdamage, on the mineralization in bone.

In spite of the large amount of contradictory data which exists, these results have shown some strong consistencies with previous *in vitro* and *in vivo* studies, which demonstrated the ability of bisphosphonates to increase mineralization by osteoblasts (Tenenbaum *et al*, 2002, D'Aoust *et al*, 2000, Chaplet *et al*, 2004, Kim *et al*, 2009). These studies, however, have been conducted in the absence of microdamage, and so this is the first time this response has been demonstrated under such conditions. Both *in vivo* and *in vitro*, zolendronate has been shown to increase the capacity of bone to become mineralized, and this occurs at higher concentrations administered (Pozzi *et al*, 2009). Further to this, some nitrogen containing bisphosphonates, including alendronate and zolendronate, have been shown to stimulate osteoblast proliferation and differentiation, and to assist in maintaining osteoblast viability (Pan *et al*, 2004). Considering this, the results show that bisphosphonates do indeed, play a role in increased osteoblast mineralization, reflective of other studies, the magnitude of which is potentiated by the presence of microdamage. Further to this, subjects being treated with bisphosphonates for osteoporosis and osteopenia tend to demonstrate an elevated level of bone mineral density and prolonged secondary mineralization (Sebba *et al*, 2008, Westenfeld *et al*, 2006, Berenson *et al*, 2008, Bell *et al*, 2010, Boivin *et al*, 2001), yet may have a greater propensity to obtain fragility fractures (Mashiba *et al*, 2000, Odvina *et al*, 2005), which may be due to impaired bone remodeling associated with bisphosphonate treatment. This may be a significant observation for individuals with osteopenia, in terms of potentially exacerbating the risk of fracture and the development of fragile bone, due to increasing the potential for osteoclastogenesis.

Furthermore, these findings may be instrumental in demonstrating the need for an alternative or adjuvant therapy for the treatment of early osteoporotic and osteopenic bone.

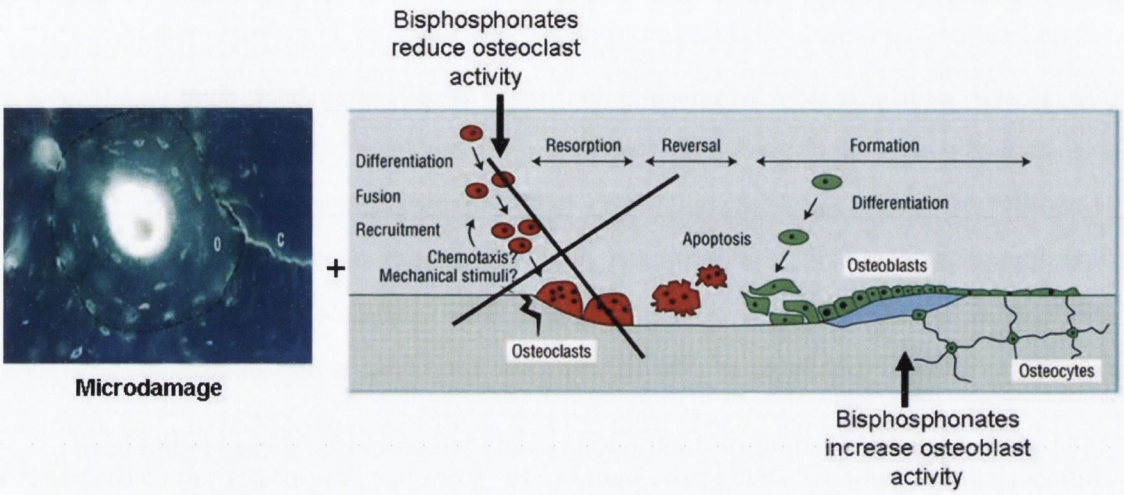


Fig. 4.12: Mechanism of action of bisphosphonates in this model of microinjury in bone. Bisphosphonates caused decreased osteoclast migration and osteoclastogenesis, and increased osteoblast activity and mineralization, in the presence of microinjury (Adapted from Taylor *et al*, 2007, and Ralston, 1997).

Considering this study as a whole, the results have demonstrated the biochemical and cellular events which occur following bisphosphonate treatment of a 3D osteocytic network subjected to microinjury, and the knock on effects that bisphosphonate treatment has throughout the various stage of bone remodeling, as depicted in **Fig. 4.12**. This chapter has effectively demonstrated that alendronate and zolendronate treatment, at an optimum dose, is capable of suppressing RANKL and OPG release, and this suppression was evident in the marked reduction of osteoclastogenesis observed. RANKL activity and osteoclastogenesis often exists concurrently, so the effect of bisphosphonate treatment may be viewed in tandem, regarding these results. It has been demonstrated that the presence of alendronate and zolendronate has no effect on, or can suppress the upregulation and successive release of RANKL and OPG (Garcia-Moreno, *et al*, 1998, Pan *et al*, 2004, Kimachi *et al*, 2011, Enjuanes *et al*, 2010). A decrease in osteoclast activity may be, as described and widely reported, due to inhibition of FPPS, and interference with the mevalonate pathway. Interestingly, ablation of OPG activity would suggest a reduction in

osteoblast activity, however, it is important to note that osteoclasts secrete a number of bone forming cytokines and growth factors, including IGF-1, TGF-β, and BMPs, and also Wnt10a and sphingosine-1-phosphate, which have been implicated in osteoclast mediated osteogenesis (Pederson *et al*, 2008). This concept may be indicative of as to why, at 400 μm, despite there being a reduction in RANKL and OPG activity, there was still an osteoclastogenic response, and subsequent osteogenic response in our model. When considering the overall results of this chapter, it may be concluded that bisphosphonates act, even in the presence of microinjury, on cellular activities, in addition to suppressing RANKL and OPG activity.

It is important to note that it has been demonstrated that, *in vivo*, osteoporotic bone tends to exhibit a greater frequency of large, linear cracks, when compared to non osteoporotic counterparts, thus, the use of a larger injury size may produce results more like what is observed in osteoporotic bone (Wasserman *et al*, 2008). Additionally, this particular study has focused on the basic events surrounding bone remodeling, such as RANKL and OPG activity, and the fundamental remodeling activities in the constitutive cells of the BMU. Bisphosphonates have been shown to effect a vast range of biochemical activities of osteocytes, osteoclasts and osteoblasts, most notably so due to the inhibition of FPPS in osteoclasts, hampering their ability to resorb bone, and also in the induction of ERKs in osteoblasts and osteocytes, facilitating maintenance of their viability and preventing apoptosis (Chen *et al*, 2005). Furthermore, this study only examined one dose of bisphosphonate for each respective drug, and so, a different response to microinjury may have been observed at greater or lesser concentrations. Overall, however, the experimental findings in this chapter have been shown that in an *in vitro* simulation of microdamage in bone, alendronate and zolendronate may inhibit microinjury mediated osteocyte apoptosis, as observed in chapter 2 (an increase in damage size causes an increase in osteocyte apoptosis) and subsequent RANKL/OPG activity. Further to this, the study has demonstrated the proposed modulatory effect of bisphosphonates on osteoclast activity, and the subsequent stimulatory potential of bisphosphonates on osteoblast mineralization in the presence of microdamage.

4.6 Conclusion

The effects of treatment of osteoporosis with bisphosphonates is well documented, however, at present, much research has been performed on *in vivo* models of osteoporosis, or on examining the effects of bisphosphonates on cellular activity in the absence of microinjury. This study has demonstrated the potential of bisphosphonates to change the normal response of cells to microinjury, through the significant suppression of RANKL and OPG release, and also had shown to modulate osteoclastogenesis, however, the levels of osteogenesis remain consistent, if not, elevated, following bisphosphonate treatment. This may be indicative as to why bone mineral density increases following bisphosphonate treatment, yet, bone quality remains poor and susceptible to fracture. In terms of addressing the aims of this study, we have been shown that bisphosphonates act at all three stages of bone remodeling, from precursor cell migration, to osteoclastogenesis and ultimately, osteogenesis. To conclude, this study has shown that bisphosphonates may be capable of restoring homeostasis in terms of the reduction of bone resorption and may be a possible mediator involved in increasing bone mineral content following microinjury.

Chapter 5

Response to microinjury is not solely governed by RANKL and OPG activity

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5.1 Introduction

As described in the introductory chapter and subsequent studies, microdamage mediated bone remodeling is regulated by the RANKL-RANK-OPG signaling triad. Repair of microdamage from bone is determined by the release of RANKL and the rate of RANKL-RANK binding. Maintenance of bone mass has been found to be governed by the presence of OPG, which is the decoy receptor for RANKL, and it functions in stopping the progression of bone resorption to a state of excess, and facilitates osteoblast activation and bone formation. Homeostasis between both of these cytokines is paramount in facilitating skeletal equilibrium, and any deviation from the norm can cause severe disease pathologies, such as the manifestation of osteoporosis, osteopetrosis, tooth eruption, paget's disease and various myelomas. It is important to note that the RANKL-RANK-OPG signaling pathway is a central moiety in preserving the balance between bone resorption and formation by the BMU but there are a number of intricate network of cell signaling pathways are independent to or linked to the production and release of RANKL, and subsequent OPG activity, which need to be explored.

RANKL, as described in chapters 1 and 2, is released following microinjury in bone, and this is related to the "Scissors Model" concept, utilised by our group, as a hypothesised mechanism of release *in vivo*. Compressive loading on bone, such as that which arises from walking and running may cause the bone to undergo damage in a shearing like mechanism, causing cell processes to be ruptured and cut, like with a pair of scissors. Shearing of cellular processes may allow the release of bone remodeling cytokines into a localised system, whereby a remodeling response can ensue. Binding of RANKL to RANK is, as described, is one of the principal steps in initiating osteoclastogenesis, and kick starting the events involved in the bone remodeling triad of RANKL-RANK-OPG interaction. A number of factors, such as interleukins, most notable IL-1, IL-6, IL-7, IL-11 and IL-17, in addition to TNF α, activins and IFN γ are responsible for potentiating the RANKL-RANK interaction, as detailed in Fig. 5.1 (Hofbauer *et al*, 1999, Ishida *et al*, 2002, Nakashima *et al*, 2000). Binding of RANKL to its receptor increases the expression of genes, resulting in RANK mediated osteoclastogenic signaling. This starts by the binding of TNF receptor associated factor proteins (TRAF) and RANKL-RANK mediated activation of NFκB pathways, which has been shown to be critical

in initiating osteoclastogenesis (Wei *et al*, 2001). The presence of IFN β and IFN γ has shown also to regulated RANKL-RANK mediated osteoclastogenesis, in both positive and negative feedback loops, respectively.

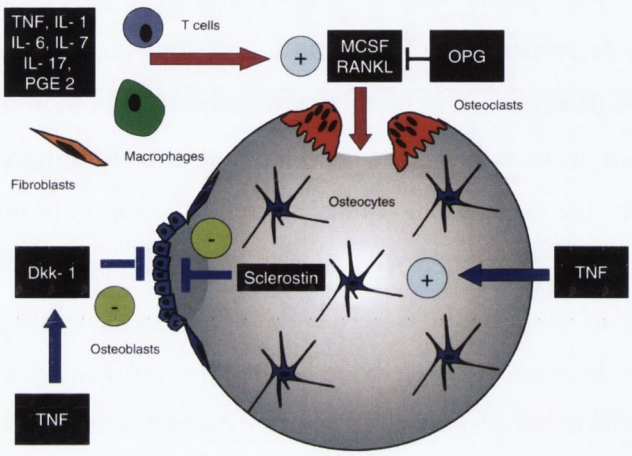


Fig 5.1 Interactions of TNF α and interleukins in the RANKL-RANK mediated activation of osteoclasts (Schett *et al*, 2010).

These interactions permit a constant stream of osteoclast mediated bone resorption, and thus, without an antagonistic receptor, a loss of bone homeostasis would prevail. Hence, OPG is recruited, under the control of both RANKL/RANK and Wnt/β-catenin pathways. Furthermore to this, downstream products of RANKL-RANK interaction such as TNF-α, IL-1α, TGF-β and IL-18, in addition to oestrogen, which is not directly involved in the microdamage mediated pathway of bone remodeling, function in the upregulation of OPG (Hofbauer *et al*, 1998, Vidal *et al*, 1998, Brandstrom *et al*, 1998). Further to sequestering RANKL and stimulating its action, OPG functions in preventing further activity of pro-resoprtive agents, including IL-1, TNF-α, and PTH (Morony *et al*, 1999). However, the more intricate details of the other molecular and biochemical participants involved in OPG signaling have yet to be determined.

Connected to the RANKL-RANK-OPG signaling pathway are several other pathways, most notably of which are the Interleukin, NFκB pathways and Wnt/ß-catenin pathways. These function synergistically and antagonistically with the RANKL-RANK-OPG pathway, but also function in the maintenance of bone homeostasis. The NFκB pathway is one

of the fundamental mechanisms utilised by bone to stimulate bone remodeling, and functions both at an osteoclastic and osteoblastic level in bone. Initiation of bone remodeling following microdamage facilitates the upregulation of NFκB via TNF-α and TACE activity (Black *et al*, 1997), which has been shown to promote osteoclastogenesis and decrease osteoclast apoptosis (Boyce *et al*, 2007). Furthermore, it has been shown to increase levels of TRAP in cells due to the synergistic activity of TNF-α and RANKL activity, as shown in **Fig 5.2** (Fretz *et al*, 2007). This additionally stimulates canonical Wnt signaling, via TRAF6 and IKKα and β activation, which bears an effect on osteoblast activity, and will be discussed in further detail later on in this chapter (Boyce *et al*, 2007). IKKβ activation, in turn, modulates osteoclastic signaling, and stimulates RANKL, IL-1 and TNF-α mediated osteoclastogenesis (Ruocco *et al*, 2005).

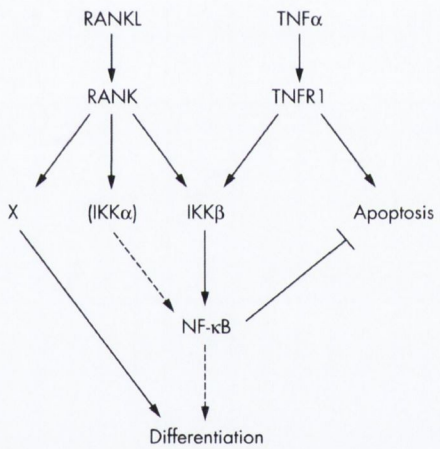


Fig. 5.2 Principal interactions of TNF α and its implications in NFκB induced osteoclastogenic differentiation (Ruocco *et al*, 2005).

The Wnt/β-catenin pathways, as shown in **Fig. 5.3**, act more so at the osteoblast level more than the osteoclast level, working synergistically with RANKL-OPG signaling. Wnts encompass a large family of lipid modified proteins (LRP) capable of binding to numerous receptors, such as the frizzled G protein coupled receptor related protein (Miller *et al*, 2002, Logan *et al*, 2004). Wnts are the functional moiety responsible for initiating osteoblast activity and a subsequent osteogenic response. The Wnt/ β-catenin or canonical pathway signals

through lrp-5, which activates disheveled (Dsh), inhibiting glycogen synthase kinase (GSK), which ultimately maintains the functionality of glycogen synthase kinase 3β, and thus β-catenin, maintaining osteoblast longevity (Miller *et al*, 2002, Logan *et al*, 2004). Further to this, non canonical pathways activated by Wnts include c-Jun NH2-terminal kinase (JNK) pathway, which is also under the control of Dsh (Woradx *et al*, 1998, Miller *et al*, 2002, Logan *et al*, 2004). Wnt signaling is a key factor in maintaining osteoclast and osteoblast homeostasis, and this, BMU activity, and thus, possesses numerous regulatory factors such as Wnt inhibitory factors (WIFs), secreted frizzled-related proteins (SFRPs), dickkopfs (DKKs) (Kawano *et al*, 2003, Radawi *et al*, 2005).

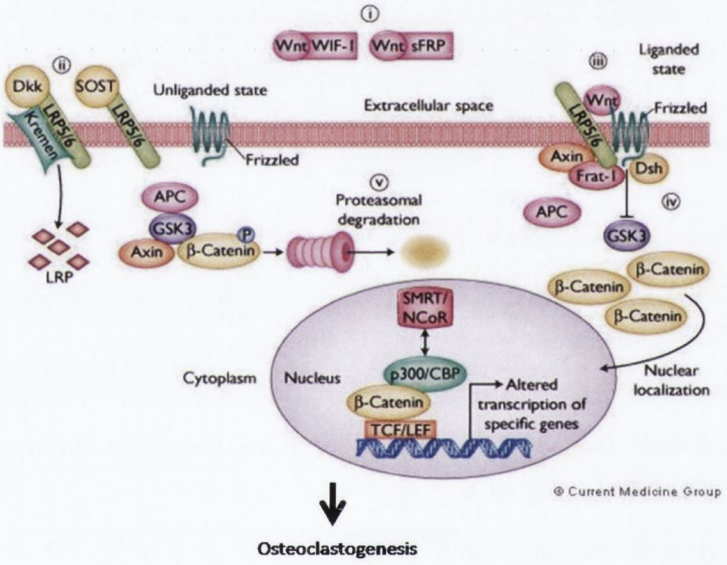


Fig. 5.3 The Wnt/ β-catenin pathway. Without Wnt, β-catenin becomes phosphorylates leading to signal inactivation. Following Wnt binding to LRP and frizzled receptors, osteoblastic gene expression ensues. DKK-1 is secreted and counteract LRP activity ceasing Wnt activity and stimulating osteoclastogenesis. (Adapted from Krishnan *et al*, 2006).

Wnts have been shown to prevent osteoblast apoptosis, which has been displayed by the work of Ameida *et al*, 2005, whereby MC3T3-E1 cells were subjected to treatments with canonical and non canonical Wnts, and displayed a Src/Erk (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase)/AKT mediated anti apoptotic effect. DKK proteins exist in 4 main forms, however, the role of DKKs in Wnt suppression has been more greatly characterised in DKK 1 and 2 (Kawano *et al*, 2003). Excessive production of DKK-1

has been shown to promote excessive bone loss via stimulation of osteoclasts, however, blocking of DKK-1 has been shown to restore homeostasis through increasing BMP-2 signaling, thus stimulating bone formation (Liu *et al*, 2006, Tian *et al*, 2003). Furthermore, Wnt has been shown to be antagonised by SOST/Sclerostin, again, through LRP-5 receptor binding (Ellies *et al*, 2006).

Microdamage, as described in chapter 4, is present at high levels in osteoporotic bone, whereby osteocyte integrity has been shown to be compromised. It has been suggested, and subsequently shown, that the mevalonic pathway is affected by bisphosphonate treatment, in addition to suppressing RANKL and OPG activity (Kimichi *et al*, 2011, Giner *et al*, 2010). However, the more intricate biochemical factors attributing to the action of bisphosphonates, like the effect of microinjury, has yet to be determined. The results of chapter 4, however, gave a clear outline as to why this study should be performed, as it demonstrated that, in spite of the ability of bisphosphonates, namely alendronate and zolendronate, to suppress RANKL and OPG activity, microinjury still bore the ability of stimulate osteoclastogenesis and osteogenesis, in a damage size dependent manner.

5.2 Aims of the study

Based on the findings of chapters 2, 3 and 4, the objective of this study was to determine the effect of microinjury on the other biochemical pathways involved in bone remodeling, and to determine a broader scope of what other biochemical factors contribute to microinjury mediated bone remodeling response. As described above, there is vast evidence that osteoclast and osteoblast activity is not just a RANKL-RANK-OPG phenomenon; however, the role which microdamage/microinjury plays in initiating these other pathways has yet to be elucidated. Considering the finding of chapter 4, where a bone remodeling response was observed in the absence of RANKL and OPG, the effects of bisphosphonates on the release and activity of alternative "bone remodeling" factors will also be determined.

Considering this, the specific aims of this particular study were as follows:

1. To block RANKL activity using recombinant proteins, to establish whether microinjury-initiated osteoclastogenesis is solely mediated by RANKL

2. To use a proteomic approach to elucidate the biochemical signaling pathways resulting from osteocytes, following microinjury by performing an antibody array, assessing 308 proteins and elucidating their relationship to bone remodeling.

3. To assess the effect of microinjury on BMP-2 activity through use of ELISA.

4. To determine the effects of bisphosphonate treatment on the biochemical pathways involved in a bone remodeling response following microinjury using a proteomics based antibody array and ELISA.

5.3 Methods

5.3.1 Materials

Cell culture materials including α-modified essential medium (α MEM), foetal bovine serum (FBS), calf serum (CS), penicillin/streptomycin, Dulbeccos phosphate buffered saline (1X), Trypsin/EDTA, Dulbeccos modified eagles medium (DMEM), 0.1 N Sodium Hydroxide solution, Trypan Blue stain, Leukocyte acid phosphatase staining kit, sodium citrate, PnPP, sodium chloride, sodium tartarate, Alizarin Red, Cetylpyridinuim chloride, Alendronate and B-D Precisionglide 21 and 26 gauge syringe needles were all purchased from Sigma Aldrich Ireland Ltd, Airton Rd, Tallaght, Dublin 24. OptiMEM reduced serum media was purchased from Gibco. Zolendronate was purchased from Alphatech technologies, Blessington, Co. Wicklow, Ireland. Hematoxylin, Eosin and Nuclear Fast Red were purchased from Lennox Laboratory Supplies, Naas rd, Dublin. Matrigel basement membrane matrix and rat tail collagen were purchased from BD Biosciences. Classic Plus acupuncture needles (160 μm, and 400 μm) were purchased from Harmony Medical Distributions Ltd, Leytonstone, London, United Kingdom. RANK-Fc and OP-Fc and BMP-2 ELISA were purchased from R&D systems. RayBio Biotin Label-based Mouse Antibody Array 1 was purchased from Insightbio. Cell culture plastic-ware was purchased from Sarstedt Ltd, Sinnottstown, Wexford, Ireland. MLO-Y4 cells were provided by Professor Lynda Bonewald, University of Missouri, Kansas, U.S.

5.3.2 Cell culture

As described in previous chapters, MLO-Y4 cells were maintained in α Modified Eagles medium (Biosera) supplemented with 5% foetal bovine serum, 5% iron supplemented calf serum and 1% antibiotics (penicillin/ streptomycin). Cells were cultured in collagen coated flasks (0.15 mg/mL rat tail collagen type 1) at 37 °C at 5% CO_2. Preosteoclasts (RAW 264.7 cells, ATCC) were cultured in high glucose Dulbecco's Modified Eagles Medium supplemented with 10% heat inactivated foetal bovine serum and 1% antibiotics (penicillin/ streptomycin).

5.3.3 Blocking RANKL activity with RANK-Fc and OPG-Fc

As described, RANKL release from osteocytes is fundamental in stimulating an osteoclastogenic response following microdamage, and much debate exists regarding the other biochemical factors involved in the stimulation of osteoclastogenesis and bone remodeling. In order to determine this, it was necessary to block RANKL activity in conditioned media, and this was achieved by the utilisation of the two principal receptors of RANKL- its functional receptor RANK, and its decoy receptor OPG, in an *in vitro* simulation of microdamage to osteocytic networks. As previously described, (chapter 2, section 2.3.3.1), MLO-Y4 cells were embedded in a rat tail collagen type I /Matrigel construct (BD Biosciences), to simulate an *in vitro* environment, facilitating cell process network formation. The collagen / Matrigel constructs containing 1 x 10^6 cells/mL of gel were cultured in 24 well plates and incubated for 1 hour to allow gels to solidify, prior to addition of culture media supplemented with 100 ng/ml RANK-Fc (Menaa *et al*, 2000) and 100 ng/ml OPG-Fc (Zhao *et al*, 2002), to block RANKL activity. This was just to determine if an osteoclastogenic response ensued following microinjury, and was not used in subsequent experiments. Following treatment with RANK-Fc and OPG-Fc, cell process formation was of normal morphology. After a 5 day incubation to facilitate cell process formation, 400 μm damage was applied (see **Fig. 2.3**, chapter 2).

To determine if blocking of RANKL with RANK-Fc and OPG-Fc was successful, and whether osteoclastogenesis is mediated by RANKL activity or whether other biochemical factors were involved, preosteoclasts were incubated in conditioned media from RANK-Fc and OPG-Fc treated media. At 7 day intervals, TRAP activity was quantified as previously described, in chapter 3, section 3.3.6. 20 μl of 1M NaOH (Sigma Aldrich) was added to stop the reaction and absorbance was read at 405 nm by spectroscopy. n = 3 cultures were assayed in each case.

5.3.4 Determination of the other biochemical factors involved in initiation of osteoclastogenesis: RayBio Biotin Label-based Mouse Antibody Array 1

In order to assess what other biochemical factors were involved in the stimulation of osteoclastogenesis following microinjury, and furthermore, the effect of bisphosphonate treatment on osteoclastogenic potential of microinjury, it was necessary to use a tool which

can measure a vast quantity of proteins, hence, in this study a RayBio Biotin Label-based Mouse Antibody Array 1 was used. This allows for the simultaneous analysis of 308 proteins, which include cytokines, growth factors, chemokines, proteases, soluble receptors and soluble adhesion molecules in culture media. The principal of the experiment is that following biotinlyation of the primary amine in the respective protein in the cell culture supernatant, labelling of the protein allows it to become adhered to its respective receptor on the membrane. The membrane is then labelled with HRP-streptavidin, which labels each protein to a certain intensity and each protein can then be detected by chemiluminescence. The result is a membrane displaying all the proteins, at different intensities, which can be quantified using optical densitometry.

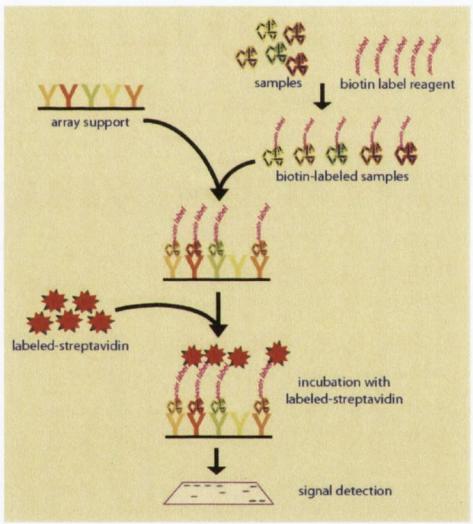


Fig. 5.4 Schematic of how the RayBio Biotin Label-based Mouse Antibody Array 1 works

(www.raybiotech.com)

The initial step of this experiment was to collect culture media from gel embedded MLO-Y4 cells, 72 hours after the application of damage, and 3 ml of each sample was dialysed overnight. Microinjury was applied (400 μm) to samples and undamaged samples were also used as a control, in the absence of RANK-Fc and OPG-Fc. Samples were places in a dialyzer and dialyzed with 2 litres of 1X PBS. This was followed by a second dialysis step for 3 hours in fresh 1X PBS. This was performed under constant stirring conditions. Following this, samples were removed from the dialyzer and 2 ml of dialyzed sample placed in a clean tube for Biotin labelling. Under manufacturer's instructions, 100 μl of 1x PBS was added to

the internal control tube and pipetted to ensure thorough mixing. 40 µl of this was then added to the dialyzed sample. 100 µl of 1x PBS was added to the labelling reagent, followed by vortexing to dissolve the powder. 50 µl of labelling reagent was added to the dialyzed sample and this was incubated at room temperature for 30 minutes under shaking conditions. 5 µl of stop solution was then added to the reaction. Following this, free biotin was removed from the dialyzed sample by centrifuging through a spin column, having undergone 3x rinses with 1x PBS, for 3 minutes at 1,000 g.

While biotin labelling is being performed, each membrane was placed in a tray (as provided by the manufacturer), and 8ml blocking buffer applied. This was followed by a 1 hour incubation at room temperature to block the membranes. The excess blocking buffer was decanted from the trays. The biotin labelled samples were diluted in blocking buffer and the volume increased to 8ml. 8 ml of samples was then added to each membrane, and incubated for 2 hours under shaking conditions. Samples were decanted from the membranes, and this was followed by 3 x rinses in 1X wash buffer 1 and 3 x rinses 1X wash buffer 2, both of which were performed under shaking conditions. 8ml of 500 fold HRP- conjugated streptavidin was added to each membrane, followed by an overnight incubation at 4 ºC.

Following this incubation, the 500 fold HRP- conjugated streptavidin was decanted from the membranes, and a 4ml solution of 1:1 detection buffer C and detection buffer D was applied to the membranes, to facilitate chemiluminescence of the samples. It is imperative that detection is performed within 40 minutes of the addition of the detection solution, and the membranes must not dry out. Using forceps, the membranes were placed, correct side up, on a plastic sheet. This was covered with another plastic sheet; in order to eliminate as many bubbles as possible. The signal was detected by means of x-ray film (fugifilm), exposing membranes for 40 seconds. Several exposures were repeated to gain the best result. Membranes were then stored at -20 ºC for future reference. X-ray films were then scanned and the amount of protein present established by optical densitometry by scanning with an Epson scanner and performing densitometry using GeneTools by Syngene software. Results were further analysed using optical densitometry and protein levels expressed in terms of pixel values.

5.3.5 Quantification of BMP-2 activity following microinjury

BMP-2, as described, is one of the principal factors facilitating osteogenesis and mineralization of MC3T3-E1 cells. Considering that microinjury to osteocytes stimulates *in vitro* osteogenesis, in the absence of osteogenic media, it was necessary to determine the amount of BMP-2 produced by cells following damage. Gel embedded MLO-Y4 cells were incubated for 72 hours in OptiMEM serum free media and the samples used were undamaged and those having received 400 μm damage. BMP-2 was quantified by means of single site specific ELISA (Mouse BMP-2 ELISA) under manufacturers' instructions (see section 2.3.4). n=3 cultures were assayed at each microinjury level, in addition to non-injured controls.

5.3.6 Determining the effects of bisphosphonate treatment on the other biochemical factors involved in initiation of osteoclastogenesis using a RayBio Biotin Label-based Mouse Antibody Array 1

As described above, gel embedded osteocytes were incubated in standard media supplemented with 10^{-5} M concentration of either Alendronate or Zolendronate. Again, after a 5 day incubation to facilitate cell process formation, 400 μm damage was applied by inserting a needle vertically into the centre of the hydrogel (see **Fig. 2.3**, chapter 2) and drawing it through at a right angle, creating a defect with dimensions of 7 mm in length x 5 mm in depth. After 72 hours, conditioned media was collected from the constructs and pooled accordingly. A RayBio biotin label-based Mouse antibody array was performed, as described under manufacturer's instructions (section 5.3.3).

5.3.6 Quantification of BMP-2 activity following bisphosphonate treatment to osteocytes following microinjury

Gel embedded MLO-Y4 cells were incubated for 72 hours in OptiMEM serum free media supplemented with 10^{-5} M concentration of either Alendronate or Zolendronate. The samples used were undamaged and those having received 400 μm damage. BMP-2 was quantified by means of single site specific ELISA (Mouse BMP-2 ELISA) under manufacturers' instructions, as described above (section 5.3.4).

5.3.7 Statistics

All data was analysed for significance ($p \leq 0.05$) using two way ANOVA, post hoc Tukey test, to compare means.

5.4 Results

5.4.1 Osteoclastogenesis is not exclusively a RANKL alone mediated phenomenon

Treatment with both 100 ng/ml RANK-Fc and OPG-Fc caused no significant difference ($p \geq 0.8$) in TRAP release in undamaged samples, as shown in **Fig. 5.5** and control samples cultured in standard culture media at all time points over a 21 day period. At 400 μm damage, treatment with both cytokines showed to significantly decrease ($p \leq 0.05$) TRAP activity in preosteoclasts, yet, TRAP activity still remained significantly higher ($p \leq 0.02$) in treated samples when compared to undamaged samples, demonstrating a mild suppression of osteoclastogenesis, yet, there still was an osteoclastogenic response, suggesting an alternative mechanism for stimulating osteoclastogenic activity following microinjury.

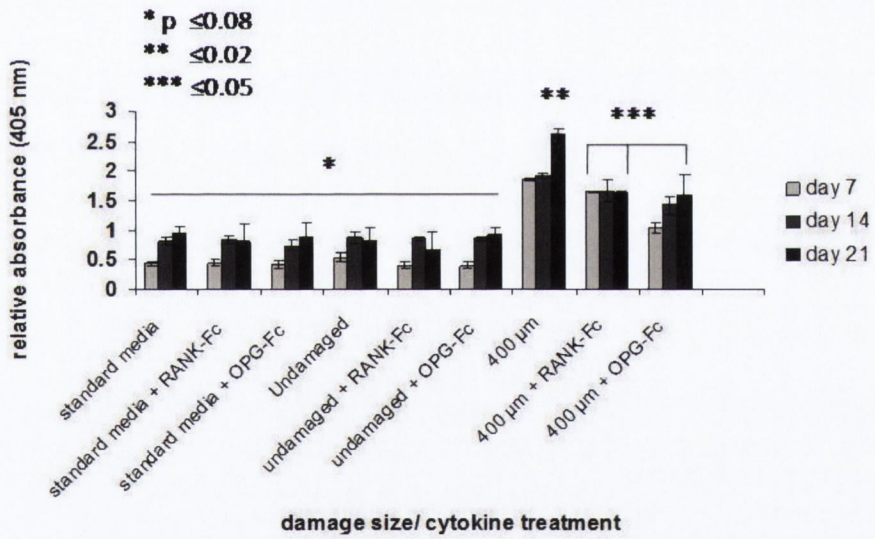
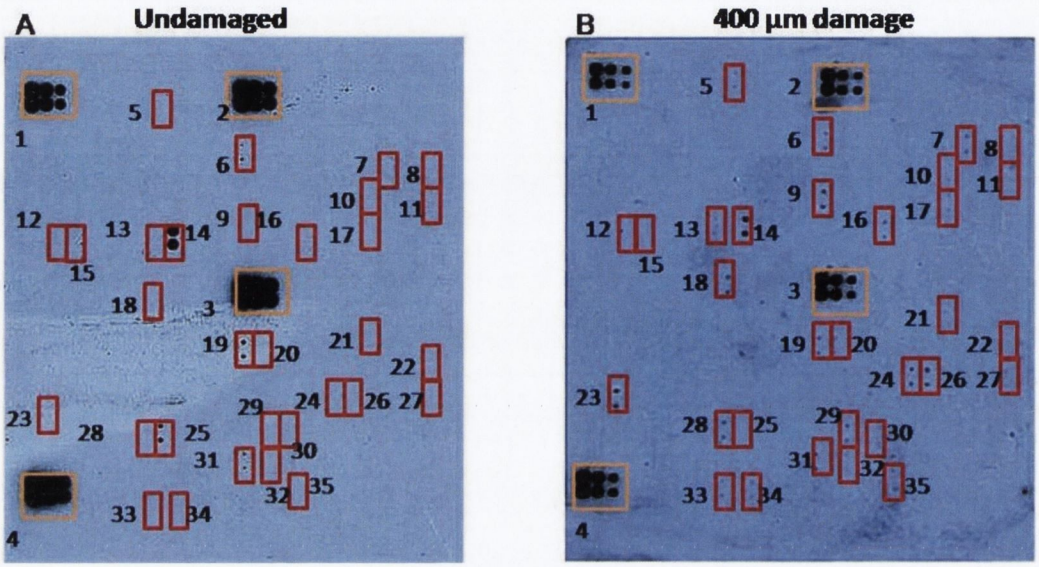


Fig. 5.5 Osteoclastogenesis is not exclusively a RANKL alone mediated phenomenon. As shown in this figure, there was no significant difference in TRAP activity in preosteoclasts cultured in cell culture media or in the presence of conditioned media from undamaged osteocytes treated with RANK-Fc and OPG-Fc. A significant increase was observed in 400μm samples when compared to undamaged samples with and without RANK-Fc and OPG-Fc, this activity was suppressed by cytokine treatment, but not abolished. * denotes $p \leq 0.08$ when comparing significance between undamaged samples and control samples with and without cytokine treatment ** denotes $p \leq 0.02$ when comparing significance between 400 μm with and without RANK-Fc and OPG-Fc. *** denotes $p \leq 0.05$, when comparing between 400 μm damage samples with cytokine treatment to undamaged and standard media samples. Error bars are indicative of standard deviation, n = 3.

5.4.2 Microinjury causes a significant increase in the activity of cytokines, chemokines, growth factors and soluble factors associated with bone remodeling



1.P-1a, P1-b & P-1C	12. Gremlin*	23. Osteopontin*	32. TWEAK*
2.P-2a, P2-b & P-2c	13. DKK-1*	24. MMP-2*	33. V-Cam-1*
3.P-3a, P3-b & P-3c	14. Frizzled 1*	25. PDGF	34. Urokinase*
4. 2.P-4a, P4-b & P-4c	15. FGF-R4*	26. OPG*	35. WIF-1*
5. Activin A	16. IGFBF-2*	27. Osteoactivin A*	
6. CD-30*	17. GFP-alpha-4*	28. RANKL*	
7.Cryptic*	18. IL-1- alpha*	29. TNF-alpha*	
8. CXCL-14/ BRAK*	19. IL-27	30. TNF-beta*	
9. IFN-beta*	20. IL-29*	31. Tie-2	
10. Epiregulin*	21. IL-17*		
11.GM-CSF*	22-IL-28*		

Fig. 5.6 A and B Dot Blot analysis of protein expressed in 400 µm damage and undamaged samples (A- Undamaged, B- Damaged) Fig 5.6 A As is depicted in the figure above, few proteins were expressed in undamaged samples, when compared to damaged samples. **Fig 5.6 B** As is depicted in the figure above, a greater number of proteins were expressed following microinjury to an osteocytic network, when compared to undamaged samples (**Fig. 5.6 A**). * denotes proteins upregulated in 400 µm samples.

To determine the other biochemical factors involved in mediating an osteoclastogenic and subsequent osteogenic response, a RayBio Biotin Label-based Mouse Antibody Array 1 was used. Results were determined using optical densitometry and expressed as relative protein level in terms of pixel density. All samples analysed were from undamaged constructs and 400 μm, 72 hours after damage. There was a vast increase in the number of proteins expressed in 400 μm when compared to undamaged constructs, as demonstrated in **Figs. 5.6 A and B**. The signals of the control were labelled in orange rectangular boxes, and signals of proteins labelled with red rectangular boxes. The value from scans was adjusted based on the intensity of positive control spots on each membrane. All microinjury samples had a tendency to display high levels of interleukins, TNF-α and TNF-β, DKK1, Activin A, GM-CSF, RANTES, MMP2 and RANKL compared to undamaged conditioned medium. Expression of RANKL also showed to confirm the results of the RANKL ELISA, as shown in chapter 2.

As demonstrated in **fig 5. 7**, the presence of microinjury had a tendency to significantly increase ($p \leq 0.01$), the presence of Interleukins (IL-1 α, IL 17, IL-27, IL-28 and IL-29), in addition to the increased expression of members of the Wnt/ β-catenin pathway, such as Frizzled 1, WIF-1 and DKK-1. With regard to the RANKL-OPG signaling pathway, RANKL expression was significantly increased ($p \leq 0.01$), in 400 μm samples when compared to undamaged samples, in which RANKL was found to be absent. OPG expression was also increased in 400 μm, whereas in undamaged samples, it was also found to be absent. GM-CSF, which functions similarly to M-CSF, and MMP-2 activity, which has shown to be involved in cleavage and activation of RANKL from membrane bound to soluble form, respectively, also showed to be significantly increased ($p \leq 0.01$), in 400 μm samples. The expression of angiogenic factors such as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), are indicative of an osteogenic response, and these showed to be downregulated in 400 μm samples. Furthermore, TNF-α RANTES, IFN-β and Activin, which have been implicated in stimulating osteoclastogenesis, all showed an increase in activity following microinjury, when compared to undamaged samples. It is significant to note that all proteins expressed have been implicated in the bone remodeling pathway.

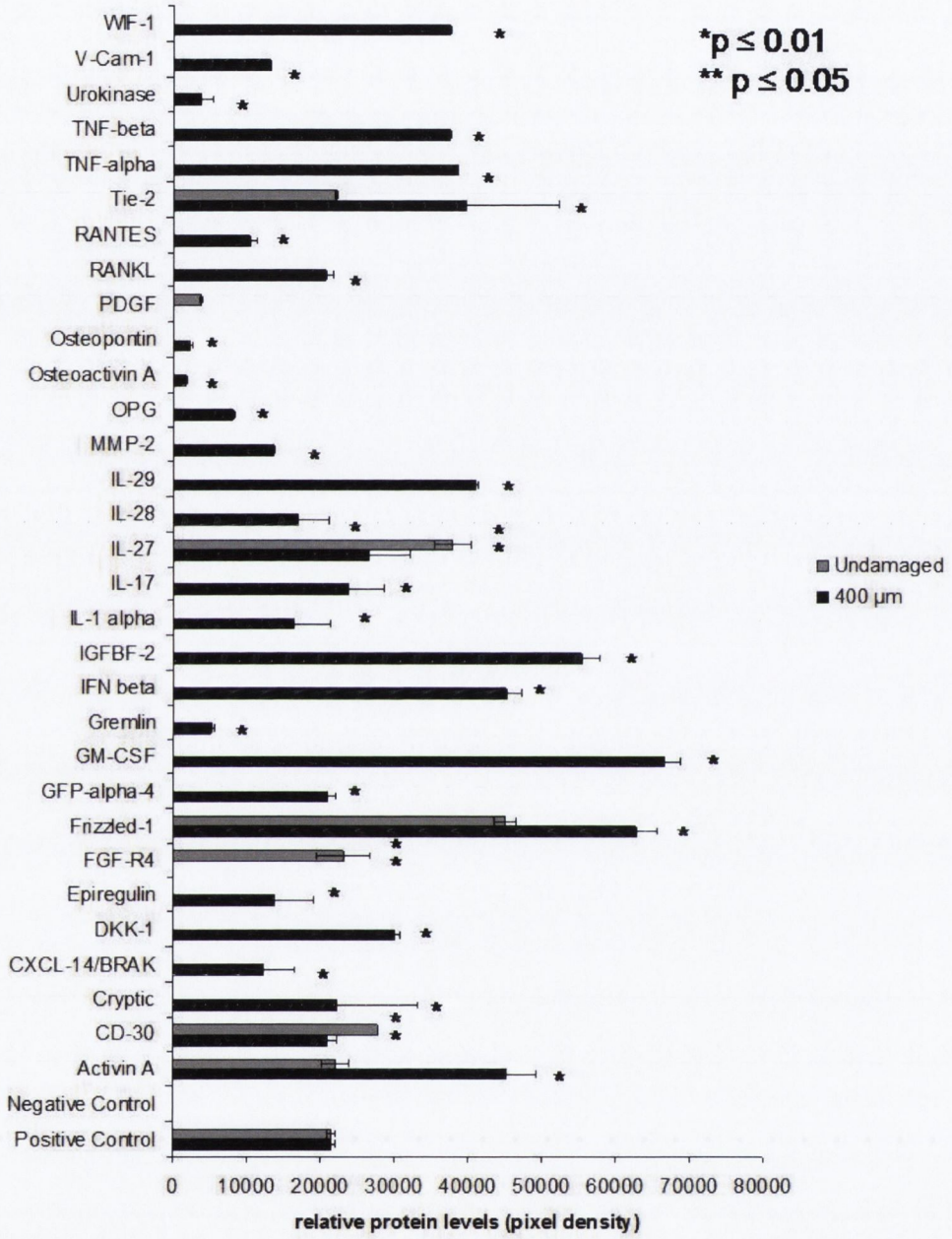


Fig 5.7 Microinjury increase the number of bone remodeling factors expressed in networks of osteocytes. On comparing undamaged to 400 µm damaged samples, a vast increase in protein expression is observed. The incidence of pro osteoclastogenic factors (TNF-α, RANKL, RANTES, Frizzled 1, DKK-1, IL-1 α, IL-17 and IFN-β) is significantly higher in damaged samples, whereas they tend to be absent or significantly less in undamaged samples. * denotes $p \leq 0.01$ when comparing significance between protein expression in 400 µm samples to undamaged samples. ** $p \leq 0.05$ when comparing significance between protein expression in undamaged samples to 400 µm samples. Error bars are indicative of standard deviation, n = 3.

5.4.3 Microinjury increases BMP-2 activity in networks of osteocyte cells

To quantify the amount of BMP-2 released following microinjury to an osteocytic network, an ELISA was performed using cell culture supernatants from undamaged and 400 μm samples, 48 and 72 hours after microinjury. As shown in **Fig. 5.8**, there was a significant increase ($p \leq 0.05$) in all samples, at both 48 and 72 hours after microinjury between undamaged and 400 μm microinjury samples.

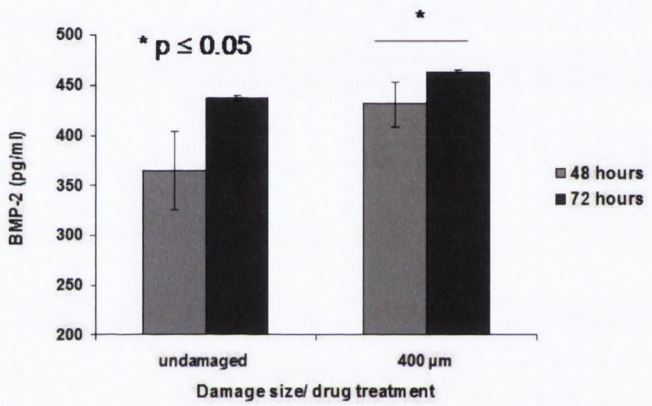


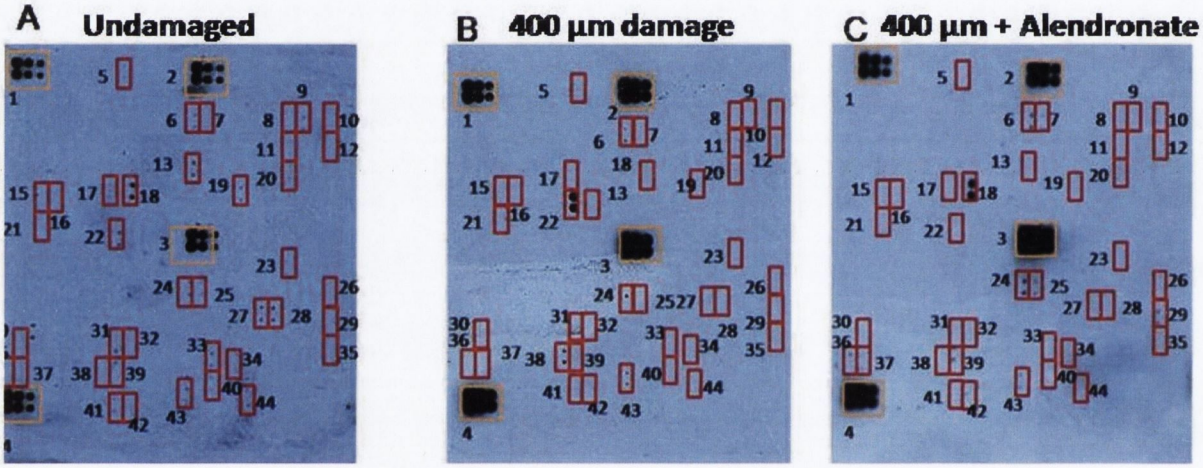
Fig. 5.8 Microinjury increases BMP-2 activity in networks of osteocyte cells in the presence of bisphosphonates. As shown in **Fig. 5.8**, there was a significant increase ($p \leq 0.05$) in BMP-2 activity at 72 hours when compared to samples at 48 hours. There was a significant increase in BMP-2 activity was observed in 400 μm samples when compared to undamaged samples. * denotes $p \leq 0.05$ when comparing significance between 400 μm and undamaged samples. Error bars are indicative of standard deviation, n =3.

5.4.4 Bisphosphonate treatment suppresses RANKL and OPG activity, yet maintains the activity of pro-osteoclastogenic factors

As was observed in the previous chapter, bisphosphonate treatment caused an almost full suppression of RANKL and OPG activity, yet osteoclastogenesis and osteogenesis was maintained, albeit to a lesser extent. To determine the whether other biochemical factors were involved in mediating an osteoclastogenic and subsequent osteogenic response following bisphosphonate treatment, conditioned media from microinjury experiments treated with alendronate and zolendronate were again analysed using an antibody array. Results were determined using optical densitometry and expressed as relative protein value in terms of pixel

density. All samples analysed were from undamaged constructs and 400 μm, 72 hours after damage, treated with 10^{-5}M concentration of alendronate and zolendronate.

5.4.4.1 Alendronate treatment suppresses some of the factors involved in the microinjury response in osteocytes yet maintains other osteoclastogenic and osteogenic factors.



1.P-1a, P1-b & P-1C
2.P-2a, P2-b & P-2c
3.P-3a, P3-b & P-3c
4. 2.P-4a, P4-b & P-4c
5. Activin A
6. CD-30*
7. CD-40 **
8.Cryptic *
9. CXCL-14/ BRAK *
10. IFN-beta*
11. Epiregulin*
12. GM-CSF *
13. Gremlin*
14. GFR-alpha 4**
15. DKK-1*
16. Frizzled 1
17. FGF-R4 *
18. IGFBF-2 *
19. GFP-alpha-4*
20. IL-1- alpha*
21.IL-11**
22.IL-12
23. IL-27
24. IL-29*
25. IL-17*
26. IL-28*
29. PDGF
31. Osteoactivin A*
27. Osteopontin*
30. OPG
28. MMP-2*
32. RANKL*
33. Spinosin Ectodomain**
34. Tie-2
35. TACI**
36. TGF- beta **
37. TNF-alpha*
38. TNF-beta*
39. TROY **
40. TWEAK*
41. V-Cam-1*
42. Urokinase*
43. V-Cam-1*
44. WIF-1*

Fig. 5.9 A, B and C Dot Blot analysis of protein expressed in 400 μm damage to osteocytes treated with 10^{-5}M Alendronate compared to non bisphosphonate treated samples. (A- Undamaged, B- Damaged, C- Alendronate) 5.9 A As is depicted in the figure above, few proteins were expressed in undamaged samples, when compared to damaged samples. As is depicted in the **fig 5.9B** , a greater number of proteins were expressed following microinjury to an osteocytic network, when compared to undamaged samples. **Fig 5.9 C** As is depicted in the figure above, fewer proteins were expressed following microinjury to an osteocytic network, when compared to 400 μm non bisphosphonate treated samples and undamaged samples. * denotes proteins upregulated in 400 μm samples, ** denotes proteins upregulated in bisphosphonate treated samples alone.

Treatment with alendronate demonstrated the expression of a greater number of proteins than the undamaged constructs, as shown in **Fig 5.9 A, B** and **C.** Again, the signals of the control were labelled in orange rectangular boxes, and signals of proteins labelled with red rectangular boxes. All microinjury samples treated with alendronate had a tendency to display high levels of IL-11, IL-12 and IL-17, Osteoactivin A, Frizzled 6, TGF- β, CD 30 and CD 40, when compared to non alendronate treated 400 μm and undamaged samples. As shown in **fig 5.10**, the treatment of osteocytes with alendronate followed by the presence of microinjury had a tendency to significantly increase ($p \leq 0.01$), the presence of IL-11, IL-17 and IL-27, and furthermore, proved to stimulate the expression of CD 30 and CD 40, Frizzled 1, GFP-alpha 4, which is responsible for mediating cell-cell interactions, osteopontin, osteoactivin and TGF- β. In comparison to the non bisphosphonate treated constructs, there was no RANKL or OPG expressed, in addition to an absence of MMP-2, TNF- α, DKK-1, IL-1, Activin A, all of which have been implicated in stimulating osteoclastogenesis.

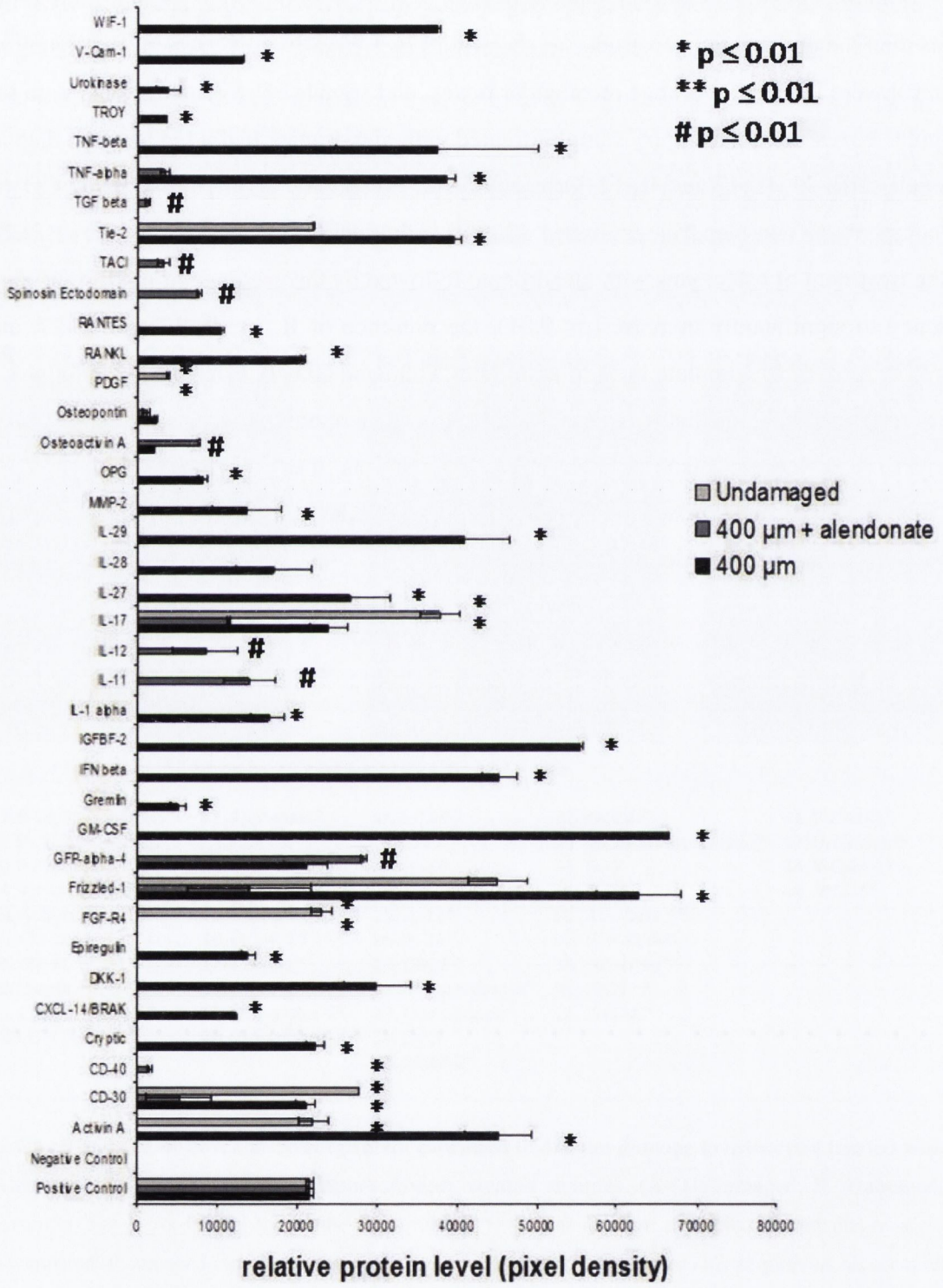
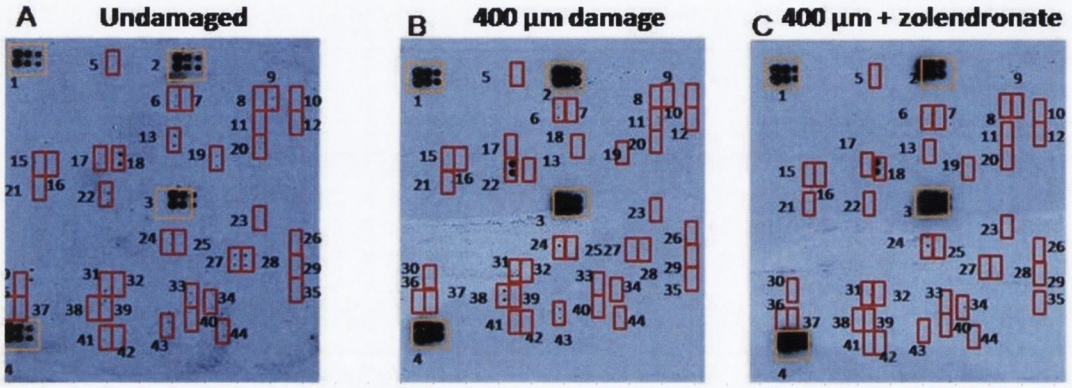


Fig 5.10 Alendronate treatment suppresses some of the factors involved in the microinjury response in osteocytes, yet maintains other osteoclastogenic and osteogenic factors. Please see figure legend of next page.

Fig 5.10 Alendronate treatment suppresses some of the factors involved in the microinjury response in osteocytes, yet maintains other osteoclastogenic and osteogenic factors. On comparing 400 μm non alendronate treated samples an increase in the presence of osteoclastogenic and osteogenic factors, such as CD-40, Frizzled-1, IL-11, IL-12, TGF- β, osteopontin and osteoactivin are observed in damaged following alendronate treatment. However, when comparing bisphosphonate treated samples to non bisphosphonate treated undamaged sample, a significant increase in frizzled 1 and PDGF was observed, the latter of which has been implicated in bone repair. * denotes $p \leq 0.01$ when comparing significance between protein expression in 400 μm samples to undamaged samples. ** $p \leq 0.01$ when comparing significance between protein expression in undamaged samples to 400 μm samples. # $p \leq 0.01$ when comparing significance between protein expression in alendronate and both non alendronate treated undamaged and 400 μm samples. Error bars are indicative of standard deviation, $n = 3$.

5.4.4.2 Zolendronate treatment suppresses some of the factors involved in the microinjury response in osteocytes yet maintains other osteoclastogenic and osteogenic factors.



1.P-1a, P1-b & P-1C
2.P-2a, P2-b & P-2c
3.P-3a, P3-b & P-3c
4. 2.P-4a, P4-b & P-4c
5. Activin A
6. CD-30*
7. CD-40 **
8.Cryptic *
9. CXCL-14/BRAK *
10. IFN-beta*
11. Epiregulin*
12. GM-CSF *
13. Gremlin*
14. GFR-alpha 4**
15. DKK-1*
16. Frizzled 1
17. FGF-R4 *
18. IGFBF-2 *
19. GFP-alpha-4*
20. IL-1- alpha*
21.IL-11**
22.IL-12
23. IL-27
24. IL-29*
25. IL-17*
26. IL-28*
29. PDGF
31. Osteoactivin A*
27. Osteopontin*
30. OPG
28. MMP-2*
32. RANKL*
33. Spinosin Ectodomain**
34. Tie-2
35. TACI**
36. TGF- beta **
37. TNF-alpha*
38. TNF-beta*
39. TROY**
40. TWEAK*
41. V-Cam-1*
42. Urokinase*
43. V-Cam-1*
44. WIF-1*

Fig. 5.11 A, B and C Dot Blot analysis of protein expressed in 400 µm damage to osteocytes treated with 10^{-5}M zolendronate compared to non bisphosphonate treated samples. (A- Undamaged, B- Damaged, C- Zolendronate) 5.11 A As is depicted in the figure above, few proteins were expressed in undamaged samples, when compared to damaged samples. **Fig 5.11 B** As is depicted in the figure above, a greater number of proteins were expressed following microinjury to an osteocytic network, when compared to undamaged samples. **Fig 5.11 C** As is depicted in the figure above, fewer proteins were expressed following microinjury to an osteocytic network, when compared to 400 µm non bisphosphonate treated samples and undamaged samples. * denotes proteins upregulated in 400 µm samples, ** denotes proteins upregulated in bisphosphonate treated samples alone.

Treatment with zolendronate demonstrated the expression of a greater number of proteins than the undamaged constructs, as shown in **Figs. 5.11, A, B** and **C,** and **5.12.** Again, the signals of the control were labelled in orange rectangular boxes, and signals of proteins labelled with red rectangular boxes. All microinjury samples treated with zolendronate had a tendency to display high levels of IL-12, IL-17 and IL-28, Osteoactivin A, Frizzled 1, TGF-

β, TROY, WIF-1, GFP-α-4, CD 30 and CD 40, whereas undamaged samples demonstrated a more suppressed array of expression, and showed an elevation in factors such as Frizzled -1, IFN-α, IL-27 and PDGF. As demonstrated in **fig 5.12**, the treatment of osteocytes with zolendronate in a simulation of microinjury to an osteocytic network had a tendency to significantly increase ($p \leq 0.05$), the presence of IL-12, IL-17 and IL-28, and furthermore, proved to stimulate the expression of CD 30 and CD 40, Frizzled 1, GFP-α 4, osteopontin, osteoactivin and TGF- β, all of which have been shown to demonstrate osteoclastogenic and osteogenic properties. In comparison to the non bisphosphonate treated constructs, there was no RANKL or OPG expressed, in addition to an absence of MMP-2, TNF- α, DKK-1, IL-1, Activin A, all of which have been implicated in stimulating osteoclastogenesis. Further to this, on comparing zolendronate to alendronate treatment of an osteocytic network following microinjury, there was no expression of the pro osteoclastogenic cytokine, IL-11, where as the pro osteogenic factor, WIF-1 was absent from alendronate treated samples. However, there was predominantly consistency observed in the proteins expressed between both bisphosphonate groups.

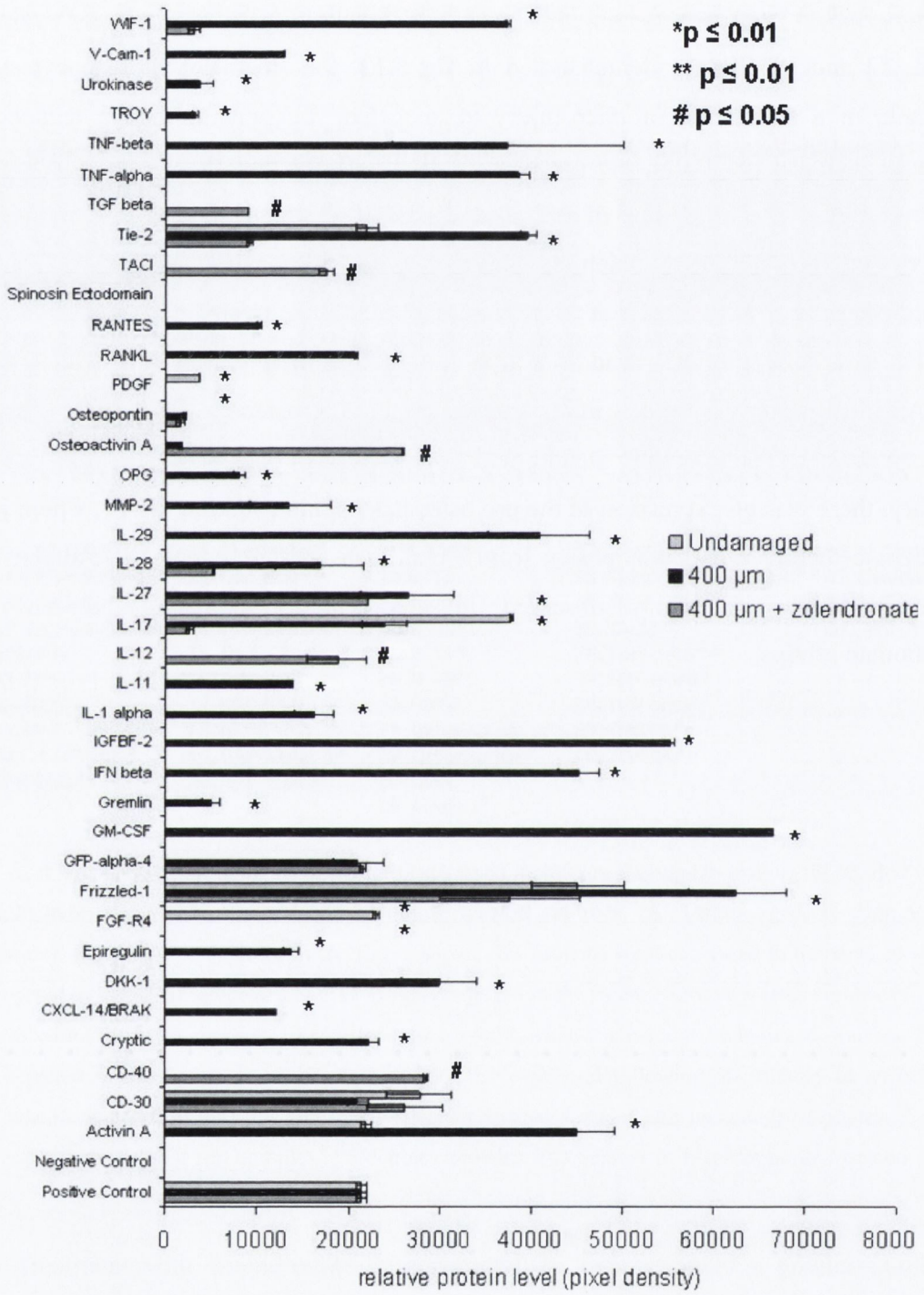


Fig 5.12 Zolendronate treatment suppresses some of the factors involved in the microinjury response in osteocytes, yet maintains other osteoclastogenic and osteogenic factors. Please see legend on next page.

Fig 5.12 Zolendronate treatment suppresses some of the factors involved in the microinjury response in osteocytes, yet maintains other osteoclastogenic and osteogenic factors. On comparing 400 μm non alendronate treated samples an increase in the presence of osteoclastogenic and osteogenic factors, such as CD-40, Frizzled-1, IL-11, IL-12, TGF- β, osteopontin and osteoactivin are observed in damaged following zolendronate treatment. However, when comparing bisphosphonate treated samples to non bisphosphonate treated undamaged sample, a significant increase in frizzled 1 and PDGF was observed, the latter of which has been implicated in bone repair. * denotes $p \leq 0.01$ when comparing significance between protein expression in 400 μm samples to undamaged samples. ** $p \leq 0.01$ when comparing significance between protein expression in undamaged samples to 400 μm samples. # $p \leq 0.05$ when comparing significance between protein expression in alendronate and both non alendronate treated undamaged and 400 μm samples. Error bars are indicative of standard deviation, $n = 3$.

5.4.4 Microinjury increases BMP-2 activity in networks of osteocyte cells treated with bisphosphonates

To quantify the amount of BMP-2 released following microinjury to an osteocytic network treated with 10^{-5}M concentrations of alendronate and zolendronate, an ELISA was performed using cell culture supernatants from undamaged and 400 μm samples, 48 and 72 hours after microinjury. As shown in fig x, there was a significant increase ($p \leq 0.05$) in all samples, at both 48 and 72 hours after microinjury between undamaged and 400 μm microinjury samples.

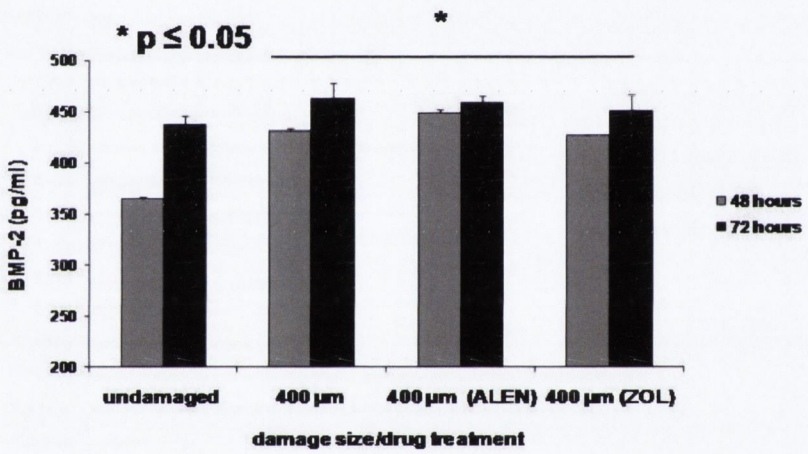


Fig. 5.13 Microinjury increases BMP-2 activity in networks of osteocyte cells in the presence of bisphosphonates. As shown in **Fig. 513**, there was a significant increase ($p \leq 0.05$) in BMP-2 activity at 72 hours when compared to samples at 48 hours. Further to this, no significant difference in BMP-2 activity was observed between alendronate treated and zolendronate treated samples at 400 μm. * denotes $p \leq 0.05$ when comparing significance between 400 μm with and without bisphosphonate treatment and non drug treated undamaged samples with and without bisphosphonate. Error bars are indicative of standard deviation, n =3.

5.5 Discussion

For many years, it was taken that RANKL and OPG function synergistically in maintaining skeletal homeostasis, alone. More recently, the roles of alternative factors and pathways involved in bone remodeling have been considered, however the relationship between microdamage and these factors has yet to be elucidated. In previous chapters, the role which microinjury plays, most notably the significance of microinjury size, on RANKL-OPG activity and physiological events in the cells of the BMU, was well established. The previous chapter demonstrated that bisphosphonate treatment altered the normal activity of RANKL and OPG, and initiated an almost entire suppression of these cytokines. Interestingly, however, the rate of osteoclastogenesis regulated by damage size, as did the rate of osteogenesis. Considering this, it posed the question- Is microdamage induced bone remodeling exclusively a RANKL-OPG mediated phenomenon, or do other factors play a role in stimulating this activity?

The ability of microdamage to stimulate bone remodeling has been shown over the years through the studies of Verborgt, Martin and Burr, Taylor, and Schaffler, whereby BMU activity has been observed in close proximity to microdamage, or, where an elevated level of apoptotic cells is present, thus eliciting a remodeling response, have been found on the periphery of microdamage in bone (Burr *et al*, 1985, Verborgt *et al*, 2000, Martin *et al*, 2000, Cardoso *et al*, 2010). As described in the previous three chapters, and consistent with existing literature, osteoclastogenesis and osteogenesis is regulated by damage size. In addition to this, the increase in RANKL activity paralleled with osteoclastogenesis, and thus, the relationship between damage size and cytokine activity was reinforced. In order to determine whether these activities are dependent greatly on RANKL activity, it was necessary to remove RANKL from the equation. RANKL has shown to be modulated by numerous osteotrophic factors, which include TNF- α mediated release of IL-1, IL-6, IL-8, IL-11, IL-15, IL-17 and IL-28. These multitude of interleukins also may act in the modulation of OPG activity following RANKL-RANK binding, and these tend to be isolated more so to IL-1, IL-6 and IL-11 (Hofbauer *et al*, 1999, Nakashima *et al*, 2000, Wei *et al*, 2002, Moreno *et al*, 2003, Moonga *et al*, 2002, Palmqvist *et al*, 2002, Bendre *et al*, 2003). Activity of RANKL has also been shown to be controlled by CD-40, and CD44, in a cell-cell based interaction, to furthermore

demonstrate the additional factors attributing to RANKL mediated and RANKL independent osteoclastogenesis (Okada *et al*, 2000). Interactions between RANKL and RANK activity, modulated by these such factors result in determining the fate of preosteoclasts, in that they bear the potential to stimulate their recruitment and differentiation, and thus, maturation into bone resorbing cells. Interestingly, there is evidence that RANKL is not the only direct mediator of osteoclastogenesis. It has been shown that TNF-α, IL-1 and TGF-β can act on preosteoclasts, stimulating their differentiation into osteoclasts (Fox *et al*, 2000, Kudo *et al*, 2002, Itonaga *et al*, 2004). On the contrary, however, it has been demonstrated that IL-1 activity depends on the presence of RANKL, so the role of RANKL as the principal stimulator of osteoclast activity is rather contradictory.

As has been well established, RANK and OPG function as being the principal receptors for RANKL, and thus, the use of recombinant forms of these proteins was fundamental in blocking RANKL activity. Varying concentrations of both cytokines was used to supplement the standard culture media, based on the amount of RANKL released in previous studies, and based on previous studies (Menaa *et al*, 2000, Zhao *et al*, 2002). This was applied to a 3D model of microdamage and conditioned media from microinjury experiments was subsequently applied to preosteoclasts, and culture for 21 days, determining the TRAP activity at 7 day intervals. As has been described, damage applied to an osteocytic network, such as in the proposed "Scissors Model", (Taylor *et al*, 2007), or alternative mechanisms whereby cell processes become ruptured in a shearing type mechanism (Cowin *et al*, 1991, Carter *et al*, 1987, Verborgt *et al*, 2000, Reilly *et al*, 2000), cause "bone remodeling" factors to be released into the surrounding environment, or in the case of this study, into the culture media. Indeed, there was suppression in the degree of osteoclast activity; however, this activity was not entirely diminished, which indicated that indeed, an alternative factor was present, capable of stimulating and initiating the bone remodeling process.

In this study, a myriad of growth factors, cytokines, chemokines, soluble factors and proteins were determined by means of a multiplex antibody array, in addition to determining the stimulus for osteogenesis. It is significant to note that this is the first time that this large number of factors released from a model of microdamage, has been determined. As described, RANKL activity depends on the presence of several factors, principally M-CSF, which results

from IL-1 and TNF- α activity, allowing the solubilisation and activation of RANKL (Lum *et al,* 1999, Kimble *et al*, 1996), which facilitates the upregulation of a major osteogenic factor, BMP-2. BMP-2 functions through the activity of the Smad signaling pathway, which are mediated by activin A and TGF β. Additionally, interleukins, primarily IL-1, functions, in addition to TNF, in upregulating RANTES, which has shown to be a stimulator of osteoclastogenesis. Further to this, other members of the TNF superfamily, such as TRAFs, have shown to stimulate an osteoclastogenic response, in conjunction with NF κ B, Leukemia Inhibitory factor and c-Fos (Boyle *et al*, 2003, O'Brien *et al*, 1999, Palmqvist *et al*, 2004). Further to this, as described above, the Wnt/ß-catenin pathway has been implicated in stimulating a bone remodeling response (Miller *et al*, 2006). This pathway encompasses numerous factors, such as Wnt inhibitory factors (WIFs), secreted frizzled-related proteins (SFRPs), dickkopfs (DKKs) (Kawano *et al*, 2003, Rawadi *et al*, 2005). These proteins function in either stimulating osteoclast activity or osteoblast activity, through a negative feedback loop. This is but a very small example of the known, and potential factors involved in bone remodeling, and, as described, their role and the potency of activity, and how this related to microdamage, has yet to be determined.

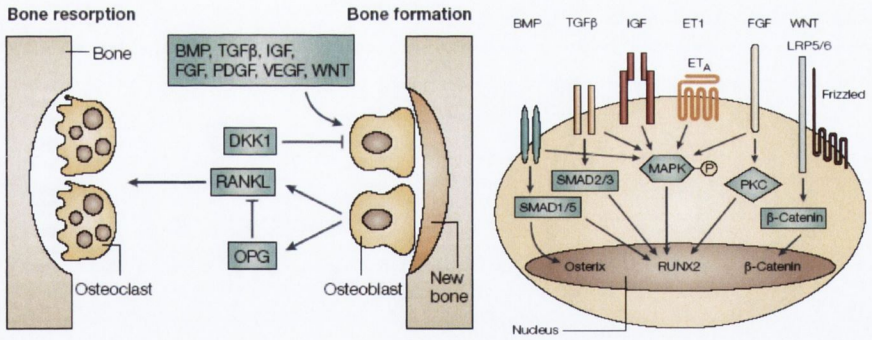


Fig 5.14 Signaling pathways in bone remodeling. Normally, bone formation and resorption involves a highly regulated process controlled by RANKL-OPG signaling. Further factor involved in this process include BMP, TGF- β, PDGF and WNT, which regulate osteoblast activity. Further to this, DKK blocks Wnt activity, and subsequent BMP activity, inhibiting osteoblast activity and stimulating osteoclastogenesis. BMP binding to its receptors is responsible for upregulating Smads, which further determine the functioning of Runx2 and osterix, regulating osteoblast activity. Furthermore, TGF-β also affects Runx signaling. FGFs and ILGF function in phosphorylating Smads and activating the MAPK pathway in osteoblasts, which, in turn, regulated osteoclastogenesis. Wnt proteins, on the other hand, interact with its receptors, frizzled or LRP, stabilizing β-catenin, initiating either an osteoclastogenic or osteoblastic response (Logothetis *et al*, 2005).

The results of the proteomics study demonstrated that following microinjury, 22 proteins were upregulated and 4 proved to be downregulated, when compared to the 6 which were determined in the undamaged construct. As expected, in 400 μm samples, RANKL activity was elevated, when compared to the undamaged construct, and in addition to this, there was an increase in MMP-2 activity, which is a gelatinase, and one of the principal matrix metalloproteases responsible for the cleavage of RANKL from its membrane bound form to soluble form (Kusano *et al*, 1998). Additionally, MMP-2 activity has been show to be regulated though binding of Ephrin B2 to Eph B4 in the bidirectional signaling pathway, which, as discussed in the introductory chapter, is responsible for regulating osteoclasts and osteoblast activity (Tat *et al*, 2008, Zhao *et al*, 2006). Interestingly, OPG activity was found to be unregulated in damaged samples, whereas it was found to be suppressed in undamaged samples. This is contradictory to the findings of chapter 2; yet, it supports the theory proposed in chapter 2, and may be considered an experimental anomaly. Further to this, some RANKL was found to be present in undamaged constructed, as detailed in chapter 2, which is suggestive that a proteomic approach may not be as sensitive at detecting certain proteins.

Interleukin pathway

As described in the introduction, RANKL activity is regulated by TNF- α and subsequent interleukin activity, principally IL-1, IL-6, IL-11, IL-17 and IL-28. As determined by the antibody array, microinjury stimulated the expression of IL-1 α, which has been suggested to be regulated by RANKL activity, and so may play a role in a RANKL mediated osteoclastogenic response (Lee *et al*, 2006, Lee *et al*, 2010, Kim *et al*, 2010). Further to this, expression of IL- 17, L-28 and IL-29 was shown to be increased following microinjury. IL-17 has vastly shown to function synergistically with RANKL in mediating bone loss (Won *et al*, 2011, Lubberts *et al*, 2003). IL-28 and IL-29 levels were also increased in 400μm samples; however, their role in either the stimulation of inhibition of both osteoclastogenesis and osteogenesis has yet to be determined. As was demonstrated in both damaged and undamaged samples, an increase in IL-27 was observed, and the increase in IL-27 was significantly greater in undamaged samples. It is significant to note that IL-27 has been shown to be a potent inhibitor of osteoclastogenesis (Kimaya *et al*, 2007, Kimaya *et al*, 2010, Kalliolas *et al*, 2011), through suppression of RANKL mediated NFAT expression and subsequent abrogation of

RANK expression on preosteoclasts. Activin A is another factor harnessed by RANKL to stimulate osteoclastogenesis (Fuller *et al*, 1999, Sugatani *et al*, 2003), and again, at 400 μm, has shown to be greatly upregulated, when compared to undamaged samples, where Activin A was present, but to a lesser extent. Inhibition of activin A has proved to enhance bone formation; yet, large amounts of activin have shown to enhance bone formation and fracture healing in rodents (Chantry *et al*, 2010). This may be a principal finding, demonstrating as to, again, why little osteoclastogenesis occurs in undamaged samples and may be due to the suppressed expression of a reduced level of RANKL, MMP-2, Activin A, TNF-α and IL-1 α.

Wnt/ β-catenin pathway

As described, the Wnt/ β-catenin pathway has strongly been associated with regulation of bone remodeling, through the mechanistic functioning of its receptors, DKK-1 and SFRPs, and antagonists, Frizzled proteins and WIFs, as demonstrated in **Fig. 5.3** (Miller *et al*, 2002, Kawano *et al*, 2003, Rawadi *et al*, 2005, Bodine *et al*, 2008). Wnts, as described, function to reduce levels of osteoblast apoptosis, as described. Furthermore, treatment of osteoblastic cells with PTH, which has shown to also upregulate RANKL, increased β-catenin protein levels, and that this effect occurred through modulation of both the protein kinase A and protein kinase C pathways, thus reducing apoptosis of osteoblastic and osteocytic cells (Tobimatsu *et al*, 2006). A study by Bodine *et al,* 2004, showed that suppression of Wnt/β-catenin lead to a downregulation of the transcription factor, Runx2, which is essential for the maturation of osteoblasts, and in the ultimate expression of type I collagen, alkaline phosphatase (ALP), bone sialoprotein and osteocalcin. Following antibody array analysis of both 400 μm damage and undamaged samples, the expression of some of the factors involved in the Wnt/ β-catenin pathway was increased. An expression of Frizzled 1, the co receptor of Wnt and which is responsible for Wnt signaling initiation, showed to be upregulated in both damaged and undamaged samples, however, its activity was found to be greater at 400 μm (Yerges *et al*, 2008). It has been shown that mechanical loading, which has been hypothesised to cause shearing of osteocyte cell processes, requires Wnt dependent activation of LRP5, initiating an osteogenic response. Antagonism of this activity, but such factors as frizzled 1 and DKK-1, initiates an osteoclastogenic response, and thus, may further demonstrate as to why osteoclastogenesis is elevated in 400 μm samples, even following the ablation of RANKL

activity (Sawakami *et al*, 2006). In addition to this, the DKK-1, which is also an inhibitor of Wnt, demonstrated an increased expression in 400 μm samples, whereas it was absent from undamaged samples. DKK-1 functions as a receptor for Wnt, stabilising the Wnt/ β-catenin pathway, initiating an osteogenic response (Levasseur *et al*, 2005). Conversely, excessive DKK-1 has shown to initiate an alternative route of activity, by blocking osteogenesis (Li *et al*, 2005) and has additionally shown to increase OPG levels, thus demonstrating a reason as to why OPG levels are increased in this model of microinjury in bone (Diarra *et al*, 2007). A further participant in the Wnt/β-catenin pathway is WIF-1, which inhibits the action of Wnt, subsequently preventing osteoblast activity and stimulating bone resorption, leading to reduced bone density (Minear *et al*, 2010, Holmen *et al*, 2005 Cho *et al*, 2008). In 400 μm samples, a large increase in WIF-1 activity was observed indicating an increase in osteoclastogenesis, whereas in undamaged samples, this protein was not detected. The presence of DKK-1 in 400 μm samples may furthermore provide the rationale as to why both osteoclastogenesis and osteogenesis are both increased, and may furthermore show the need for homeostasis and additionally, the functional relationship between DKK-1, WIF-1 and frizzled-1, in maintaining bone remodeling.

Bone Morphogenic Protein 2

BMP-2, a member of the TGF- β superfamily, has long been implicated in the initiation of osteogenesis and in promoting mineralisation in bone. This protein acts as a disulphide linked homodimer, capable of inducing bone and cartilage formation, and thus has been used therapeutically in bone tissue engineering. The role which BMP-2 plays, following microinjury has yet to be determined. BMP-2 has shown to be a critical factor in the induction of fracture repair (Seeherman *et al*, 2005), and abnormalities in BMP-2 expression can lead to low BMD and increased fracture risk (Styrkarsdottir *et al*, 2006). Additionally, BMP-2 has harboured the ability to initiate differentiation of cells from a mesenchymal to an osteoblastic lineage of cells (Abe *et al*, 2000). Further to this, the Wnt/ β-catenin pathway has been implicated in regulating the initiation and correct functionality of BMP-2, and in turn, Frizzled 1 has shown to be regulated by BMP-2 (Roman *et al*, 2004, Chen *et al*, 2007). Conditioned media from microinjury experiments was analysed for BMP-2 activity using a single site specific ELISA, 48 and 72 hours after damaged, and an increase in BMP-2 activity was

determined following microinjury. This indicated that, indeed, microinjury to an osteocytic network is capable of initiating BMP-2 activity, and in turn, furthermore demonstrates the reason as to why microinjury increases osteogenesis in preosteoblastic cells. Furthermore, these results complement the findings of the antibody array, whereby increased levels of DKK-1, Frizzled-1 and WIF-1 were observed. These results may demonstrate a relationship, complementary to those of Qiang *et al*, 2008, whereby it was shown that Wnt signaling along is not sufficient to stimulate osteogenesis. This study furthermore illustrated the requirement for BMP-2 to be present to stimulate osteoblast differentiation and activation.

The effects of bisphosphonate treatment of alternative pathways stimulating bone remodeling

The next section of this chapter was to determine the effects of bisphosphonate treatment on the various cell signaling pathway involved in microinjury induced bone remodeling. As described and subsequently demonstrated in the previous chapter, bisphosphonate treatment interferes with RANKL and OPG activity (Engel *et al*, 2003, Kimachi *et al*, 2011) and also has shown to suppress the normal RANKL-OPG response to microinjury. However, in spite of the almost full ablation of RANKL and OPG activity following microinjury, osteoclastogenesis and osteogenesis remained consistent with previous findings, albeit to a decreased degree (Mulcahy *et al*, 2011), and proved to increase accordingly with microinjury size. This, again with the results demonstrated above, regarding blocking RANKL activity with its receptor proteins, RANK and OPG, indicated furthermore that another signaling mechanism was in place to facilitate the maintenance of bone remodeling and ultimately, skeletal integrity. As described in the previous chapter, bisphosphonates function in the reduction of osteocyte apoptosis, in the depletion of osteoclast activity, reducing their potential to resorb bone, and by increasing bone mineral density, reducing fracture risk and providing a more stable, tenacious skeletal structure (Kennedy *et al*, 2007, Giner *et al*, 2010, Hughes *et al*, 1995, Recker *et al*, 2008, Brennan *et al*, 2010).

With regard to determining the release of pro-remodeling factors in response to microinjury, conditioned media from osteocytes treated with 10^{-5}M alendronate or zolendronate, 72 hours following microinjury of 400 μm was subject to proteomic analysis using an antibody array. An initial observation was the marked suppression and absence of many of the proteins expressed in the non bisphosphonate treated samples, most significantly

RANKL and OPG, and its associated factors, MMP- 2, IL-1 α, TNF-α and DKK-1, all of which are responsible for stimulating osteoclastogenesis. A study by Asahi *et al*, 2006, demonstrated that bisphosphonate treatment suppressed mRNA levels of both MMP-2 and RANKL, and have shown to elicit and inhibitory role on IL-1 and TNF-α expression (Penannen *et al*, 1995) as well as various dikkopf proteins (Ruggiero *et al*, 2007). As described in the previous chapter, an osteoclastogenic response was observed following microinjury. In both alendronate and zolendronate treated samples, there was an increase in CD-40, which has been implicated in binding to CD-44 on other cell types, such as preosteoclasts, and thus initiating an osteoclastogenic response (Okada *et al*, 2000). Furthermore, there was an increased incident in the levels of Frizzled 1, in damaged and undamaged samples, indicating the propensity for bisphosphonate treatment to indeed suppress RANKL induced osteoclastogenesis yet still maintain an osteoclastogenic response, and facilitate a degree of bone remodeling. This showed consistency with previous studies which demonstrated the effects of bisphosphonates on frizzled 4, and frizzled related proteins, which can act on osteoclasts (Streeten *et al*, 2008). Further to this, there was found to be an increase in pro osteoclastogenic IL-11, IL-12 and IL-17, which have, as described above, been shown to be potent stimulators of osteoclastogenesis, independently of RANKL. There was an increase in expression of WIF-1 in zolendronate treated samples, which, was also upregulated in non bisphosphonate treated samples, which, as previously described, bears the potential to halt osteoblast activity and osteogenesis, and switch on the signaling process associated with osteoclast recruitment and activation, which may explain why osteoclast recruitment and osteoclast activity was greater in zolendronate treated samples than alendronate treated samples. On comparing bisphosphonate treated sample to non bisphosphonate treated sample, following microinjury, a vast suppression in the expression of pro osteoclastogenic factors, such as IL-1, IL-6, IL-11, IL-17 and IL-28, in addition to TNF-α and DKK1 was observed in bisphosphonate treated groups. This indicates a reason as to why osteoclastogenesis was reduced in the studies of the previous chapter, but not fully impeded.

With regard to the expression of osteogenic factors, there was, in both alendronate and zolendronate treated samples, following damage, a marked increase in IL-27, which has, as described, been implicated in the inhibition of osteoclastogenesis and initiation of osteoblast activity (Kalliolas *et al*, 2011). Further to this, an increased level of osteoactivin A

and osteopontin was present in 400 μm bisphosphonate treated samples, when compared to non bisphosphonate treated samples under the same parameters. Osteoactivin A is associated with the maturation and differentiation of osteoblasts, and functions as a downstream mediator of BMP-2 activity (Abdelmajid *et al*, 2003). Osteopontin, or bone sialoprotein, is expressed in bone as a marker of osteogenesis, and is expressed by all bone cell types, predominantly osteoclasts and osteocytes, and is under the control of the Runx2 and Osx signaling pathways (Merry *et al*, 1993, Ducy *et al*, 1997). Osteopontin has furthermore been implicated in initiating osteoblast recruitment and in stimulating mineralisation in osteoblastic cells (Oldberg *et al*, 1986), which would be indicative as to why there are elevated levels of osteoblast recruitment and osteoblast mineralisation in bisphosphonate treated samples following microinjury. To further demonstrate the osteogenic potential of bisphosphonates in the absence of RANKL and OPG, again, conditioned media samples were analysed for BMP-2 release using ELISA. Treatment with bisphosphonate showed no significant difference in BMP-2 activity when compared to non bisphosphonate treated osteocytes, however, there was an increase in BMP-2 activity observed again in 400 μm samples, when compared to undamaged samples. This again indicated that bisphosphonate treatment does not affect BMP-2 activity, and that either the presence of osteogenic proteins released following microinjury, or that the ability of bisphosphonates to stimulate spontaneous mineralisation of preosteoblasts, may be the principal causes of increased mineralisation following microinjury.

When considering the cumulative results of this section of the study, it is evident that microinjury to an osteocytic network induces a larger quantity of pro osteoclastogenic than osteogenic factors. This demonstrates that, in spite of the absence of RANKL, osteoclastogenesis can occur, via inhibitions of Wnt through DKK1 and WIF, through increased levels of TNFα, and IL-1 α and IL-17. It may seem that this would lead to an environment whereby excessive bone resorption would prevail, yet, it is paramount to note that, *in vivo*, by products of bone resorption, such as PTH, estrogens, TRAP and TGF-β, which function in stimulating osteoblast activity. Further to this, the presence of IL-27, IFN β, Frizzled 1, and BMP-2, the latter of which works synergistically with the Wnt/ β-catenin pathway, provides a platform to stimulate osteoblast activity and osteogenesis. Further to this, treatment of osteocytes with bisphosphonates and the application of microinjury to this system proved to suppress a number of the factors involved in osteoclastogenesis and osteogenesis,

such as OPG, RANKL, MMP- 2, IL-1 α, TNF-α and DKK-1, yet caused an increase in osteoclastogenic factors including WIF-1, Frizzled 1, IL-11 and IL-17, in addition to osteogenic factors such as osteopontin, IL-27 and osteoactivin A. This again may be an important finding in terms of reducing concerns associated with using bisphosphonates, such as an increased risk of vertebral fractures due to impediment of the normal bone remodeling process (Arlot *et al*, 2008, Fazallari *et al*, 1998). Further to this, the use of new generation osteoporosis therapeutics, such as denosomab, a monoclonal antibody which targets RANKL, reducing its activity and thus potentially inhibiting bone resorption and subsequent bone formation (Pageau *et al*, 2009, McClung *et al*, 2006, Ellis *et al*, 2008). The partial presence of an alternative reparative mechanism utilized by bone, as demonstrated by the results of this study, allows osteoclast activity to be reduced to an extent, yet still maintain a baseline level of bone remodeling, which would thus reduce excessive bone resorption, whilst reducing the risk of fracture which would also be fundamental in maintaining skeletal strength and durability. An additional aspect of this study is the identification of other factors involved in microdamage induced bone remodeling, which may prove useful as a therapeutic target in the development of new therapeutics for osteoporosis, and other such bone diseases.

A limiting factor to this study is that the use of multiplex antibody arrays, while facilitating the expression of a vast quantity of proteins, still neglected to show the expression of other important proteins expressed, which are related to, and involved in both the RANKL-OPG signaling pathway, and also the wnt/ β-catenin pathway, the latter of which has shown great promise in terms of being an alternative mechanism for facilitating bone remodeling. Further to this, it may have proved useful to target certain proteins and quantify their activity using ELISA or PCR. Additionally, it may have proved useful to analyze the conditioned media resulting from microinjury induced osteoclastogenesis, to determine if there is a greater frequency of osteogenic factors in place to further demonstrate why there is an increase in mineralization in 400 μm damage samples. Again, this study only examined one dose of bisphosphonate for each respective drug, and so, a difference response to microinjury may have been observed at greater or lesser concentrations. Overall, the experimental findings in this chapter have shown that in an *in vitro* simulation of microdamage in bone, there are alternative pathways in place to stimulate and regulate bone remodeling. Furthermore, treatment with bisphosphonates has been shown to reduce the number of osteoclastogenic and

osteogenic factors released following microinjury, yet still maintain the presence of other cytokines to regulate bone remodeling.

5.6 Conclusion

In conclusion, this study has demonstrated that microinjury may be critical in the instigating bone remodeling through release of biochemical factors capable of stimulating osteoclastogenesis and osteogenesis. This chapter has shown that RANKL activity and OPG secretion may not be exclusively involved in stimulating microinjury induced osteoclastogenesis, in terms of TRAP activity in preosteoclasts, and thus, may pose an impact on subsequent osteogenesis. Additionally, this study has shown that microinjury instigates RANKL independent osteoclastogenesis through the action of interleukins, TNF-α, and the Wnt/ β-catenin pathway. Further to this, the use of bisphosphonates has been shown to suppress some of the proteins involved in initiating osteoclastogenesis and osteogenesis, in addition to RANKL and OPG, however still maintaining some of the factors involved in bone remodeling. Finally, BMP-2 activity has also been shown to be regulated by the presence of damage size, and may be a mediator of bone formation following microinjury. In summary, this study shows that bone remodeling may not exclusively be a RANKL-OPG mediated phenomenon, and further demonstrates that microdamage may be a principal mediator of bone remodeling.

Chapter 6

Discussion

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6 Discussion

Based on previous findings in this research group, a large emphasis has been placed on the concept of crack size and the number of cellular processes ruptured following microdamage, and how this affects bone remodeling, and ultimately how this maintains structural and functional homeostasis in bone. On commencement of this study, two simple questions were posed; **1.** How does bone detect microcracks? & **2.** On detection of these microcracks, how does bone repair them? These questions gave rise to the central hypothesis of this study; If the size of the crack in bone is related to the number of osteocyte cell processes ruptured, then crack size would influence the biochemical stimuli which initiate bone remodeling. This would pose an impact on the behaviour of the cells involved in the BMU, ultimately governing the repair of cracks in bone. Relating to the first question posed in this thesis, how does bone detect microcracks, this study focused on the principal mechanism by which the BMU is stimulated, maintained and mediated via the RANKL-RANK-OPG signaling pathway. Using a set of novel experiments, in the absence of any other stimulus, such as repetitive cyclic loading or fluid flow, the roles of these cytokines were evaluated as a function of damage size in a 3D simulation of microdamage in bone. This then leads to the incorporation of the second question posed in this thesis; on detection of these cracks, how does bone repair them. Cytokine cascades and cellular responses using the constitutive cells of the BMU, were evaluated in another set of original experiments. Furthermore, the role of osteocyte rupture in an environment with elevated microinjury, such as what is observed in osteoporosis, was determined. Additionally, whether bisphosphonate treatment can potentiate or impede bone remodeling following microinjury was also clarified. The results obtained in this thesis demonstrate, for the first time, the effects of microinjury on numerous biochemical pathways. In turn, this was again, the first time, a "remodeling response" was observed in the cells of the BMU.

6.1 Microinjury results in cellular apoptosis and stimulates RANKL and OPG activity in a damage size dependent manner

The initial portion of this thesis has shown the relationship between microinjury size and bone remodeling, which is reflective of previous work by other researchers, whereby it was suggested that RANKL and OPG release, and ultimately, bone remodeling, is dependent on microinjury size. On the development and implementation of an *in vitro* model for the study of microdamage in bone, the role of microinjury size, in relation to RANKL and OPG activity, was established. It was found that an increase in damage size, up to 400 μm, caused an increase in RANKL activity and a decrease in OPG activity; a reciprocal relationship was observed in undamaged and smaller damage sizes. Further to this, at 800 μm damage size, the pattern of RANKL/OPG activity varied significantly from the normal pattern of activity, suggesting that an alternative route of bone repair is utilised. These results also demonstrated, as a whole, that a fine line exists between microdamage and what may be considered failure, and that microinjury size is fundamental in initiating the release of bone remodeling cytokines. Additionally, microinjury, using this *in vitro* model, has been shown to cause an apoptotic response, with regard to caspase 3 and 7 activity, again, in a damage size dependent manner. In summation, chapter 2 has shown that microinjury may be a principal mediator of bone remodeling due to its effect on RANKL and OPG activity.

6.2 Microinjury mediates the bone remodeling functions of the constitutive cells of the BMU in a damage size dependent manner

As was found in chapter 2, microinjury of a given size, mediates the biochemical and apoptotic events in osteocytic cells, which demonstrates the mechanostimulatory potential of osteocytes. Damage to an osteocytic network, which has been shown to affect RANKL and OPG activity, may pose an effect on the cellular functioning of the BMU, *in vivo*, and may affect the rate of BMU activity in terms of progenitor cell recruitment, differentiation and maturation, osteoclastogenesis and osteogenesis. As described in the introduction, RANKL release stimulates osteoclastogenesis, and when sufficient bone resorption has occurred, a reversal phase is observed, whereby OPG is released on osteoclast apoptosis, and this binds to RANKL, preventing further osteoclastogenesis and

subsequent osteogenesis. This concept lead us to the hypothesis of this study, whereby variations in damage size would cause changes in the degree of precursor cell migration, osteoclastogenesis and osteogenesis, in the constitutive cells of the BMU. Thus, this hypothesis facilitated the development of a study plan: to segment the events in bone remodeling, which involve recruitment and activation of preosteoclasts, and thus osteoclastogenesis, and subsequent recruitment, activation and maturation of preosteoblasts, with subsequent osteogenesis.

Using the same model of microdamage as described in chapter 2, conditioned media obtained from microinjury to an osteocytic network, of varying size, was used as a stimulus for preosteoclast recruitment and as a stimulator of osteoclastogenesis, to determine the relationship between damage size and osteoclastogenesis and osteogenesis. The use of an *in vitro* simulation of microdamage to an osteocytic network was shown to stimulate all the stages observed in bone remodeling. Preosteoclast recruitment and subsequent maturation into TRAP positive osteoclasts, and consequent preosteoblast recruitment, maturation, and successive osteogenesis, in the constitutive cells of the BMU, were stimulated following microinjury to an osteocytic network, in a damage size dependent manner, most principally so, at 400 μm. Combining the results of chapter 2 and 3 have shown, for the first time, that microinjury size, most principally at 400 μm, mediates a bone remodeling response, demonstrating that damage size is a critical factor impacting on the initiation of bone remodeling. In summation, the study shows that microinjury is capable of stimulating osteoclastogenesis and osteogenesis *in vitro*, the degree of which is also injury size dependant.

6.3 Bisphosphonates change the normal response to microinjury but maintain osteoclastogenesis and osteogenesis

An elevation of microdamage in bone is a characteristic attribute of osteoporosis, and this result is due to an imbalance in RANKL-OPG signaling, caused by a reduction in estrogen, most notably during menopause. The net result of this is an increase in osteoclastogenesis and osteoclast activity, and no subsequent bone formation will occur. The net result of these cumulative activities results in an increase in the risk of fragility fractures. Bisphosphonates are a common treatment of osteoporosis, and function in

suppressing osteoclast activity and increasing bone mineral density. Further to this, it has been suggested that bisphosphonates function in the inhibition of RANKL. However, the exact mechanisms of action of bisphosphonates, and how this relates to microinjury, which is a characteristic property of osteoporotic bone, required examination. The hypothesis of this study was that the effect of microinjury, which has been shown to induce both osteoclastogenic and osteogenic responses, would be affected by the presence of bisphosphonates. Bisphosphonate treatment to an *in vitro* simulation of microdamage may present variations in the degree of precursor cell migration, and may decrease osteoclastogenesis, and ultimately, increase the levels of osteogenesis, in the constitutive cells of the BMU. Additionally, it was thought that treatment may also alter the release and activity of RANKL and OPG. The aim of this study was to determine the synergistic role of microinjury and bisphosphonate treatment, in terms of RANKL and OPG activity, and the subsequent bone remodeling activities using the constitutive cells of the BMU.

The results of this chapter, as a whole, have shown that bisphosphonates function by modulating the normal "microdamage effect" in osteoclasts, and results again demonstrate, microinjury size is fundamental in initiating bone remodeling responses. Treatment of an osteocytic network with alendronate and zolendronate, suppressed RANKL and OPG activity and reduced preosteoclast migration and osteoclastogenesis in the absence of microinjury. However, at a critical damage size, i.e. 400 μm, RANKL and OPG activity remained modulated, however, a level of osteoclast activity was maintained. Further to this, osteogenesis proved to be stimulated by bisphosphonates, in conjunction with microinjury. The overall conclusive result of this study was that bisphosphonates pose an effect with microdamage, on the mediation of bone remodeling activities, *in vitro*.

Considering these results, this study has provided the basis for the studies involved in the following chapter. The demonstration that RANKL and OPG activity are almost entirely suppressed, yet, osteoclastogenesis and osteogenesis occur, in the absence of any other stimulatory factor, suggests an alternative signaling mechanism used by bone. This was the focus of the final chapter, whereby other biochemical factors involved

in the stimulation of bone remodeling were characterised following microinjury and bisphosphonate treatment.

6.4 The response to microinjury is not solely governed by RANKL and OPG activity

As described in chapters 2 and 3, RANKL and OPG have long been implicated as the principal factors in bone remodeling, yet other biochemical factors have been implicated in the activation, functioning and longevity of the BMU. The findings of chapters 2, 3 and 4 demonstrated that microinjury size is a critical factor contributing to the stimulation of RANKL and OPG, and subsequent bone remodeling activities in the constitutive cells of the BMU. A proteomics approach was employed to determine the proteins which may be involved in stimulating the bone remodeling pathway outside of the RANKL-RANK_OPG pathway. BMP-2 was also analysed to determine whether it was a factor involved in stimulating the osteogenic response observed after microinjury. Results of this study showed that microdamage induced bone remodeling is not exclusively a RANKL/OPG mediated response; the release of interleukins and TNF α, which have been implicated in osteoclastogenesis, occurred following microdamage. Additionally, the presence of other factors, such as dikkopf, wif 1 and frizzled 1, suggest an alternative mechanism of mediating bone remodeling, such as the wnt/ β cadherin pathway which may also be involved in regulating the osteoclastogenic and osteogenic response to microdamage. Furthermore, an increase in BMP-2 activity was demonstrated, and this shows that this protein is a prime candidate involved in microdamage induced osteoclastogenesis.

A further aspect of this study was to determine the effect of bisphosphonates on the normal "microdamage response" in osteocytes. As shown in chapter 4, treatment of injured osteocytes with alendronate and zolendronate inhibited RANKL and OPG, yet still maintained an, although suppressed, osteoclastogenic and subsequent osteogenic response. Drug treatment suppressed some of the pathways involved following microinjury, as observed in non bisphosphonate treated cells. However, a number of interleukins and members of the wnt/ β-catenin pathway were upregulated following microinjury in the presence of bisphosphonates, suggesting an alternative remodeling mechanism. It can be deduced from the results of this experiment, that the suppression of

RANKL and OPG following bisphosphonate treatment, can still stimulate an osteoclastogenic and osteogenic response.

The overall results of this chapter suggest that microinjury to osteocytes induce a number of osteoclastogenic factors, and furthermore, osteogenic factors. It is evident that alternative pathways to the RANKL-OPG pathway are employed, including the interleukin pathway, TNF- α pathway, and Wnt/ β-catenin pathway, even following bisphosphonate treatment. As previously discussed, this may have clinical implications in terms of using these factors as drug targets in osteoporosis therapeutics. A concluding aspect of this study shows that alternative mechanisms may be utilised by bone to maintain homeostasis, and furthermore demonstrates the critical impact of microdamage in initiating a bone remodeling response.

Conclusion

The cumulative results of this thesis have demonstrated that microdamage and *in vitro* microinjury regulates the activity of RANKL and OPG, and can initiate alternative biochemical cascades to stimulate bone remodeling. Furthermore, it has been shown that microdamage, and *in vitro* microinjury stimulates all the principal stages of bone remodeling in the constitutive cells of the BMU. A final aspect of this thesis illustrated that bisphosphonates, in spite of mediating the suppression of RANKL and OPG, maintain a degree of bone remodeling responses in the cells of the BMU in our model. This again, may be due to the findings that alternative bone remodeling pathways are upregulated following bisphosphonate treatment when microdamage is present. The principal conclusion, however, on completing this thesis, is that microdamage size may be critical in mediating a remodeling response in bone.

6.5 Limitations of this thesis

The principal limitation of this thesis is the question of how similar is the model to the *in vivo* scenario. However, it has to be noted, as described in chapter 2, that the cells used experimentally, MLO-Y4 cells, differ somewhat in morphology and properties to that of endogenous osteocytes, and so, the given the calculations detailed , it was found that crack size was quite analogous to what was observed *in vivo*. The development of a

theoretical model, which is being performed by another researcher in the group, facilitates the extrapolation of the results of this study to varying damage sizes, however, it must be noted that the experiments in this thesis, in spite of the requirement for refinement, have shown, for the first time, the effects of damage alone, on RANKL and OPG activity, in addition to other biochemical pathways, on bone remodeling activities of the BMU, to an osteocytic network. The size of the damage, as discussed, is quite similar to what is observed *in vivo*, however, whether it is the cell processes that are being ruptured, or damage to entire cells, or populations of cells, requires further examination. However, the results of the experiments demonstrated that, regardless of the type of damage which ensues following this method, there is a relationship between cellular damage and a "remodeling response".

Secondary limitations of this thesis include the following; that the constitutive cells of the BMU are not contained all together in one system, and the osteoclastogenic and osteogenic activities which occur do not function in an environment analogous to that of bone. However, the use of experiments has shown the stimulatory potential of microdamage in bone in terms of the bone remodeling capabilities of preosteoblasts and preosteoclasts. Chapter 4 has outlined the more basic events initiating bone remodeling activity following bisphosphonate treatment and microinjury to an osteocytic network. Both RANK-OPG activities were determined, however, a more detailed method of analysing the biochemical responses may prove more insightful in elucidating how bisphosphonates interact with cells on a biochemical level. A further limiting factor of this study is that the use of proteomics based assays, which, while maintaining ample potential to detect a vast array of proteins, do not provide a quantitative result. It may have proved viable to target certain proteins and quantify their activity using ELISA or PCR.

Chapter 7

Future Work

The studies encompassed within this thesis have demonstrated the fundamental activities involved in an *in vitro* model of microdamage induced bone remodelling, from a biochemical and cellular perspective. There is still the potential to explore further mechanisms by which bone maintains its structure following microdamage.

Firstly, further characterisation of the MLO-Y4 cell line would provide a greater scope in validating our experimental models, regarding the number and length of cell processes, and also to determine their spatial distribution within the 3D matrix. This could be achieved through confocal microscopy of the gel embedded osteocytes, by counting the number of cell processes before and after the application of damage, and also by calculating more intricate parameters such as cell spacing over a broader range of cells, numbers of cell processes affected by damage, and number of cells disturbed following passage of the needle through the matrix.

A major focus of a future study would be to refine the damage size used in experiments. The current system has shown to be a useful tool as a "testbed" for future experimentation. The development of a rig or device to produce smaller damage sizes, under cyclic conditions, could provide a more realistic scenario relating to what happens *in vivo*. Further to this, the use of a laser may assist in cutting fewer, or single cellular processes. From this, the amount of RANKL and OPG on a singular process could be quantified. In addition to this, it may prove more clinically relevant to isolate and culture human osteocytes, osteoclasts and osteoblasts, and perform similar experiments.

As was described by Cardoso, *et al*, 2009, suppression of osteocyte apoptosis by means of caspase inhibitors, in our *in vitro* system may provide useful data with regard to further validating our model and also to determine whether a similar response was observed. Cardoso, *et al*, described how a use of apoptosis inhibitors reduced the amount of osteocyte apoptosis considerably, and a reflection of this in our model would further

show how our model reflects the in vivo scenario, and thus would further demonstrate the potential of injury to cells as a mechanism of stimulating bone remodeling.

The use of a tissue engineered scaffold, such as collagen glycosaminoglycan or a collagen hydroxyapatite composite, seeded with a co culture of osteocytic and osteoblastic cells may facilitate mineralisation of the 3D matrix, and may provide a more realistic environment. Introduction of osteoclastic cells, prior to, or following the application of focal microinjury may provide an environment more similar to that observed *in vivo*. Using co culture methods may also allow for observing the cell-cell interactions, such as the bidirectional signaling pathway of ephrin B2-EphB4 interaction, and also may facilitate the microscopic analysis of how various cell types interact with each other, in terms of the formation of an *in vitro* "BMU". Furthermore, the stiffer material may also allow for the application of cyclic microdamage, due to a lesser propensity for matrix degradation, which has been observed using collagen-matrigel constructs. Additionally, for this reason, the use of such constructs may facilitate a longer period of analysing responses to microdamage and microinjury.

The "Scissors Model" of microdamage in bone may be furthermore explored, in terms of cellular apoptosis, in addition to biochemical responses. Using ex vivo bone slices, subject to compressive loading to stimulate shearing of cell process rupture, followed by immunostaining for RANKL and OPG activity, in addition to immunohistochemical staining for caspase 3 activities. The experimental model used in this thesis has shown to produce an apoptotic effect, and so, evaluation of the apoptotic potential of the shearing mechanism of bone cells would reinforce the validity of this model as a means of studying microdamage in bone, and, in turn, may potentiate the concept of the scissors hypothesis.

The effect of microinjury on numerous growth factors, cytokines, chemokines and other soluble factors was also demonstrated, as detailed in chapter 5, by use of an antibody array. The results of this showed that microinjury stimulates interleukin, TGF-β and TNF-α mediated osteoclastogenesis, and has also shown that, through the upregulation of dikkopf and frizzled proteins, the wnt/ β-catenin regulatory pathway may also be evaluated in relation to microinjury, and also how these pathways are affected by

bisphosphonate treatment. Molecular characterisation and release profiling of these factors may provide a greater understanding of how bone detects and repairs itself following microdamage.

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