IDENTIFICATION OF RANKL-REGULATED GENES INVOLVED IN OSTEOCLAST DIFFERENTIATION AND RESORPTION

Thesis submitted in fulfillment of the requirements of the degree of Doctor of Philosophy, Griffith University.

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Submitted: June 2007

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Abstract

Peripheral blood mononuclear cells (PBMCs) are pluripotent for osteoclast and macrophage cell lineages. The differentiation of macrophages and osteoclasts from a common monocyte precursor is induced following exposure to macrophage-colony stimulating factor (M-CSF), or both M-CSF and receptor activator of nuclear factor κB ligand (RANKL) respectively. Differential gene expression resulting from cytokine treatment of PBMCs was examined over time using differential display PCR (DD-PCR) and quantitative real-time PCR (Q-PCR). Q-PCR analysis verified the expression of a new chemokine, FAM19A1, in addition to Special AT-rich binding sequence 1 (SATB1), solute carrier family 16 member 6 (SLC16A6) and LIM kinase 1 (LIMK1) in primary human osteoclasts, however, only LIMK1 was significantly up-regulated by RANKL.

Highly efficient delivery of small interfering RNA (siRNA) transfection to primary human osteoclasts was developed, and represents a technical milestone due to the inherent phagocytic tendencies of the PBMC lineage. The development of RNA interference for use in primary human osteoclasts was conducted using siRNA synthesised by Dicer enzyme, to verify the role of candidate genes in osteoclast differentiation and osteoclast bone resorption. Cathepsin K (CTSK) is the key proteinase expressed by osteoclasts, and was used as a benchmark for the optimisation of siRNA inhibition in primary human osteoclasts. Transfection of primary human osteoclasts with siRNA to CTSK significantly diminished bone resorption, with a 60% reduction in area resorbed (P=1.3x10^{-2}), and a 50% reduction in pit number (P=1.8x10^{-2}).
Normal bone remodelling is dependent on both the rate of osteoclast formation and resorption. A number of genes were examined for their contribution to osteoclast formation and resorption using siRNA. Nuclear factor of activated T cells, calcineurin dependant 1 (NFATc1) inhibition was found to significantly deplete osteoclast formation (P=4.0x10^{-3}), confirming other NFATc1 inhibition studies, and the necessity of NFATc1 in osteoclast differentiation. In pre-differentiated osteoclasts, siRNA targeting NFATc1 did not reduce osteoclast bone resorption, rather it significantly increased area resorbed (P=1.0x10^{-3}), with no significant difference in cell number. This result suggests that NFATc1 may act in accordance with its regulator calcineurin, which has been found to enhance osteoclast differentiation, but inhibit osteoclast resorption in mature cells.

The inhibition of LIMK1 by targeted small interfering RNA (siRNA) was found to significantly diminish osteoclast formation (P=1.0x10^{-3}), pits resorbed (P=4.2x10^{-2}), as well as area resorbed (P=4.0x10^{-3}). LIMK1 is a signalling kinase, identified as RANKL-regulated in murine osteoclasts, notwithstanding, this is the first study that confirms LIMK1 involvement in osteoclast formation and activity. LIMK1/cofilin-mediated actin reorganisation is critical to progenitor cell migration to stromal cells, and also regulates the stability of F-actin formation. F-actin rings were analysed in LIMK1 depleted pre-differentiated osteoclasts, which seemed to have formed properly and did not appear dissimilar from controls.

Targeted knockdown of the chemokine-like gene FAM19A1 with siRNA resulted in a significant reduction in osteoclast formation (P=2.2x10^{-2}), and resorption with significantly less pits resorbed (P=3.3x10^{-2}) and less area resorbed (P=2.0x10^{-3}).
FAM19A1 is a chemokine-like gene previously uncharacterised in osteoclasts, which like many chemokines in osteoclast biology, provides further evidence for the interaction between bone and the immune system.

SLC16A6 siRNA suppression resulted in the most potent reduction in osteoclast formation with 70% reduction in osteoclast cell count ($P=2.0 \times 10^{-3}$), as well as the most potent reduction in bone resorption, reducing pit number by 80% ($P=2.5 \times 10^{-2}$), and area resorbed by 72% ($P=2.0 \times 10^{-3}$). To date, all that is known of this molecule is that it is part of the solute carrier or major facilitator superfamily of proteins, and no detailed cellular or functional role has been attributed to SLC16A6.

The siRNA inhibition of several genes tested, resulted in poor pit formation and/or diminished rates of resorption. These genes included GA binding protein (GABP), Far upstream element (FUSE) binding protein (FBP), and Special AT-rich binding protein 1 (SATB1). GABP subunit alpha and beta were independently found to be necessary for osteoclast formation, with significant reductions in osteoclast cell formation following siRNA inhibition ($P=4.4 \times 10^{-3}$; $P=1.5 \times 10^{-2}$). Suppression of the deoxyribonucleic acid (DNA) binding subunit, GABPA, significantly reduced resorbed area ($P=2.8 \times 10^{-2}$), and siRNA inhibition of both GABP subunits reduced the size and quality of pits resorbed. FBP and SATB1 expression was not found to be necessary for proper rates of osteoclast differentiation. However, inhibition of FBP resulted in significant reductions in area resorbed ($P=2.6 \times 10^{-2}$), due to the formation of smaller pits. By contrast, SATB1 inhibition in osteoclasts resulted in significantly increased pit numbers produced in dentine ($P=1.8 \times 10^{-2}$), which were also as a result of smaller pit size.
These results suggest that many genes expressed in osteoclasts are required for both differentiation and resorption. Importantly, a number of genes identified, affected only resorption, with significant differences in the quality of bone resorbed following siRNA inhibition, possibly providing a valuable alternative to drug targets which result in total osteoclast inhibition.
I declare that the work contained in this thesis was performed within the school of Medical Science under the supervision of Dr. Nigel Morrison, and represents research performed for Doctor of Philosophy (PhD). This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Abbreviations

AEMF  Analytical Electron Microscopy Facility
Ago  Argonaute
ANOVA  Analysis of variance
AP-1  Activator protein 1
Arp2/3  Actin related protein 2/3
ATP  Adenosine triphosphate
Atp6v0d2  ATPase, H+ transporting, lysosomal v0 subunit d2
BCAR1  Breast cancer anti-estrogen resistance 1
Bcl-2  B cell CLL/lymphoma 2
BMU  Basic multicellular unit
bp  base pair
BSA  Bovine serum albumin
Cbfa-1  Core-binding factor α1
Cbl  Casitas B-lineage lymphoma
Cdc42  Cell division cycle 42
cDNA  copy DNA
CCL  CC chemokines ligand
CCR  CC chemokine receptor
ChIP  Chromatin immunoprecipitation
CLA  Conjugated linoleic acid
Ct  Cycle threshold
CT-R  Calcitonin receptor
CTSB  Cathepsin B
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>CTSK</td>
<td>Cathepsin K</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole, dihydrochloride</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxy adenosine triphosphate</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>Dendritic cell-specific transmembrane protein</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxy cytidine triphosphate</td>
</tr>
<tr>
<td>DD-PCR</td>
<td>Differential display PCR</td>
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<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region gene-8</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxy guanosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy-ribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxy nucleotide triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxy thymidine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine tetra acetic acid</td>
</tr>
<tr>
<td>EP2</td>
<td>Epididymis–specific 2</td>
</tr>
<tr>
<td>Erk</td>
<td>ELK-related tyrosine kinase</td>
</tr>
<tr>
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<td>Expressed sequence tag</td>
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<tr>
<td>FAM19A1</td>
<td>Homo sapiens family with sequence similarity 19 (chemokines (C-C motif) –like) A1</td>
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<tr>
<td>FBP</td>
<td>FUSE binding protein</td>
</tr>
<tr>
<td>Fosl</td>
<td>Fos-like antigen</td>
</tr>
<tr>
<td>Fra-1</td>
<td>Fos-related protein 1</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>FUSE</td>
<td>Far upstream element</td>
</tr>
<tr>
<td>Gab2</td>
<td>GRB2 associated binding protein 2</td>
</tr>
<tr>
<td>GABP</td>
<td>GA binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GABPA</td>
<td>GA binding protein subunit alpha</td>
</tr>
<tr>
<td>GABPB</td>
<td>GA binding protein subunit beta</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor bound protein 2</td>
</tr>
<tr>
<td>HDAC6</td>
<td>Histone deacetylase 6</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>L</td>
<td>ladder</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified essential media</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>miRNP</td>
<td>miRNA-protein complex</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia transcription factor</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix metalloproteinase 9</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NFATc1</td>
<td>Nuclear factor of activated T-cells, calcineurin dependent 1</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>OAS1</td>
<td>Oligoadenylate synthetase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>ODF</td>
<td>Osteoclast differentiation factor</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPGL</td>
<td>Osteoprotegerin ligand</td>
</tr>
<tr>
<td>OSCAR</td>
<td>Osteoclast-associated receptor</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PI4P-5</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase (PI4P-5)</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol bisphosphate 2</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Proline-rich tyrosine kinase 2</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of NFκB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of NFκB ligand</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normally T-expressed, and presumably secreted</td>
</tr>
<tr>
<td>rasiRNA</td>
<td>Repeat-associated siRNAs</td>
</tr>
<tr>
<td>RdRP</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>SATB1</td>
<td>Special AT-rich binding sequence 1</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor 1</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLC16A6</td>
<td>Solute carrier family 16 member 6</td>
</tr>
<tr>
<td>SLS</td>
<td>Sodium lauryl sarcosine</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Switch/Sucrose Non Fermentable</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetic acid EDTA</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TBE</td>
<td>Tris boric acid EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tfe3</td>
<td>Transcription factor E3</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>TRANCE</td>
<td>TNF-related activation-induced cytokine</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar-type H+-ATPase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiscott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WC</td>
<td>Water control</td>
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I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Nigel Morrison, for inspiring and encouraging me to love science, think scientifically and for teaching me to surf.

I would like to thank Chris Day for assistance with the research performed for this thesis, for all the assistance with cell culture, PCR, as well as working with me on the RNA interference publication, and synthesising NFATc1 siRNA. I would also like to thank Chris for teaching me so much, for listening to my ideas, and giving me new ones. I would like to thank Michael Kim for his assistance with cell culture, and the synthesis of FBP, GABP alpha and beta siRNA. I would like to thank Rouha Granfar for editorial assistance and figures. I would like to thank Sebastien Stephens for his help with cell staining and making me laugh. I would like to thank Cameron Flegg for his help with cell staining. I would like to thank members of Geoff Nicholson’s lab for providing all the dentine slices for bone resorption assays.

I also wish to acknowledge my parents, who taught me the power of ideas. But I would especially like to thank my partner Jesiah Davis, who is the best teacher of all. Jes, you possess a tireless sense of humor, the deepest affection I have ever known, and deserve a doctorate in common sense. Your significance is infinite (P=1.0x10^-∞).
LOOKING AT NATURE

When the familiar gaze is gone and you see the world with fresh eyes,
Nature is astonishing! Enough for a lifetimes wonder and
Exploration. Through cognitions lense comes understanding,
But thought alone is not enough. Tradition too clouds the view.

Old ideas pull us back to the comfortable, worn spaces of complacency.
To reveal the world, look past tradition's texts,
Beyond the wisdom of the folk, build on the work of others,
To further insights won by labour and by thought.

Culture without science is life without a future,
Children playing in the park,
Never growing up.

Knowledge is a palimpsest
We yearn to read.

Andrew Selinger
Chapter 1

Introduction
1.1 Bone homeostasis.

Bone tissue is a highly specialised and dynamic organ undergoing constant renewal (Boskey & Posner, 1984; Buckwalter & Cooper, 1987). The characteristic of renewal is a product of the bone remodelling process performed by osteoclasts and osteoblasts, and permits the maintenance of mineral homeostasis. Bone also functions as an internal support structure for organs and as a depository for haematopoietic stem cells and cells of the immune system (Dexter, 1979). The cells of the bone marrow microenvironment are harmoniously regulated by a complex array of cytokines, chemokines and local factors, and it is the goal of many studies to determine how signalling factors organise the components of bone biology and maintain bone turnover, by specifically regulating resident cells.

The most important cells involved in the maintenance of bone turnover and homeostasis are osteoblasts and osteoclasts. Osteoblasts synthesise bone whereas osteoclasts resorb bone. It is generally when the break down of bone by osteoclasts outpaces the formation of bone by osteoblasts that many bone remodelling diseases arise.

1.2 Bone Types.

Bone is a specialised connective tissue, composed of an organic matrix made up of predominantly type I collagen and to a lesser extent proteoglycans and numerous noncollagenous proteins including osteocalcin, osteopontin, osteonectin and bone sialoprotein (Roach, 1994). Bone serves as the principal reservoir of various mineral salts stored as hydroxyapatite crystals (Williams & Frolik, 1991), and contains ions of calcium, phosphorus, sodium, magnesium and carbonate (Green, 1994). Bone can be
characterised into two morphological forms: cortical (compact) and trabecular (spongy) (Figure 1.1). The most abundant bone is cortical, found in the shafts of long bones. Cortical bone has a high mineral content, although its principal function is mechanical (Augat & Schorlemmer, 2006). Cortical bone tissue is made up of a number of cylindrical units, the osteons, at the centre of which is a Haversian canal containing blood vessels and nerves (Buckwalter & Cooper, 1987). The canal is surrounded by up to half a dozen layers of bone, described as lamellae (Price, 1993). Trabecular or spongy bone is composed of a lattice of fine bone plates filled with haematopoietic marrow, fat containing marrow or blood vessels. Trabecular bone can be found in vertebral bodies, flat bones and in the epiphyses of adult long bones (Goldstein, 1987). Trabecular bone functions to reduce skeletal weight without compromising strength (Price, 1993). The multiple surfaces of trabecular bone are important sites of osteoblast and osteoclast activity for the purpose of bone remodelling.
Figure 1.1 Bone structure of cortical bone and trabecular bone, and bone resorption. Frontal midsection of the neck of the femur bone (A) (Gray, 1918), showing the structure of cortical bone (i) and trabecular bone (ii). A scanning electron micrograph (500X magnification) of bone resorption on the surface of dentine as a result of osteoclast resorption *in vitro* (B).
1.3 Skeletal morphogenesis and growth.

Osteoclasts are crucial in the development of bone, or bone modelling. During skeletal development (intramembranous ossification), bone increases in diameter as an individual’s height increases. This is achieved by new bone being laid down by the osteogenic layer of the periosteum (Price, 1993). However, intramembranous ossification does not significantly increase the skeletal mass of long bone, because there is resorption of bone on the inner surface by osteoclasts. Osteoclasts resorb bone to form an erosion cavity, which is filled by mononuclear cells that differentiate into osteoblasts. Osteoblasts lay down matrix, with mature osteoblasts secreting matrix for 14 days (Choi et al., 1996; Price, 1993). The cycle of bone resorption and formation (bone remodelling) continues throughout life, with the uncoupling of formation from resorption leading to skeletal diseases.

1.4 Site of haematopoiesis.

Bone marrow is also the site of haematopoiesis, which, in addition to blood cell production, includes production of stem cells and precursor cells of the bone microenvironment (Weiss, 1981), including osteoclast and osteoblast progenitors. Within the bone marrow, osteoclasts have been found to mediate stem cell mobilisation, enabling stem cells to shift from the marrow into blood circulation (Kollet et al., 2006). Metalloproteinase 9 (MMP-9) mediated deactivation of the chemokine stromal cell-derived factor 1 (SDF-1), is involved in this process (Kollet et al., 2006). In mice, osteoblasts have been shown to provide a supportive microenvironment for haematopoietic stem cells (Adams & Scadden, 2006). In this way, osteoblast-osteoclast cell coupling is a major regulator of haematopoiesis and stem cell turnover. Many cells of the haematopoietic system, such as activated
immune cells, also provide critical feedback and cellular communication to regulate bone remodelling (Lorenzo, 1991).

1.5 Bone Remodelling.

Sites of active remodelling are termed basic multicellular units (BMUs). BMUs are transitory complexes of bone cells, which remove portions of bone matrix (Jilka, 2003) and is followed by bone regeneration. Bone remodelling is the result of a dynamic equilibrium between bone resorption and bone formation and is a tightly regulated process, influenced by cytokines, chemokines, inflammatory factors and hormones (Kollet et al., 2006). Bone remodelling is essential for the refinement of shape during growth, the adaption of bone to mechanical stresses, to repair microdamage, and to maintain chronic serum mineral levels (Blair et al., 2002; Jiang et al., 1999). Osteoclast resorption is more vigorous than osteoblast bone synthesis, with a single osteoclast able to resorb the equivalent mineralised tissue synthesised by 100 osteoblasts (Remedios, 1999). As a consequence, rapid bone turnover following osteoclast resorption results in the replacement of 10% of the bone structure after one year (Alliston & Derynck, 2002). Hence, a small imbalance favouring osteoclast activity can drastically affect the equilibrium of bone turnover, potentially resulting in disease.

Bone sustains continual site-specific degradation via osteoclast bone resorption, which is offset by parallel bone production by osteoblasts (Bentolila et al., 1998; Mori & Burr, 1993). However, bone turnover takes place at a rate that notably exceeds that required for growth or repair. The mineralisation requirements of the body influence
bone turnover, mainly for the purpose of liberating Ca\(^{2+}\), PO\(_{3}^{3-}\) and OH\(^{-}\) via osteoclast degradation of the bone matrix (Price, 1993).

Thus, bone remodelling serves to regulate shape, structure, repair, mineral homeostasis and blood production, of which the balanced interplay between osteoclasts and osteoblasts is a central component.

1.6 Cell Biology of Bone: cellular components

The cellular components of bone consist of precursor cell lineages, bone lining cells, osteoblasts, osteocytes and osteoclasts.

1.6.1 Monocyte/macrophage cell lineage.

Peripheral blood monocytes are cells of the immune system that possess migratory, chemotactic and phagocytic characteristics (Territo & Cline, 1975). Upon migration into tissues, monocytes can undergo further differentiation to become tissue macrophages or, within a suitable bone microenvironment, differentiate into osteoclasts (Udagawa et al., 1990). Monocytes are known to be chemotactically attracted to resorbed products of the bone matrix (Malone et al., 1982). Osteoclast precursor cells migrate via the vascular pathway to the skeleton where osteoblasts, osteocytes, endothelial cells, and stromal cells provide the necessary environment for directing the precursors to the site of bone remodelling. In vitro, monocytes can be induced to differentiate into macrophages by treating them with macrophage-colony stimulating factor (M-CSF) (Stanley et al., 1983). The addition of both M-CSF and receptor activator of NF-κB ligand (RANKL) to monocyte precursors in vitro induces
complete osteoclastogenesis (Quinn et al., 1998). The genetic program of osteoclastogenesis induced by these cytokines is yet to be completely outlined.

Macrophages are mononuclear phagocytes responsible for numerous homeostatic, immunological and inflammatory processes. Osteoclasts share many morphological, cytochemical, and immunophenotypic characteristics with macrophages (Athanasou et al., 1986; Sminia & Dijkstra, 1986). Macrophages have a long life span, and under the influence of local cellular and humoral factors within the bone microenvironment, they can be induced to differentiate into osteoclasts (Takeshita et al., 2000; Udagawa et al., 1990). Therefore macrophages share some genetic similarity with the osteoclast. RANKL is thought to be a key molecule in the macrophage/osteoclast lineage switch (Miyamoto, 2006). For this reason, comparing gene expression in macrophages and osteoclasts has become a useful technique for distinguishing important genes regulating osteoclastogenesis.

1.6.2 Bone lining cells.

Bone lining cells cover most trabecular bone surfaces. Activated bone lining cells remove the thin layer of matrix underlying them (Hall & Chambers, 1996), and are signalled to retract from the bone surface by osteoclasts in preparation for bone resorption (Perez-Amodio et al., 2004). This process forms an important initial step in bone remodelling (Parfitt, 2006).
1.6.3 Osteoblasts and osteocytes.

Bone remodelling involves the formation of bone matrix by osteoblasts, which later become mineralised. Osteoblasts are derived from pluripotential mesenchymal stem cells, which can be found in the stromal fibroblastic system of the bone marrow, and in other connective tissues such as the peristeum (Aubin, 1998). Osteoblasts synthesise a collagen-rich matrix, which mineralises to form mature bone (Ducy et al., 2000; Hohling et al., 1997). The osteoblast contributes a number of functions, in addition to the well known role of synthesising the organic components of bone matrix. Osteoblasts are crucial for the stimulation of osteoclast formation, as well as the activation of mature osteoclasts to resorb bone, which is a cell-to-cell contact-dependent process (Jimi et al., 1996). Osteoblasts occupy a central role in a system of cell-cell communications by responding to circulating hormones, growth factors, and other cells within the marrow. Products of osteoblasts mediate intercellular communication, and also act upon osteoclasts and osteoclast precursors to induce resorption (Teti et al., 1991). Osteoblasts express on their surface RANKL, which promotes the differentiation and resorptive activity of osteoclasts (Udagawa et al., 1999). Osteoblasts also secrete M-CSF, which provides the osteoclast signals for survival and proliferation (Weir et al., 1993). Therefore osteoblast and osteoclast functions are intimately related.

Osteocytes derive from osteoblasts having synthesised bone matrix, and become buried within the mineralised matrix of bone (Manolagas, 2000). Osteocytes are the most abundant of the bone cells, being ten times more plentiful than osteoblasts (Parfitt, 1990). Osteocytes maintain the ongoing cellular activities of bone tissue, such as the exchange of nutrients and wastes with the blood (Parfitt, 1990). Osteocytes
have been proposed to act as sensors of mechanical strain to produce signals that induce bone remodelling (Bonewald, 2006; Kogianni & Noble, 2007). Further evidence also suggests that osteocytes can play a role in modifying the bone microenvironment, causing local hypomineralisation (Lane et al., 2006). In this way, osteocytes also participate in the regulation of bone remodelling.

1.6.4 Osteoclasts.

The osteoclast is a specialised multinucleated polykaryon giant cell, which resorbs bone, and in doing so, regulates the homeostasis of skeletal mineral. The osteoclast is the only cell type that functions to resorb mineralised matrix (Price, 1993). Macrophages can remove bone, but this activity seems to be restricted to dead bone, unlike osteoclasts, which resorb vital bone (Rifkin et al., 1979). Therefore, osteoclasts are pivotal cells for bone remodelling. The rate of osteoclast differentiation as well as the rate of bone resorption by osteoclasts influences the pace of bone remodelling. Osteoclasts are located on the bone surfaces tightly associated with the calcified matrix (Price, 1993), and form via fusion of mononuclear precursors (Zambonin Zallone et al., 1984). Osteoclasts are rich in mitochondria, free polysomes, coated transport vesicles, and vacuoles (Baron, 1989). Osteoclasts are highly motile cells that migrate along bone surfaces, mostly forming the interface between bone and bone marrow (endosteum) (Baron, 1989). Osteoclasts are also required during tooth eruption (Marks, 1981), where they resorb alveolar bone making way for the tooth to exit the bony crypt (Wise et al., 2002).

Osteoclasts are distinguished by the expression of a number of cellular markers such as tartrate resistant acid phosphatase (TRAP) (Hayman et al., 1996), calcitonin
receptor (CT-R) (Nicholson et al., 1986), cathepsin K (CTSK) (Gelb et al., 1996; Gowen et al., 1999), integrin α,β3 (Zambonin Zallone et al., 1989) and the presence of multiple nuclei (Parfitt, 1984).

During osteoclast differentiation the expression of monocyctic surface cell markers ceases (van de Wijngaert et al., 1987). Following the discovery of in vitro systems of mononuclear precursor-derived osteoclast differentiation with cytokines (Quinn et al., 1998), rather than precursor-stromal cell co-cultures, the osteoclast differentiation system has been developed into a sophisticated model, allowing for the observation of stages of cellular differentiation.

1.7 Osteoclast formation.

Osteoclasts differentiate from haematopoietic stem cells such as monocytes in the marrow (Akashi et al., 2000). Haematopoietic cells are recruited from the blood to the bone surface (Malone et al., 1982). Osteoclastogenesis is controlled by the proliferation and homing of monocyte presursors to bone, and their subsequent differentiation and fusion to form multinucleated cells (Boyle et al., 2003; Duong & Rodan, 2001).

During bone development, cells from the osteoblast lineage secrete molecules that in turn initiate and control osteoclast differentiation, such as parathyroid hormone (PTH), interleukin 11 and 1α,25-dihydroxyvitamin D3, which stimulate osteoblasts and stromal cells to release factors, such as M-CSF and RANKL, inducing osteoclast differentiation (Huang et al., 2004; Nakagawa et al., 1998; Teti et al., 1991). Immune and haematopoietic factors not only influence osteoclast differentiation, but also the
rate of activity of osteoclasts, with osteoclasts in turn, regulating members of the immune system and the stem cell niche (Kollet et al., 2007).

Despite being responsive to a large spectrum of cytokines, chemokines, hormones and local factors, osteoclast differentiation is principally regulated by three cytokines: M-CSF, RANKL (which is also known as osteoprotegerin ligand [OPGL] (Lacey et al., 1998), osteoclast differentiation factor [ODF] (Yasuda et al., 1998) and TNF-related activation-induced cytokine [TRANCE] (Wong et al., 1997) and osteoprotegrin (OPG) (Mizuno et al., 1998; Vidal et al., 1998).

1.8 Macrophage-colony stimulating factor.

M-CSF is produced by many cells such as osteoblasts, fibroblasts and macrophages (Chitu & Stanley, 2006). M-CSF is an important soluble factor for osteoclastogenesis, providing osteoclast precursor cells with signals for survival, proliferation (Felix et al., 1990; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990) and differentiation (Quinn et al., 1998). M-CSF induces the expression of RANK (although RANK induction is not M-CSF specific), maintains NF-κB activation (Brach et al., 1991), and increases B-cell lymphoma 2 (Bcl-2) expression – a suppressor of apoptosis (Lagasse & Weissman, 1997). M-CSF binds to its receptor c-Fms on an osteoclast precursor (Sherr et al., 1985). The induction of c-Fms is mediated by the transcription factor PU.1 (Krysinska et al., 2007). M-CSF is an absolute requirement for the differentiation of osteoclasts (Quinn et al., 1998), as exhibited by the M-CSF deficient osteopetrotic op/op mouse, which is severely deficient in osteoclasts and macrophages (Yoshida et al., 1990). Similarly, even in the presence of RANKL, functional osteoclasts are not produced in the absence of M-CSF in vitro (Matsuzaki et al.,
M-CSF alone (in the absence of RANKL) induces precursor cells to differentiate into macrophages, often regarded as the default pathway of macrophage differentiation (Arai et al., 1999). The cytoskeletal protein, breast cancer anti-estrogen resistance 1 (BCAR1), functions downstream of c-Fms and adhesion-related signals (Nakamura et al., 2003) and has been found to also bind TNF receptor-associated factor 6 (TRAF6) (Nakamura et al., 2002). M-CSF is also important for the survival, proliferation and differentiation of monocytes and macrophages (Brach et al., 1991).

1.9 Receptor activator of NF-κB ligand.

RANKL is a member of the tumor necrosis factor superfamily that induces osteoclast differentiation and activates mature osteoclasts to resorb bone (Fuller et al., 1998; Lacey et al., 1998; Yasuda et al., 1998). RANKL is an osteoclast differentiation factor, which in combination with M-CSF stimulates osteoclastogenesis (Quinn et al., 1998). RANKL is found on stromal cells, osteoblasts and T lymphocytes, but can also be found as a soluble molecule in the bone microenvironment (Karsenty, 1999). RANKL binds the receptor activator of NF-κB (RANK) receptor on osteoclasts and osteoclast precursors (Galibert et al., 1998). The intracellular domain of RANK contains a binding site for TRAFs (Galibert et al., 1998). When RANK receptors on osteoclasts are activated, signals are mediated by TRAFs (Galibert et al., 1998), allowing RANKL to activate several signalling pathways for osteoclast maturation, including the transcription factors NF-κB and Akt, and the protein kinases Jun N-terminal kinase (JNK), c-Src and mitogen-activated protein kinase (MAPK) (Arron & Choi, 2000; Davies et al., 2005). Fos proteins are components of the transcription factor activator protein 1 (AP-1) and are necessary for osteoclastogenesis. RANKL induces transcription Fos-like antigen 1 (Fosl1) and in a manner dependent on c-Fos.
(Matsuo et al., 2000). RANKL induces auto-inhibition of c-Fos, by the induction of interferon β (IFN-β) and protein kinase R (PKR), which inhibit c-Fos (Takayanagi et al., 2002b). Another signalling protein essential to RANK function is growth factor receptor bound protein 2 (GRB2) associated binding protein 2 (Gab2), which mediates the activation of NF-κB, Akt and JNK (Wada et al., 2005). In addition, RANKL activates microphthalmia associated transcription factor (MITF), via the MAPK kinase 6 (MKK6)/p38 signalling pathway (Mansky et al., 2002; So et al., 2003), which induces the expression of key osteoclast genes TRAP, CTSK and osteoclast-associated receptor (OSCAR) (Luchin et al., 2000; Motyckova et al., 2001). OSCAR is a costimulatory receptor for osteoclast differentiation (Kim et al., 2002; Koga et al., 2004). RANKL also acts directly on fully differentiated, mature osteoclasts, inducing a rapid rearrangement of the actin cytoskeleton into actin rings and subsequently inducing multiple cycles of bone resorption (Burgess et al., 1999). RANKL represents a crucial trigger of genetic events necessary for osteoclast differentiation and function, and provides a means to examine the formation of osteoclasts.

### 1.10 Osteoprotegerin.

OPG is a soluble member of the tumor necrosis factor receptor (TNFR) family, and has profound inhibitory effects on osteoclast differentiation and bone resorption (Mizuno et al., 1998; Vidal et al., 1998). OPG is known as a “decoy” receptor that binds to RANKL, preventing its interaction with RANK on haematopoietic cells (Mizuno et al., 1998). The prevention of interaction makes signalling between T cells/stromal cells/osteoblasts and osteoclast precursors via RANKL and RANK impossible. The secretion of OPG by osteoblasts and stromal cells is regulated by
numerous hormones and cytokines, such as members of the transforming growth factor β (TGFβ) superfamily, and the transcription factor core-binding factor α 1 (Cbfa-1) (Thirunavukkarasu et al., 2000; Thirunavukkarasu et al., 2001). In mice, OPG inhibits osteoclastogenesis, and animals lacking OPG have accelerated osteoclastogenesis, displaying defective osteoclast regulation and osteopetrosis (Mizuno et al., 1998). The quantity of bone resorbed relies on the ratio between the stimulator of osteoclastogenesis- RANKL and the inhibitor of osteoclastogenesis- OPG.
Figure 1.2 Osteoclast differentiation via M-CSF and RANKL. Osteoclast and osteoblast precursors derive from stem cells of the mesenchymal lineage. RANKL and M-CSF are produced by osteoblast precursors and osteoblasts. RANKL binds to the RANK receptor, and M-CSF binds to the c-FMS receptor, on osteoclast precursor cells, stimulating the activation of osteoclast differentiation. OPG is a decoy receptor for RANKL, and is produced by osteoblasts. M-CSF stimulates the up-regulation of a number of factors upon binding to the c-FMS receptor. RANKL upon binding to RANK, induces a number of genes, of which the OPG decoy receptor is able to block by binding also to RANK. Upon activation by M-CSF and RANKL, osteoclast precursors fuse to become multinucleated osteoclasts, followed by polarisation and resorption of bone, before undergoing apoptosis.
1.11 Osteoclast research models.

Both human and murine models of osteoclast formation are used to perform research on osteoclast differentiation and osteoclast resorption. Human osteoclast models are frequently based on mononuclear cells derived from peripheral blood cultured in vitro (Nicholson et al., 2000; Shinoda et al., 2003; Yang et al., 2004). Human osteoclast models are advantageous as they can be representative of human biology and disease. However, mouse in vivo models can provide more comprehensive information, where genes can be studied in a 'whole organism' context, providing complex interactions and phenotypes. In vitro mouse culture models include mononuclear cells derived from mouse bone marrow (Takayanagi et al., 2000; Yang et al., 2004) and the RAW264.7 cell line. The RAW264.7 cell line is unique in that it does not require the addition of M-CSF for osteoclast formation (Hirotani et al., 2004).

1.12 Regulatory factors.

There are many other important regulatory factors involved in osteoclastogenesis such as various cytokines, activated B cells, hormones, regulatory genes and transcription factors. Cytokines are important inducers of osteoclast differentiation. Cytokines such as interleukins (ILs) are implicated in osteoclast differentiation such as IL-1, IL-6, oncostatin-M and IL-11. These proinflammatory cytokines are implicated in rheumatoid arthritis and transduce their signals through a composite receptor containing a signal-transducing glycoprotein gp130 subunit (Karsenty, 1999), which itself is crucial for bone formation and osteoclast resorption (Kawasaki et al., 1997; Shin et al., 2004). IL-6 and soluble IL-6 receptor is elevated in the serum and in the synovial fluid of affected joints of patients with rheumatoid arthritis (Bertazzolo et al., 1994; Dasgupta et al., 1992; Holt et al., 1991; Houssiau et al., 1988; Robak et al.,
Mouse models bearing a gp130 mutation that induces excess IL-6 signalling, develop rheumatoid arthritis-like joint disease (Atsumi et al., 2002). IL-1 is also able to cause erosive arthritis when the antagonist IL-1ra is genetically deleted in rodent models (Horai et al., 2000). IL-1 and tumor necrosis factor-α (TNF-α) are key players in inflammatory joint disease, and both are currently targeted for anti-inflammatory therapy in rheumatic diseases (Bresnihan et al., 1998; Gartlehner et al., 2006). Oncostatin-M appears to contribute to IL-1 and TNF-α induced inflammatory arthritis by increasing the expression of RANK and RANKL in inflammatory cells (Hui et al., 2005). IL-11 is both a stimulatory factor for osteoblast formation, binding to receptors on the osteoblast surface, and osteoclast formation by stimulating RANKL expression in osteoblasts (Horwood et al., 1998; Romas et al., 1996).

Activated B cells express many of the osteoclastogenic cytokines required, such as RANKL, TNF-α, and IL-6 (Choi et al., 2001). Activated B cells along with CD4+ T cells have been found to induce osteoclastogenesis in the presence of M-CSF alone (Choi et al., 2001), by expressing RANKL and serving as osteoclast progenitors (Manabe et al., 2001). Whereas CD8+ T cells profoundly suppress osteoclastogenesis (Choi et al., 2001). TNF-α is an important stimulant of osteoclastogenesis in M-CSF treated mouse models (Li et al., 2004). In vitro cultures of human osteoclast precursors stimulated with TNF-α differentiate into functional osteoclasts, however they are only capable of weakly resorbing bone (Kudo et al., 2002). TNF-α induces the resorptive activity of osteoclasts by affecting the production of RANKL and OPG by osteoblast/stromal cells, as well as the activity and proliferation of cells of the osteoclast lineage (Boyce et al., 2005).
The fusion of osteoclast precursors during osteoclast differentiation involves the activation of dendritic cell-specific transmembrane protein (DC-STAMP), which is expressed after RANKL stimulation (Yagi et al., 2006), and is also essential for bone resorption (Miyamoto, 2006). Another gene required for pre-osteoclast fusion is the d2 isoform of vacuolar H+ adenosine 5'-triphosphate (ATP)ase V(0) domain or Atp6v0d2. Atp6v0d2 simultaneously promotes osteoclast fusion and inhibits bone formation (Lee et al., 2006).

1.12.1 Transcription factors.

Global gene profiling of osteoclast differentiation has highlighted the capacity of a combination of ubiquitously expressed transcription factors, in addition to cell signalling pathways to control osteoclast differentiation, rather than the consequence of a set of osteoclast-specific factors.

Pu.1 is a transcription factor that controls both macrophage and osteoclast differentiation (Tondravi et al., 1997), and is expressed in monocytic and B lymphoid lineages (Klemsz et al., 1990). Pu.1 knockout mice are osteopetrotic, displaying arrested formation of osteoclasts and all cells of the macrophage/monocyte cell lineage (Tondravi et al., 1997). Pu.1 regulates the transcription of c-fms, the gene encoding the receptor for M-CSF (Sherr et al., 1985). The chromatin remodeller, Special AT-rich binding protein 1 (SATB1) positively regulates Pu.1 expression in hematopoietic progenitor cells, with targeted disruption of SATB1 in mice leading to a reduction in Pu.1 transcription in granulocyte-macrophage and megakaryocyte-erythrocyte progenitors (Steidl et al., 2007).
c-fos is another transcription factor involved in osteoclast differentiation. It is a major component of the AP-1 transcription factor (Chiu et al., 1988) and is the cellular homologue of the v-fos oncogene (Miller et al., 1984). Interestingly the over-expression of c-fos in transgenic mice alters osteoblastic gene expression and causes chondroblastic osteosarcoma tumors (Grigoriadis et al., 1993). But the deletion of c-fos in mice leads to the early arrest of osteoclast differentiation, with functional osteoblast gene expression intact (Johnson et al., 1992; Wang et al., 1992). Another member of the fos gene family is the Fos-related protein 1 (Fra-1), which directs osteoclast differentiation in osteoclast-macrophage precursor cell lines (Owens et al., 1999), by dimerising with AP-1 proteins (Bakiri et al., 2007).

The transcription factor nuclear factor-κ B (NFκB) is involved during early osteoclast differentiation (Karsenty, 1999). The NFκB/Rel family of transcription factors includes p65 (RelA), c-Rel, RelB, NFκB1 (p50/p105) and NFκB2 (p52/p100) (Bonizzi & Karin, 2004; Ghosh & Karin, 2002; Hayden & Ghosh, 2004). Each transcription factor contains an N-terminal Rel homology domain, which is responsible for dimerisation, DNA binding, and interaction with the inhibitory IκB proteins. Three of these transcription factors, p65, c-Rel, and RelB possess C-terminal transcriptional activation domains necessary for activating target gene expression. Homodimers of p50 and p52 are unable to drive transcriptional activation, however, the absence of p50 or p52, arrests osteoclast differentiation ((Franzoso et al., 1997; Iotsova et al., 1997). TRAF6 is also essential for NFκB signalling, with RANK-mediated NFκB induction completely inhibited when the membrane-proximal TRAF6 interaction domain is deleted (Darnay et al., 1998; Wong et al., 1998). NF-κB-inducing kinase controls processing of p100 (a prototranscription factor) to generate
p52 to regulate NFκB signalling (Novack et al., 2003). RANKL signalling induces NFκB, via activation of c-Fos and nuclear factor of activated T cells, calcineurin dependent 1 (NFATc1) to stimulate osteoclast differentiation (Yamashita et al., 2007). Selective inhibition of NFκB in mice using a peptide inhibitor of the IκB-kinase complex was found to reduce the severity of collagen-induced arthritis by reducing levels of TNFα and IL-1β (Jimi et al., 2004).

MITF regulates gene expression in several cell types, but in particular MITF has been found to critically regulate osteoclast differentiation and function as evidenced by mutant mice carrying dominant negative MITF, which display severe osteopetrosis (Hershey & Fisher, 2004). Osteoclasts from MITF mutant mice, are significantly smaller (Holtrop et al., 1981; Steingrimsson et al., 2002), are less multinucleated (Mansky et al., 2002; Thesingh & Scherft, 1985), and have diminished bone resorption activity (Thesingh & Scherft, 1985), with poorly developed ruffled membrane borders (Holtrop et al., 1981; Marks & Walker, 1981). Mutations in MITF in people suffering pycnodysostosis, a disease in which sufferers have increased bone mineral density, uncovered the role of MITF in regulating cathepsin K expression (Motyckova et al., 2001). Transcription factor E3 (Tfe3), is part of the same family of transcription factors and can form a homo or heterodimer with other family members (Hemesath et al., 1994). Tfe3 and MITF play a functionally redundant role in osteoclasts (Steingrimsson et al., 2002). A combined loss of both transcription factors results in severe osteopetrosis, whereas mice deficient either transcription factor alone possess normal appearing osteoclasts (Steingrimsson et al., 2002). MITF binds PU.1 to activate TRAP (Luchin et al., 2000) and osteoclast-associated receptor (OSCAR) (So et al., 2003). MAPK-phosphorylation of MITF and p38 MAPK, following
RANKL and M-CSF stimulation, induces increased expression of CTSK and TRAP via Switch/Sucrose Non Fermentable (SWI/SNF) chromatin-remodelling complex promoter association (Sharma et al., 2007).

A number of transcription factors have found to be activated during osteoclastogenesis. Two transcription factors: Far upstream element (FUSE) binding protein (FBP) and c-Jun, are induced early in osteoclast differentiation (Day et al., 2004). FBP binds FUSE, which induces c-Myc expression to regulate a large range of factors (He et al., 2000). c-Jun, along with c-Fos, makes up the transcription factor AP-1. AP-1 is modulated by JNK1 phosphorylation of c-Jun, which is essential for efficient osteoclastogenesis (David et al., 2002). Phosphorylation of c-Jun on serines 63 and 73 by JNKs increases the transcriptional activity of AP-1 (Chang & Karin, 2001; Davis, 2000). JNK1 activity also protects monocytes from RANKL-induced apoptosis during osteoclastogenesis (David et al., 2002).

NFATc1 is a calcineurin-mediated transcription factor that is required for osteoclast differentiation (Day et al., 2005; Takayanagi et al., 2002a). Calcineurin stimulates osteoclast differentiation via activation of NFATc1, but also inhibits bone resorption in mature osteoclasts (Sun et al., 2007). NFATc1 is induced by c-Fos via the p38 MAPK signalling pathway following RANKL stimulated osteoclast differentiation (Huang et al., 2006). NFATc1 is sufficient to induce bone-resorbing osteoclasts in murine cells (Hirotani et al., 2004; Takayanagi et al., 2002a), as well as osteopenia in NFAT1 overexpressing mice. However, abundant NFATc1 is expressed in monocyte chemotactic protein-1 (MCP-1) stimulated osteoclasts, which fail to resorb bone (Kim et al., 2006a).
GA binding protein (GABP) is a heteromeric Ets transcription factor that is expressed during osteoclast differentiation (Day et al., 2004), with widespread tissue distribution. GABP activates a number of genes including: several ribosomal protein genes (Genuario & Perry, 1996), enhancer intron of TNF-α (Tomaras et al., 1999) and interleukin 16 (IL-16) (Bannert et al., 1999). GABP is also required for cell cycle progression (Yang et al., 2007). The functional significance of GABP expression during osteoclast differentiation is yet to be clarified.

1.12.2 Osteoclast chemokines.

A number of chemokines have been found to act on osteoclast precursors to regulate differentiation. Chemokines are small secretory proteins that are ligands for highly related chemokine receptors and are grouped into two main subfamilies, CC and CXC chemokines, based on the positions of the first two of four conserved cysteine residues (Baggiolini, 2001). Chemokines are involved in the coordination of cellular trafficking and the activation of immune responses, via the activation of both G-protein and protein tyrosine kinase-coupled signalling pathways (Ward & Westwick, 1998). At sites of inflammatory bone disease, chemokines act as chemoattractants for monocytes and macrophage-like cells (Choi et al., 2000).

MCP-1 binds the CC chemokine receptor 2 (CCR2), a G protein coupled receptor, which is expressed in osteoclast precursors following RANKL stimulation (Kim et al., 2006a). MCP-1 is a chemotactic factor inducing fusion of osteoclast precursors in the presence of M-CSF and induces the expression of a number of osteoclast markers such as TRAP, CT-R and NFATc1 (Kim et al., 2006a). MCP-1 is regularly found at
sites of tooth eruption, rheumatoid arthritic bone degradation and bacterially induced bone loss (Wise et al., 2002).

Regulated upon activation, normal T-cell expressed and presumably secreted (RANTES) binds CCR1, CCR3 and CCR5. RANTES is expressed during osteoclast formation (Cappellen et al., 2002; Ishida et al., 2002), and can induce the differentiation of TRAP positive multinucleated pre-osteoclasts (Kim et al., 2006b). RANTES is secreted in osteoclasts in response to elevated calcium, and acts as an osteoblast chemoattractant and pro-survival factor acting via phosphatidylinositol 3-kinase (PI3-K) signalling and phosphorylation of Akt (Yano et al., 2005).

Macrophage inflammatory protein-1 (MIP-1) α, β and γ play a role in regulating osteoclast number and function. Blocking MIP-1α action, reduced myeloma cell growth, homing and bone destruction in mice (Choi et al., 2000). Blocking MIP-1α has also been shown to inhibit the formation of TRAP positive osteoclast precursors and multinucleated osteoclasts, accompanied by an increase in macrophage-like cells (Scheven et al., 1999). MIP-1α induces the formation of TRAP positive multinucleated cells in the absence of RANKL (Kim et al., 2006b) as well as the formation of bone resorbing osteoclasts in human bone marrow cultures (Han et al., 2001; Kukita et al., 1997). MIP-1α has been shown to be chemotactic for osteoclasts (Fuller et al., 1995). MIP-1α plays a role in adhesion between myeloma and stromal cells, and results in increased expression of osteoclast activating factors such as PTH related protein (PTHrP), RANKL and 1α,25-dihydroxyvitamin D₃ (Roodman, 2001; Roodman & Choi, 2004). MIP-1α and β are secreted by multiple myeloma cells, and enhance osteoclast bone resorption in vitro and in vivo (Abe et al., 2002). When the
chemokine receptor CCR5 is activated by MIP-1α and β in stromal cells, RANKL expression is induced, thereby prompting osteoclast differentiation (Abe et al., 2002). MIP-1γ promotes RANKL-induced osteoclast differentiation, cytoplasmic motility, polarisation and survival in mouse models (Lean et al., 2002; Okamatsu et al., 2004).

IL-8 or CXC chemokine ligand 8 (CXCL8) stimulates osteoclast motility and spread (Fuller et al., 1995) as well as osteoclast differentiation independent of RANKL (Bendre et al., 2003). IL-8 is secreted by osteoclasts (Rothe et al., 1998) and contributes to bone metastasis (Bendre et al., 2002).

SDF-1 or CXCL12, has been shown to regulate haematopoietic stem cell migration to the bone marrow (Dar et al., 2006), and promote chemotactic recruitment, differentiation and survival of human osteoclasts (Wright et al., 2005).

In contrast to the action of many other chemokines, the chemokine CXCL11, which is up-regulated by IFNβ, was found to inhibit osteoclast differentiation in human primary monocytes (Coelho et al., 2005).

The expression of chemokines by osteoclasts suggests that osteoclasts coordinate precursor cell recruitment to sites of bone remodelling, and inflammatory conditions which favour chemokine production may influence the rate of bone degradation.

1.12.3 Hormones.

Of the hormones involved in bone physiology, the sex steroid hormones, such as estrogen, play an important role. Receptors for estrogen have been identified on
osteoclasts and osteoblasts, such as the estrogen receptor protein (Bord et al., 2001) and the estrogen receptor-related receptor α (Bonnelye et al., 2002). Estrogen is important for the maintenance of bone health as a deficiency in estrogen, as seen during menopause, is associated with bone loss (Bord et al., 2001). Estrogen stimulates apoptosis in the pre-osteoclast population through expression of TGF-β, and promotes the formation of IL-1β decoy receptors and OPG (Krassas & Papadopoulou, 2001; Zallone, 2006). Estrogen decreases the expression of osteoclastogenic cytokines in stromal cells (Michael et al., 2005), and blocks PTH-induced osteoclast formation (Kaji et al., 1996).

The pituitary glycoprotein, follicle stimulating hormone (FSH), has also been found to play a key role in osteoclastogenesis, stimulating osteoclast formation and function through Gi2α-coupled receptor signalling to activate ELK-related tyrosine kinase (Erk), NF-kB and Akt (Sun et al., 2006).

1.13 Osteoclast function.

The functional cycle of the osteoclast consists of three stages:

- matrix adherence
- matrix resorption, and
- migration to a new site of degradation

1.13.1 Osteoclast matrix adherence.

Osteoclast attachment takes place once surface proteins have been removed by collagenase (an enzyme secreted by osteoblasts) (Holliday et al., 1997). The signals which localise osteoclasts to their site of resorption are largely unknown, however
MMP activity and vascular endothelial growth factor (VEGF) are important factors directing adhesion to the extracellular matrix (Engsig et al., 2000). The attachment of osteoclasts to bone involves the interaction between the bone matrix protein osteopontin and vitronectin receptors in the osteoclastic cell membrane (Reinholt et al., 1990). Osteopontin and bone sialoprotein facilitate the binding of osteoclasts via the arg-gly-asp motif (Razzouk et al., 2002; Roach, 1994).

Attachment is aided by adhesion molecules such as integrins. The $\alpha_\nu\beta_3$ integrin is important in forming the sealing zone, a tight ring-like zone of adhesion between the osteoclast and bone (Baron et al., 1993), and transmitting bone matrix-derived signals prompting intracellular events such as cytoskeleton reorganisation. An activated form of $\alpha_\nu\beta_3$ accumulates in the motile areas of the plasma membrane of non-resorbing osteoclasts, promoting osteoclast migration to osteopontin (Faccio et al., 2002). The binding of $\alpha_\nu\beta_3$ integrin directs the formation of a proline-rich tyrosine kinase 2 (Pyk2)/Src/ casitas B-lineage lymphoma (Cbl) complex (Sanjay et al., 2001). Cbl is one of the key regulators of Src kinase activity and of cell adhesion and migration (Sanjay et al., 2001). Activated by RANKL binding to the RANK receptor, TRAF6 enhances the kinase activity of c-Src, which leads to the downstream phosphorylation of signalling molecules such as c-Cbl and the formation of the ruffled membrane (Wong et al., 1999). A molecular complex consisting of the tyrosine phosphorylated proteins Src and Cbl, and the calcium-dependent tyrosine kinase Pyk2 regulates osteoclast cell adhesion and motility upon activation of integrin $\alpha_\nu\beta_3$ (Sanjay et al., 2001).
A deficiency in TRAF6 or c-Src blocks RANKL mediated activation of osteoclasts (Wong et al., 1999). The attachment zone is rich in podosome structures, which are crucial adhesion structures made up of F-actin and associated actin-binding proteins, and assemble to form a podosome belt (Vaanen et al., 2000). Rho activation localises a histone deacetylase 6 (HDAC6)/mDia2 complex to microtubules to stimulate microtubule deacetylation, and in doing so regulates podosome patterning and osteoclast differentiation (Destaing et al., 2005). Microtubule dynamics regulate Rho GTPases (Ory et al., 2002), which signal F-actin and adhesion structure rearrangements (Etienne-Manneville & Hall, 2003).

Figure 1.3 Osteoclast attachment to the matrix takes place once surface proteins are removed by collagenases. Attachment involves the binding of osteopontin in the bone matrix to the vitronectin receptor on the osteoclast cell membrane. Bone sialoproteins also aid osteoclast adhesion. Integrin binding directs the formation of a Pyk2/Src/Cbl complex. RANKL binding to RANK, results in TRAF6 enhancement of c-Src kinase activity, which phosphorylates c-Cbl and results in formation of the ruffled membrane.
A sealed microenvironment is formed within the attachment zone where hydrogen ions, acid hydrolases, proteases and other enzymes are secreted via the ruffled border for degradation of the bone surface and subsequent removal of degraded bone matrix (Baron, 1989). The sealing zone is associated with an f-actin ring, made up of integrated podosomes near the apical membrane (Destaing et al., 2003). Actin is removed basolaterally by filament cleavage proteins such as gelsolin (Destaing et al., 2003), which also trigger actin ring formation (Chellaiah et al., 1998; Chellaiah et al., 2001). Actin related protein 2/3 (Arp2/3) complex is also required for actin ring formation, where it localises with and is activated by cortactin (Hurst et al., 2004).

1.13.2 Osteoclast resorption.

Once attached, the osteoclast develops a specialised cytoskeleton to assist in forming an isolated microenvironment between itself and bone (Teitelbaum, 2000). The osteoclast can polarise on bone, and form a ‘ruffled membrane’- a complex infolding of the plasma membrane. The ruffled membrane appears only when the osteoclast is attached to bone and acts in the transport of acidifying vesicles along microtubules and their insertion into the plasma membrane (Teitelbaum, 2000).

c-Src is required for osteoclast bone resorption, regulating cell adhesion, movement and proliferation (Miyazaki et al., 2006). c-Src kinase activity acts as a membrane-associated molecular switch in the plasma membrane (Thomas & Brugge, 1997). When located in the mitochondria, c-Src regulates cytochrome c oxidase activity important for normal actin ring formation and bone resorption (Miyazaki et al., 2003). c-Src is also implicated in sealing zone formation (Saltel et al., 2004). Cortactin, a c-Src target, associates with the sealing zone and is an essential component of osteoclast
podosomes (Luxenburg et al., 2006). Cortactin depletion results in complete loss of sealing zone and bone resorption in murine osteoclasts (Tehrani et al., 2006). Arp2/3 is also essential for sealing zone formation associated with bone resorption (Hurst et al., 2004).

Figure 1.4 The polarised osteoclast forms a tight sealing zone between the plasma membrane and the bone matrix. The ruffled membrane aids in the transportation of vesicles such as lysosomes to the resorption lacunae. The binding of RANKL to its receptor RANK, stimulates osteoclast bone resorption, via TRAF6 signalling. When M-CSF is bound to c-Fms, c-Src and PI3K signal transduction results in increased motility, bone resorption and survival. The binding of calcitonin to its receptor inhibits osteoclast resorption, by relocating Pyk2 and promoting co-localisation of Pyk2/Src in the central region of osteoclasts. The process of matrix degradation involves the secretion of protons by H⁺-ATPases to create an acidic environment, as well as a variety of enzymes such as TRAP, CTSK and MMP-9, which degrade the bone matrix.
As previously discussed, osteoblasts are crucial regulators of osteoclast differentiation, but also act as a stimulator of osteoclast bone resorption. Under the influence of hormones for bone resorption, osteoblasts release RANKL, which not only commits progenitor cells to the osteoclast phenotype, but stimulates osteoclastic resorption of bone (Burgess et al., 1999). The activated osteoclast synthesises several proteolytic enzymes, which are then transported and secreted into the extracellular bone compartment, in between the ring of attachment and the bone matrix (Baron et al., 1993). Matrix degradation then occurs, involving acidification of the isolated microenvironment. The process of matrix degradation is mediated by a vacuolar H\(^+\)-ATPase in the cell’s ruffled membrane (Teitelbaum, 2000). The acidic environment, which is maintained at pH 4.5 by HCO\(_3^-\)/Cl\(^-\) exchange at the antiresorptive surface, mobilises bone mineral. Many enzymes are crucial for bone resorption. The demineralised organic component is degraded by cathespin K (Drake et al., 1996), which also degrades collagen (Price, 1993). Another enzyme abundant within osteoclasts is the acid phosphatase enzyme, TRAP, which is often used as a cytochemical marker (Hayman et al., 1996). MMP-9, also called Gelatinase B, is a highly expressed protease in the developing osteoclast, and can cleave several collagens and elastin (Rice et al., 1997). Carbonic anhydrase II is also a necessary enzyme involved in osteoclast activity (McMahon et al., 2001). Functional differences of osteoclast resorption in long bone and calvariae have been identified, based on different enzyme systems (Everts et al., 1999). As resorption occurs, endocytosis of bone material takes place. Degraded bone matrix is swallowed up by the membrane of the ruffled border, which pinches-off to allow transcytosis of the endocytotic vesicle through the cytoplasm (Salo et al., 1997). Opposite the basolateral membrane, the
endocytotic vesicle is expelled and its contents released to the extracellular space, of the paratrabecular sinusoids in the bone (Salo et al., 1997).

Calcitonin inhibits osteoclast bone resorption and motility (Chambers & Magnus, 1982; Suda et al., 1992). Calcitonin receptor is used as a marker of osteoclast differentiation and maturation (Hattersley & Chambers, 1989; Lee et al., 1995). Calcitonin inhibits bone resorption by decreasing motility, inducing retraction, disassembling podosomes, and disrupting the actin ring, by relocating Pyk2 and promoting colocalisation of Pyk2/Src in the central region of osteoclasts (Shyu et al., 2007). Dephosphorylating Pyk2 at the peripheral adhesion region, in addition to the phosphorylation of Pyk2 and dephosphorylation of Src in the central zone of osteoclasts, promotes podosome reassembly and detachment (Shyu et al., 2007).

Cytoskeletal organisation is crucial for osteoclast polarisation in preparation for bone resorption. The features of the resorbing osteoclast such as podosomes and the actin ring are made up of highly organised actin filaments (Jurdic et al., 2006). Actin ring formation requires Rho and cell division cycle 42 (Cdc42), and is enhanced by the interaction of Cdc42 with the Arp2/3 complex (Chellaiah, 2005). Rho GTPases interact with phosphatidylinositol bisphosphate 2 (PIP$_2$), along with Cdc42, to activate Wiscott-Aldrich syndrome protein (WASP), a scaffolding protein which complexes with Arp2/3 (Chellaiah, 2005; Higgs & Pollard, 2000; Rohatgi et al., 1999; Rozelle et al., 2000; Sechi & Wehland, 2000). WASP is a critical component of podosomes, with cultures derived from WASP-null mice exhibiting impaired podosomes, actin rings and defective bone resorption (Calle et al., 2004). $\alpha$ $\beta$3 integrin signalling causes downstream Rho GTPase activation of phosphatidylinositol 4-phosphate 5-
kinase (PI4P-5) and PI3-K, as well as induction of phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2) (Chellaiah, 2006). Interaction of PI(4,5)P2 with gelsolin and WASP is necessary for podosome regulation and actin ring formation in osteoclasts (Chellaiah, 2006), summarised in Figure 1.3. Localised within the core of the podosome are the actin binding proteins α-actin, fimbrin (Marchisio et al., 1987), cortactin and gelsolin (Chellaiah et al., 2000). Many other proteins localise around the actin core, such as integrins, integrin-associated proteins such as paxillin, talin, vinculin and p130Cas, and the endocytic proteins dynamin and endophilin (Bruzzaniti et al., 2005; Ochoa et al., 2000). Vav3, a Rho family guanine nucleotide exchange factor, is essential for osteoclast activation and bone mass in mice, with Vav3-deficient osteoclasts showing dysfunctional downstream signalling from c-Fms and αvβ3 resulting in multiple cytoskeletal defects and impaired bone resorption (Faccio et al., 2005). In addition to these structures, the cytoskeletal framework (which also is constructed from highly organised actin filaments) is thought to be involved in the transport of vacuolar H+-ATPase to the ruffled border (Lee et al., 1999). Ruffled border projections contain a highly interwoven meshwork of filaments (Akisaka et al., 2006).

Through mechanisms unknown, osteoclasts recognise and respond differently to different resorption substrates, with osteoclasts cultured on glass and mineralised bone displaying distinct differences in morphology and function (Saltel et al., 2004). Demineralised bone cannot be resorbed (Jones et al., 1984). Mechanisms which dictate where and when an osteoclast commences resorption remain unclear.
Figure 1.5 Cytoskeletal reorganisation pathway. Scheme representing the signaling events leading to cytoskeletal reorganisation, podosome production and actin ring formation. Activation of c-Fms and RANK receptors signals downstream events required for podosome and actin ring formation. αvβ3 integrin signalling activates c-Src and causes downstream Rho GTPase activation of PI4P-5 and induction of PI(4,5)P2. Interaction of PI(4,5)P2 with gelsolin and WASP, and complex formation of WASP with Arp2/3 results in podosome regulation and actin ring formation. Vav3 signals (via c-Fms and αvβ3), require Rac1 and Cdc42, and is enhanced by the interaction of Cdc42 with the Arp2/3 complex. Cdc42 is also able to activate WASP.
1.13.3 Osteoclast migration.

After the osteoclast resorbs to a certain depth, determined by mechanisms that remain to be explained, the osteoclast detaches and moves along the bone surface before reattaching and forming another resorption pit (Baron et al., 1993). Unlike the actively resorbing osteoclast, the non-resorptive migrating osteoclast is spread, allowing inchworm-like migration (Saltel et al., 2004). Molecular features distinguishing migrating osteoclasts from resorbing osteoclasts, are largely unknown.

1.14 Abnormal osteoclast activity.

During the process of resorption a certain volume of bone matrix is removed, which is then replaced, under normal circumstances, by osteoblasts secreting newly formed bone matrix a few days later (Baron et al., 1993). The resorbed bone is completely restored by osteoblasts in young individuals (Teitelbaum, 2000). As an individual ages, the amount of bone deposited in resorption lacunae is less than that previously removed by osteoclasts. When pronounced, this can lead to clinically significant osteoporosis, which is distinguished by irregular formation of bone marrow cavities and low bone density (Teitelbaum & Ross, 2003; Tolar et al., 2004).

The impact of bone degenerative diseases dominate the field of bone biology, and genetic studies have contributed much to the current understanding of abnormal osteoclast activity. It is generally agreed that as we age or as a consequence of disease, imbalances between osteoclast and osteoblast activities arise. An imbalance in the ratio of bone formation and bone resorption leads to abnormally dense (osteopetrosis and osteosclerosis) or porous bone (osteoporosis).
Because adult osteoporosis always implies enhanced bone resorption, understanding the disease and formulating treatment requires an understanding of osteoclast biology. Genetic factors are now recognised to be one of the most important determinants of osteoporosis (Ralston, 1997). The identification of genes involved in normal osteoclast development may lead to further insights into general bone biology with repercussions for understanding abnormal bone resorption. Considering the increasing population of patients suffering from disorders of the skeleton, it is important for both physicians and scientists to further understand the biology of bone and how osteoclasts contribute to disease.

For the study of osteoclast biology, primary human culture models represent an authentic system for the study of osteoclast differentiation and function. Primary osteoclast models have been used to characterise genes required for development, however, the construction of an in vitro cell culture model which can be used to distinguish genes contributing to bone resorption distinct from cellular differentiation has not been well established. The employment of siRNA inhibition with primary human osteoclasts, may provide a method by which this can be achieved.

1.15 RNAi mediated gene silencing.

The determination of gene function has been aided by the discovery of new technologies, with the characterisation of hereditary diseases, transgenic models, antisense techniques and genome sequencing contributing to our discoveries of gene function. RNA interference (RNAi) is a flourishing field, which has significantly improved our understanding of gene regulation. RNAi is a promising tool for the functional analysis of genes, providing an efficient and robust mechanism for
assigning phenotypes in a loss-of-function context. This method can easily be scaled up to perform genome wide screening and therapeutic target validation, providing that careful optimisation is performed with the model of interest. For these reasons, siRNA provide a useful way in which to test candidate genes for osteoclast differentiation and resorption in the primary osteoclast cell model.

RNAi is a process observed in all eukaryotes and plants, involving the degradation of sequence-specific message RNA (mRNA) species directed by double-stranded RNA (dsRNA) (Hannon, 2002; Hutvagner & Zamore, 2002). Aside from what was thought to be the original purpose of RNAi in protecting against rogue genetic elements and viruses, RNAi is currently recognised to be involved in the regulation of transcription (Yekta et al., 2004), translation (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993), mRNA and DNA stability/degradation (Jing et al., 2005), genome integrity and chromatin structure (Hall et al., 2002; Volpe et al., 2002), as well as being critical for regulating cell growth, differentiation and apoptosis (Bartel, 2004; Gregory & Shiekhattar, 2005; Lu et al., 2005).

All RNA silencing pathways involve processing of dsRNA into 21-30 nucleotide fragments which integrate into a 'silencing complex' to repress the function of a particular genetic outcome (Jackson & Standart, 2007).

siRNAs are produced exogenously by chemical synthesis, or endogenously by the RNAi pathway, when adenosine triphosphate (ATP)-dependant Dicer-2 enzyme processively cleaves dsRNA in the cytoplasm (Bernstein et al., 2001; Hutvagner &
siRNAs are processed from perfectly matched dsRNA intermediates in the RNAi pathway.

microRNAs (miRNAs) are endogenous small RNAs cropped by Drosha-DiGeorge syndrome critical region gene-8 (DGCR8) complex to form a 70 nucleotide transcript (Lee et al., 2003) that forms imperfectly base-paired hairpin structures, which are processed by the miRNA pathway (Kim, 2005). The pre-miRNA hairpin is exported from the nucleus into the cytoplasm, where it is cleaved by Dicer-1 into 21 residue RNAs (Bernstein et al., 2001; Hutvagner et al., 2001). The binding of miRNA to imperfectly matched mRNAs results in translational repression of protein-coding genes (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993) or cleavage of complementary mRNAs (Yekta et al., 2004). The Argonaute protein Ago1 is involved in miRNA biogenesis (Miyoshi et al., 2005; Okamura et al., 2004).

Both pathways share some cellular machinery with functional mechanisms occurring in a similar, if not identical way. More specifically, miRNA has been found to cleave perfectly homologous mRNAs (Yekta et al., 2004), and exogenously introduced siRNAs have been found to translationally repress imperfectly matched mRNAs (Doench et al., 2003; Saxena et al., 2003). Both siRNAs and miRNAs possess 3' extensions of 2 nucleotides and a 5' phosphate (Jackson & Standart, 2007). The strand of the RNA duplex which has lower stability base-pairing at its 5' end (the guide strand) both activates, and is assembled into, the RNA-induced silencing complex (RISC), termed siRISC in the context of siRNAs and termed miRISC or the miRNA-protein complex (miRNP) in the context of miRNAs (Tomari & Zamore, 2005). The RISC loaded with a guide strand binds to target mRNA resulting in degradation of
perfectly homologous mRNAs by Argonaute2 (Ago2) (Liu et al., 2004; Meister et al., 2004), or translational repression of mRNA by binding with imperfect complementarity to the 3’ untranslated region (UTR) of target mRNA (Doench et al., 2003; Saxena et al., 2003) and localises to cytoplasmic mRNA processing bodies (p-bodies) (Liu et al., 2005; Pillai et al., 2005; Sen & Blau, 2005). The other strand (the passenger strand) is degraded (Matranga et al., 2005). RISC components such as Ago1 and Ago2, localise to p-bodies, where translationally repressed mRNA-protein complexes and rck/p54 interact to mediate translational repression (Chu & Rana, 2006; Liu et al., 2005).

Another distinct small RNA pathway involving RNA strands derived from repetitive sequences in the genome, termed repeat-associated siRNAs (rasiRNAs), silence retrotransposons and repetitive sequences in a Dicer-independent manner (Gunawardane et al., 2007; Vagin et al., 2006).

The silencing effects of small RNAs are potent and specific, and for this reason have been aggressively adopted for loss-of-function studies. The mechanism behind the efficiency of small RNAs is yet to be completely elucidated, however, a number of aspects have been hypothesised. The ‘Dicing’ of long dsRNAs into many 21-23 nucleotide siRNAs is thought to provide a level of amplification. RISC may catalyse multiple targeted cleavage reactions (Rana, 2007). RNA-dependent RNA polymerase (RdRP) is necessary for gene silencing in plants, fungi and worms (Cogoni & Macino, 1999; Dalmay et al., 2000; Mourrain et al., 2000), and a model for siRNA-primed RdRP-style reactions where mRNA is converted into dsRNA for further Dicer cleavage, has also been proposed (Lipardi et al., 2001; Sijen et al., 2001). However,
studies with mammalian cells have shown that the free 3’-OH group, which is necessary for RdRP reactions, is not necessary for RNAi activity (Chiu & Rana, 2002; Czauderna et al., 2003; Schwarz et al., 2002).

Highly differentiated primary human cells such as osteoclasts do not perform comparably to immortalised cell lines, with the uptake of DNA, RNA and virus historically representing technical obstacles (Laitala-Leinonen, 2005; Taylor et al., 2007). The optimisation of siRNA-mediated gene silencing in primary human osteoclasts has the potential to accelerate gene discovery and the assignment of function to novel genes, as well as validate candidate therapeutic targets for skeletal disorders.
1.16 Aims and Hypothesis

The essential capability of organisms to be multicellular is their ability to express only certain collections of genes in particular cells at particular times. Differential gene expression among cell types can be compared to reveal genes that are common and exclusive to each cell type. Time-course differential display PCR (DD-PCR) and quantitative real-time PCR (Q-PCR), is to be used to outline genes differentially expressed in the primary human osteoclast cell model.

Genetic factors distinguishing bone resorbing osteoclasts from differentiating or migrating osteoclasts are not completely understood. A more comprehensive knowledge of genes which regulate bone resorption is vital for understanding skeletal diseases and the principles of bone remodelling. Genetic control of osteoclast differentiation and bone resorption is to be examined separately, using differentiating and pre-differentiated osteoclasts in combination with RNAi. By setting up comparisons of gene expression in differentiating osteoclasts distinct from bone resorbing osteoclasts, the genetic control of formation and activity in the osteoclast can be clarified.

RNA interference of uncharacterised genes is an untested method for mass screening of osteoclast candidate genes. This method would be of value to studies for discerning gene involvement in osteoclast formation and/or function, as well as simple loss-of-function assays.

Therefore, the hypothesis that RANKL induces the expression of genes, which are crucial for osteoclast differentiation and bone resorption, and that these genes can be
identified using the differential display PCR method, is to be tested. Furthermore, differentially expressed genes can be verified using real-time PCR. It would be useful to examine if genes expressed in osteoclasts would be required for differentiation, or required for resorption, and whether some genes are instrumental for both processes. It was hypothesised that siRNA inhibition of candidate genes could be used in a novel way to distinguish genes that are necessary for osteoclast differentiation independently from genes which are necessary for bone resorption, by using two cell culture systems. One system involves inhibiting gene expression in precursor populations as they differentiate through RANKL and M-CSF cytokine induction. The second system involves the use of pre-differentiated mature osteoclasts, that are freshly plated onto dentine in the presence of siRNA inhibiting the gene of interest, so that resorptive activity can be assayed.
Chapter 2

Materials and Methods
2.1 Materials

21 x 21cm glass plate (backing plate)
21 x 15.1cm glass plate (front plate)
21 x 1.4 x 0.4cm teflon spacer
21 x 1.4 x 0.8cm teflon spacer
Amersham Life Sciences Hypercassette 24x30cm Light Excluding Cassette
Amersham Life Sciences Hypercassette 35x43cm Light Excluding Cassette
Amersham Pharmacia Biotech Ficoll-Paque Plus
Amersham Pharmacia Redivue α-32P dCTP
Amresco Ammonium persulfate
Amresco N,N’-Methylene-bis acrylamide
Amresco Phosphate buffered saline (PBS)
Amresco SDS
Amresco Tris base
Art Pipette DNase/RNase/Pyrogen-free tips 10uL
Art Pipette DNase/RNase/Pyrogen-free tips 20uL
Art Pipette DNase/RNase/Pyrogen-free tips 200uL
Art Pipette DNase/RNase/Pyrogen-free tips 1000uL
Astral EDTA (trisodium salt)
Axygen PCR strip tubes 0.2mL
Beckman Instruments Polyallomer centrifuge tubes
Beckman Ultra Centrifuge
Becton Dickinson BioCoat collagen I coated 6-well plate
Becton Dickinson cell scraper
Becton Dickinson Nucleospin RNA II
Becton Dickinson serological pipet 5mL
Becton Dickinson serological pipet 10mL
Becton Dickinson serological pipet 25mL
Becton Dickinson tissue culture plate 96 well
Becton Dickinson tissue culture plate 24 well
Becton Dickinson tissue culture plate 12 well
Becton Dickinson tissue culture flask 25cm²
Biorad 500/200 volt power supply
Biorad Agarose molecular grade
Biorad iCycler iQ Real-time detection system software version 3.0
Biorad iCycler iQ System
Biorad/Kodak Imaging Screen –K 20x25cm
Biorad Personal Molecular Imager FX
Biorad Screen Eraser –K
Biorad Screen Guard Protective Film
Biorad SYBR Green I Supermix
Centrifuge 3K15 from Sigma
Clontech Delta Differential Display kit
Corning 15mL Centrifuge Tubes
Eppendorf 1.5mL Microfuge Tubes
Eppendorf Microfuge-Centrifuge 5402
Falcon 50mL Centrifuge tubes
Fermentas 1Kb+ DNA Marker
Fisher Biotech gWIZ GFP plasmid
Fisher Biotech Dicer siRNA Generation kit
Gilson Pipetteman P2
Gilson Pipetteman P20
Gilson Pipetteman P200
Gilson Pipetteman P1000
ICN Cesium chloride ultra pure grade
Invitrogen Alexa fluor 568 phalloidin
Invitrogen Dissociation buffer
Invitrogen Fetal bovine serum (FBS) (heat inactivated)
Invitrogen Lipofectamine 2000
Invitrogen Minimum essential media (MEM)
Invitrogen Oligofetamine
Invitrogen Penicillin-streptomycin
Invitrogen Phalloidin (Rhodamine)
Invitrogen Superscript II RNase H Reverse Transcriptase
New England Biosciences Bovine serum albumin (BSA) 100x
New England Biosciences ShortCut RNase III
New England Biosciences T7 RNA polymerase
Nikon Coolpix digital camera
Nikon Inverted research-grade microscope TE-2000U
Pepro Tech Human M-CSF cytokine
Pepro Tech Human RANKL cytokine
Polyplus transfection JetPEI
Primers custom-made from Invitrogen and Proligo
Promega 10x PCR buffer
Promega Guanidine thiocyanate
Promega MgCl₂ 25mM
Promega Nick Translation System
Promega RQ1 DNase 1
Promega rNTP mix
Promega set of dATP, dCTP, dGTP, dTTP 100mM
Promega Taq DNA Polymerase
Promega Wizard™ Series 9600 Miniprep Reagent System
Promega Wizard™ Series PCR Purification System
Roche FuGENE 6
Sarstedt 3.5mL transfer pipettes (sterile)
Sarstedt 10mL centrifuge tubes
Sigma-Aldrich 99.9% Acrylamide
Sigma-Aldrich Diethyl pyrocarbonate (DEPC)
Sigma-Aldrich N,N,N',N’-Tetramethylethylenediamine (TEMED)
Sigma Boric Acid
Sigma Dithiothreitol (DTT) 1mM
Sigma Leukocyte acid phosphatase staining kit
Sigma Tris Base
Teflon well forming comb 18 x 6 x 0.4cm – 18 well spaces (0.7cm width)
### Table 2.1. Primers used in DD-PCR, Q-PCR and siRNA

<table>
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<th>Gene</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
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<td><strong>DD-PCR &amp; Q-PCR Primers</strong></td>
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Table 2.2 Standard curves constructed from real time Q-PCR

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2.2 Isolation of PBMCs and cell culture- differentiating osteoclasts and macrophages.

Whole blood was obtained from healthy lab member volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated for culture using a modified method described by Nicholson et al., (2000). An equal volume of phosphate buffered saline (PBS)-minimum essential media (MEM) solution (1:1) was added to whole blood, and layered over 15mL of Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), then centrifuged (1500rpm for 30 minutes). The PBMC layer was removed and washed with PBS-MEM solution, and centrifuged (1000g for 10 minutes). Isolated PBMCs were resuspended in MEM supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen, Carlsbad, CA, USA), and 1% penicillin-streptomycin. PBMCs were plated at 1.5x10⁶ cells per mL and incubated at 37°C with 5% CO₂. After two hours incubation, non-adherent cells were removed by washing twice with MEM. Adherent monocytes were supplemented with 25ng/mL M-CSF, or 40ng/mL RANKL and 25ng/mL M-CSF, with media and cytokine replacement every 3-7 days for up to 3 weeks. Cell cultures for expression studies and cell staining were plated onto plastic. Cell cultures for bone resorption studies were cultured on sperm whale dentine slices. Fluorescence staining assays were performed on glass coverslips.

2.3 Pre-differentiated osteoclasts for siRNA bone resorption assays.

PBMCs were isolated as above, and plated onto BioCoat collagen I coated 6-well plates (BD Biosciences, Franklin Lakes, NJ, USA). Cells were washed and media replaced at 7 days. At 2 weeks, cells were washed and treated with dissociation buffer (Invitrogen, Carlsbad, CA, USA) for 15 minutes at 37°C. Remaining cells were dislodged by gently tapping the culture dish and applying a cell scraper. Cells were
centrifuged (100g for 5 minutes) through a 20% serum cushion in MEM, and plated out on sperm whale dentine slices at approximately 50% confluency with 25ng/mL M-CSF and 40ng/mL RANKL.

2.4 Cell Marker Staining

2.4.1 Fixation

Cells were washed with PBS, before fixing in 10% formalin for 5-10 minutes. After rinsing with PBS, cells were permeabilised with 0.5% Triton X-100 for 10 minutes.

2.4.2 Tartrate resistant acid phosphatase stain.

TRAP staining was performed using the leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA). Fresh stain was made up following the manufacturers protocols, and added to formalin-fixed or non-fixed cells and incubated for 1 hour at 37° C. Stain was replaced with PBS prior to bright field microscopy. TRAP positive cells stain purple revealing the TRAP enzyme precipitate located within osteoclasts.

2.4.3 Nuclear stain.

To visualise multiple nuclei within osteoclasts a 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) stain (Sigma-Aldrich, St. Louis, MO, USA) was used. The DAPI stock solution was diluted in PBS (1:50,000) stain and was applied to non-fixed cells without media and incubated at 37° C for 5 minutes. DAPI stained nuclei fluoresce blue. Cells were optionally fixed after staining. Cells were washed with PBS and analysed with bright field microscopy.
2.4.4 F-actin stain.

F-actin staining was performed on cells fixed with 10% formalin, using Alexa fluor 568 phalloidin (Invitrogen, Carlsbad, CA, USA). The phalloidin stock was diluted 1:500 with a bovine serum albumin (BSA)-PBS solution (1:40). Cells were incubated with stain for 10 minutes in darkness, washed with PBS and analysed with bright field microscopy.

2.5 Multinucleated TRAP positive cell counts.

Osteoclast cell counts were calculated from TRAP stained, fixed, 96 well cultures. A minimum of three nuclei was designated multinucleated. Cells which stained purple were identified as TRAP positive and cells stained brown were identified as TRAP negative.

2.6 Bone Resorption Assays.

Pits resorbed on dentine slices were visualised with light microscopy and scanning electron microscopy (SEM). Cellular debris and stain were removed from dentine slices by agitation in chloroform/methanol solution (2:1). A layer of ink was applied to the surface of the dentine slice using an Artline 70 marker. The ink layer was immediately removed with absorbent paper to reveal ink stained resorption pits within the bone as described in Hodge et al., (2004). Pits were analysed by light microscopy and individually counted, with continuous trench-like pits counted as one individual pit. Area resorbed on dentine was determined, using a 552 square grid, which was layered over high resolution SEM micrographs (80X magnification) using Adobe Photoshop. Squares containing resorption were divided by the total squares to obtain percentage resorbed.
SEM was performed at the Analytical Electron Microscopy Facility (AEMF) at the Queensland University of Technology on chloroform/methanol cleaned dentine slices that were gold sputter coated, using a Bio-Rad sputter coater, by quanta SEM.

2.7 Cesium chloride gradient RNA isolation.

Adherent cells were physically dislodged from the flask, plate or dentine slice using a pipette tip and/or cell scraper, in a 4M guanidine thiocyanate/1% sodium lauryl sarcosine (SLS) solution. The RNA isolation protocol described has been adapted from Glisin et al., (1974). A 23 gauge needle was used to homogenise the cell lysate, before layering onto a 3.5mL cushion of 5.7M CsCl-0.1M ethylene-diamine tetra acetic acid (EDTA) and centrifuged (27,000rpm for 16hrs at 22°C in SW41 rotor). The upper phase was removed with a transfer pipette, the tube inverted and the bottom 1cm section of the tube sliced off with a sterile scalpel and rinsed with 70% ethanol. The pellet was dissolved with 60μL Tris EDTA (TE)- sodium dodecyl sulfate (SDS) (10mM Tris-Cl, 1mM EDTA pH 8.0 – 1% sodium dodecyl sulfate). The tube was rinsed with 40μL TE to dissolve any remaining RNA in the tube. The RNA solution was transferred into an RNase-free microfuge tube with 250μL ice cold 100% ethanol and 10μL of sodium acetate, and centrifuged (14,000rpm for 15 minutes) to precipitate the RNA. The RNA pellet was rinsed with 75% ethanol, centrifuged (14,000rpm for 5 minutes), and dried before resuspending in RNase-free H₂O.
2.8 Spin-column RNA isolation.

Total RNA was also isolated from guanidium thiocyanate cell lysates using a spin-column system with the Nucleospin® RNA II Kit (BD Biosciences, Franklin Lakes, NJ, USA) as per instructions.

2.9 First-strand cDNA synthesis

1μg of total RNA and 2.5μg of oligo dT primer were combined in tubes up to a final volume of 5μL with RNase-free H₂O. For 3 minutes the tubes were incubated at 70°C, after which they were cooled on ice for 2 minutes. A master mix was then prepared for all cDNA synthesis reactions. Per reaction the master mix contained: 1X reaction buffer, 1mM dNTP (Promega, Madison, WI, USA), 2.5mM MgCl₂ and 200 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Five microlitres of RNA solution was added to 15μL of master mix and incubated at 42°C for 60 minutes. Reverse transcription was inactivated by incubating at 95°C for 3 minutes.

2.10 Differential Display PCR

DD-PCR was optimized in previous studies using the Delta Differential Display Kit (Clontech, Palo Alto, CA, USA). The use of extended primers, increased the specificity and reproducibility of the experiments. DD-PCR experiments confirmed that of the 7 primer combinations tested, ‘P1’ and ‘T1’ primers were the combination of primers providing the most differential amplification between cDNAs. Duplicated DD-PCR experiments revealed highly reproducible banding patterns. Care was taken to use consistent concentrations of RNA in each DD-PCR, to maintain reproducibility. The inherent reproducibility of DD was verified by the recurrence of the same
sequence, amplified using cDNAs from cells of the same treatment from independent experiments (Table 2.1).

<table>
<thead>
<tr>
<th>Sequence Identity</th>
<th>Genbank Accession</th>
<th>Bands</th>
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<th>DD Primers</th>
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<td>3</td>
<td>P1-T1</td>
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<tr>
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<td>20, 47</td>
<td>2</td>
<td>P1-T1</td>
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<td>PRO1859</td>
<td>AF132202</td>
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<td>4</td>
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<td>233, 247</td>
<td>2</td>
<td>P1-T1</td>
</tr>
</tbody>
</table>

Table 2.3 Frequency of DD-PCR products in duplicated experiments. Identical sequences were identified in repeat DD-PCR experiments using the same primers and cDNAs from cells of the same treatment, but from independent experiments.

DD-PCR was performed using the Delta differential display kit (Clontech, Palo Alto, CA, USA). Per reaction the following was added: 200-500ng of cDNA, 0.25μM Clontech P primer, 0.25μM Clontech T primer, 1X PCR reaction Buffer, 125μM dNTP solution containing dATP, dTTP, dGTP, [α-33P]dCTP and 2 units Taq DNA polymerase. The PCR conditions were 94°C for 5 minutes, 40°C for 5 minutes, 72°C for 5 minutes for 1 cycle; 94°C for 2 minutes, 40°C for 5 minutes, 72°C for 5 minutes for 1 cycle; 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes for 4 cycles; and 72°C for 7 minutes for 1 cycle.
2.11 Polyacrylamide and Agarose Gel Electrophoresis

Six percent (for DD-PCR visualisation) and twelve percent (for reamplification PCR visualisation) polyacrylamide gels were prepared for DD-PCR visualisation, general PCR validation, re-amplification PCR, and for checking Dicer enzyme digestion and siRNA quality. 1X TBE was used in addition to 100μL of TEMED and 400μL of 10% APS per gel, which was poured between glass plates separated by 4mm thick spacers. Gels were left to set for 20-30 minutes. For each reaction 10μL of the PCR product was combined with 1X loading dye (0.25μM Xylene Cyanol, 0.25μM bromophenol blue and 50% glycerol). Gels were electrophoresed at 200V for 2-3 hours.

After electrophoresis, DD-PCR polyacrylamide gels were transferred to Whatman paper and carefully dried under vacuum in a gel drier. The dried gel was exposed to a phosphor screen in a light-excluding cassette for up to 12 hours. A Bio-Rad molecular imager was then used to scan the image. A transparency printout of the resulting image was aligned to the original gel and taped to keep the autoradiograph firmly aligned. A test band was excised and the gel exposed to a phosphor screen to check correct alignment of the gel with the transparency. Using a sharp pin, amplicons were marked by poking holes through the film and into the gel beneath. A sterile scalpel was then used to excise each of the amplicons from the gel using the pin marks as guides. Each fragment was placed in a microcentrifuge tube containing 50μL of RNase/DNase-free H₂O. Alternatively, after electrophoresis, other polyacrylamide gels were stained with ethidium bromide and visualised via ultraviolet transillumination.
One to two percent agarose gels, with 1X TAE Buffer and 1μg ethidium bromide per gel, were used to visualise PCR products. Per well, 10μL of the PCR product was combined with 1X loading dye (0.25μM Xylene Cyanol, 0.25μM bromophenol blue and 50% glycerol). Gels were electrophoresed at 200 volts for 30 minutes, and visualised by ultraviolet transillumination.

2.12 Reamplification PCR

The DD-PCR displayed on the gel produces an array of amplicons of different sizes (Figure 3.0.). These amplicons are cut from the gel with a sterile scalpel blade and placed in 50μL of sterile RNase/DNase-free H₂O. The amplicon is left to diffuse into the water overnight and this is then used as template for subsequent re-amplification reactions.

To re-amplify the DNA, a PCR was performed using 2-8μL of the liquid containing eluted PCR product. A master mix was made so that each reaction contained: 1X PCR Buffer, 125μM dNTPs, 0.25μM of ‘P’ primer and 0.25μM of ‘T’ primer used in the DD-PCR, 0.5-4mM MgCl₂, 1 unit of Taq DNA polymerase, and up to 20μL with MQ-H₂O. Optimal MgCl₂ concentrations were tested using a typical range of 1 to 4mM. The PCR was performed in a Biorad iCycler Thermal Cycler under the following conditions: 94°C for 5 minutes; (94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes) for 35 cycles; and 72°C for 7 minutes.

The reaction results were visualised on a 12% polyacrylamide gel to allow sufficient resolution to determine if an amplicon contains multiple sized fragments. PCR products that consisted of one single amplicon were directly purified and sequenced.
PCR products that contained more than one amplicon were separated by polyacrylamide or agarose gel purification.

2.13 Polyacrylamide Gel Purification

Upon running 10µL of PCR amplicon on 12% polyacrylamide gel to visualise the results of the PCR, multiple sized amplicons of DNA contained in a single PCR reaction could be separated. Polyacrylamide gel purification was performed by carefully slicing the section of gel containing single-sized amplicons of DNA with a sterile scalpel and placing the fragment in 200µL of H₂O to elute the DNA. This DNA was then used in subsequent PCR reactions to amplify amplicons of a single-size before performing sequencing.

2.14 Exo-Sap Purification

Exo-Sap contains 1:100 dilution of exonuclease I and a 1:10 dilution of shrimp alkaline phosphatase to degrade unused primers and dNTPs. 1µL of Exo Sap-IT (Amersham Biosciences, Uppsala, Sweden) was combined with 6µL of DNA to be purified and placed in the thermocycler. The cycler conditions were: 37°C for 30 minutes and 80°C for 15 minutes.

2.15 DNA Sequencing

Purified DNA was sequenced along with a positive control (pGEM cloning vector) using the following reaction mix: 8µL of Terminator Ready reaction mix (Applied Biosystems, Foster City, CA, USA), with 1µg of template DNA, 3.2pmol of forward primer used in previous reactions, and up to 20µL with MQ-H₂O. The cycle conditions for the reaction were as follows: (96°C for 30 seconds, 50°C for 15 seconds
and 60°C for 4 minutes) repeated 25 times. The labelled DNA was then precipitated with 1:10 the reaction volume of 3M sodium acetate (pH 5.2) and 2.5 times the reaction volume of 95% ethanol. The solution was then incubated at room temperature for 15 minutes and centrifuged for 20 minutes at 14,000rpm. The supernatant was then poured off and 250µL of 70% ethanol added. A further spin for 10 minutes at 14,000rpm was performed. The supernatant was poured off and all traces of ethanol removed. The pellet was then left to dry or vacuum dried for 5 minutes. The sequencing reaction was performed at Griffith Sequencing Facility, Nathan Campus. Sequence information was viewed using the software Chromas 2 (Technelysium Pty Ltd), and compared with databases of NCBI Genbank, UCSC Human BLAT Search, and ENSEMBL.

2.16 Real-time quantitative PCR

To validate DD-PCR products and measure specific levels of gene expression within each profile, Q-PCR was performed using 10ng cDNA (synthesised as previously described) in a reaction mix containing: 10µL SYBR Green Supermix (100mM KCl, 40mM Tris-HCl, pH 8.4, 0.4mM of each dNTP [dATP, dCTP, dGTP and dTTP], 50 units/mL iTaq DNA polymerase, SYBR Green I, 20nM fluorescein, and stabilisers), 125nM each of forward and reverse primers and up to 20µL of H2O. A negative control was incorporated into the reaction where sterile H2O replaced the cDNA, and a positive control was also incorporated where cDNA with known gene expression or genomic DNA replaced the test cDNA. Ribosomal 18S was used as a standard control, due to its consistent level of expression across cDNAs of equal concentrations from different cell types (data not shown). Q-PCR was performed with the Biorad iCycler iQ with Real-time Detection System Software version 3.0 under the following
cycling conditions: 94°C for 2 minutes, (94°C for 30 seconds, 50-60°C for 45 seconds, 72°C for 1 minute) x 45 cycles; followed by a melt curve analysis from 55°C to 95°C in 0.5°C increments.

The cycle threshold (Ct) for each PCR reaction was used to determine relative gene expression using the ΔΔCt method as per Livak and Schmittgen (2001). The mean ΔCt of each culture condition was compared to calculate fold change in gene expression. Standard error was calculated for the mean ΔCt of each culture group.

Standard curves were generated for all primer sets using target DNA standards from $10^0$ copies to $10^{12}$ copies. Copy number of the unknown product was calculated from the equation representing the slope of the line ($y = mx + c$), where $y$ is the Ct of the gene, $m$ is the slope of the line, $x$ is the log copy number, and $c$ is the y intercept. The equation is solved for $x$, and the copy number derived from the inverse of $x$. The copy number of the gene was divided by the copy number of 18S for 1ng of cDNA.

2.17 siRNA synthesis

siRNA were generated from dsDNA matching a 500-1000bp region of the gene of interest amplified by PCR. Forward and reverse primers were designed with flanking T7 RNA polymerase promoter sites. The PCR was assembled to contain: 2μL of genomic DNA, 1X PCR Buffer, 0.5μM dNTP mix (dCTP, dGTP, dATP, dTTP), 125nM of each forward primer and reverse primer, 1.6-3mM MgCl$_2$ and 2 units Taq DNA Polymerase. The total final volume was made up to 20μL with Milli-Q H$_2$O. A gradient of 1.6mM-3mM of MgCl$_2$ with 0.2mM steps was used to determine optimum MgCl$_2$ concentrations. PCR was performed with the Biorad iCycler iQ system under
the following cycling conditions: 94°C for 3 minutes, (94°C for 30 seconds, 50-65°C for 1 minute, 72°C for 2 minute) x 35 cycles, 72°C for 5 minutes. The amplified product was purified using the Promega Wizard PCR Purification Kit (Promega, Madison, WI, USA).

dsRNA was generated from the PCR product. Each dsRNA synthesis reaction was assembled to contain: 1X buffer, 5mM DTT, 19mM MgCl₂, 1X BSA, 40 units RNase OUT, 4mM rNTP mix, 300 units T7 RNA polymerase, 2μg PCR template DNA, and up to 60μL Nuclease-free water. The reaction was mixed and incubated at 37°C for 3 hours. Five units of RQ1 DNase (Promega, Madison, WI, USA) was added to each reaction upon completion, and incubated for a further 30 minutes at 37°C. dsRNA yield was visualised on a 2.5% agarose gel.

dsRNA was ethanol precipitated, by adding 1/10th volume of 3M sodium acetate (pH 5.5) and 2 volumes of cold 95% ethanol. The solution was chilled on ice for 15 minutes, and centrifuged (14,000rpm for 15 minutes). The RNA pellet was washed with 80% ethanol, centrifuged for 5 minutes at 14,000rpm, before air-drying. The RNA pellet was resuspended in nuclease-free water, and incubated for 5 minutes at 70°C to dissolve the RNA. RNA was quantitated by UV light absorbance and agarose gel electrophoresis.

Purified dsRNA was digested into siRNA using RNase-III ‘Dicer’ enzyme (New England Biolabs, Ipswich, MA, USA). Each Dicer Reaction contained: 10μg dsRNA, 1X MnCl₂, 1X ShortCut Reaction Buffer, 10 units ShortCut RNase III enzyme, and up to 100μL with nuclease-free water. This reaction was incubated for 20 minutes at
37°C. EDTA was added to stop the reaction. Complete digestion was checked by running product on a 12% polyacrylamide gel.

siRNA was purified by ethanol precipitation. One tenth volume of 3M sodium acetate (pH 5.5), 2μL of RNase-free glycogen and 3 volumes of cold 95% ethanol, was added to the siRNA, and placed at -80°C for 30 minutes. The solution was centrifuged (14,000rpm for 15 minutes), washed with 80% ethanol and centrifuged again (14,000rpm for 5 minutes) before air-drying. The siRNA was then resuspended in RNase-free H₂O, and quantitated by ultraviolet light absorbance.

2.18 Transfection of siRNA

PBMCs were plated out to approximately 50-70% density for gene-specific siRNA and green fluorescent protein (GFP)-specific siRNA transfection.

Complexes between siRNA and the transfection reagents were prepared according to the particular specifications of the individual manufacturers of each reagent. For each transfection reagent, after siRNA-reagent complexes had formed, the mixture was exposed to cultures and incubated for 6 hours before replacing the M-CSF/RANKL supplemented media. Both the siRNA transfection reagent comparison and the siRNA dose-response cultures were performed using siRNA concentrations of 25 nM and 0-25 nM, respectively. FuGENE 6 (Roche, Penzberg, Germany) was used as per guidelines for 24-well plates with a volume of 3μl FuGENE 6 per well. One microliter of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 3μl Oligofectamine (Invitrogen, Carlsbad, CA) was used per well following the manufacturer’s procedures. Two microliters of JetPEI (Polyplus-transfection, Illkirch, France) was
used per well following the appropriate protocol for 24-well plates. Transfection in 96-well plates for bone resorption assays were performed as per the 24-well plates scaled for smaller final volumes. All bone resorption assays were performed using FuGENE 6 as a transfection reagent.

The efficacy of gene-specific siRNA knockdown was monitored by Real-time PCR quantification of cDNA, synthesised from mRNA isolated from transfected cells. Interferon response was monitored by comparing the expression of interferon induced genes in non-transfected cells to the expression in siRNA transfected cells.

2.19 Statistical analysis

Statistical analysis was performed using Excel and SPSS 10.0 for cell counts, resorption pit number, resorbed area, fold gene expression and absolute gene expression. Data is presented plus or minus the standard error of the mean. Significant differences were calculated using paired sample T-tests and analysis of variance (ANOVA), using data from 3 or more separate experiments.
Chapter 3

Characterisation of RANKL-regulated genes in osteoclasts using DD-PCR
3.0 Introduction

Recent rapid advances in the field of osteoclast biology can be attributed to the discovery of genes, which are essential to osteoclast formation and function. Much is to be gained from discovering further genes regulating these processes. Microarrays have proved to be a valuable screening tool for determining key genes regulated in the osteoclast. Unlike microarrays, where expression profiling is reliant on the hybridisation of gene products to probe sequences, differential display PCR (DD-PCR) is essentially a randomised gene profiling tool, in which completely novel gene isoforms can be identified. Traditional Affymetrix microarrays use one or more probesets containing around 11 perfect-match and 11 mismatch probes which are homologous to the 3' region of mRNA transcripts. High throughput EST and full length cDNA sequencing has revealed that mammalian transcripts contain extensive variations at the 3’ region (Moucadel et al., 2007). Altered 3’ end mRNA isoforms are often related to differential stabilities and translation rates (Moucadel et al., 2007). DD-PCR, therefore, has the potential to detect alternative mRNA splice variants. Recently, microarrays have been developed which no longer ignore large areas of mRNA transcripts, and target all the annotated and predicted exons in the genome. DD-PCR was originally selected as a tool to identify novel genes regulated during osteoclast differentiation.

DD-PCR is a technique that reverse transcribes poly-A mRNAs into cDNAs using a 3’ oligo(dT)-anchored primer followed by PCR with the 3’ anchored primer and a 5’ arbitrary primer. The cDNA products are visualised side by side on a high resolution gel, allowing immediate comparison of differential gene expression between control and experimental samples. DD-PCR simultaneously screens for up-regulated and
down-regulated transcripts in multiple populations in a semi-quantitative manner. This method samples the transcriptome with the advantage of amplifying mRNA unbiased by current limitations of sequence information.

In order to distinguish genes that are involved during osteoclast differentiation, distinct cellular stages of osteoclast formation were first characterised, after which the identification of transcriptional events corresponding to these stages were profiled using DD-PCR. Real time quantitative PCR was used to validate gene expression of a selection of gene products identified by DD-PCR.
3.1 Results

3.1.1 Defining cellular stages of differentiation.

Primary human PBMCs are pluripotent and differentiate into macrophages or osteoclasts in response to cytokine signalling. PBMC precursors differentiate into macrophage cells in the presence of M-CSF (Munn & Cheung, 1990), whereas the combination of M-CSF and RANKL stimulate differentiation of PBMCs into osteoclasts (Quinn et al., 1998; Takahashi et al., 1999). Osteoclast precursors express tartrate-resistant acid phosphatase (TRAP) at three days of differentiation (Day et al., 2004; Lee et al., 1995), and a high number of calcitonin receptors at 7 days of differentiation (Lee et al., 1995). RANKL-regulated genes were determined by parallel comparison of DD-PCRs from untreated PBMCs, M-CSF treated PBMCs and M-CSF & RANKL treated PBMCs.

Four time points were chosen for analysis within each cytokine treatment: 0 days (untreated), 3 days, 7 days and 21 days. These cellular stages of differentiation were determined from *in vitro* observations (Figure 3.1).

- At the initial time point of 0 days, untreated PBMCs (Figure 3.1a) were TRAP negative, small and spherical.

- By 3 days, early differentiation events have taken place in response to surface signalling of either c-fms receptor or RANK receptor and various downstream genes are activated. At 3 days of differentiation, small visible differences can be seen between PBMC precursors and cytokine treated cells. Both macrophages and osteoclasts at 3 days differentiation appear as round TRAP negative cells slightly larger than PBMCs, osteoclasts have visible nuclei (Figure 3.1b, 3.1e).
- After 7 days, macrophages have formed and osteoclast cell fusion has begun. Round and spindle-shaped TRAP negative macrophages are present in both M-CSF (Figure 3.1c) and M-CSF & RANKL (Figure 3.1f) treated cultures, however in M-CSF & RANKL cultures premature osteoclasts cells have fused and some multinuclear TRAP positive cells are present (Figure 3.1f).

- Mature macrophages and osteoclasts have formed by 21 days, TRAP negative macrophages have increased in size (Figure 3.1d) and abundant TRAP positive multinuclear osteoclasts are present (Figure 3.1g).

Cell cultures for each time point were grown for each cytokine combination to provide gene expression profiles for each condition.
Figure 3.1. Stages of differentiation were observed in primary human PBMC cultures following cytokine addition. All cells were live TRAP stained and viewed at 10x magnification. A) untreated PBMCs at 0 days were small, round and TRAP negative. PBMCs treated with 25ng/mL M-CSF increased in size marginally at 3 days (B). At 7 days M-CSF treated PBMCs develop into spindle-shaped and round cell populations (C), which increase in size after 21 days (D). M-CSF treated cells were TRAP negative (B, C, D). PBMCs treated with 25ng/mL M-CSF and 20-40ng/mL RANKL increased in size after 3 days (E) resembling 3 day macrophages and were TRAP negative. After 7 days (F), M-CSF and RANKL treated PBMCs have developed into spindle-shaped cells and small irregularly shaped osteoclasts. TRAP positive cells containing 1-3 nuclei were abundant. Mature M-CSF and RANKL treated cells at 21 days (G) differentiation appear large, TRAP positive and irregularly shaped, with 3-50 nuclei per cell. Arrowheads show osteoclast cells. Bar represents 100μm.
3.1.2 Genes regulated by M-CSF and/or RANKL identified by DD-PCR.

DD-PCRs were performed to provide expression profiles for the effects of M-CSF and M-CSF & RANKL on PBMC precursors, at a variety of time intervals during cellular differentiation. Cytokine regulation during osteoclast and macrophage differentiation can be seen in the semi-quantitative banding patterns displayed in Figure 3.2.

Twenty differentially expressed products were sequenced, matching unknown and established genes. A number of false positives were also generated, which represented genomic DNA. The obtained nucleotide sequence data was evaluated by comparing it with nucleotide and expressed sequence tag (EST) sequences registered in the NCBI, UCSC BLAT Server and ENSEMBL databases. Many of the sequenced cDNA products were complementary to ESTs with unknown or hypothetical gene products. LIM kinase 1 (LIMK1), was found to be up-regulated in 7 day M-CSF and RANKL treated cultures, and is an established RANKL-regulated gene, with reported expression in murine osteoclast cells (Cappellen et al., 2002). The presence of this gene in the study, validates the DD-PCR methodology, for its ability to correctly detect genes regulated by RANKL. Many of the genes identified by DD-PCR represented regulatory factors such as DNA binding proteins and signalling kinases. Cytoskeletal and motor proteins were also identified, with two kinesins in particular, expressed following M-CSF and RANKL treatment at 7 days of differentiation. A number of transmembrane proteins were identified, with biliary glycoprotein 1 and solute carrier family 16, member 6A expression present following M-CSF and RANKL treatment. Two immune related factors, the chemokine-like gene Homo sapiens family with sequence similarity 19 (chemokine (C-C motif) -like) A1
(FAM19A1) and the beta-defensin-like gene epididymis-specific 2 (EP2), were amplified at 7 days in M-CSF and RANKL treated PBMCs. These results show that the composition of regulated genes expressed during differentiation and proliferation of macrophage and osteoclast cells contains a large proportion of genes that have not been characterised in these cells.
Figure 3.2. DD-PCRs were amplified from cells grown under the following conditions: M-CSF (M) treated cells and M-CSF & RANKL (MR) treated cells at time points of 3 days, 7 days and 21 days. 0hr untreated PBMC control (0hr), water control (WC) and a DNA ladder (L) was included.
Table 3.1 Genes identified from time course DD-PCR

<table>
<thead>
<tr>
<th>Identity</th>
<th>GenBank Accession No.</th>
<th>Locus</th>
<th>Regulation</th>
</tr>
</thead>
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<td></td>
<td></td>
</tr>
<tr>
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<td>AK057375</td>
<td>9 p11.2</td>
<td>↑ 7d, 21d MR</td>
</tr>
<tr>
<td>Special AT-rich binding protein 1</td>
<td>AC144521.9</td>
<td>3 p23.3</td>
<td>↑ 0d, 3d MR</td>
</tr>
<tr>
<td>CTC-280C13 / CTD-2311A18 Similar to Chromokinesin</td>
<td>XM_209695.2</td>
<td>5 q33.2</td>
<td>↑ 3d MR</td>
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<tr>
<td>BAC RP11-335M11 EAP30 subunit of ELL complex</td>
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<td><strong>Signalling kinases</strong></td>
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<td></td>
</tr>
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<td>FER (tyrosine kinase)</td>
<td>J03358</td>
<td>5 q21</td>
<td>↑ 7d, 21d M</td>
</tr>
<tr>
<td>LIM Kinase 1</td>
<td>U63721.1</td>
<td>7 q11.23</td>
<td>↑ 7d MR</td>
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<tr>
<td><strong>Cytoskeletal proteins</strong></td>
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<tr>
<td>RP11-234K24 Contains gene EPB41L1</td>
<td>AL121895.26</td>
<td>20 q11.2</td>
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<td>17 q24.3</td>
<td>↑ 0d, all MR</td>
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<td><strong>Immune-related proteins</strong></td>
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<td>3 p14.1</td>
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<td>8 p23.1</td>
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<td><strong>Ribosomal proteins</strong></td>
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<tr>
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<td>AC006369.3</td>
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Table 3.1 DD-PCR products were sequenced and local alignments were performed to DNA sequence databases (Genbank, UCSC, ENSEMBL). Gene identities were assigned with a minimum threshold homology of 95%. DD-PCR products which were obtained from cultures exposed to M-CSF (M) or M-CSF & RANKL (MR) at 0 days (0d), 3 days (3d), 7 days (7d) and 21 days (21d) of differentiation, displayed up-regulation (↑) or down-regulation (↓) at distinct time points in PBMCs or in response to cytokine treatments. Regulation was assigned based on the presence or absence of a distinct PCR product.
3.1.3 Quantification of gene expression using Real-time PCR.

Five genes were selected for investigation with real-time Q-PCR, to further quantify and validate the regulation of genes. Genes selected for Q-PCR analysis included three previously uncharacterised genes, FAM19A1, EP2 and Solute carrier family 16 member 6 (SLC16A6), as well as more established genes, such as LIMK1 and Special AT-rich binding sequence 1 (SATB1). A minimum of three culture replicates were used with an average of 5 paired cell culture replicates per time point. PBMCs treated with M-CSF and PBMCs treated with M-CSF & RANKL were each compared with untreated PBMCs, at three time points: 3 days, 7 days and 21 days (Figure 3.3) to parallel DD-PCR observations. Q-PCR validated gene regulation observed in the DD-PCR experiment. Paired Student's t-tests were performed comparing M-CSF and M-CSF & RANKL treatments, at each time point. Some genes that were expressed in M-CSF & RANKL treated cultures in the DD-PCR, were not significantly regulated when analysed by Q-PCR. Also, some genes were regulated by M-CSF and RANKL treatment, but relative to PBMC expression, were repressed. These results highlight the semi-quantitive nature of DD-PCR. Increased time points and further cell culture repeats may have generated additional statistical power with which to detect significant differences in cytokine treatments over time.
Fold regulation compared to untreated PBMCs

**EP2**

- 3 day 7 day 21 day

-70 -60 -50 -40 -30 -20 -10 0 10

M-CSF M-CSF & RANKL

**FAM19A1**

- 3 day 7 day 21 day

-50 -40 -30 -20 -10 0 10 20

M-CSF M-CSF & RANKL

**LIMK1**

- 3 day 7 day 21 day

-3 -2 -1 0 1 2 3 4 5

M-CSF M-CSF & RANKL

*
Figure 3.3 Time course fold gene expression of selected DD-PCR products. Time course real-time Q-PCR was performed for genes selected from the DD-PCR study at 3 day, 7 day and 21 day time points, and compared to 0 day/untreated PBMCs. Gene expression was normalised to 18S rRNA expression. Fold change in gene expression in M-CSF treated PBMCs (grey) and M-CSF & RANKL treated PBMCs (black) was derived from mean ∆Cycle threshold (Ct) of each group compared to the mean ∆Ct of untreated PBMCs and fold was calculated using the $2^{-\Delta\Delta Ct}$ method. Error bars represent +/-SE of mean ∆Ct, * denotes P≤0.05.
EP2 is also known as human epididymal secretory protein, and sperm-associated antigen 11. The sequence identified using DD-PCR was similar to an alternatively spliced human EP2 gene. The translated protein sequence showed highest similarity to an EP2L protein variant found in *Macaca mulatta* or Rhesus monkey species. The closest human protein relative was the EP2C protein, which was 75% homologous to the EP2L variant. The EP2C peptide has the 6-cysteine distribution pattern characteristic for β-defensins (Frohlich *et al.*, 2000). β-defensins have been shown to play roles in inflammation, wound repair, cytokine expression, histamine production and enhancement of antibody responses (Lillard *et al.*, 1999). Compared with PBMC expression, EP2 is repressed in M-CSF and M-CSF & RANKL treated PBMCs until late stage differentiation (Figure 3.3). EP2 is not significantly regulated in either macrophages or osteoclasts compared with PBMCs.

A cysteine rich chemokine-like gene, FAM19A1, was found to be regulated by RANKL. This gene was also known as TAFA1, and was identified using clustering algorithms, with verified expression in the central nervous system (Tom Tang *et al.*, 2004). This gene is repressed at 3 days in both M-CSF and M-CSF & RANKL treated PBMCs (Figure 3.3) relative to untreated PBMCs. At 7 days and 21 days expression is up-regulated in osteoclasts relative to PBMCs by 3 fold (p=6.6x10^{-2}) and 11 fold (p=1.8x10^{-1}) respectively, however the regulation was not statistically significant.

LIMK1 is a signalling kinase up-regulated by RANKL during osteoclast differentiation (Figure 3.3). LIMK1 phosphorylates and inactivates cofilin’s actin depolymerising activity (Yang *et al.*, 1998), which enhances the formation of stable F-actin. LIMK1 is up-regulated 4 fold (p=3.1x10^{-1}) and 2 fold (p=6.4x10^{-1}) compared
with PBMCs at 7 days and 21 days respectively (Figure 3.3). When compared with M-CSF treated PBMCs, expression of LIMK1 in osteoclasts is significantly different at 7 days differentiation by 4 fold \((P=4.0\times10^{-3})\), and at 21 days is regulated 3 fold \((P=4.5\times10^{-2})\).

SATB1 organises chromatin architecture to regulate multiple genes over large distances (Yasui et al., 2002). SATB1 is up-regulated in 3 day osteoclasts 4.4 fold \((p=6.3\times10^{-1})\) compared with PBMCs, and 2 fold \((p=5.6\times10^{-1})\) compared with macrophages (Figure 3.3). Expression is repressed in both macrophages and osteoclasts at 7 and 21 days relative to PBMCs.

SLC16A6 family members regulate pH and normal cellular metabolism by exporting lactic acid generated from glycolysis and facilitating its import in cells undergoing gluconeogenesis and respiratory metabolism (Halestrap & Meredith, 2004). SLC16A6 is repressed in macrophages and osteoclasts compared with PBMC expression. However, comparing M-CSF & RANKL treated PBMCs with M-CSF only treatment, as is commonly the case with osteoclast studies, SLC16A6 is up-regulated 2 fold \((p=7.5\times10^{-2})\) and 3 fold \((p=2.8\times10^{-1})\) at 7 days and 21 days differentiation (Figure 3.3). An InterProScan of the SLC16A6 peptide sequence was performed (http://www.ebi.ac.uk/InterProScan/). Peptide analysis revealed that SLC16A6 contains protein motifs belonging to the major facilitator superfamily, which are involved in the transport of sugars, inositols, drugs, and metabolites (Pao et al., 1998).
3.1.4 Further characterisation of FAM19A1.

Chemokines regulate cellular trafficking, proliferation, cellular adherence and chemotaxis, and have been shown to also regulate osteoclast formation (Baggiolini, 2001; Roodman, 2001; Roodman & Choi, 2004). The regulation of FAM19A1 by RANKL in osteoclasts led to a hypothesis that FAM19A1 is involved in osteoclast formation and/or function. Before functional analysis was undertaken, further investigation into the coding sequence, and sequence homology with other CC-chemokine family members was carried out.

The full coding sequence of FAM19A1 was sequenced following PCR amplification using primers designed to flank the coding region of all homologous expressed sequence tags. Sequencing confirmed that the coding sequence of the osteoclast expressed transcript of FAM19A1 is not altered from the GenBank mRNA sequence (accession BC025746) (Appendix 1).

Peptide alignments were performed in order to compare FAM19A1 with other CC-chemokine members, using ClustalW software (http://www.ebi.ac.uk/clustalw/). Despite having the four cysteine residues characteristic of CC-chemokines, FAM19A1 is distinct from any other CC-chemokine when aligned with other CC family members, as unlike other CC-chemokines where cysteine residues spatially align, the cysteine residue positions in the FAM19A1 amino acid sequence are spatially unique (Figure 3.4), as FAM19A1 has a larger N-terminus. Alignment scores, representing matches of amino acids of the FAM19A1 peptide sequence with the rest of the CC-chemokine family, showed the FAM19A1 peptide was most similar
to CC chemokine ligand 3 (CCL3) or macrophage inflammatory protein 1 alpha (MIP-1α) (Table 3.3). A phylogenetic tree was calculated using the neighbour-joining method in ClustalW. The neighbor-joining method is statistically consistent under many models of evolution, and with sufficient sequence length, neighbor-joining will reconstruct the true tree with high probability (Gascuel & Steel, 2006; Saitou & Nei, 1987). The phylogenetic tree between FAM19A1 and CC-chemokines aligns FAM19A1 with CCL27 and CCL28 (Figure 3.5). When FAM19A1 is aligned with the six most similar chemokines (CCL3, CCL4, CCL7, CCL14, CCL18, CCL27) its four cysteine residues align, revealing a large extended domain near the end of the peptide (Figure 3.6).

Peptide motif analysis of FAM19A1 did not reveal any conserved domains within the extended regions. Further peptide sequence alignment analysis with the NCBI protein database, did not reveal any major homology or conservation with other protein families.
Table 3.2 ClustalW alignment scores for FAM19A1 compared with 24 other CC-chemokines.

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<th>Name of Sequence B</th>
<th>Amino Acid Length of Sequence B</th>
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<td>CCL1</td>
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Table 3.2 Pairwise alignments were performed between the FAM19A1 peptide sequence and all other human CC chemokine ligand (CCL) peptide sequences using the multiple sequence alignment program ClustalW. Scores are assigned to pairwise alignments, which incorporate weighting of near-duplicate regions to evaluate sequences that possess regions of similarity and regions that diverge. The six top scores have been highlighted, with the six highest matches (CCL3, CCL4, CCL7, CCL14, CCL18, and CCL27) in bold.
Figure 3.4 ClustalW alignment of the 24 members of the CC chemokine family with FAM19A1. Pairwise alignments were performed using the multiple sequence alignment program ClustalW, comparing all human CC chemokine peptide sequences with the FAM19A1 peptide sequence. The Clustalx colour scheme highlights structured regions containing similar amino acid residues.
**Figure 3.5** Phylogram comparing peptide sequences of all human CC chemokines with the FAM19A1 peptide sequence. The lengths of the phylogram branches and distance scores listed at each branch are proportional to the evolutionary change inferred by sequence similarity between the 24 CC chemokine family members and FAM19A1.
Figure 3.6 ClustalW alignment of FAM19A1 with the six most similar CC chemokines (CCL3, CCL4, CCL7, CCL14, CCL18, CCL27). Arrows denote cysteine residues definitive for CC chemokines.
To confirm if FAM19A1 is a secreted protein equivalent to other members of the CC-chemokine family, analysis of the peptide sequence was undertaken. SignalP software (http://www.cbs.dtu.dk/services/SignalP/) was used to analyse the peptide sequence of FAM19A1 for a signal peptide pattern. The FAM19A1 peptide sequence contains a predicted cleavage site at position 19-20 (Figure 3.7), which is indicative of secreted proteins. The predicted cleavage site is similar to the cleavage sites of most other CC-chemokines.

**Figure 3.7** SignalP prediction plot for the FAM19A1 peptide. The SignalP plot using ‘neural networks’ indicates that FAM19A1 has a signal sequence that is most likely cleaved between position 19 and 20.
3.2 Discussion

RANKL provides crucial stimulus for osteoclast differentiation. Several genes were regulated by RANKL in osteoclasts during distinct time points of cellular differentiation. All genes tested with Q-PCR verified expression in cDNAs used for DD-PCR amplification. However, most genes examined showed small fold inductions by RANKL.

The signalling kinase, LIMK1, was significantly regulated by RANKL. LIMK1 has reported expression in murine osteoclasts (Cappellen et al., 2002), and its regulation by RANKL in human osteoclasts, identified by DD-PCR, and confirmed by Q-PCR, validates this study. LIMK1 was induced by RANKL significantly at the fusion stage of osteoclast differentiation (P=4.0x10^{-3}) and remains regulated in mature osteoclasts (P=4.5x10^{-2}). LIMK1 phosphorylates and inactivates the actin binding/depolymerising factor cofilin (Yang et al., 1998), for F-actin regulation important for cytoskeletal rearrangements. The cycling of cofilin phosphorylation by LIMK1 is hypothesised to be important for osteoclast fusion, cell spreading, podosome and actin ring formation, which depend on proper cytoskeletal organisation. LIMK1 has also been associated with breast cancer tumor invasion and osteolytic lesions (Yoshioka et al., 2003).

At all time points of osteoclast differentiation, the gene SLC16A6 is expressed. Relative to PBMC precursors, SLC16A6 is repressed in M-CSF treated and M-CSF & RANKL treated cells. However, relative to M-CSF only treated cells, SLC16A6 appears to be regulated by RANKL at all time points of osteoclast differentiation. SLC16 member 6 has not been characterised and to date is attributed no known
function. An InterProScan of the SLC16A6 peptide sequence revealed that it contains protein motifs belonging to the major facilitator superfamily. The major facilitator superfamily is one of the largest and most diverse families of membrane transport proteins, involved in the transport of sugars, inositols, drugs, and metabolites (Pao et al., 1998). SLC16 family members regulate pH and normal cell metabolism by exporting lactic acid generated from glycolysis and facilitating its import by cells undergoing gluconeogenesis and respiratory metabolism (Halestrap & Meredith, 2004). The requirement of acid exchange by H+-ATPase to the ruffled border for osteoclast bone resorption, suggests a putative role for SLC16A6 in pH regulation and metabolism.

During the 7 day stage of osteoclast differentiation where fusion events have taken place, the chemokine-like gene FAM19A1 is regulated by RANKL. Expression of FAM19A1 is up-regulated further in mature osteoclasts at 21 days. Several chemokines induced by RANKL, such as MCP-1, RANTES and MIP-1\(\alpha\) (Kim et al., 2006b), are important for multinucleation steps of osteoclastogenesis. Further sequence analysis of FAM19A1 revealed that it has features characteristic of CC-chemokines, such as the four defining cysteine residues and a peptide signal sequence indicative of secreted proteins, but is not a closely conserved member of the CC-chemokine family. Of all the CC chemokine members, FAM19A1 most closely aligns with MIP-1\(\alpha\). The ability of osteoclasts and osteoblasts to migrate to sites of bone remodelling presumably involves chemotactic and haptotactic factors, such as chemokines and integrins, of which FAM19A1 may play a role.
One gene was found to be induced during early differentiation at 3 days, SATB1, which was up-regulated following M-CSF & RANKL treated PBMCs relative to untreated PBMCs. SATB1 acts by recruiting chromatin-remodelling factors and enabling specific histone modifications to regulate genes over long distances. SATB1 is a global gene regulator (Yasui et al., 2002) that has been shown to be involved in T-cell development (Alvarez et al., 2000; Yasui et al., 2002) and cytokine regulation (Cai et al., 2006). The attachment of the SATB1 network upstream of the Myc promoter has been shown to be crucial for proper induction of Myc transcription (Cai et al., 2003). Expression of c-Myc is required for osteoclast differentiation (Battaglino et al., 2002). SATB1 and FBP bind this region 1.5kb upstream of Myc, and it has been proposed that SATB1 may also function to prepare chromatin domains for correct regulation by transcription factors such as FBP (Cai et al., 2003), which itself is highly up-regulated in osteoclastogenesis (Day et al., 2004). The role of SATB1 expression early in osteoclast differentiation and in monocyte precursors may be to regulate the binding regions of transcription factors for proper differentiation.

One gene was confirmed to have expression in M-CSF and RANKL treated PBMCs, however, this expression was not regulated relative to other culture conditions. EP2 was a gene found regulated by less than 2 fold between M-CSF treated PBMCs and M-CSF & RANKL treated PBMCs. Relative to PBMCs, EP2 was repressed in both cytokine treatments. EP2 has been shown to have antimicrobial activity and is thought to be part of the innate epithelial defense system of the epididymal duct (von Horsten et al., 2002). The expression of EP2 in PBMC populations suggests that EP2 may be a beta-defensin-like gene, which plays a role in immune defense by peripheral blood
monocytes. DD-PCR, is a semi-quantitative technique, and therefore, false positive results such as EP2 are yielded.

The inclusion of PBMC precursor gene expression analysis is rarely included in osteoclast differential expression profiling. This study has outlined that a comparison between cytokine stimulated PBMCs and unstimulated PBMCs reveals relative repression, where otherwise gene regulation would appear between cytokine treatments. The majority of genes analysed by Q-PCR were regulated by RANKL, at low levels, with significant up-regulation of LIMK1. The data presented is the result of successive culture repeats of at least 3 culture replicates per time point and 10 separate experiments per gene, and has revealed genes that display moderate fold induction in response to cytokine treatment. A secondary method for determining the requirement of candidate genes in differentiating osteoclasts was designed to resolve, in particular, the involvement of FAM19A1 and LIMK1 in osteoclast formation and function.
Chapter 4

Optimised gene silencing in primary human osteoclasts: siRNA inhibition of cathepsin K
4.0 Introduction

Determining the relevance of novel and uncharacterised genes in primary human osteoclasts requires a convincing system of analysis. Many novel genes and newly identified genes lack detailed information and convenient inhibitors with which to analyse gene function in osteoclasts. Small interfering RNA (siRNA) is a post-transcriptional silencing phenomenon that involves the introduction of short specific double-stranded RNA fragments to silence expression of complementary mRNA species (Hannon, 2002). siRNA silencing of genes provides a useful indicator of the relevance of candidate genes in loss-of-function assays. In vivo, siRNAs are produced when ATP-dependent Dicer-1 enzyme processively cleaves dsRNA (Bernstein et al., 2001; Hutvagner & Zamore, 2002). Further cleavage results in 19-21bp duplexes containing 2-nucleotide 3’ overhangs. These duplexes bind and allow activation of the RISC resulting in targeted degradation of homologous mRNAs (Hannon, 2002). By making use of this technique in vitro, gene silencing can be induced, even for genes with minimal sequence information. siRNA is highly specific and its effects are more prolonged and less prone to degradation than antisense oligonucleotides (Bertrand et al., 2002).

Primary human osteoclasts are non-dividing, adherent, terminally differentiated cells that are reputedly difficult to transfect. The intrinsic phagocytic tendencies of the PBMC cell lineage is thought to be an obstacle for effective siRNA transfection. Careful optimisation of siRNA transfection into osteoclasts was performed, successfully targeting the principal protease expressed abundantly in osteoclasts, in order to gauge the efficacy of the method for this cell model, for the purpose of investigating further candidate genes.
A number of different methods are available for siRNA synthesis and siRNA transfection. siRNA can be designed as a specific single fragment (chemically synthesised or in vitro synthesised), or a pool of siRNA fragments can be generated by ‘Dicer’ enzyme digestion. Specificity and siRNA delivery were taken into consideration. Single siRNA fragment design requires testing of up to three fragments on average to establish specificity and potency of knockdown, whereas the introduction of a pooled population of siRNA fragments has been generally found to be specific and potent (Kawasaki et al., 2003; Kim et al., 2005a; Myers et al., 2003), with increased potency as a result of using Dicer substrates.

Previous experiments revealed plasmid transfection to be very difficult in primary osteoclast cells. However, the transient lipid cation transfection method has been used successfully in primary human osteoclasts previously for transfecting dominant negative proteins (data not shown), and so was trialed for use with siRNA inhibition.

Before testing putative candidate genes identified by DD-PCR, siRNA inhibition was tested for efficacy and specificity by first targeting one of the most abundant and critical genes for osteoclast biology, to benchmark the RNA silencing technique for this in vitro model.

CTSK is the key proteinase produced by osteoclasts (Drake et al., 1996). It is a widely accepted marker of osteoclast formation and a parameter of bone resorption (Gowen et al., 1999; Saftig et al., 1998). Bone resorption by osteoclasts occurs with demineralisation followed by degradation, a process dependent on cysteine proteases
(Gowen et al., 1999; Hill et al., 1994), where CTSK knockout results in pycnodysostosis, an osteopetrotic state (Ho et al., 1999). CTSK is abundantly and selectively expressed in osteoclasts (Drake et al., 1996), and is released from lysosomes at the ruffled border into the resorption lacunae where it degrades type I collagen, specifically type I and type II collagen (Garnero et al., 1998) and type II collagen (Kafienah et al., 1998). The successful inhibition of CTSK provides a proof of principle model that siRNA knockdown can be used effectively in primary osteoclast cells, to silence even the most abundant of transcripts.

The effectiveness of diced siRNA in primary human osteoclasts was ascertained using a number of transfection reagents and 'Diced' siRNA concentrations. Off-target effects such as Cathepsin B (CTSB) expression and the expression of an interferon response gene, 2’ ,5’-Oligoadenylate synthetase 1 (OAS1), were monitored. Changes in CTSK gene expression as a result of siRNA inhibition and the ability of mature osteoclasts treated with siRNA to resorb bone were examined. In this way, silencing of genes during differentiation, and silencing of genes post-differentiation during osteoclast bone resorption, provided criterion for determining genes involved in formation and function.
4.1 Results

4.1.1 Optimisation of siRNA conditions in differentiating primary human osteoclasts.

Having successfully transfected primary human osteoclasts by cationic lipid transfection previously, a selection of four cationic lipid reagents were tested for their ability to transfect 25nM CTSK siRNA (Figure 4.1). Antibiotic and serum free media was used for all siRNA transfections. Lipofectamine 2000 resulted in the greatest inhibition (98%), followed by FuGENE6 (87%), Oligofectamine (79%) and JetPEI (67%) (Figure 4.1). Typical siRNA knockdown experiments aim for 70% knockdown or greater, and these results show that the use of common lipid cation transfectants approach or exceed this rate using Dicer-siRNA. The transfection of CTSK siRNA using JetPEI, also resulted in the death of some osteoclasts. It is reported that the toxicity of various polycations is induced as a result of the individual structure and charge (Hill et al., 1999) of the transfectant. The data for comparison of transfection reagents was derived from one experiment.
Comparison of siRNA inhibition using lipid cation transfection reagents

- Control
- JetPEI
- Oligofectamine
- FuGENE 6
- Lipofectamine 2000

Relative CTSK expression (%)

Figure 4.1 Comparative knockdown of CTSK expression using four lipid cation transfection reagents.

Non-specific silencing of CTSK using lipid cation transfectic reagents

- Control
- FuGENE 6
- Lipofectamine 2000

Relative CTSK expression (%)

* Error bars represent +/- standard error of the mean.

Figure 4.2 Non-specific knockdown of CTSK expression was analysed by Q-PCR following transfection with 25nM GFP siRNA. The use of Lipofectamine 2000 to transfect non-specific control siRNA into primary human osteoclasts resulted in significantly silenced CTSK expression (* P=2.0x10^{-4}).
Peak silencing transfectants, Lipofectamine 2000 and FuGENE6, were used to test for non-specific knockdown of CTSK in osteoclasts by transfecting with 25nM of non-specific siRNA designed for Green fluorescent protein (GFP) (Figure 4.2). Lipofectamine 2000 was associated with a significant down-regulation of CTSK (P=2.0x10^{-4}) despite the use of non-specific siRNA. Lipid-induced inhibition of CTSK expression has been reported in osteoclasts via the influence of conjugated linoleic acid (CLA) (Rahman et al., 2006). CLA is a lipid found primarily in the meat and dairy products of ruminants, and has been found to suppress RANKL signalling in the murine cell line RAW264.7, resulting in down regulation of CTSK, TRAP, CT-R, MMP-9, c-Fos and NFAT (Rahman et al., 2006). Non-specific down regulation of CTSK expression following transfection with Lipofectamine 2000, may be a result of the same inhibitory mechanisms induced by CLA. FuGENE 6 is a non-liposome transfectant, and did not significantly reduce expression of CTSK (P=1.3x10^{-1}), therefore it was used for all subsequent siRNA experiments in this study.
Figure 4.3 Primary human osteoclasts were transfected with various concentrations of CTSK siRNA using FuGENE 6. CTSK expression diminished with increasing concentrations of siRNA, relative to untreated control cells analysed by Q-PCR.

A range of concentrations, between 2.5nM and 25nM of CTSK siRNA, were tested for potency of inhibition in primary human osteoclasts following transfection with FuGENE 6. CTSK expression was monitored using real-time PCR. Maximal down-regulation of 91% was achieved with 25nM CTSK siRNA (Figure 4.3). Down-regulation of 70% and 68% compared with control osteoclast culture occurred using 20nM and 12.5nM of CTSK siRNA respectively (Figure 4.3). The transfection of 2.5nM CTSK siRNA did not inhibit CTSK expression substantially.
Figure 4.4 Interferon response to CTSK siRNA transfection. OAS1 expression in osteoclasts transfected with 12.5nM, 20nM and 25nM CTSK siRNA, was compared with control transfection with no CTSK siRNA by Q-PCR analysis. Concentrations of 20nM and 25nM significantly increased OAS1 expression relative to control osteoclasts (* P=2.0x10^{-2}, and P=1.0x10^{-2} respectively). Error bars represent +/- standard error of the mean.

In mammalian cells, cytoplasmic dsRNAs longer than 30bp can induce the expression of dsRNA dependent protein kinase PKR and OAS1, resulting in translation inhibition and non-specific degradation of mRNA (Pebernard & Iggo, 2004). These genes are expressed as part of the interferon response to dsRNA. The expression of OAS1 was monitored post-siRNA transfection, as an indicator of non-specific interferon response to siRNA (Figure 4.4). OAS1 expression was significantly increased after transfection with 20nM and 25nM CTSK siRNA (P=2.0x10^{-2}, P=1.0x10^{-2}).
Transfection of 12.5nM CTSK siRNA did not significantly change OAS1 expression (P=3.9x10^{-1}).

**Figure 4.5** Specificity of CTSK siRNA. Transfection of control non-specific GFP siRNA and CTSK siRNA did not significantly alter the expression of CTSB as measured by Q-PCR. Error bars represent +/- standard error of the mean.

CTS NB is a lysosomal cysteine protease expressed in osteoclasts, related to CTSK (Inaoka et al., 1995). To check for non-specific repression of cathepsins, CTSB expression was analysed after CTSK siRNA treatment (Figure 4.5). No significant change in CTSB expression was found in cells treated with CTSK siRNA compared with GFP siRNA treated control cells (P=5.0x10^{-1}) and compared with untreated cells (P=1.8x10^{-1}).
Transfection of osteoclasts with 12.5nM CTSK siRNA significantly reduced CTSK expression relative to control siRNA treatment (*P=2.0x10^{-2}) analysed by Q-PCR. No significant difference in CTSK expression occurred in osteoclasts transfected with GFP siRNA compared with osteoclasts transfected with no siRNA (P=9.5x10^{-1}). Error bars represent +/- standard error of the mean.

To achieve specific, potent knockdown without inducing up-regulation of the interferon response gene OAS1, 12.5nM CTSK siRNA was used. Significant inhibition of CTSK expression was achieved with CTSK siRNA compared with untreated osteoclasts (P=2.0x10^{-2}) and compared with GFP siRNA treated osteoclasts (P=1.7x10^{-2}), with 60% knockdown (Figure 4.6). GFP siRNA had no significant effect on CTSK expression (P=9.5x10^{-1}) relative to osteoclasts transfected with no siRNA. Osteoclast formation was not impaired by CTSK silencing, with multinucleated cells also staining positive for TRAP expression.
4.1.2 siRNA inhibition in pre-differentiated osteoclasts: disruption of bone resorption.

GFP siRNA and CTSK siRNA were used to treat pre-differentiated mature osteoclasts, which were recovered from collagen I coated plates and seeded onto dentine chips. Non-siRNA treated and siRNA treated osteoclasts were left for 96hrs on dentine chips before observing cell morphology and performing scanning electron microscopy (Figure 4.7). All cultures contained abundant multinuclear TRAP positive osteoclasts. Cultures were performed in triplicate in three separate experiments. Non-treated osteoclast cells and GFP siRNA treated osteoclast cells produced abundant pits in dentine and average pit number was not significantly different (P=9.3x10^{-1}, P=9.1x10^{-1}) (Figure 4.8). CTSK siRNA treated osteoclasts resorbed less bone surface, with a 60% reduction in area resorbed (P=1.3x10^{-2}) (Figure 4.8). CTSK siRNA treatment also resulted in a 50% reduction in average pit number (P=1.8x10^{-2}) (Figure 4.8). Despite a reduction in cell numbers, the remaining cells and resorption pits did not appear atypical compared with control cultures (Figure 4.7). Total cell count was not significantly different between CTSK siRNA treatment and controls (P=8.5x10^{-1}).
**Figure 4.7** Cellular morphology of non-siRNA treated and siRNA treated cells (white arrows) stained for TRAP, visualised on dentine with bright field microscopy (A, C, E), and SEM micrographs of resulting bone resorption pits (yellow arrows) (B, D, F) corresponding to siRNA treatment. Small pits (yellow arrows) can also be seen on dentine visualised by light microscopy (A, C). Control osteoclasts transfected with no siRNA displayed TRAP positive multinucleated cells (A), which resorbed dentine pits (B). Similarly, GFP siRNA treated osteoclasts were TRAP positive (C), multinuclear, bone resorbing cells (D). CTSK siRNA treated osteoclasts were indistinguishable from other TRAP positive (E), multinucleated cells, however they did not produce pits as abundantly (F). The bar represents 100μM.
Figure 4.8 Effects of CTSK siRNA on bone resorption. CTSK siRNA knockdown of CTSK expression in osteoclasts results in significant reduction in area of resorbed bone ($P=1\times 10^{-2}$), and number of pits resorbed ($P=2.0\times 10^{-2}$). No significant difference in pit number or area resorbed was detected between osteoclasts transfected with no siRNA and osteoclasts transfected with GFP siRNA ($P=9.3\times 10^{-1}$, $P=9.1\times 10^{-1}$). Error bars represent +/- standard error of the mean.
4.2 Discussion

siRNA is used for its ability to specifically and potently inhibit the expression of almost any gene. The transfection of primary cells with siRNA has proved problematic, especially the transfection of large adherent terminally differentiated osteoclast cells. Through careful screening and optimisation of a variety of transfection reagents and concentrations, an effective combination of siRNA conditions for the inhibition of osteoclast genes were identified for primary human cells for the first time. These conditions avoided apoptosis and resulted in osteoclasts that expressed osteoclast-specific gene markers and resorbed bone.

With an increase in new gene targets obtained from microarrays, DD-PCR and gene mining tools, there has never been a greater need for methods, which can determine the functions of unknown targets on-mass. siRNA is routinely used for gene target inhibition in cell lines, however, until now it has not been successful for use in primary osteoclast cells. The 'Dicer' method of siRNA inhibition, is a valuable format for specifically inhibiting genes of interest, and addresses the need for a mass screening tool for the primary osteoclast cell model.

The lipid cationic transfection reagent, Lipofectamine 2000, was found to significantly reduce CTSK expression in the absence of specific siRNA. It is surmised that Lipofectamine suppresses RANKL signalling, resulting in downstream inhibition of CTSK, as found with other lipids such as CLA (Rahman et al., 2006). This suggests that the use of lipid transfection reagents in osteoclast cultures, should proceed accompanied by the scrutiny of off-target gene expression effects, such as those downstream of RANKL signalling.
Off-target effects following siRNA transfection were triggered by increasing siRNA concentration, with siRNA levels above 12.5nM significantly inducing the expression of the interferon response gene OAS1. At 12.5nM, CTSB expression is not altered by the presence of CTSK-specific siRNA. Despite testing the expression of OAS1, one of the principal genes induced as part of the IFN response pathway, it could not be ruled out that further off-target effects were induced as a consequence of siRNA transfection, without further experimental investigation.

The osteoclast serves to resorb bone, using proteases such as CTSK to degrade collagen and the organic matrix. The knockdown of CTSK by 60%, using siRNA, did not completely halt resorption or impair the expression of other osteoclast gene markers, however, the number of pits and surface area of bone resorbed was significantly diminished. This result not only confirms the role that CTSK plays in osteoclast bone resorption, but also provides a model system for further siRNA studies into genes specific for bone resorption. The use of pre-differentiated osteoclasts plated onto dentine permits the isolation of osteoclast bone resorption loss-of-function studies. Evidence provided from this study, warrants the use of siRNA to further investigate the role of candidate genes, such as LIMK1 and FAM19A1, in osteoclast formation and function assays, with the ability to monitor impaired osteoclast differentiation or bone resorption independently.
Chapter 5

Multiple screening of genes for osteoclast formation and function using siRNA
5.0 Introduction

Normal bone remodelling is dependent on both osteoclast formation and resorption. Having identified a collection of candidate genes regulated by RANKL in osteoclasts, it was speculated as to whether any of these genes would be essential for the osteoclast, and if so whether they would be involved in osteoclast formation or resorption. To test this possibility, siRNA inhibition was used to individually silence genes of interest from candidate genes identified by DD-PCR, as well as genes identified by microarray studies performed previously (data not shown). siRNA inhibition of gene targets was employed in such a way as to outline whether genes were involved in aspects of osteoclast differentiation or function. Most importantly, targeted knockdown of genes using siRNA was used to clarify both the role of established genes and novel genes. With the only requirement being to possess a minimum of 23 nucleotides of transcript information, siRNA technology provides a useful means of determining gene function in the absence of detailed functional protein domains or commercial inhibitors.

siRNA was synthesised to inhibit gene expression of 7 genes, some with established and putative functions in osteoclasts, along with a GFP siRNA control. Cell counts, cell structure, f-actin staining, and scanning electron microscopy of osteoclast resorption were employed to determine cellular and functional attributes following candidate gene inhibition.
5.1 Results

5.1.1 siRNA inhibition of RANKL-regulated genes in differentiating osteoclasts: genes required for proper osteoclast formation.

siRNA was synthesised for FAM19A1, FBP, GA-binding protein transcription factor, alpha subunit (GABPA), GA-binding protein transcription factor, beta subunit (GABPB), LIMK1, NFATc1, SATB1 and SLC16A6, with GFP siRNA used as a non-specific control. Q-PCR was performed to assess gene expression, measuring mRNA abundance relative to the internal control 18S rRNA. Mean cycle thresholds were compared using one-tailed ANOVA and Student's t-tests, as only a one-direction hypothesis of siRNA knockdown was tested. Knockdown was achieved using 12.5nM siRNA, resulting in significant reductions (α=0.05) in gene expression for all non-control genes tested. Knockdown presents the residual expression in siRNA treated cultures as a percentage of original total expression analysed in GFP siRNA control cultures. Significant knockdowns were achieved for siRNA experiments targeting FAM19A1 (96%, P=1.0x10^-6), FBP (94%, P=1.0x10^-6), GABPA (85%, P=9.0x10^-4), GABPB (90%, P=1.6x10^-4), LIMK1 (90% P=1.7x10^-4), NFATc1 (88% P=3.6x10^-3), SATB1 (96%, P=3.0x10^-6) and SLC16A6 (99%, P=8.9x10^-10) (Figure 5.1).
**Figure 5.1.** siRNA knockdown of multiple genes in differentiating osteoclasts was monitored by Q-PCR. Expression of each gene was examined individually in gene-specific siRNA treated osteoclasts compared with GFP siRNA treated osteoclasts, using 12.5nM siRNA. Asterisk denotes significance (P ≤ 0.05). Error bars represent +/- standard error of the mean.
For monitoring osteoclast formation, gene expression was inhibited throughout a 14 day differentiation period. Osteoclast formation assays were performed by immunohistochemistry, with osteoclasts stained for TRAP using leukocyte acid phosphatase stain. Cultures treated with non-specific siRNA for GFP, resulted in TRAP positive multinucleated osteoclast cell counts, which did not differ from cultures that received no siRNA treatment (Figures 5.2, 5.3). Targeted siRNA inhibition of 6 of the 8 genes tested resulted in significantly reduced osteoclast cell number. Statistics were performed, using Student's t-test. Total osteoclast cell count was reduced by 64% following treatment with FAM19A1 siRNA (\(P=2.2\times10^{-2}\)), 37% with GABPA siRNA (\(P=4.4\times10^{-3}\)), 38% with GABPB (\(P=1.5\times10^{-2}\)), 63% with LIMK1 siRNA (\(P=1.1\times10^{-3}\)), and 44% with NFATc1 siRNA (\(P=3.6\times10^{-4}\)) and 70% with SLC16A6 siRNA (\(P=1.7\times10^{-3}\)) (Figure 5.2). Diminished levels of each of these genes have revealed their contribution to proper osteoclast formation. The most potent reduction in osteoclast formation (70% less formation) was achieved with SLC16A6 siRNA. Cultures treated with siRNA targeting FBP had 23% less TRAP positive multinucleated osteoclasts (\(P=5.7\times10^{-2}\)) and siRNA targeting SATB1 reduced TRAP positive multinucleated osteoclast cell number by 14% (\(P=2.4\times10^{-1}\)), resulting in a difference which was below statistical significance (Figure 5.2).
Figure 5.2 siRNA treatment effects on formation of multinucleated TRAP positive osteoclasts. Cells were stained for TRAP and visualised by light microscopy. In the presence of GFP siRNA, abundant multinucleated TRAP positive osteoclasts formed (A). No significant difference in multinucleated TRAP positive cell count was observed between cells transfected with GFP siRNA (A), no siRNA (B), FBP siRNA (D) and SATB1 siRNA (I). In the presence of siRNA targeting FAM19A1 (C), GABPA (E), GABPB (F), LIMK1 (G), NFATc1 (H) and SLC16A6 (J), a statistically significant reduction in the formation of multinucleated TRAP positive osteoclasts was observed ($P < 0.05$). Bar represents 100 μm. Arrows distinguish multinucleated osteoclasts.
Figure 5.3 Effects of gene-specific siRNA treatment on the number of multinucleated TRAP positive osteoclasts formed. Significant reductions in multinucleated TRAP positive cell counts were observed following treatment of osteoclasts with siRNA targeting FAM19A1 (A), GABPA (C), GABPB (D), LIMK1 (E), NFATc1 (F), SLC16A6 (H) relative to GFP siRNA treatment (*P<0.05). Reductions in multinucleated TRAP positive osteoclast cell formation were also observed as a result of treatment with FBP siRNA (B), and SATB1 siRNA (G), however these reductions were not statistically significant. Error bars represent +/- standard error of the mean.
5.1.2 siRNA inhibition of RANKL-regulated genes in pre-differentiated osteoclasts: genes required for osteoclast bone resorption.

The resorptive ability of 14 day differentiated osteoclasts, which had properly formed without any prior siRNA inhibition, were tested following siRNA inhibition of selected genes. This would clarify whether these genes play a role in late stage osteoclast function. A significant reduction in bone resorption was observed, with a number of genes tested. Using scanning electron micrographs (SEM) at 80X magnification, resorption was analysed. Resorption pits were counted individually. Single pits and long continuous trenches were counted as one pit. Resorption area was calculated by dividing the dentine SEM into a 552 square grid and counting the percentage of the grid resorbed.

The inhibition of some genes in mature osteoclasts clearly resulted in diminished bone resorption, indicating that these genes play a role in osteoclast resorptive activity. Osteoclasts treated with FAM19A1 siRNA resorbed 44% less pits than GFP siRNA control (P=3.3x10^{-2}), and 70% less area was resorbed (P=2.0x10^{-2}) compared with control. LIMK1 siRNA reduced resorption by 50% (P=4.2x10^{-2}) less pits relative to GFP siRNA control, and area resorbed was reduced by 60% (P=4.3x10^{-3}) compared with control. SLC16A6 siRNA reduced pit number by 80% (P=2.5x10^{-2}) relative to GFP siRNA control, and area resorbed was reduced by 72% of control (P=1.5x10^{-3}). Both the number of pits produced, and the total surface of dentine excavated was significantly reduced following highly potent siRNA silencing of FAM19A1, LIMK1 and SLC16A6, indicating that properly differentiated mature osteoclasts cannot effectively resorb bone without the sustained expression of these genes.
The inhibition of some genes appeared to mainly affect the average size of the pits produced in dentine (data not shown). FBP siRNA treatment did not significantly change pit number resorbed, with a 4% reduction in pit number (P=4.8x10^{-1}). However, diminished FBP expression in mature osteoclasts significantly reduced the total resorbed area by 39% (P=2.6x10^{-2}). This suggests that despite producing a similar number of pits in dentine that the average pit size was smaller, resulting in reduced total resorbed area compared with the control. Similarly, GABPA siRNA did not result in a large reduction in pit number, 11% reduction (P=5.0x10^{-2}), however a 53% reduction in area resorbed was observed (P=2.8x10^{-2}), which was statistically significant. Silencing of the GABPB subunit similarly affected pit size, resulting in abundant small dot-like pits. However, a significant increase in pit number was observed (P=1.9x10^{-2}), whilst resorbed area was not significantly different from control (P=7.4x10^{-1}). Silencing of the chromatin remodelling gene SATB1 also affected the size of pits produced, with a significant increase in pit number (1.8x10^{-2}), with no significant change in the total area resorbed (P=7.6x10^{-1}).

The disparity between the effects on osteoclast resorptive activity following siRNA inhibition of the two GABP subunits may be attributable to the abundant number of small pits. In the case of GABPA and GABPB, these smaller pits appeared to be produced by osteoclasts, which were less spread, and with fewer nuclei than those present in control cultures, and this is also apparent in the osteoclast cell formation assay (Figure 5.2). FBP and SATB1 reductions did not affect the formation of multinucleated TRAP positive osteoclasts and so the aberrant rates of resorption are mainly as a result of changes in osteoclast activity produced in osteoclast cells, which do not appear defective. For these cellular outcomes where pit size was changed, total
resorbed area appears to be a more useful indicator of the contribution of these genes to osteoclast bone resorption.

NFATc1 silencing did not result in a significant reduction in pit number (86% increase in pit number, $P=2.2\times10^{-3}$) or area resorbed (16% increase in resorbed area, $P=1.2\times10^{-3}$) with diminished NFATc1 expression resulting in significantly increased levels of bone resorption. This result suggests that although NFATc1 expression is clearly necessary for proper osteoclast differentiation, mature osteoclasts do not require NFATc1 to effectively resorb bone.
Figure 5.4 Effects of targeted siRNA knockdown on osteoclast pit resorption and area resorbed were analysed using scanning electron microscopy. Arrows denote resorption pits. Mature osteoclasts treated with GFP siRNA resorbed pits in dentine (A), and did not differ significantly in the number of pits formed or area resorbed, compared to cultures treated with no siRNA. Both pit number and area resorbed was significantly decreased in mature osteoclast cultures treated with siRNA targeting FAM19A1 (B), LIMK1 (F) and SLC16A6 (I). Osteoclasts treated with GABPB siRNA resorbed significantly less pits than the GFP siRNA control. Percentage area resorbed relative to GFP siRNA control differed significantly for FBP (C) and GABPA (D) siRNA treatments. NFATc1 siRNA treatment (G) resulted in significantly greater pit number and area resorbed relative to GFP siRNA control. SATB1 siRNA treatment (H) also resulted in significantly greater pit number resorbed. Error bars represent +/- standard error of the mean.
5.1.3 F-actin formation in siRNA-treated pre-differentiated osteoclasts.

Osteoclast differentiation and bone resorption requires cytoskeletal reorganisation (Lakkakorpi & Vaananen, 1996). In particular LIMK1 has been implicated in influencing actin cytoskeleton rearrangement, downstream of Rho GTPases (Scott & Olson, 2007). A prerequisite for bone resorption is cytoskeletal reorganisation and actin ring formation, and therefore it was hypothesised that the reduction in bone resorption as a result of siRNA silencing was due to dysfunctional f-actin ring formation. Nuclei and F-actin were visualised in pre-differentiated osteoclasts (Figure 5.5) treated with siRNA for 72hrs targeting GFP (A, E, I), FAM19A1 (B, F, J), LIMK1 (C, G, K), and SLC16A6 (D, H, L). Cells treated with gene specific siRNA displayed typical osteoclast cell structure, nuclei and F-actin rings compared with the GFP control (Figure 5.5). This suggests that abnormal levels of bone resorption due to siRNA silencing of FAM19A1, LIMK1, and SLC16A6, cannot be attributed to abnormal actin ring formation. Despite having the appearance of typical f-actin rings, it could not be established as to whether these actin rings contributed to a fully functional sealing zone or not.
**Figure 5.5** Gene-specific siRNA effects on F-actin ring formation. Pre-differentiated osteoclasts were treated with siRNA for 72hrs before staining nuclei (A-D, blue) and F-actin (E-H, red). Images (I-L) are merged nuclei and F-actin. siRNA treatments targeting GFP (A, E, I), FAM19A1 (B, F, J), LIMK1 (C, G, K), and SLC16A6 (D, H, L) resulted in osteoclasts with actin rings (red), podosome belts (arrowheads) and typical osteoclast cellular formation.
5.2 Discussion

A complex network of genetic events contribute to cellular differentiation. The task of identifying new genes and their functional roles in osteoclast biology can be furthered by using gene screening techniques, such as these methods, which can distinguish genes for osteoclast formation or resorption. This is the first developed siRNA method for the primary osteoclast cell model. In addition, this method provides a means for distinguishing whether genes are implicated in osteoclast differentiation, osteoclast resorption, or both.

These experiments reveal that genes such as FAM19A1, SLC16A6 and LIMK1, are required for osteoclast formation, and osteoclasts demand sustained expression for proper bone resorption. These genes, however, are not essential for cell survival or basic cell maintenance, as PBMCs cultures in the absence of these genes, do not die, and display the morphology typical of precursor cell populations.

The function of a number of newly identified genes such as FAM19A1 and SLC16A6, have been clarified in human primary osteoclasts for the first time. It has been shown that chemokines can regulate the migration (Fuller et al., 1995) and differentiation of progenitor cells into pre-osteoclasts (Kim et al., 2005b), and mediate cross-talk between the immune system and bone (Choi et al., 2000). FAM19A1 is a new chemokine-like gene, which until now, has only verified expression in the nervous system (Tom Tang et al., 2004), and has not been functionally investigated previously. FAM19A1 was silenced using siRNA and despite its predominantly late stage expression in osteoclasts, a significant reduction in osteoclast cell formation during the first two weeks of differentiation was observed. siRNA knockdown in
mature osteoclasts also resulted in a potent reduction in osteoclast bone resorption. FAM19A1 is a new type of chemokine, which is necessary for proper osteoclast differentiation and resorption, and warrants further in vitro and clinical investigation.

Another previously uncharacterised gene, which this study has proved to be involved in osteoclast differentiation and resorption is SLC16A6. SLC16 family members regulate pH and normal cellular metabolism by exporting lactic acid generated from glycolysis and facilitating its import by cells undergoing gluconeogenesis and respiratory metabolism (Halestrap & Meredith, 2004). SLC16A6 was cloned from a circulating blood cDNA library and contains 10 to 12 transmembrane domains (Price et al., 1998), but until now, has not been functionally characterised. Upon silencing SLC16A6 with siRNA, a potent reduction in TRAP positive multinucleated osteoclast cells was observed. Similarly, siRNA inhibition of SLC16A6 in mature osteoclasts resulted in a reduction in resorbed pits and resorbed area in dentine. Cell stain analysis of F-actin rings revealed normal actin ring formation. The requirement of acid exchange by H+-ATPase to the ruffled border for osteoclast bone resorption, suggests a putative role for SLC16A6 in pH regulation and metabolism for proper osteoclast function.

Another gene that requires sustained expression for osteoclast differentiation and resorption is LIMK1. LIMK1 is a signalling kinase up-regulated significantly by RANKL during osteoclast differentiation. LIMK1/cofilin-mediated actin reorganisation has been found to be critical in the regulation of haematopoietic progenitor cell migration to stromal cells in vitro and for the homing of haematopoietic progenitor cells to bone marrow in vivo (Konakahara et al., 2004).
Upon knockdown of LIMK1, a significant reduction in both osteoclast cell number and osteoclast bone resorption was observed, indicating that osteoclast function is impaired in LIMK1 siRNA treated cells. In osteoclasts with diminished LIMK1, the area of bone resorbed is reduced, and pit number is reduced by half. Resorbing osteoclasts modify their cytoskeleton and it has been reported that LIMK1 is essential for cytoskeletal rearrangement (Konakahara et al., 2004). LIMK1 is phosphorylated by the Rho-associated kinase ROCK, to mediate actin cytoskeleton remodelling (Maekawa et al., 1999). Despite this association, no significant impairment in osteoclast actin rings was observed, however this may have been a result of adequate expression of LIMK2, which has also been shown to mediate actin cytoskeleton reorganisation and the regulation of actin assembly (Vardouli et al., 2005). siRNA inhibition of both LIMK1 and LIMK2, in osteoclasts would clarify their involvement in cytoskeletal organisation and actin ring formation.

A number of DNA binding proteins were inhibited, such as FBP, GABP, and SATB1, which resulted in osteoclasts which produced pits that were much smaller on average than pits produced by control osteoclast cultures. The absence of these genes appeared to affect the quality of bone resorption produced by osteoclasts. Less trench-like resorption pits were formed, and on average pits excavated were shallow in depth.

One of these transcription factors, FBP, was previously reported to be expressed in osteoclasts (Day et al., 2004), however the functional significance for this gene in osteoclast biology was not investigated. siRNA inhibition has shown that FBP is not required for the proper development of multinucleated TRAP positive osteoclasts. A study involving siRNA inhibition of FBP in primary human fibroblasts revealed a
profile of downstream genetic events (Chung et al., 2006), which included calcineurin. Calcineurin is a necessary downstream mediator of osteoclast differentiation (Sun et al., 2007), and was downregulated following FBP inhibition (Chung et al., 2006). This is consistent with a role for FBP in regulating osteoclast specific genes. However, it suggests that the absence of FBP, would fail to induce calcineurin. The inhibition of calcineurin results in significant reductions in osteoclast differentiation (Sun et al., 2007). Further experiments, such as chromatin immunoprecipitation of FBP, to confirm DNA binding targets such as c-Myc and calcineurin during osteoclast differentiation, would have potentially provided clarification of this paradox. None-the-less, the involvement of FBP in regulating proper osteoclast resorption can be inferred from these experiments.

Another transcription factor, GABP, was investigated in the osteoclast cell model. GABP is a transcription factor that consists of two subunits, alpha and beta. GABPA is the DNA binding subunit, whereas GABPB contains the transcription activation domain (Gugneja et al., 1995) and can homodimerise and bind GABPA, increasing its DNA binding affinity (LaMarco et al., 1991). The GABP transcription factor is involved in the regulation of mitochondrial enzymes (Batchelor et al., 1998; Gugneja et al., 1995), cellular differentiation and oncogenesis (Hsu et al., 2004). GABP has been shown to regulate cell-cycle control genes, with GABPA null mice showing defective cellular proliferation and reduced cell growth (Yang et al., 2007). In addition, both GABPA and GABPB subunits are enriched in haematopoietic stem cells (Ivanova et al., 2002). GABP cooperates with Sp1 and PU.1 to increase CD18 expression in myeloid cells (Rosmarin et al., 1995; Rosmarin et al., 1998), an integrin associated with monocyte spreading and adhesion (Keizer et al., 1987), and was found
to be RANKL-regulated (Day et al., 2004) in osteoclasts. Suppression of both subunits of GABP resulted in significant reductions in osteoclast differentiation, with concordant reductions in osteoclast cell numbers following inhibition of each individual subunit. Diminished GABPA significantly reduced the total area resorbed by osteoclasts. Diminished GABPB, did not reduce osteoclast resorption. GABPA is the DNA binding subunit of the Ets transcription factor and so the specific reduction of osteoclast resorption following inhibition of only the GABPA subunit reflects the functional significance of this subunit of the transcription factor. GABPB has been found to increase the binding affinity of GABPA. Inhibition of the GABPB subunit did not impair the total area resorbed as a consequence of gene inhibition. Average pit size appeared to be smaller following inhibition of each subunit of GABP. The smaller size of pits formed in dentine, resulted in increased pit formation following GABPB inhibition, whilst not significantly changing the total area resorbed. GABPA knockdown, despite also resulting in smaller pit size, amounted to a decrease in overall resorbed area. This suggests that GABP may regulate genes in osteoclasts which contribute to cell spreading and adhesion. The significant reduction of osteoclast formation and area resorbed by mature osteoclasts in osteoclasts deficient in GABPA, suggests that GABP is a transcription factor required for osteoclast differentiation and subsequent resorption.

The DNA binding protein, SATB1, was also investigated. SATB1 is a matrix binding protein that functions to organise chromatin architecture to regulate multiple genes over large distances (Yasui et al., 2002). The absence of SATB1 as a result of siRNA inhibition in osteoclast cultures, did not prevent osteoclast differentiation, despite displaying predominantly early expression at 3 days of osteoclast differentiation, with
numbers of TRAP positive multinucleated osteoclasts resembling that of control cultures. The absence of SATB1 did however, significantly increase the number of pits formed by mature pre-differentiated osteoclasts, and affected the quality of pits resorbed. SATB1 inhibition appeared to reduce the average pit size, in a similar manner to FBP, whilst the total area resorbed did not significantly differ from that produced by control cultures. This result suggests that SATB1 may affect normal osteoclast cell spreading, encouraging proper cell dimensions, and/or affect the depth and size of bone resorptive activity. Further investigation of SATB1 regulation of FBP, would have clarified the putative interaction between these genes in the osteoclast cell model.

In addition to the above DNA binding proteins, the established osteoclast transcription factor, NFATc1, was investigated for involvement in osteoclast differentiation and resorption. NFATc1 is the major NFAT variant expressed in human osteoclasts (Day et al., 2005), and is crucial for osteoclast differentiation (Takayanagi et al., 2002a). siRNA silencing of NFATc1 in differentiating osteoclasts resulted in a significant reduction in the formation of TRAP positive multinucleated osteoclasts. This result confirms previous reports that NFATc1 is crucial for proper osteoclast formation, and validates the Dicer siRNA method in primary human osteoclasts. Inhibition of NFATc1 in pre-differentiated osteoclasts, however, did not significantly reduce pit number or area resorbed, suggesting that NFATc1 does not play a role in osteoclast bone resorption. Interestingly, the area resorbed by NFATc1 siRNA treated osteoclasts, significantly increased. This result parallels the findings of the NFATc1 regulator, calcineurin by Sun et al., (2003), where calcineurin was found to enhance osteoclast differentiation, but inhibit osteoclast resorption in mature cells.
siRNA is a useful tool with which to characterise the role of genes in primary osteoclast cell systems. These results reveal genes that are necessary for normal osteoclast formation and are required for optimum levels of bone resorption, and can also affect the quality of bone resorbed. siRNA inhibition provides a method for high throughput screening of novel genes for functional significance in the osteoclast cell model. Primary human osteoclast cultures serve as an important model system for the analysis of osteoclast differentiation, osteoclast activity, gene function and drug discovery, as primary osteoclast cultures more closely resemble in vivo systems compared with immortalised cell lines. Typical transfection efficiencies are low in primary human osteoclast cultures. Effective delivery of siRNA into primary human osteoclasts represents a significant achievement towards the development of new tools for the screening of genes for loss-of-function phenotypes. The Dicer synthesised siRNA method has shown to suppress target gene expression with exceptional potency, with most genes silenced by greater than 90%, in a highly specific manner. However, further experiments with western blots would verify the levels of proteins present. The inhibition of CTSK and NFATc1 using this siRNA method in primary osteoclasts provided results that corresponded to published findings, offering validation of the experimental results. siRNA silencing in primary osteoclast cultures has revealed functions in seven genes analysed, using an approach which distinguishes contributions to osteoclast differentiation and bone resorption.

Mouse knockouts have provided useful insights into the primary functions of genes in a whole organism context. Genes quite often have more than one function, and in a mouse knockout model a complex phenotype may arise as the result of abnormalities
in more than one organ or cell type. The primary cell culture model is useful in distinguishing the contribution of a gene specifically to osteoclast differentiation and bone resorption, as well as in a human cell context.

The relative expression of these genes in bone *in vivo* could be investigated by immunohistochemistry of bone tissue sections. Expression could be confirmed in bone resorbing osteoclasts, and in non-resorbing or differentiating osteoclasts. Populations of osteoclasts may be compared between subjects with normal bone density and abnormal bone density as well as the percentages of osteoclasts expressing these genes.
Chapter 6

Conclusions and Future Directions
Advances in understanding the complex system of osteoclast differentiation and activity, have largely been due to gene discovery, recombinant mouse models, and loss-of-function assays. Primary human culture models represent an authentic system for the study of osteoclast differentiation and function. Although cell-screening of candidate genes is already an essential tool in drug discovery, the use of relevant human disease-oriented primary cell models is seen as an important component for developing valid drug research. The use of primary cells has been historically hampered by difficulties encountered in manipulation and transfection efficiency. The optimisation of siRNA inhibition in primary human osteoclasts, provides an important innovation, in particular for the characterisation of multiple targets amassed from microarrays, DD-PCR or similar studies, and for characterising their role in osteoclast biology. In addition, this study provides a novel method for distinguishing gene involvement in osteoclast differentiation independently of osteoclast bone resorption, and vice versa, as well as the quality of bone resorption produced.

A subset of DD-PCR genes, analysed with Q-PCR, showed differential expression induced by RANKL, however, only LIMK1 was statistically significant. DD-PCR produced a number of false positives and the semi-quantitative products produced on the DD-PCR gel, exaggerated the regulation of a number of targets. Q-PCR analysis of a selection of DD-PCR products, revealed expression patterns produced by cytokine treatment, that displayed low fold inductions and variation in gene expression. More refined time points and additional repeats may have generated further statistical power with which to detect significant differences in cytokine treatments over time. Secondary verification of putative osteoclast genes was performed, with siRNA inhibition used to clarify gene involvement in osteoclast
formation and function. Despite low fold induction for many genes investigated, inhibition resulted in defective osteoclast formation and/or defective osteoclast activity. This suggests that many genes which display moderate induction in osteoclasts, are none-the-less instrumental in osteoclast biology. Genes investigated did not prove to be merely necessary for general cell function and maintenance, as the inhibition of these genes did not induce apoptosis, and many siRNA inhibition assays resulted in cultures which resembled precursor cell populations.

The distinction between genes which regulate osteoclast differentiation and those which regulate osteoclast bone resorption, or function to regulate both, was undertaken (Table 6.0). This study reveals that the majority of genes investigated are important for both differentiation and resorption. FAM19A1, LIMK1 and SLC16A6 are genes which were necessary for both osteoclast formation and osteoclast function. Inhibition of FAM19A1, LIMK1 and SLC16A6 resulted in cultures with abundant small cells which resembled PBMC precursors, and displayed a significantly reduced ability to resorb bone. However, some genes affected mainly differentiation or resorption. NFATc1, predominantly influences differentiation, and FBP predominantly influences resorption. These distinctions suggest that two pathways of osteoclast regulation occur, involving genes which control the differentiation of multinucleated osteoclasts, and genes which control the rate and quality of bone resorption, with a large overlap of function amongst most genes involved.

The inhibition of a number of genes affected the pit size or quality of bone resorption. These genes include FBP, GABP and SATB1, and potentially may regulate normal osteoclast cell spreading, encouraging proper cell dimensions, which affect the depth
and size of bone resorptive activity. The quality of bone resorption is a factor which can contribute to changes in bone mass. Alterations in bone microstructure as a result of poor resorption is an undetermined area of osteoclast research, and may provide clues to individual changes in bone density and fracture risk. In the context of drug therapy, total inhibition of osteoclast resorption is an undesirable outcome, which is attributed to necrosis of the jaw in patients treated with strong osteoclast inhibitors such as bisphosphonates, involving tooth extraction (Mortensen et al., 2007). Suppressing the rate or quality of osteoclast resorption may present more favourable outcomes than inhibition of osteoclast formation altogether. It would be interesting to ascertain whether mutations of FBP, GABP or SATB1 contribute to changes in bone mass or bone structure in mouse models or in population studies.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Necessary for differentiation</th>
<th>Necessary for proper rates of resorption</th>
<th>Regulates pit size</th>
<th>Regulate differentiation or resorption</th>
</tr>
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<tbody>
<tr>
<td>FAM19A1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Both</td>
</tr>
<tr>
<td>FBP</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Resorption</td>
</tr>
<tr>
<td>GABPA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Both</td>
</tr>
<tr>
<td>GABPB</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>Both</td>
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<tr>
<td>LIMK1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Both</td>
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<tr>
<td>NFATc1</td>
<td>+</td>
<td>*</td>
<td>-</td>
<td>Differentiation</td>
</tr>
<tr>
<td>SATB1</td>
<td>-</td>
<td>*</td>
<td>+</td>
<td>Resorption</td>
</tr>
<tr>
<td>SLC16A6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Both</td>
</tr>
</tbody>
</table>

Table 6.1 Genes involved in regulating differentiation, resorption or both. Plus symbols (+) denote the necessity of genes for involvement in differentiation or resorption. Asterisks (*) denote that siRNA inhibition resulted in increased rates of bone resorption.

Two new genes, the chemokine-like gene FAM19A1 and the solute transporter gene SLC16A6, were characterised for the first time in this study. Chemokines are important mediators of osteoclast precursor recruitment and osteoclast differentiation. The expression of a variety of chemokines by osteoclasts suggests that osteoclasts coordinate precursor cell recruitment to sites of bone remodelling. Until now, FAM19A1 was a chemokine-like protein, which had no known functional role and was not known to be expressed in osteoclast cells. This study has uncovered the involvement of a novel type of chemokine, which is not highly conserved with any other chemokine or protein family, which plays a role in osteoclast differentiation and resorption, and may prove to be a useful clinical target. Further functional analysis of
FAM19A1, is warranted, to further elucidate its binding receptor and subsequent downstream events. Construction of a FAM19A1 protein expression vector and specific antibody would allow for immunoprecipitation and subsequent identification of the FAM19A1 binding receptor via mass spectrometry or protein microarray.

The functional examination of SLC16A6, has previously not been undertaken. SLC16A6 family members regulate pH and normal cellular metabolism (Halestrap & Meredith, 2004), so it is reasonable that siRNA inhibition of SLC16A6 in osteoclasts resulted in a reduction in osteoclast formation and resorption. The requirement of acid exchange by H+-ATPase to the ruffled border for osteoclast bone resorption, suggests a putative role for SLC16A6 in pH regulation and metabolism for proper cellular function, but particularly for the osteoclast, which is specialized to maintain a local acidic environment for bone resorption. SLC16A6 contribution to osteoclast pH maintenance could be investigated using a pH-sensitive indicator with the microfluorometric method described by Nordstrom et al. (1995).

Genes such as NFATc1 and LIMK1 have previously been reported in osteoclast cells, and have validated the Dicer siRNA technique. NFATc1 is an important osteoclast transcription factor, and its requirement for proper osteoclast formation parallels previous findings in NFATc1 inhibited osteoclasts (Day et al., 2004; Sun et al., 2007; Takayanagi et al., 2002a). Calcineurin stimulates osteoclast differentiation via activation of NFATc1, but also inhibits bone resorption by mature osteoclasts (Sun et al., 2007). Inhibition of NFATc1 in mature osteoclasts did not diminish bone resorptive activity, with the area resorbed by NFATc1 inhibited osteoclasts significantly increased. This result parallels the findings of the NFATc1 regulator,
calcineurin by Sun et al., (2003), where calcineurin was found to enhance osteoclast differentiation, but inhibit osteoclast resorption in mature cells. NFATc1-silenced mature osteoclasts have further clarified the role of NFATc1 in osteoclast bone resorption activity, providing an interesting link to the finding of increased bone mass in mice expressing constitutively active nuclear NFATc1 in osteoblasts (Winslow et al., 2006). It seems that NFATc1 simultaneously regulates bone remodelling through signalling osteoclast differentiation in osteoclast precursors, and promoting osteoblast differentiation in osteoblast precursors, and may mitigate the resorptive activity of osteoclasts, facilitating the coupling of bone formation with bone resorption.

A previous report of LIMK1 expression in osteoclast cells (Cappellen et al., 2002) has been validated by this study, along with the role of LIMK1 in osteoclast biology for the first time. The cycling of cofilin phosphorylation by LIMK1 was hypothesized to play a role in osteoclast fusion, cell spreading, podosome and actin ring formation, which depend on proper cytoskeletal organisation. LIMK1 plays a significant role in osteoclast differentiation and resorption, however the reduction of bone resorptive activity in LIMK1 silenced mature osteoclasts was found to not be a result of disrupted actin ring or podosome formation. It remains to be determined as to whether LIMK2 plays a redundant role alongside LIMK1, to regulate the formation of a functional sealing zone. Simultaneous siRNA inhibition of LIMK1 and LIMK2 in osteoclasts, may further clarify the role of these signalling kinases in osteoclast function.

Further analysis of the DNA binding targets of FBP, GABP, NFATc1 and SATB1, for example by using chromatin immunoprecipitation (ChIP)-on-chip to compare
osteoclast cultures treated with siRNA compared with control siRNA, would further clarify the downstream gene activation by these DNA binding proteins.

Despite the inclusion of siRNA controls and monitoring two off-target gene effects, these controls are insufficient to completely rule out the induction of off-target cellular effects. One study has shown that dicer generated siRNA can induce inflammatory and interferon-response genes non-specifically in HeLa cells (Kettner-Buhrow et al., 2006). Further studies have indicated that off-target effects can sometimes be generated by siRNA in the absence of interferon activation (Jackson & Linsley, 2004). The siRNA method used in this study did not generate a recurring non-specific effect, as the differentiation and resorption assays produced a variety of results. In addition, the inhibition of CTSK and NFATc1 provided results that corresponded to published findings. Even so, these results should be taken with caution until further investigation into the potential non-specific effects of siRNA in this model are further investigated. Microarray analysis comparing each gene inhibition treatment would satisfy whether any results were produced in a manner that does not correspond to inhibition of the target of interest, and would determine whether other key pathways or factors were affected non-specifically. It is possible that when one gene is silenced, a compensatory result is triggered resulting in a phenotype that is not specifically the outcome of the gene targeted.

Despite confirming the functional contribution many genes provide for osteoclast cell formation and activity, these results merely offer evidence warranting further functional investigation in the osteoclast model, and understanding further downstream events are crucial for outlining the true significance of these genes.
Future works involving the use of expression vectors for these genes incorporated into various culture models would further verify the capacity and significance of these genes in osteoclast biology. Overexpressing these genes in monocyte cultures, as well as in the presence of M-CSF, M-CSF and RANKL and pre-differentiated osteoclasts, would determine if these genes are able to induce differentiation or increase the rate of differentiation. Similar experiments could be performed to determine the effects of overexpression on bone resorption. In the presence of drug inhibitors of important osteoclast signalling pathways, such as calcineurin (cyclosporin A), PI3K (LY294002), and MEK1 (U0126), overexpression would determine whether any of these genes are able to rescue osteoclast differentiation or resorption.

Successful silencing of genes in primary human osteoclasts has not previously been undertaken, due to the difficulties associated with transfection into non-dividing, adherent, terminally differentiated primary cells. These experiments have revealed that primary osteoclasts can be successfully transfected with siRNA using a transient transfection reagent and a precise optimised concentration of siRNA. Furthermore, separating siRNA treatments for into pre-differentiated and post-differentiated osteoclast gene inhibition, allows for the designation of genes for formation and/or function. Despite the high efficiency of siRNA studies, gene expression is not completely suppressed. It is considered that cells which are effectively transfected with siRNA quell gene expression entirely, and a proportion of cells escape transfection and contribute to normal cell formation counts and resorbing activity. Total knockouts of these genes may have revealed much greater necessity for differentiation or resorption. Regardless of limitations of incomplete inhibition, siRNA silencing is a useful method for the mass screening of candidate genes for
formation or function discernment within the *in vitro* osteoclast model, as potent and significant gene suppression has shown to result in remarkable reductions in proper osteoclast formation and function. Both rates of formation and resorption, are relevant to diseases involving abnormal osteoclast activity, and current clinical treatments are based on current knowledge of these processes. Further investigations to elucidate the role of novel genes in bone remodelling, based on these preliminary findings, may well extend our understanding of bone biology. In particular siRNA inhibition of candidate genes can be scaled up to rapidly assign functional significance to large numbers of gene targets identified via microarray studies, and provide a basis for further analysis into roles in osteoclast differentiation and bone resorption.
Chapter 7

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Chapter 8

Appendices
8.1 FAM19A1 full coding sequence

FAM19A1 sequence 104
FAM19A1 sequence 102
8.2 Publications from PhD

