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THE STUDIES OF NOVEL REGULATORS OF BONE CELL FUNCTION

Jian-ming Lin

A thesis submitted in partial fulfilment of the requirements for the Degree of Philosophy, The University of Auckland, 2004

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The current study investigates novel factors in bone cell biology by using both \textit{in vitro} and \textit{in vivo} models. The results show that murine receptor activator of nuclear factor-\kappaB ligand (muRANKL) and rat RANKL (rRANKL) stimulated, while human osteoprotegerin (hOPG) inhibited, $^{45}$Ca release and $[^3H]$-thymidine incorporation in rat calvarial organ cultures, osteoclastogenesis from mouse bone marrow cultures and bone resorption in rat isolated mature osteoclast assays. Interestingly, truncated rRANKL(246-318) became an antagonist for non-truncated rRANKL in isolated osteoclast assays.

Fibroblast growth factor (FGF)-8a at 25 ng/mL or greater potently stimulated osteoblast proliferation and mildly inhibited osteoblast differentiation. An autocrine mechanism may be involved in the regulation of osteoblasts based on detection of the expression of FGF-8 in these cells. Osteoclastogenesis was significantly reduced by FGF-8a at 0.05—25 ng/mL, while the ratio of RANKL/OPG was elevated by this agent.

$\alpha$-melanocyte stimulating hormone ($\alpha$-MSH) markedly stimulated both osteoblast proliferation (at $10^{-9}$ M or greater) and osteoclastogenesis (at $10^{-8}$ M or greater) \textit{in vitro}. The stimulatory effect on osteoclasts was associated with the increased ratio of RANKL/OPG. Systemic administration of $\alpha$-MSH resulted in a decrease in trabecular volume in tibiae. $\alpha$-MSH also stimulated canine chondrocyte proliferation, but had no direct interaction with mature osteoclasts.

Milk basic fraction (MBF)-1, MBF-2, milk acidic fraction (MAF)-1 and MAF-2 were found to be mitogenic to osteoblasts \textit{in vitro}. While MAF-1 inhibited and MAF-2 stimulated bone resorption in calvarial cultures, the peptic hydrolysates of the both fractions showed inhibitory effects in this model. Among the individual components identified from the milk fractions, lactoferrin was found to be an interesting novel anabolic factor, potently stimulating osteoblast proliferation at 1-10 $\mu$g/mL or greater in both isolated osteoblast and calvarial organ cultures, as well as potently inhibiting
osteoclast formation from bone marrow cells via a RANKL/OPG-independent mechanism.

In summary, this study has revealed the possibility of the development of a RANKL antagonist through truncation of the molecule. In addition, the osteoblastic and osteoclastic effects of FGF-8a, α-MSH and lactoferrin have been demonstrated for the first time. However, further study is still needed to better understand the mechanisms of their functioning and to evaluate their potential for clinical application.
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<td>1,25(OH)$_2$D$_3$</td>
<td>1α,25 dihydroxyvitamin D$_3$</td>
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<tr>
<td>ºC</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ABF-1</td>
<td>activated B-cell factor-1</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropin</td>
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<td>AIGF</td>
<td>androgen-induced growth factor</td>
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<td>activator protein-2</td>
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<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>colony-forming unit granulocytic-macrophages</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CT</td>
<td>calcitonin</td>
</tr>
<tr>
<td>Cx43</td>
<td>connexin 43</td>
</tr>
<tr>
<td>DDH</td>
<td>death domain homologous</td>
</tr>
<tr>
<td>Dex</td>
<td>dexamethasone</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
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<td>deoxynucleotides</td>
</tr>
<tr>
<td>DPM</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DR3</td>
<td>death receptor 3</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>median effective concentration</td>
</tr>
<tr>
<td>ED$_{50}$</td>
<td>median effective dose</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
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</table>
EGF  epidermal growth factor
ELISA  enzyme-linked immuno sorbent assay
ER  endoplasmic reticulum
FACIT collagen  fibril-associated collagen with interrupted triple helices
FasL  Fas ligand
FBS  foetal bovine serum
FDA  the U.S. Food and Drug Administration
FDCR-1  follicular dendritic cell receptor 1
FEO  familial expansile osteolysis
FGF  fibroblast growth factor
FGFR  FGF receptor
Fig  figure
g  gram
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GM-CSF  granulocyte-macrophage colony-stimulating factor
GMP  glycomacropeptide
GST  glutathione S-transferase
HDL  high-density lipoproteins
HMG  high mobility group
hOPG  human OPG
HPLC  high performance liquid chromatography
hRANKL  human RANKL
IFN  interferon
Ig  immunoglobulin
IGF  insulin-like growth factor
IL  interleukin
JNK  c-Jun N-terminal kinase
kb  kilo base pairs
kD  kilo Dalton
L  liter
LDL  low-density lipoproteins
LFA-1  lymphocyte function-associated antigen-1
LT-α  leukotriene-α
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MAF</td>
<td>milk acidic fraction</td>
</tr>
<tr>
<td>MBF</td>
<td>milk basic fraction</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MC1-R</td>
<td>melanocortin-1 receptor</td>
</tr>
<tr>
<td>αMEM</td>
<td>Minimum Essential Medium Alpha Medium</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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<td>mL</td>
<td>milliliter</td>
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<td>mm</td>
<td>millimetre</td>
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<td>MMP-9</td>
<td>matrix metalloproteinase 9</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>α-MSH</td>
<td>α-melanocyte stimulating hormone</td>
</tr>
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<td>muRANKL</td>
<td>murine RANKL</td>
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<tr>
<td>NF</td>
<td>nuclear factor</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>OAF</td>
<td>osteoclast-activating factor</td>
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<tr>
<td>OCIF</td>
<td>osteoclastogenesis inhibitory factor</td>
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<tr>
<td>Oct-1</td>
<td>octamer transcription factor-1</td>
</tr>
<tr>
<td>ODAR</td>
<td>osteoclast differentiation and activation receptor</td>
</tr>
<tr>
<td>ODF</td>
<td>osteoclast differentiation factor</td>
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<tr>
<td>ODFR</td>
<td>osteoclast differentiation factor receptor</td>
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<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
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<td>OPGL</td>
<td>osteoprotegerin ligand</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Paget's disease of bone</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>proteoglycan</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
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<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
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<td>proteose peptone component 5</td>
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<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>parathyroid hormone-related peptide</td>
</tr>
</tbody>
</table>
rAmylin  rat amylin
RANK  receptor activator of nuclear factor-κB
RANKL  receptor activator of nuclear factor-κB ligand
RNA  ribonucleic acid
rRANKL  rat RANKL
RT-PCR  reverse transcription polymerase chain reaction
sCT  salmon calcitonin
Shh  sonic hedgehog
SOFA  stromal osteoclast forming activity
SPARC  secreted protein acidic and rich in cysteine
TACE  TNF-α convertase
TCA  trichloroacetic acid
TFIID  transcription factor IID
TGF  transforming growth factor
TNF  tumor necrosis factor
TNFR  tumor necrosis factor receptor
TNFRSF  tumor necrosis factor receptor superfamily
TR-1  TNFR-like molecule 1
TRAF  TNFR-associated factor
TRAIL  TNF-related apoptosis-inducing ligand
TRAP  tartrate-resistant acid phosphatase
μm  micrometer
μl  microliter
UV  ultraviolet
WPC  whey protein concentrate
CHAPTER 1: LITERATURE REVIEW AND RESEARCH SCOPE

Bone disorders are a common threatening factor to the health of humankind. These disorders include osteoporosis, Paget's disease and osteocarcinoma. It is reported that 200 million people are affected worldwide by osteoporosis (Mundy 1999b). An incidence of 3-5% for Paget's disease was scored among the people over the age of 55 in the United Kingdom, Australia and New Zealand (Sharpe 1996; Kanis 1998). In the United States, bone metastases occurs in two-thirds of people who died of cancers, making the skeleton the third most favored site for metastasis of solid tumors (Mundy 1999b). Therefore, the skeleton has been the focus of clinical and basic research. In the combat against these common diseases, an increased understanding of bone biology and the factors involved in them are an important scientific question.

PART A: BONE AND BONE CELL BIOLOGY

A1.1. INTRODUCTION

Bone is a highly specialised form of connective tissue consisting of four types of cells and a highly mineralized extracellular matrix. It has three major functions: (1) providing the site for the attachment of muscles and offering support to the body; (2) housing bone marrow and protecting the organs, which are vital to the life of the organism; (3) serving as the reservoir of ions and growth regulators, which regulate the functions and activities of cells and tissues in the body (Baron 1999).

Mammalian bone is developed from the primitive mesenchymal tissue. Two models exist for the development of bone. One is the replacement of pre-existing cartilage by bone (endochondral ossification), including the formation of a cartilage and then the calcification of cartilage matrix followed by the death of chondrocytes. Most of the skeleton is formed by this way (e.g. long bones). The other model is the direct mineralization of the template made of highly vascular connective tissue membrane.
(intramembranous ossification, e.g. skull bones). Throughout life, bone is under constant remodeling: resorption by osteoclasts and formation by osteoblasts (McCarthy and Frassica 1998; Scheuer and Black 2000).

### A1.2. THE MORPHOLOGY AND STRUCTURE OF BONE

#### A1.2.1. The organization of bone at macroscopic level

Morphologically, bones fall into four categories: long bone (femur, tibia, humerus and radius), short bone (tarsal bone), flat bone (skull bone, scapula and ilium) and irregular bone (vertebra and maxilla) (Ma et al. 2000). A typical structure of bone can be seen in a long bone (Fig. 1-1). Externally, a long bone displays two wider extremities and a cylindrical tube. From the longitudinal dissection, a long bone displays (from the end toward the middle) epiphysis, epiphyseal cartilage (growth plate, for longitudinal growth in growing bone), metaphysis and diaphysis. Based on the density and strength, a long bone consists of cortical bone (compact bone) and trabecular bone (cancellous bone or spongy bone) (Baron 1999).

Cortical bone is made of thick and dense calcified tissue and provides the structural strength for the bone/skeleton frame. It is thicker in the diaphysis and progressively getting thinner toward metaphysis and epiphysis (Baron 1999). The inner and outer surfaces of the cortical bone are covered by endosteum and periosteum respectively. The endosteum and peristeme are membranes made of layers of osteogenic cells, which are capable of proliferation and are responsible for bone repair (McCarthy and Frassica 1998). The trabecular bone appears as a network structure and mainly locates in metaphysis and epiphysis. The space enclosed by the trabecular bone is in continuity with the medullary cavity enclosed by the cortical bone in the diaphysis. These spaces are filled with blood vessels and hematopoietic or fatty bone marrow, which consists of fat, hematopoietic cells, osteoprogenitor cells and fibroblasts (Sharpe 1996; McCarthy and Frassica 1998; Baron 1999). Consistent with its mechanical and protective function, 80% to 90% of cortical bone is calcified. In contrast, only 15% to 25% of trabecular bone is calcified, and the rest is occupied by bone marrow, blood vessels and connective tissue. Trabecular bone is mainly responsible for metabolic function (Baron 1999).
Since the surface area of the trabecular bone is much larger than that of the cortical bone, it is the major site for bone remodeling and calcium exchange (McCarthy and Frassica 1998).

**Fig. 1-1.** Schematic view of a longitudinal section through a growing long bone (Adopted from Baron 1999)

In a growing long bone, there are two segments of cartilage: articular cartilage and epiphyseal cartilage. Articular cartilage covers the surface of epiphysis and contributes, together with epiphysis, to the formation of the joint. Articular cartilage remains soft and non-calcified, providing a smooth surface and good elasticity to facilitate the movement in relation to another bone (Ma et al. 2000). Epiphyseal cartilage (also called epiphyseal plate or growth plate) is located between the epiphysis and metaphysis, and is responsible for the longitudinal growth of bone. At the end of growth period, it becomes completely calcified and is replaced by bone (Baron 1999).

In addition to the supporting tissues periosteum and endosteum, bones have an extensive network of blood vessels for nutrient supply and gas exchange. A larger nutrient artery (diaphyseal artery) enters the bone via the canal in the diaphysis. Several other arteries enter the bone from the epiphysis and metaphysis. The diaphyseal artery does not branch in the canal within the cortical bone, but when it reaches the medullary cavity, it divides into several branches, some of which are open ended and some of which anastomose with
the branches of metaphyseal vessels. In the vicinity of the metaphyseal zone, the branches of the medullary vessels terminate in helical loops and are aligned vertically to the growth plate. In addition, many small blood vessels enter the bone from the periosteum (McCarthy and Frassica 1998; Scheuer and Black 2000). However, there is no vasculature within the articular cartilage, whose nutrient supply is through the osmosis of the blood vessels in the epiphysis under it and from the joint fluid (Ma et al. 2000).

A1.2.2. The organization of bone at microscopic level

Bone, as a tissue, contains two major components: bone cells and extracellular matrix, (Baron 1999). Based on the stage of bone development and the structure of matrix, bone tissue can be divided into non-lamellar bone and lamellar bone (Ma et al. 2000). Non-lamellar bone is also referred to as woven bone (Ross et al. 1989). Woven bone is an immature type of developing bone and can be seen in stages of embryonic osteogenesis and bone repair, and in some pathological states such as Paget’s disease (Sharpe 1996; Ma et al. 2000; Scheuer and Black 2000). Woven bone is formed quickly and is relatively weak. Its name is derived from the organization of the collagen fibers, which appear to be similar to the weaves of fabric under the microscope (Ma et al. 2000; Scheuer and Black 2000). In this type of bone, the collagen fibers, which can be up to 13 μm in diameter, are not preferentially orientated. The osteocytes in woven bone are larger in size compared to those in lamellar bone. Lamellar bone is known as secondary bone tissue and is the mature status of bone (Ma et al. 2000). The collagen fibers in lamellar bone are oriented in a preferential direction and aggregated to form a layered structure called lamella, which is a few μm thick (Sharpe 1996). The fibers in the same lamella are parallel to each other whilst the collagen fibers in the adjacent lamella are at right angles. This laminated matrix forms the basis of the strong lamellar bone (Sharpe 1996; Baron 1999).

The horizontal dissection of cortical bone of a long bone reveals three zones from the outside toward the inside: periosteal bone, Haversian bone and subcortical bone. Periosteal bone, the outermost of which is covered by the periosteum, is made of layers of bone lamellae arranged circumferentially around the bone shaft. The thickness of
periosteal bone is from one tenth to one fifth of the cortical bone. The inner aspect of periosteal bone is being continuously changed to Haversian bone (Sharpe 1996). Many small canals called Volkmann’s canals, which run through the lamellae, can be seen in the cortical bone (Ma et al. 2000). Volkmann’s canals run perpendicular to the Haversian systems and connect the Haversian canals to the marrow cavity or to the periosteum. Blood vessels also run through the Volkmann’s canals (Fig. 1-2) (McCarthy and Frassica 1998).

The majority of the cortical bone is called Haversian bone, which is located between periosteal bone and subcortical bone. This part of cortical bone is made up of many subunits known as Haversian systems (or osteons). Haversian systems (first identified by Clopton Havers in 1691) are oriented parallel to the long axis of the cortical bone. Each Haversian system consists of a Haversian canal and 8 to 15 concentric bone lamellae, which are around the canal (McCarthy and Frassica 1998). The branches of Haversian canals join each other to form a network and anastomose with the Volkmann’s canals. Blood vessels and nerves run through the canals. This system supplies nutrients to
osteocytes deep within the bone matrix in small osteocytic lacunae (Baron 1999). In a developing Haversian system, the bone lamellae are still under calcification and the surface of the Haversian canal is lined with active osteoblasts. In a mature Haversian system, the number of bone lamellae is increased and the spindle-shaped cells (osteocytes) on the surface of the Haversian canal develop processes (called canaliculi, in the innermost Haversian lamella) to allow the contact with other osteocytes (Ma et al. 2000). Canaliculi are the small canals formed during the formation of the matrix by the long processes of osteocytes. They are organised into a network permeating the entire bone matrix (Baron 1999). Bone-resorbing osteoclasts can be found on the wall of the canal in the resorptive Haversian system. Around the Haversian system is a cement line (also called remodeling lines), which is basophilic and is made of matrix containing a higher proportion of bone salts and less collagen fibers. In mature bone, cement lines can be seen due to their stronger refraction (McCarthy and Frassica 1998; Ma et al. 2000). Osteocytes are located in the small holes called lacunae evenly distributed throughout the bone matrix (McCarthy and Frassica 1998). Between the plasma membrane of osteocytes and the bone matrix (including lacunae and canaliculi) is the periosteocytic space filled with extracellular fluid (Baron 1999; Ma et al. 2000).

The inner surface of the Haversian bone is covered with a thin layer of subcortical bone surrounding the marrow cavity. The spicules of trabecular bone arise from the subcortical bone and form an interconnecting network of plates of bone within the marrow cavity (Sharpe 1996; McCarthy and Frassica 1998).

A1.3. THE COMPOSITION OF BONE MATRIX

The majority of bone is extracellular matrix made of four major components: type I collagen, noncollagenous proteins, nonproteinaceous organic material and crystalline hydroxyapatite \([3Ca_3(PO_4)_2](OH)_2\) (Sharpe 1996; Baron 1999). The organic matrix of bone is called osteoid (McCarthy and Frassica 1998). It is synthesized and deposited by the cells in the osteoblastic lineage. The precipitation of carbonate-containing hydroxyapatite in bone matrix results in the mineralization of bone (Robey et al. 1993).
A1.3.1. Collagens

The primary component of bone matrix is type I collagen, which accounts for about 90% of total matrix protein (Sharpe 1996). In addition to type I collagen, traces of type III and type V collagen, and fibril-associated collagen with interrupted triple helices (FACIT collagen) can also be detected in bone matrix (Robey et al. 1993). At present, more than 20 types of collagen have been identified based on their structure and function (Myllyharju and Kivirikko 2001). Type I, type II, type III, type V and type XI collagen are fibrillar proteins, while some others collagens, such as type IX, type XII, and type XIV collagens, which are less common and have specialised functions and distribution, fall into the category of FACIT collagens (Rossert and de Crombrugghe 1996; Woolf and Dixon 1998). FACIT collagens are associated with fibrillar collagens via one of their three domains and interact with other extracellular components or cells via the other domains without the triple helical structure (Rossert and de Crombrugghe 1996). Type I collagen is overwhelmingly predominant in bone matrix though it is also present in skin, tendons and ligaments. Type II collagen is the major constituent of cartilage along with type XI collagen (Rossert and de Crombrugghe 1996; McCarthy and Frassica 1998).

Type I collagen is a triple helix composed of two identical α1 polypeptides encoded by the gene COLIA1 and another structurally different α2 polypeptide encoded by COLIA2. The COLIA1 and COLIA2 are located in human chromosome 17q21.3-q22 and chromosome 7q21.3-q22, respectively (Rossert and de Crombrugghe 1996; Woolf and Dixon 1998). The primary translation product is called procollagen, which is processed to form procollagen within the cell via the steps including hydroxylation of proline and lysine to form hydroxyproline and hydroxylysine, assembly of two α1 chains and one α2 chain into a triple helical structure, and finally glycosylation. A feature of type I collagen molecule is the repeating triplet of Gly-X-Y in the polypeptide chains. X is usually proline while Y is usually hydroxyproline, which is essential in stabilizing the triple helix. This triplet repeat of amino acids allows the peptide to coil into a left-handed helix. Procollagen is then secreted into the extracellular space, where a couple of modifications occur. Firstly, the N- and C- termini (totally about one third of the mass) of the procollagen are cleaved by the specific procollagen aminopeptidase and
procollagen carboxypeptidase and gives rise to a mature collagen molecule, which is much less soluble compared to its propeptide (Rossert and de Crombrugghe 1996; Woolf and Dixon 1998). Then each of the mature collagen molecules is staggered quarterly to the adjacent one and spontaneously aggregates into collagen fibrils. Finally, interchain aldehyde cross-links, which considerably stabilize the fibril structure, are formed by some of the lysine and hydroxylysine residues between the collagen molecules (Rossert and de Crombrugghe 1996; McCarthy and Frassica 1998).

The amino acids, hydroxyproline and hydroxylysine, and the interchain aldehyde bond are mainly confined to bone collagen. These features have been used as the indicators for bone resorption in clinical diagnosis. When collagen is digested during bone resorption by osteoclasts, hydroxyproline and hydroxylysine are released from the collagen molecules, and a compound known as pyridinoline is formed from the aldehyde cross-links between the lysine and hydroxylysine. The released amino acids and pyridinoline are passed into the blood stream and secreted in urine. Therefore, abnormally high levels of these molecules in the urine are the indication of an abnormal rate of bone resorption, which is due to the increased osteoclast activity associated with some diseases, such as Paget's disease and hyperparathyroidism (McCarthy and Frassica 1998; Woolf and Dixon 1998).

**A1.3.2. Noncollagenous proteins**

In addition to the major component collagen, bone matrix also contains noncollagenous proteins, which make up about 10% of the organic matrix (McCarthy and Frassica 1998). Most of the noncollagenous proteins found in the bone matrix are produced by bone-forming cells. Although many of them have been characterised biochemically and a few of them have been found to have significant biological activities, their functions are still not fully understood (Baron 1999; Mundy 1999b). The major noncollagenous proteins include proteoglycans (such as biglycan and decorin), glycoproteins (such as osteonectin, osteopontin and alkaline phosphatase), osteocalcin and some growth regulators, such as insulin-like growth factor (IGF)-I, IGF-II and transforming growth factor (TGF)-β (McCarthy and Frassica 1998; Woolf and Dixon 1998; Mundy 1999b). The
chromosomal locations of the genes for many of the noncollagenous proteins have been identified (Robey 1996).

A1.3.2.1. Proteoglycans

This class of molecule is particularly abundant in cartilage matrix. However, they are also found in bone matrix (McCarthy and Frassica 1998). Proteoglycan (PG) is a complex macromolecule comprised of a central core protein and long acidic polysaccharide chains (glycosaminoglycans) with varying compositions and repeating disaccharide subunits. The polysaccharide chains are covalently attached to the central core protein. The members of proteoglycans include biglycan (PG-I) and decorin (PG-II), both of which are the most abundant proteoglycans in mineralized matrix. Biglycan is composed of a ~45 kD core protein and two chondroitin sulfate glycosaminoglycans chains attached to the proximity of the N-terminus of the core protein. Decorin has a 38 kD core protein with one chondroitin sulfate chain attached. Though the core proteins in biglycan and decorin are the products of two distinct genes, they are highly homologous and contain repeating (11 times) leucine sequences. The exact function of biglycan and decorin is still not clear. However, they seem to be involved in the regulation of collagen fibril growth. The leucine rich repeat sequence in the core proteins may mediate the binding of the proteoglycan molecules to matrix constituents. These proteoglycans have been found to bind to TGF-β, suggesting a role in regulating the activity of the growth factor (Young et al. 1992; Robey et al. 1993; Robey 1996).

A1.3.2.2. Osteocalcin

Osteocalcin (previously known as bone Gla-protein), one of the first noncollagenous proteins isolated and identified from bone, has been intensively studied. It is a single-chain protein with a molecular weight of 6000 Dalton. It is relatively abundant in bone matrix, accounting for about 20% of the total noncollagenous proteins. Osteocalcin is produced by the cells in the osteoblastic lineage, including osteoblasts and osteocytes. The gene for osteocalcin was mapped to human chromosome 1 (Robey 1996). Osteocalcin peptide contains three glutamic acid residues, which are γ-carboxylated
during the post-translational modification via a vitamin K-dependent mechanism. These \(\gamma\)-carboxylated amino acids confer the calcium-binding ability to the peptide (Woolf and Dixon 1998; Mundy 1999b).

The function of osteocalcin is obscure. However, it has been postulated as the retardant of bone mineralization and chemoattractant for osteoclast progenitors (attracting the cells to bone surface). Rats chronically (8 months) treated with warfarin, a vitamin K antagonist, had only 2% of normal levels of osteocalcin. The reduced levels of osteocalcin were associated with the excessive mineralization of bone, the closure of proximal tibial growth plate and cessation of longitudinal growth (Price et al. 1982). Another strong evidence for the function of osteocalcin was from the osteocalcin-deficient mice, in which the cortical bone thickness was increased (Ducy et al. 1996). It can be speculated from these results that osteocalcin may be a down-regulator of bone formation.

Although osteocalcin was also found in osteocytes, platelets and megakaryocytes, it has been widely used as a marker of osteoblast activity due to its predominant production by osteoblasts. The high levels of osteocalcin in serum are indicative of high bone turnover, which can be seen in some diseases, such as high-turnover osteoporosis and renal osteodystrophy (McCarthy and Frassica 1998; Woolf and Dixon 1998; Mundy 1999b).

A1.3.2.3. Osteonectin

Another well-characterised noncollagenous protein is osteonectin. It was first identified by Termine and coworkers from the bovine bone as a 32 kD molecule (Termine et al. 1981) and then found to be identical to secreted protein acidic and rich in cysteine (SPARC), and basement membrane protein-40 (BM-40) based on the cDNA sequence comparisons (Mann et al. 1987; Motamed 1999). As one of the most abundant glycoproteins in bone matrix, osteonectin accounts for about 15% of the total matrix noncollagenous proteins (McCarthy and Frassica 1998). It is synthesized by the osteoblasts, however, its presence is not confined to bone matrix. It can also be detected in other tissues, such as hypertrophic cartilage, \(\alpha\)-granules of platelets (Robey et al.
The expression of osteonectin is regulated spatially and temporally during development. High levels of the mRNA and protein can be seen in the tissues undergoing remodeling or in response to injury and “culture shock”. The levels of osteonectin mRNA and protein can be augmented in some situations or stress conditions, such as endotoxin stimulation, heat shock, cellular aging, wound repair and serum starvation in culture (Motamed 1999).

Osteonectin is a phosphorylated glycoprotein rich in cysteine (17 residues) and with the acidic feature conferred by its N-terminus (Young et al. 1992). It contains three domains, which play various kinds of functions including high-affinity and low-affinity calcium-binding, inhibition of cell spreading and cell proliferation (Motamed 1999).

The early observation that osteonectin strongly binds to type I collagen and hydroxyapatite has led to the speculation that osteonectin might be involved in the initiation of mineralization (Termine et al. 1981). In bovine osteogenesis imperfecta, characterised as multiple bone fractures and friable teeth, the osteonectin levels in the bones are less than 2% of normal levels (Termine et al. 1984). Decreased levels of osteonectin are also seen in the osteoblasts isolated from the patients with osteogenesis imperfecta and in the bones from the frol/fro mice with fragilitas ossium mutation (Muriel et al. 1991; Vetter et al. 1991). Delany et al further found that bone formation, as well as the number of osteoblasts and osteoclasts, are decreased in osteonectin-null mice displaying osteopenia. This suggested that osteonectin deficiency was associated with the decreased bone remodeling with a negative mineralization balance (Delany et al. 2000).

Recent studies showed that osteonectin is also involved in the inhibition of cell spreading and proliferation, and the regulation of cell cycle progression and cell shape (Robey 1996; Motamed 1999). Osteonectin exerts an arrest of the cells in the G1 phase and hence inhibits cell proliferation. In addition, osteonectin-deficient mice develop severe early-onset cataract, suggesting a role of osteonectin on lens transparency (Brekken and Sage 2000).
A1.3.2.4. Osteopontin

Osteopontin is a secreted glycoprotein and is highly phosphorylated (13 phosphorylation sites in bovine) in the serines and threonines. Osteopontin also has the following structural features: (1) two conserved N-terminal domains involved in the interaction with extracellular matrix, fibronectin, collagen, osteocalcin and integrin $\alpha_6\beta_1$ (Giachelli and Steitz 2000); (2) a polyaspartic acid-containing domain; and (3) an arginine-glycine-aspartate (RGD)-containing domain which is recognized by cell surface receptors, integrins $\alpha_6\beta_1$, $\alpha_9\beta_3$, $\alpha_6\beta_3$ (Robey et al. 1993; Giachelli and Steitz 2000); (4) a serine-valine-valine-tyrosine-glutamic acid-leucine-arginine (SVVYGLR)-containing domain, which can interact with integrin $\alpha_9\beta_1$ after osteopontin is subjected to thrombin cleavage (Giachelli and Steitz 2000). Osteopontin is similar to bone sialoprotein (another bone matrix glycoprotein) in molecular weight (~85 kD) and in containing stretches of acidic amino acid residues (polyaspartate in osteopontin, polyglutamic in bone sialoprotein) (Robey 1996). The acidic amino acid residues, together with the phosphorus groups, endow osteopontin a highly acidic feature. Osteopontin has been found to bind calcium and hydroxyapatite crystals with high affinity, suggesting its role in bone mineralization (Giachelli and Steitz 2000).

Osteopontin exists in bone extracellular matrix in an immobilised form and in most body fluids as a cytokine. In addition to bone, high levels of osteopontin can be found in the kidney (Giachelli and Steitz 2000; Denhardt et al. 2001b). It is expressed in osteoblasts, osteoclasts, osteocytes, hypertrophic chondrocytes and most epithelial lining cells (Denhardt and Noda 1998; Yoshitake et al. 1999; Giachelli and Steitz 2000; Denhardt et al. 2001a). Its expression is up-regulated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), TGF-$\beta$ and bone morphogenetic protein (BMP)-7, the factors promoting bone formation, and down-regulated by bisphosphonates, which inhibit bone resorption (Sodek et al. 1995).

One of the major roles of osteopontin seems to be in facilitating recovery of an organism from injury and infection (Denhardt and Noda 1998). It have been shown by many
studies that the expression of osteopontin was markedly up-regulated under the conditions of inflammation and wound healing, and in response to some pathological conditions, such as cancer, arterial restenosis, atherosclerosis, renal tubulointerstitial fibrosis, stroke and tuberculosis, etc (Giachelli and Steitz 2000). The function of osteopontin in inflammation and injury response has been demonstrated in recent years through its effect on macrophage (white blood cell) infiltration, a key process for recovery. It was found that macrophage accumulation induced by the potent chemotactic peptide (N-formyl-met-leu-phe) was greatly inhibited by the neutralizing anti-osteopontin antibodies (Giachelli et al. 1998). Macrophage influx in the obstructed kidneys with induced tubulointerstitial disease was threefold to fivefold lower in the osteopontin-null mice than in the wild type mice (Ophascharoensuk et al. 1999). When subjected to the mutagen to induce cutaneous squamous cell carcinoma, osteopontin-deficient mice showed a higher rate of tumor growth and lower degree of macrophage infiltration than the wild-type animals (Crawford et al. 1998).

Osteopontin is a functional matrix component in bone remodeling. It can be cross-linked by transglutaminase and binds to other matrix components including type I collagen, osteocalcin and fibronectin. The interactions between these molecules are likely to increase the strength of bone matrix (Denhardt and Noda 1998). The role of osteopontin in bone metabolism has been investigated. It was reported that significantly increased osteoclastogenesis was seen from the co-cultures of spleen cells from osteopontin-deficient mice and calvarial osteoblasts from normal mice, indicating that endogenous osteopontin is not necessary for osteoclastogenesis (Rittling et al. 1998). Osteopontin-null mice exhibited resistance to ovariectomy-induced bone loss. It was suggested from this result that strategies to block the action of osteopontin could be the pathways to suppress osteoporosis (Yoshitake et al. 1999). In ectopic implantation experiments, calvarial bone discs from the osteopontin knockout mice showed lower bone loss and osteoclast number (Asou et al. 2001). Recent research found that parathyroid hormone (PTH) and receptor activator of nuclear factor-κB ligand (RANKL) failed to stimulate osteoclast number and bone resorption in cultured long bones from osteopontin-deficient mice (Ihara et al. 2001).
Over all, the biological function of osteopontin is still not fully understood. Further work is required to elucidate its function in bone remodeling as well as in other areas (Asou et al. 2001).

A1.4. THE CELLS OF BONE

In addition to mineralized extracellular matrix, bone is composed of four different types of cells: osteoblasts, osteocytes, bone lining cells and osteoclasts. Osteoblasts, bone lining cells and osteoclasts locate on the surface of bone, while osteocytes are trapped deep inside the bone matrix (Marks and Popoff 1988; Marks and Hermey 1996). Osteoblasts and osteoclasts, the most important cell types involved in bone development and remodeling, are responsible for the formation and resorption of bone respectively. The coupling of the activity of osteoblasts and osteoclasts determines the net effect of bone modeling.

A1.4.1. Osteoclasts

A1.4.1.1. Origin and development of osteoclasts

It is well documented that osteoclasts are originated from the hematopoietic stem cells in the tissues such as bone marrow, spleen and peripheral blood. This is supported by the evidence including cure of the osteopetrotic mice through parabiotic union with the normal littermates, parabiotic union showing the osteoclasts derived from the nonirradiated rat rather than the lethally irradiated counterpart, and cure of osteopetrosis by transplantation of hematopoietic tissues (Roodman 1996; Suda et al. 1996). In addition to these in vivo experiments, osteoclast formation from the in vitro co-cultures also further indicates the hematopoietic origin of osteoclasts. These include co-cultures of bone marrow cells and fetal bone rudiments lacking pre-osteoclasts (Burger et al. 1982; Scheven et al. 1986), and co-cultures of mouse osteoblastic cells and spleen cells (Takahashi et al. 1988a). However, the precise hematopoietic lineage of the osteoclast and the mechanism(s) by which the differentiation is regulated is still not well established (Scheven et al. 1986; Roodman 1996). The most favored hypothesis is that the hematopoietic stem cell gives rise to the colony-forming unit granulocytic-macrophages.
(CFU-GM), which is in the monocyte-macrophage lineage and is the earliest identifiable precursor for the osteoclast. CFU-GM then differentiates in sequence to early precursor (proliferative), committed precursor, immature osteoclast and mature osteoclast. The mature osteoclasts are formed by the fusion of their mononuclear precursors and are terminally differentiated multinucleated cells (Fig. 1-3). However, other researchers also proposed that osteoclasts are not derived from CFU-GM, but from the more primitive cells giving rise to a different osteoclast lineage without going through CFU-GM (Roodman 1996).

![Fig. 1-3. Stages of osteoclast differentiation (Adopted from Roodman 1996, with modification).](image)

The regulation of osteoclast differentiation is still not fully understood. But significant progress has been made in recent years. Previous studies showed that in in vitro cultures, osteoclast formation from spleen cells and peripheral blood monocytes required the direct contact with the supporting osteoblastic cells and the presence of 1α,25 dihydroxyvitamin D₃ [1,25-(OH)₂D₃] (Takahashi et al. 1988a; Martin and Udagawa 1998; Riggs et al. 2000). In mouse bone marrow cultures, which contained mixed population of both osteoclast precursors and supporting osteoblastic cells, 1,25-(OH)₂D₃ or PTH addition was necessary for osteoclastogenesis (Takahashi et al. 1988b). It is now clear that 1,25-(OH)₂D₃ and PTH, as well as interleukin (IL)-11, stimulate osteoblasts to produce a mediator called RANKL, which in turn stimulates osteoclastogenesis (Nakagawa et al. 1998). RANKL, together with macrophage colony-stimulating factor (M-CSF), can replace the need for the supporting cells and other agents to induce
osteoclast formation from spleen cells or peripheral blood monocytes (Quinn et al. 1998; Shalhoub et al. 1999).

In addition to the primary cells from hematopoietic tissues, osteoclasts can be generated, in the presence of necessary agents or supporting cells, from some cell lines including the murine monocytic cell line RAW 264.7 (Xu et al. 2000), human monoblastic cell line UG3 (Ikeda et al. 1998), mouse macrophage cell line BDM-1 (Shin et al. 1995), mouse hematopoietic cell line FDCP-mix A4 (Hattersley and Chambers 1989), human promyeloblastic leukemia cell line HL60 (Nagai et al. 2000) and other cell lines (Chambers et al. 1993; Orlandini et al. 1995; Hentunen et al. 1998; Hentunen et al. 1999; Roodman 1999; Sakiyama et al. 2001).

A1.4.1.2. Characteristic features for osteoclasts

Osteoclasts are large multinucleated cells up to 100 μm in diameter and with 2 to 100 nuclei per cell. The most striking morphological feature, which distinguishes them from other multinucleated giant cells such as macrophage polykaryons or megakaryocytes, is the ruffled border. The ruffled border is formed in the central area by highly infolded plasma membrane and provides sufficient surface for interaction between the cell and resorption lacunae. Around the ruffled border is the clear zone, an organelle-free area of the plasma membrane, which enables the osteoclast to seal to the bone surface. Active osteoclasts contain large numbers of lysosome vesicles, a high density of mitochondria and extensive Golgi complexes (Marks and Hermey 1996; Roodman 1996; Vaananen et al. 2000; Compston 2001).

In addition to the morphological features, osteoclasts can be recognized by the enzyme/protein markers, including tartrate-resistant acid phosphatase (TRAP), calcitonin (CT) receptor, matrix metalloproteinase 9 (MMP-9, also termed 92 kD gelatinase or type IV collagenase), vitronectin receptor, carbonic anhydrase II and cathepsin K (also termed cathepsin O) (Roodman 1996). The most commonly used marker is TRAP, which provides a convenient histochemical staining for osteoclasts. TRAP, whose physiological role is not fully understood, is also expressed in the tissues in gut, kidney and lung, but its levels are highest in bone (Roodman 1996). MMP-9 expression precedes the expression
of TRAP and is the earliest expressed marker for cells in the osteoclast lineage, while CT receptor (not found in avian osteoclasts) expresses only when the cells are committed to osteoclast lineage. Therefore, MMP-9 and CT receptor serve as the markers of the differentiation stages of osteoclasts. Antibodies against surface proteins, such as 23c6, which reacts with the vitronectin receptor, have been used for identification of osteoclasts. The osteoblast markers, alkaline phosphatase and osteocalcin are not detectable in osteoclasts (Roodman 1996; Suda et al. 1996; Roodman 1999).

Generally, the criteria used to identify a mature osteoclast are: multinucleation, TRAP-positive and the ability to resorb bone.

Fig. 1-4. Postulated steps of osteoblast differentiation (Adopted from Aubin 1998)

A1.4.2. Osteoblasts
A1.4.2.1. Origin and development of osteoblasts

Osteoblasts are differentiated from pluripotent (multipotential) mesenchymal stem cells, which are defined by their capability of unlimited self-renewal and to repopulate many differentiated lineages including, along with osteoblasts, chondrocytes, adipocytes, myoblasts and fibroblasts (Aubin and Liu 1996; Triffitt 1996; Compston 2001).
the development of osteoblasts, a number of transitional steps have been recognized. As shown in Fig. 1-4, the first stage in the osteoblast lineage is the recruitment of the committed osteoprogenitor cells from the pluripotent mesenchymal stem cells. The osteoprogenitor cells further differentiate to preosteoblasts and then to mature osteoblasts, the phenotype of which is defined by their ability to make a tissue recognizable as bone. Both osteoprogenitor cells and preosteoblasts retain limited potential of proliferation, while mature osteoblasts are post-proliferative (Aubin and Liu 1996). Following maturation, the active osteoblasts have three subsequent pathways: undergoing apoptosis, becoming bone lining cells or osteocytes. Bone lining cells and osteocytes are thought to be the final mature stage of osteoblasts (Compston 2001).

The commitment to the osteoblastic lineage is a key step to generate osteoblastic cells. Though the mechanism for this is still not fully understood, it is known that this process is under the regulation of some factors, such as core binding factor a-1 (Cbfal), BMPs, fibroblast growth factors (FGFs), TGF-β, glucocorticoids and 1,25-(OH)₂D₃ (Compston 2001). Among these factors, the most important one is Cbfal (also termed osteoblast stimulating factor 2 or Osf 2, or runx 2), which was characterised in recent years as a nuclear transcription factor (Ducy 2000; Compston 2001; Karsenty 2001). The key role of Cbfal in osteoblast differentiation was demonstrated by the generation of Cbfal-deficient mice, which exhibited complete lack of bone formation due to the maturational arrest of osteoblasts (Komori et al. 1997; Otto et al. 1997). No alternative pathway is available to overcome the consequence of Cbfal-deficiency. In addition, Cbfal also regulates the rate of bone matrix deposition by osteoblasts and binds to the promoters of the osteoblastic genes, including osteocalcin, bone sialoprotein, osteopontin and type I collagen genes (Ducy 2000). Cbfal also regulates the differentiation of hypertrophic chondrocytes (Karsenty 2001). Recently, osterix, whose expression is Cbfal-dependent, has been found to be another critical transcription factor for osteoblast development. In the osterix-deficient mice, osteoblast differentiation was severely impaired (Nakashima et al, 2002).

A1.4.2.2. Characteristic features for osteoblasts

Osteoblasts can be distinguished from other types of cells by morphological, histochemical and biochemical criteria. Structural characteristics for osteoblasts include
a round nucleus at the base of the cell, a prominent Golgi apparatus, well-developed rough endoplasmic reticulum and cytoplasmic processes, which join the osteocyte canalicular network inside the bone matrix. The cytoplasm of osteoblasts is highly basophilic (Aubin and Liu 1996; Marks and Hermey 1996; Compston 2001).

Mature osteoblasts can be recognized by their ability to synthesize mineralized matrix and to express the relatively specific molecules, such as alkaline phosphatase, type I collagen, osteonectin, bone sialoprotein, osteopontin and osteocalcin. The response to PTH is also a characteristic of osteoblasts, which express the receptor for this hormone (Aubin et al. 1995). Osteoblastic cells at different development stages may express different levels and different combinations of the molecules (Table. 1-1). These markers may be used to identify the osteoblastic cells in the developmental sequence both in vivo and in vitro (Aubin et al. 1995; Aubin and Liu 1996; Compston 2001).

**Table. 1-1.** Some markers expressed in the differentiation stages of osteoblasts

<table>
<thead>
<tr>
<th></th>
<th>MSC</th>
<th>Early OP</th>
<th>Late OP</th>
<th>Pre OB</th>
<th>OB</th>
<th>Osteocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>?</td>
<td>?</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>?</td>
<td>?</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Bone sialoprotein</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD44</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>?</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cbfa 1</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

MSC, mesenchymal stem cell; OP, osteoprogenitor; OB, osteoblast; “?” not identified or not known; “-”, not present; “+”, present; “++”, present at higher levels (Aubin et al. 1995; Aubin 1998)

However, these markers are not restricted to osteoblasts. For example, hypertrophic chondrocytes can also express bone sialoprotein, osteopontin, osteonectin, PTH receptor,
alkaline phosphatase, osteocalcin, CD44 and galectin-3 (Aubin et al. 1995). CD44 is a glycoprotein receptor associated with cell adhesion, migration, tumor growth and metastasis. Its role in cell-cell, cell-matrix interactions and signal transduction is mainly through its principle ligand hyaluronan and likely through other ligands such as osteopontin and collagens (Cichy and Pure 2003). Galectin-3 is a β-galactoside-specific lectin. It has been found to be associated with pre-mRNA splicing, cell adhesion and cell growth regulation (Stock et al. 2003).

A1.4.3. Osteocytes and bone lining cells

Osteocytes are small-flattened cells terminally differentiated from osteoblasts by a mechanism that is not well understood. It is proposed that they form from some (10-20%) of the surface-lining osteoblasts, which become encased during the deposition of bone matrix (Aubin and Liu 1996; Nijweide et al. 1996; Compston 2001). As the most numerous specialised cell type in bone, the number of osteocytes is about 10 times as many as osteoblasts (Nijweide et al. 1996). The most striking phenotype of osteocytes is their typical morphology: cytoplasmic extrusions (canniculi), which form in vivo within bone matrix and in vitro on culture support. In bone, each osteocyte body is situated within an individual lacuna, the space in the bone matrix. Its long slender processes extend and radiate throughout the canalicular network to communicate, via gap junctions, with other osteocytes and with osteoblastic lining cells on the surface of bone (Marks and Hermey 1996; Nijweide et al. 1996; Compston 2001).

During the transition of the cells, osteocytes lose some osteoblastic phenotypes. They are smaller and have lower alkaline phosphatase activity than osteoblasts (Aubin and Liu 1996). Osteocytes express CD44 which is not detected in osteoblasts and bone lining cells (Hughes et al. 1994b; Nijweide et al. 1996), but is expressed in other cell types including osteoclasts (at lower level). The antibody against CD44 is used for isolation of osteocytes. Other osteocyte-specific monoclonal antibodies, some of which have unknown antigens, have also been produced (Nijweide et al. 1996).
Embedded osteocytes are thought to be at the end stage of differentiation in the osteoblastic lineage and may ultimately undergo apoptosis or be phagocytosed during bone resorption by osteoclasts (Compston 2001). They have limited ability to synthesize and resorb bone (Marks and Hermey 1996). Their central role is thought to be in sensing mechanical stimuli based on the recent observation that mechanical strain modulates the expression of the signaling messengers such as glucose-6-phosphate dehydrogenase, glutamate transporters, nitric oxide, and insulin-like growth factor in osteocytes (Noble and Reeve 2000; Compston 2001).

Bone lining cells are flat elongated cells with spindle-shaped nuclei and few cytoplasmic organelles. They are located on the surfaces of quiescent bone that is not undergoing remodeling, and is inactive in terms of bone matrix synthesis. According to their location, they may serve, together with the endosteal membrane, as the protection layer for the bone surface. Little is known about their physiological function. They are thought to be post-proliferative. However, under some circumstances, they can revert to an osteoblastic phenotype (Aubin and Liu 1996; Marks and Hermey 1996; Noble and Reeve 2000; Compston 2001).

### A1.4.4. Bone remodeling

Bone modeling is a process to construct bone, occurring in the period of the individual growth or during bone fracture healing. In contrast, bone remodeling provides a mechanism to maintain the mechanical integrity of bone and to modulate the homeostasis of ions, particularly calcium and phosphate. During bone remodeling, both bone resorption and formation take place. The coupling of the formation and resorption is important for the normal status of bone (Compston 2001).

Bone remodeling by osteoclasts and osteoblasts includes a series of events occurring in order: activation of bone surface from quiescent status, resorption, formation, mineralization and quiescence. The activation phase involves retraction of bone lining cells and digestion of the endosteal membrane to expose the mineralized bone surface (Compston 2001). The resorption cycle by osteoclasts includes migration and attachment of osteoclasts to the resorption site, polarization and formation of ruffled border and
sealing zone, resorption of bone matrix, and removal of the degradation products from the resorption lacuna (Vaananen et al. 2000). The attachment of osteoclasts to bone surface is facilitated by integrins such as αvβ3, which recognizes the Arg-Gly-Asp motif in the bone matrix proteins (Blair 1998; Compston 2001). The ruffled border formed by the highly infolded plasma membrane on the side of bone surface penetrates the bone matrix. The cytoskeleton of actin is organised into an attachment ring surrounding the ruffled border to form the sealing zone (Vaananen et al. 2000). Within the sealing zone, bone mineral is dissolved by the secreted HCl and bone organic matrix is degraded by lysosomal enzymes including collagenase and cathepsins released from osteoclasts to the resorption lacuna. Hydrogen ions for HCl are generated by carbonic anhydrase II (Blair 1998). The degradation products are transported by transcytotic vesicles through the osteoclasts from the resorption lacuna to the secretory domain and released to the extracellular space (Fig. 1-5) (Lerner 2000; Vaananen et al. 2000).

Bone resorption by osteoclasts is coupled both spatially and temporally by bone formation by osteoblasts. "In the normal adult skeleton, bone formation occurs only where bone resorption has previously occurred" (Baron 1999). After the cessation of the resorption phase, bone formation by osteoblasts begins. As the result of bone resorption, many factors are released from the bone matrix. These factors include TGF-β, BMPs, collagen and osteocalcin. They are likely to serve as chemotactic factors to attract osteoblasts or their precursors to the sites of the resorption defect, where osteoblast precursors proliferate and then differentiate to mature osteoblasts (Mundy 1999b). The osteoblasts converging at the bottom of resorption cavities deposit bone matrix (osteoid) into the resorption cavities until the cavities are filled. The osteoid is then mineralized.
In the normal adult skeleton, bone formation and resorption are almost quantitatively equivalent, thus maintaining the normal bone mineral density (Compston 2001).

**PART B: REGULATORS OF BONE CELL DIFFERENTIATION AND ACTIVITY**

**B1.1. INTRODUCTION**

The balance of bone remodeling requires the precise coupling of bone resorption and bone formation. Though the coupling mechanism is still not fully understood, significant progress has been made with the identification of many factors involved in these processes, particularly a trio of peptides identified in 1997 and 1998: osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL) and receptor activator of nuclear factor-κB (RANK) (Yasuda et al. 1999).

Via the production of regulators, cross-talk is established between osteoclasts and osteoblasts. Based on the producer cells and target cells, the action patterns of hormones are described as autocrine or paracrine. Autocrine is defined as denoting the effect of a hormone on cell that produces it, while paracrine is defined as that the effects of a hormone are localized to adjacent or nearby cell (Anderson et al. 1998).

**B1.2. OPG, RANKL AND RANK**

The studies of this trio of peptides have been focused on their molecular characteristics, expression patterns, biological activities and their involvement in bone diseases. The progresses in this area have been documented by many review articles in recent years (Hofbauer and Heufelder 1998; Green and Flavell 1999; Kong et al. 1999a; Suda et al. 1999; Takahashi et al. 1999; Yasuda et al. 1999; Aubin and Bonnelye 2000; Hofbauer et al. 2000; Marie et al. 2000; Riggs et al. 2000; Lories and Luyten 2001; Teitelbaum and Ross 2003).
B1.2.1. Identification and nomenclature

B1.2.1.1. Identification of OPG

OPG was identified independently by four research groups before the discovery of its ligand RANKL (Riggs et al. 2000). In 1997, Simonet et al reported a novel protein identified by sequence homology analysis during a fetal rat intestine cDNA sequencing project. They found that this protein belonged to the tumor necrosis factor receptor (TNFR) superfamily and named it “osteoprotegerin” (OPG) because of its activity of inhibiting osteoclast differentiation and protecting bone density (Simonet et al. 1997). At the same time, Tsuda et al also identified a secreted novel protein, termed “osteoclastogenesis inhibitory factor” (OCIF), from the conditioned medium of human embryonic lung fibroblasts by heparin affinity chromatography (Tsuda et al. 1997). This research group then realised that the OCIF was identical to OPG based on the cDNA sequence they obtained subsequently (Yasuda et al. 1998a). This protein was also identified by two other groups and was named “TNFR-like molecule 1” (TR1) (Tan et al. 1997; Kwon et al. 1998) and “follicular dendritic cell receptor 1” (FDCR-1) respectively (Yun et al. 1998).

B1.2.1.2. Identification of RANKL

Four research groups working on immunology and bone metabolism have contributed to the discovery of RANKL. In an attempt to search for apoptosis-regulatory genes, a novel member (designated “tumor necrosis factor-related activation induced cytokine E”, TRANCE) of the tumor necrosis factor (TNF) ligand family was cloned from the murine T cell hybridomas and human leukemia libraries (Shalhoub et al. 1999). At the same time, Anderson et al identified a novel receptor (designated RANK) and its ligand (designated RANKL) of the TNF receptor and ligand families by direct sequencing of a cDNA library constructed with the cDNA from the human bone-marrow-derived myeloid dendritic cells (Anderson et al. 1997). According to the reports from the above two groups, TRANCE and RANKL exhibited similar activity in regulating T cell growth and dendritic cell function. Almost at the same time, this protein was identified as the ligand for OPG from the cDNA expression libraries of ST2 cells and murine myelomonocytic
cell line 32D by another two groups using OPG as a probe (Lacey et al. 1998; Yasuda et al. 1998b). This newly cloned protein was termed “osteoclast differentiation factor” (ODF) and “OPG ligand” (OPGL) by the latter two groups respectively, and was demonstrated to have a critical role on osteoclast formation. Comparison of the predicted amino acid sequence revealed that this OPG-binding protein was identical to the TRANCE/RANKL (Yasuda et al. 1998b).

B1.2.1.3. Identification of RANK

In 1997, Anderson et al. initially identified RANK from humans. They then cloned the murine RANK gene by PCR amplification of the human homology followed by colony hybridization to a murine fetal liver epithelium cDNA library (Anderson et al. 1997). Another research group with the belief of the presence of a membrane-bound receptor for RANKL (termed ODF in the paper) subsequently cloned a peptide called “osteoclast differentiation factor receptor” (ODFR) from a mouse macrophage-like osteoclast progenitor cell line, C7. Nucleotide sequence revealed that ODFR was identical to RANK (Nakagawa et al. 1998). To demonstrate that RANK is a functional receptor for RANKL and a mediator for osteoclastogenesis, the effects of soluble RANK and the antibody against the extracellular domain of RANK were studied (please refer to B1.2.4.5 “Mode of action and signaling pathway”).

B1.2.1.4. Standard nomenclature

Since these peptides were identified independently by different research groups and different names have been given to the same peptide (Table 1-2), confusion and inconvenience have occurred in the research communications. Therefore, a standard nomenclature for these peptides has been recommended by a special committee appointed by the President of the American Society for Bone and Mineral Research. These are: receptor activator of nuclear factor-κB (RANK) for the membrane receptor, RANK ligand (RANKL) for the ligand, and osteoprotegerin (OPG) for the soluble receptor (Riggs et al. 2000). In this thesis, the recommended standard names are used regardless of the name(s) used in the original publications.
Table 1-2. Names used for a trio of peptides belonging to TNF ligand and receptor families (the recommended names are in **bold**.)

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Fil names</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soluble receptor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG</td>
<td>OsteoProteGerin</td>
<td>Simonet et al. 1997</td>
</tr>
<tr>
<td>OCIF</td>
<td>OsteoClastogenesis Inhibitory Factor</td>
<td>Tsuda et al. 1997; Yasuda et al. 1998</td>
</tr>
<tr>
<td>TR1</td>
<td>Tumor necrosis factor Receptor-like molecule 1</td>
<td>Tan et al. 1997; Kwon et al. 1998</td>
</tr>
<tr>
<td>FDCR-1</td>
<td>Follicular Dendritic Cell-derived Receptor-1</td>
<td>Yun et al. 1998</td>
</tr>
<tr>
<td>TNFRSF-11B*</td>
<td>Tumor Necrosis Factor Receptor SuperFamily 11 B</td>
<td>Riggs et al. 2000</td>
</tr>
<tr>
<td><strong>Ligand</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear factor-kB Ligand</td>
<td>Anderson et al. 1997</td>
</tr>
<tr>
<td>ODF</td>
<td>Osteoclast Differentiation Factor</td>
<td>Yasuda et al. 1998b</td>
</tr>
<tr>
<td>SOFA</td>
<td>Stromal Osteoclast Forming Activity</td>
<td>Yasuda et al. 1998b</td>
</tr>
<tr>
<td>TRANCE</td>
<td>Tumor necrosis factor-Related Activation induced Cytokine E</td>
<td>Wong et al. 1997b</td>
</tr>
<tr>
<td>OPGL</td>
<td>OsteoProteGerin Ligand</td>
<td>Lacey et al. 1998</td>
</tr>
<tr>
<td>TNFSF-11*</td>
<td>Tumor Necrosis Factor SuperFamily 11</td>
<td>Riggs et al. 2000</td>
</tr>
<tr>
<td><strong>Membrane receptor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator of Nuclear factor-kB</td>
<td>Anderson et al. 1997</td>
</tr>
<tr>
<td>ODAR</td>
<td>Osteoclast Differentiation and Activation Receptor</td>
<td>Hsu et al. 1999</td>
</tr>
<tr>
<td>ODFR</td>
<td>Osteoclast Differentiation Factor Receptor</td>
<td>Nakagawa et al. 1998</td>
</tr>
</tbody>
</table>

(∗: according to the systematic nomenclature system for the TNF/TNFR super families, http://www.gene.ucl.ac.uk/nomenclature/genefamily/tnftop.html)

### B1.2.2. Molecular and biochemical characteristics

#### B1.2.2.1. OPG

OPG exists in the forms of a monomer (~60 kD) and a homodimer (~120 kD), both of which have similar specific activity in inhibiting osteoclastogenesis *in vitro* (Tsuda et al. 1997). The homodimer is formed by a disulfide bond between the two Cys**400** moieties.
Substitution of Ser for Cys⁴⁰⁰ to block the formation of homodimer did not affect its biological activity (Yamaguchi et al. 1998; Takahashi et al. 1999). The OPG precursor from the human, rat or mice consists of 401 amino acid (aa) residues and displays the following structural features (Fig. 1-6) (Itoh and Nagata 1993; Tartaglia et al. 1993; Simonet et al. 1997; Tan et al. 1997; Tsuda et al. 1997; Morinaga et al. 1998; Yamaguchi et al. 1998).

Fig. 1-6. Schematic diagram of the structures of OPG, RANKL and RANK (Adopted from Yamaguchi et al. 1998, Xu et al. 2000 and Takahashi et al. 1999, respectively, with modification).

(1) signal peptide (aa1-21), which is hydrophobic and does not exist in the mature molecule (the mature peptide has 380 aa residues);
(2) N-terminal half (aa22-194), which is highly homologous to the counterparts of all members from the TNFR superfamily, and contains four cysteine-rich domains (domains 1-4) and four potential sites of N-linked glycosylation;

(3) C-terminal half (aa195-401), which represents domains 5-7. Domains 5 and 6 are called death domain homologous (DDH) regions, which is also a structural feature of TNFR 1, Fas, death receptor 3 (DR3) and the TNF-related apoptosis-inducing ligand (TRAIL) receptors. The death domains in the TNFR family were found to be responsible for the transduction of death signals. Domain 7 at the C-terminus is responsible for heparin-binding and the dimer formation.

As a secreted protein, OPG does not contain a membrane-spanning sequence, which can be seen in other known TNFR-like molecules. Truncation experiments showed that the sequence of 22-185 aa was critical for the osteoclastogenesis activity, while the portion from the C-terminus to 194 aa was not (Simonet et al. 1997).

B1.2.2.2. RANKL

RANKL consists of 317 aa in the human, 316 aa in the mouse and 318 aa in the rat, with the molecular weight of approximately 35 kD (Anderson et al. 1997; Wong et al. 1997b; Ross 2000; Xu et al. 2000). It is a type II transmembrane protein consisting of a cytoplasmic domain (48 aa in mouse), a hydrophobic transmembrane domain (21 aa) and an extracellular domain (247 aa) (Anderson et al. 1997). The extracellular domain, which is comprised of a long stalk region and a TNF-like core region, contains two predicted N-linked glycosylation sites (Fig. 1-6) (Wong et al. 1997b; Xu et al. 2000). However, glycosylation did not seem to be necessary for the activity, since the recombinant mouse RANKL expressed in E.coli and lacking glycosylation appeared to be as potent as the natural product (Dr. Colin Dunstan, Amgen Inc, personal communication). Based on the amino acid sequence deduced from the full-length cDNA, the mouse RANKL displays 85% and 96% identity to the human and rat RANKL respectively (Anderson et al. 1997; Xu et al. 2000). Sequence comparison of the extracellular domain revealed that the
mouse RANKL had similarity with TRAIL (20%), Fas ligand (FasL) (19%) and TNF (17%) (Wong et al. 1997b).

Within the stalk region of the extracellular domain, consensus cleavage sites for TNF-α convertase (TACE; Fig. 1-6) were identified at phenylalanine 139 in mouse, isoleucine 140 or alanine 145 in human. This might allow the specific metalloprotease to cleave the molecule to generate soluble RANKL released into the circulation (Lacey et al. 1998; Xu et al. 2000). Consistent with the putative cleavage sites, it was demonstrated that, like TNF-α, the mouse membrane-anchored RANKL precursor could be released from the plasma membrane due to the cleavage by this enzyme. The cleavage of the immunoprecipitated RANKL by the enzyme was similar to that in cultured cells (Lum et al. 1999).

Indeed, RANKL was found to exist in more than one form. In human and mouse, both soluble RANKL (31 kD) and membrane-bound RANKL (45 kD) were detected (Lacey et al. 1998; Kong et al. 1999b). A recent report showed that three isoforms of RANKL were detected in a mouse bone marrow stromal cell line ST2 and a preosteoblastic cell line MC3T3-E1: the original membrane-bound RANKL, RANKL with shorter intracellular domain, and RANKL without a transmembrane domain (thought to be the soluble form) (Ikeda et al. 2001). Furthermore, a trimeric form was seen in the truncated human RANKL retaining the extracellular domain (Willard et al. 2000).

Nagai et al reported the cloning of a cDNA encoding the secreted form of RANKL from the human cancer cells responsible for humoral hypercalcemia. This cDNA is composed of an unknown 5' sequence followed by the sequence completely identical to that of the RANKL extracellular domain (Nagai et al. 2000). This suggests that the soluble form of RANKL can also be derived from transcription and/or post-transcription stage(s) in addition to a post-translation stage as seen in the cleavage of the peptide by TNF-α convertase.
B1.2.2.3. RANK

The length of RANK is 616 aa in the human and 625 aa in the mouse, and the amino acid identity between them is 70%. It is a type I transmembrane protein comprising of a signal sequence (28 aa, in the human), an extracellular domain (184 aa), a transmembrane region (21 aa) and a cytoplasmic domain (383 aa). The extracellular domain, which contains two potential N-linked glycosylation sites and four predicted cysteine-rich pseudorepeats, shares significant homology in amino acid sequence with the other members from the TNFR family, with lowest 21% identity with CD27 and highest 40% identity with CD40. In contrast, the cytoplasmic domain has little similarity to other TNFR family members and has no homology with any other known protein sequences (Fig. 1-6) (Anderson et al. 1997; Takahashi et al. 1999).

B1.2.3. The genes, chromosomal localization and expression

This section focuses on the structure of the genes, the localization and regulation of the gene expression, and the signaling pathways for these newly identified peptides. Recent investigations linking these peptides and bone diseases are also presented.

B1.2.3.1. Gene organization and chromosomal localization

B1.2.3.1.1. OPG gene

By using radiation hybrid mapping or fluorescence in situ hybridization techniques, the OPG gene was localized to human chromosome 8q24.1 (Tan et al. 1997; Ludecke et al. 1999). The OPG gene is a single-copy gene spanning 29 kb in the human genome. Among the five exons of the gene, exon 4 seems to be the duplicate of a portion of exon 5 based on the sequence homology, and they encode the two death domains in the OPG molecule. Within the gene, intron 3 intervenes between the sequence for the cysteine-rich domain and the death domain. The 5.9 kb promoter region contains 12 putative binding sites for Cbfa1, the osteoblast-specific transcription factor, and the sites for a number of other transcription factors, such as transcription factor IID (TFIID). The transcription of the OPG gene seems to be under the control of two promoters, and can
initiate at one major and two minor positions (Morinaga et al. 1998; Yasuda et al. 1998a; Thirunavukkarasu et al. 2000).

Several polymorphisms in the OPG gene (*TNFRSF11B*) have been found to be associated with some bone disorders. Within the promoter, two single base pair mutations, A163-G and T245-G, have been linked to a higher incidence of osteoporotic vertebral fractures (Langdahl et al. 2002). Another polymorphism within exon 1, G1181-C, which results in an amino acid substitution from lysine to asparagines, was suggested to be linked to low bone mineral density (Wynne et al. 2002). Homozygous deletion of OPG gene was identified in two patients with juvenile Paget's disease (Whyte et al. 2002). Recently, a 3-pb inframe deletion in exon 3 of *TNFRSF11B*, which results in the loss of an aspartate residue in position 182, was found to be associated with idiopathic hyperphosphatiasis. This mutation leads to the inactivation of recombinant OPG protein (Cundy et al. 2002).

B1.2.3.1.2. **RANKL gene**

The RANKL gene is in human chromosome 13q14 and mouse chromosome 14 (Anderson et al. 1997; Wong et al. 1997b). The RANKL gene exhibits similarity in genomic organization with the other members of the TNF ligand family, particularly the CD40 ligand. In mouse, it is a single copy gene consisting of five exons and spanning approximately 40 kb in the genome (Kodaira et al. 1999). The predicted promoter region contains inverted TATA- and CAAT- boxes, repeat half-site for the vitamin D$_3$ and glucocorticoid receptors, and the potential binding sites for transcription factors, such as Cbfa1, octamer transcription factor-1 (Oct-1), lymphocyte function-associated antigen-1 (LFA-1), activator protein-2 (AP-2), TFII-1 and activated B-cell factor-1 (ABF-1) (Kitazawa et al. 1999a; Kodaira et al. 1999). Unlike the OPG gene, the RANKL gene only contains one major transcription initiation site, identified by the primer extension of the mRNA from ST2 cells treated with 1,25-(OH)$_2$D$_3$ and dexamethasone (Dex) (Kodaira et al. 1999). In the same cell line, only one transcript with the size of approximately 2.4 kb was detected when probed with the RANKL cDNA (Yasuda et al. 1998b). Sequence analysis showed that the full-length cDNA for mouse RANKL consists of 2237 bp (Wong et al. 1997b).
B1.2.3.1.3. RANK gene and Paget's disease

The RANK gene is mapped to human chromosome 18q22.1 (Anderson et al. 1997). The chromosomal localization of RANK gene has led to the investigation of the genetic locus related to the bone disorders Paget's disease of bone (PDB) and familial expansile osteolysis (FEO). Previous study has found that FEO, a rare bone disorder with great similarity to PDB, and some familial PDB were linked to the chromosome 18q21-22 (Hughes et al. 1994a; Sharpe 1996; Haslam et al. 1998), the similar location for RANK gene (Anderson et al. 1997). To investigate the relationship between the RANK gene and these bone diseases, Hughes et al have screened the promoter region, introns, exons, 5' and 3' untranslated regions of the RANK gene (*TNFRS11A*) from the patients with FEO and PDB. As a result, they identified two similar mutations in exon 1: an 18-base duplication (84dup18) insertion and a 27-base duplication (75dup27) insertion. The first mutation was found in the gene from all the affected members from two families with FEO and from one member from the family with PDB. This mutation was not found in the unaffected members of the FEO families, 90 individuals with sporadic PDB and 158 controls. The second type of mutation was identified in one of the four PDB families, but not in the other three families or five patients with sporadic PDB. The mutations also enhanced the RANK-mediated nuclear factor-κB signaling in the co-transfection experiment using the luciferase gene as a reporter. The enhanced signaling was explained as the result of higher intracellular accumulation of the mutant RANK due to the abnormal cleavage of the signal peptide and the impaired trafficking to the cell surface (Hughes et al. 2000).

However, the subsequent analysis from another group failed to confirm the results. Sparks et al showed that the RANK gene was not responsible for PDB in their study. They found no specific abnormalities in the RANK gene from the bone lesions and peripheral blood of patients with familial and sporadic PDB (Sparks et al. 2001). Another investigation with 28 PDB patients could not detect the PDB-related mutation in the RANK gene either (Wuyts et al. 2001).
B1.2.3.2. Patterns of expression

B1.2.3.2.1. OPG

In the human, high levels of OPG mRNA were detected in the thyroid, kidney, liver, heart and spinal cord, while lower levels were detected in lung, spleen, thymus, prostate, small intestine, colon, lymph node, trachea, pancreas, adrenal gland, bone marrow, brain, placenta and skeletal muscle. It was not detected in the peripheral blood, T cells and isolated osteoclasts. Its expression in B cells is controversial (Tan et al. 1997; Yasuda et al. 1998a; Yasuda et al. 1998b; Yun et al. 1998; Myers et al. 1999).

The expression of OPG in osteoblasts was up-regulated by osteoclastogenesis inhibitors such as calcium ions (Yasuda et al. 1999) and TGF-β1 (Murakami et al. 1998), and was down-regulated by osteoclastogenesis stimulators including Dex (Hofbauer et al. 1999; Brandstrom et al. 2001), 1,25-(OH)2D3, prostaglandin E2 (PGE2), PTH and IL-1. This indicates that the control of OPG expression is at least one of the mechanisms by which the above factors regulate osteoclastogenesis (Murakami et al. 1998). In ovariectomized rats, the OPG levels in trabecular bones were significantly reduced compared to that in the sham-operated animals (Yasuda et al. 1998b). The expression of OPG in the B cells was up-regulated by CD40, a member of TNFR family, suggesting a role of OPG in the immune system (Yun et al. 1998). Discrepant sizes of mRNA for OPG have been detected as the major transcript (2.4 kb) and the products (4.2 and 6.5 kb) of alternative splicing (Morinaga et al. 1998; Yasuda et al. 1998a; Yasuda et al. 1999).

Based on the identification of the putative binding sites for Cbfal in the promoter region of the OPG gene, the regulation of OPG expression at molecular level was studied in the transient transfection assays. The results showed that the over-expression of Cbfal in the human osteosarcoma U2OS cell line increased the OPG expression by 54%. This provided the evidence, for the first time, for the direct effect of Cbfal on the expression of osteoblast-derived factors involved in the bone resorption pathway (Thirunavukkarasu et al. 2000).
B1.2.3.2.2. RANKL

High levels of RANKL expression was detected in the activated T cells (Wong et al. 1997b; Kong et al. 1999b), mature dendritic cells (Wong et al. 1997a), osteoblasts, chondrocytes, mesenchymal cells (Kartsogiannis et al. 1999), lymph nodes, thymus (Wong et al. 1997b), trabecular bone (Yasuda et al. 1998b), brain and heart (Kartsogiannis et al. 1999), whilst lower levels were found in the bone marrow, spleen (Yasuda et al. 1998b), lung, liver, kidney and intestine (Kartsogiannis et al. 1999). It is not detected in the B cells (Wong et al. 1997b), bone lining cells and mature osteocytes (Kartsogiannis et al. 1999). In isolated osteoclasts, RANKL expression was only detected in two of the eight samples (Myers et al. 1999).

The expression of RANKL was modulated by many factors. In the T cell hybridomas, RANKL expression could be induced by antigen-receptor engagement and the expression was not inhibited by cycloheximide (a translation inhibitor), indicating a rapid onset of expression (Wong et al. 1997b). The stimulators of osteoclastogenesis, such as 1,25-(OH)_2D_3, PTH, PGE_2 and IL-11, increased the levels of RANKL mRNA (Yasuda et al. 1998b; Udagawa et al. 1999; Yasuda et al. 1999). In the transient transfection experiment using the construct of the RANKL promoter and the reporter gene (luciferase gene), 1,25-(OH)_2D_3 and Dex greatly enhanced the promoter activity, consistent with the presence of the binding sites for the receptors for these agents in the promoter region (Kitazawa et al. 1999a; Kitazawa et al. 1999b). In the mouse chondrogenic cell line, ATDC5, the expression of RANKL was up-regulated by Indian hedgehog, a secreted protein expressed in early hypertrophic chondrocytes (Akiyama et al. 1999). The experiments with signaling inhibitors indicated that RANKL expression was via the protein kinase C, phosphoinositide 3-kinase and calcineurin-mediated signaling pathways (Kong et al. 1999b).

The capability of the cell lines to support osteoclast formation is related to the reciprocal expression of OPG and RANKL. The calvaria-derived cell line MC3T3-G2/PA6 and bone marrow-derived stromal cell line ST2, both of which are capable of supporting osteoclastogenesis, showed increased RANKL levels and diminished OPG levels in
response to either 1,25-(OH)₂D₃+Dex or PGE₂+Dex. Whereas MC3T3-E1, a mouse osteoblastic cell line, which does not support osteoclastogenesis, did not show decreased OPG levels when treated with the same agents (although the RANKL levels were enhanced) (Nagai and Sato 1999). In the ST2 cells, RANKL mRNA was detected exclusively on the early passages in the presence of 1,25-(OH)₂D₃ and Dex, consistent with the passage-dependent capability of ST2 cells to support osteoclastogenesis. It was suggested that this passage-dependent phenomenon was due to the reduced RANKL expression caused by the methylation of the CpG region (also called CpG island) in the promoter of the RANKL gene (Kitazawa et al. 1999a; Kitazawa et al. 1999b). It was also reported that the undifferentiated hMS (2-15), a human marrow stromal cell line, could support the osteoclast formation from mouse bone marrow cells. However, this capability was lost as the cells differentiated with 35-fold decrease in RANKL/OPG ratio (Gori et al. 2000). It was found that the suppression of osteoclast formation from mouse bone marrow cells by the mineralized MC3T3-E1 cells was associated with the dramatically decreased levels of RANKL (Deyama et al. 2000).

The prostate and breast cancer cells could also produce a soluble form of RANKL and support osteoclast formation from the RAW 264.7 cell line (Zhang et al. 2001a). When these cancer cells were co-cultured with bone marrow cells, osteoclastogenesis was enhanced with the increased levels of RANKL and decreased levels of OPG in the system (Chikatsu et al. 2000). This suggests that RANKL signaling is involved in the skeletal metastasis of the cancers. Accordingly, treatment with OPG prevented the prostate tumor growth in bone by blocking the effect of RANKL, though OPG itself did not affect the viability, proliferation and survival of the cancer cells in vitro (Zhang et al. 2001a). In the co-culture system, the breast cancer cells were found to express RANKL and inhibit the production of OPG by the bone marrow cells (Chikatsu et al. 2000). These observations reveal a mechanism by which the skeletal lesions of cancers are formed during the skeletal metastasis processes.

**B1.2.3.2.3. RANK**

RANK expression was detected in the mouse isolated osteoclast progenitors, isolated mature osteoclasts and the mature osteoclasts in vivo (Fuller et al. 1998; Burgess et al. 2000).
1999; Hsu et al. 1999; Myers et al. 1999), human skeletal muscle, thymus, liver, colon, small intestine, adrenal gland, dendritic cells, B and T cells, foreskin fibroblasts (Anderson et al. 1997; Shalhoub et al. 2000; Lories and Luyten 2001; Varsani et al. 2003) and chondrocytes (Kong et al. 1999b). High levels of RANK expression were also detected in the freshly isolated and mature dendritic cells from the mouse bone marrow, lymph nodes and spleen (Anderson et al. 1997; Wong et al. 1997a).

The expression of RANK by the human dendritic cells was up-regulated by CD40 ligand, a member of the TNF ligand family. Its expression on the surface of the peripheral blood T cells activated by phytohemagglutinin-P or anti-CD3 could be consistently induced by IL-4 and TGF-β1 in a synergistic manner (Anderson et al. 1997). TGF-β1 stimulated osteoclast formation from the murine monocytic cell line, RAW264.7, in the presence of M-CSF and RANKL. At the same time, RANK mRNA levels were also increased by about 2 to 3.5-fold (Yan et al. 2001). TNF-α and RANKL were found to synergistically stimulate RANK expression in the mouse marrow macrophages in vitro (Zhang et al. 2001b).

**B1.2.4. The biological activities of OPG, RANKL and RANK**

OPG, RANKL and RANK play a critical role on bone cells (osteoclasts) development and function. In addition, they are also involved in the regulation of the cells in immune system. Through the production of RANKL, osteoclastogenesis is also under the regulation of the cells from immune system.

**B1.2.4.1. On osteoclastogenesis and bone density**

**B1.2.4.1.1. OPG, an inhibitor**

Following the identification of OPG, Tsuda et al tested its biological activity in the unfractionated mouse bone cell cultures and in the co-cultures of the spleen cells and supporting stromal cells. OPG at 1 to 40 ng/mL inhibited osteoclast formation induced by 1,25-(OH)2D3, PTH or IL-11, and inhibited the expression of the calcitonin receptor (Tsuda et al. 1997). In contrast, addition of the monoclonal anti-OPG antibodies
enhanced osteoclast formation from the murine bone marrow cells co-cultured with the osteoblasts in the absence of exogenous OPG, indicating the presence of endogenous OPG as an osteoclastogenesis inhibitor in the culture system (Kwon et al. 1998). In the mouse fetal long bone cultures, OPG inhibited bone resorption measured as calcium release into the medium (Kwon et al. 1998; Tsukii et al. 1998).

Simonet et al demonstrated that subcutaneous injection of the recombinant murine OPG [22-401]-Fc (10 mg/kg.day) to the mice increased the trabecular bone density in the area of tibial metaphysis (by 3-fold) and distal femur, and produced cartilage retention within the bone trabeculae. Administration of OPG also prevented the bone loss induced by ovariectomy in rats (Simonet et al. 1997). Systematic treatment of the recombinant human OPG (3 or 24 mg/kg.day) to mice significantly increased bone volume and mineral density in the proximal tibiae, and concurrently decreased the active osteoclast number in the distal femoral metaphysis (Yasuda et al. 1998a).

Hepatic over-expressors of OPG were generated by transforming the mice with the rat OPG coding region under the control of human apolipoprotein E gene (ApoE) promoter and its liver-specific enhancer element. These transformants exhibited osteopetrosis with the characteristics of increased density of long bones, vertebrae and pelvis. They had increased spleen weight, but had no abnormalities in external appearance, behavior, bodyweight and tooth eruption compared to the control (Simonet et al. 1997). In comparison, OPG-deficient mice showed severe decrease in bone strength and bone density characterised by the lack of trabecular bone in their femora, the porosity in the trabecular and cortical bones, and the marked thinning of the parietal bones of the skull (Bucay et al. 1998; Mizuno et al. 1998). In addition to the effect of OPG on bone, there was associated vascular calcification (which occurs in some osteoporosis patients) observed in the aorta and renal arteries of the OPG-deficient mice, most of which died or were moribund by six months after birth. This implies that OPG has a role in preventing pathological calcification of arteries, which are also sites of endogenous OPG production (Bucay et al. 1998).
B1.2.4.1.2. RANKL, a stimulator

In contrast to OPG, RANKL is a stimulator of osteoclastogenesis. In the presence of 1,25-(OH)$_2$D$_3$, it enhanced osteoclast formation from the mouse bone marrow cells and TRAP activity in a dosage-dependent manner with $ED_{50}$ of approximately 1 ng/mL (Lacey et al. 1998). Moreover, RANKL in combination with M-CSF was sufficient to replace the role of supporting osteoblastic cells to induce osteoclast formation from the mouse spleen cells, human peripheral blood monocytes and C7 cells (Quinn et al. 1998; Shalhoub et al. 1999). In the osteoclastogenesis system, it was found that RANKL acted directly on the osteoclast progenitors, based on the specific binding of $[^{125}\text{I}]$-RANKL to C7 cells (Yasuda et al. 1998b). RANKL also stimulated the mRNA levels of cathepsin K, a lysosomal protease expressed by osteoclasts, indicating its effect on bone-resorbing activity of the cells (Shalhoub et al. 1999). The effect of RANKL on osteoclastogenesis and pit formation could be abolished by the addition of OPG (Shalhoub et al. 1999).

The role of RANKL in vivo was examined by gene targeting. Mutant mice of $opgl^{-/-}$ (deficiency of the gene for RANKL) exhibited severe osteopetrosis characterised as increased density in the long bones, vertebral bodies and ribs, failure of tooth eruption and the absence of TRAP-positive multinucleated osteoclasts in the bone sections examined. These mutant mice grew normally until weaning at three weeks after birth due to the difficulty in eating food without normal teeth (Kong et al. 1999c).

B1.2.4.1.3. Osteoclastogenesis regulated by T cells via RANKL signaling

RANKL expression was detected in the T cells activated by concanavalin A, anti-CD3 antibodies or anti-CD28 antibodies (Wong et al. 1997b; Horwood et al. 1999; Kong et al. 1999b). Consistent with this, osteoclastogenesis occurred with the support of the activated T cells as seen in the following co-culture systems: murine spleen cells with human activated T cells (Horwood et al. 1999); spleen cells from the $opgl^{-/-}$ mice with fixed activated T cells from the wild type mice (Kong et al. 1999b); human peripheral monocytes with autologous activated T cells (Kotake et al. 2001). In addition, severe osteoporosis was seen in the $ctla4^{-/-}$ mice, in which T cells were spontaneously activated.
and RANKL levels were hence likely elevated. The bone loss in these mice could be prevented by the systemic treatment of OPG, indicating that RANKL has contributed to the elevated bone resorption in these animals (Kong et al. 1999b).

The role of activated T cells in osteoclastogenesis suggests that pathological conditions leading to higher amounts of activated T cells can contribute to osteoclastic bone resorption. In fact, elevated soluble RANKL levels were detected in the synovial fluid from patients with rheumatoid arthritis (Kotake et al. 2001), and increased osteoclast numbers and bone loss were seen in adjuvant-induced arthritis rats (Kong et al. 1999b).

During osteoclastogenesis induced by T cells, the function of RANKL is balanced by interferon (IFN)-γ, which is also expressed by T cells. IFN-γ blocks RANKL signaling and inhibits osteoclast formation by inducing the degradation of TNFR-associated factor (TRAF)-6, the signal transducer downstream of RANK (receptor for RANKL) (Takayanagi et al. 2000).

RANKL-independent mechanisms for osteoclastogenesis have also been suggested. In a stromal cell free osteoclast generating system with purified monocytes, saturating concentrations of OPG failed to suppress 30% of the osteoclast formation induced by the activated T cells, and the medium conditioned by the activated T cells exhibited an additive effect on osteoclastogenesis in the presence of maximal concentrations of RANKL and M-CSF (Weitzmann et al. 2001).

B1.2.4.2. On mature osteoclast activity

In an in vivo model, hypercalcemia was seen only 1 hour after injection of RANKL into the mice. This response was too fast to be explained as the consequence of osteoclast differentiation and suggested an activation of the existing osteoclasts (Burgess et al. 1999). In isolated rat osteoclast cultures, RANKL stimulated bone resorption activity in the absence of M-CSF, a well-documented osteoclast survival factor, indicating the direct effect of RANKL on mature osteoclasts activity exclusive of cell survival (Fuller et al. 1998; Lacey et al. 1998; Burgess et al. 1999).
Along with the stimulation of osteoclast activity, changes in cellular structure and cellular contents were seen upon treatment with RANKL. A few minutes after the addition of the peptide, increased pseudopodial motility and cell spreading were observed in the isolated osteoclasts (Fuller et al. 1998). The formation of an actin ring, which is a cytoskeletal structure required for osteoclasts to form a tightly sealed compartment for bone resorption, was also very rapidly formed (within 30 minutes of treatment). In addition, similar results can be produced by the addition of the agonistic antibodies against RANK, the receptor for RANKL. Actin ring formation, induced by RANKL, can be blocked by OPG, demonstrating the effect of RANKL-RANK signaling during this process (Burgess et al. 1999). Moreover, RANKL also induced the translocation of nuclear factor (NF)-κB (p65) to the nucleus, and concurrently enhanced the intracellular ionic calcium levels, likely as a consequence of increased osteoclast activity (Myers et al. 1999). RANKL is the first agent demonstrated to stimulate mature osteoclast activity without the presence of osteoblastic cofactor(s) (Fuller et al. 1998).

In addition to the role as an antagonist of RANKL, the direct effect of OPG on mature osteoclast activity has been studied with controversial results. It was reported that OPG only inhibited the osteoclast activity induced by RANKL without significant effect on the basal level (Lacey et al. 1998; Burgess et al. 1999). In some experiments, the inhibitory trend by OPG was seen in the absence of exogenous RANKL. This was ascribed to the interaction of OPG with the endogenous RANKL (Burgess et al. 1999). In contrast, addition of OPG to the crude osteoclast preparation from mouse bone marrow culture inhibited pit formation and resulted in disruption of the F-actin rings (Kwon et al. 1998). Hakeda et al also showed the direct effect of OPG on bone-resorption and actin ring formation in a highly pure (>95%) mature osteoclast preparation. In this cell preparation, no RANKL expression was detected and, therefore, the interaction between OPG and endogenous RANKL could be excluded. Furthermore, they detected a specific OPG binding protein (140 kD) on the surface of the isolated osteoclasts. This revealed a foundation for the direct action of OPG on osteoclasts (Hakeda et al. 1998).
B1.2.4.3. On osteoclast survival

It was reported that RANKL did not affect the survival of isolated mature osteoclasts with no difference seen in the osteoclast number between RANKL treatment and control (Lacey et al. 1998; Burgess et al. 1999). However, others have shown that RANKL did affect the survival of osteoclasts (Fuller et al. 1998; Suda et al. 1999; Udagawa et al. 1999). For example, addition of each of RANKL, osteoblasts, M-CSF or IL-1 significantly increased the survival of mouse osteoclasts recovered from bone marrow culture. In the absence of these agents, most of the cells died within 24 hours (Jimi et al. 1999; Udagawa et al. 1999). The discrepancy of results from the different groups can be due to the different culture conditions (Burgess et al. 1999).

In a mouse bone marrow culture, osteoclast apoptosis was increased by OPG, OPG truncate lacking the death domain homology (less potent) and by TGF-β1 in a dose- and time-dependent manner when they were added at the later stages of culture (Akatsu et al. 1998; Murakami et al. 1998). OPG did not directly affect apoptosis of highly pure osteoclast populations (Hakeda et al. 1998). Therefore, the effect of OPG on osteoclast survival may be via the neutralization of RANKL (Akatsu et al. 1998).

B1.2.4.4. Non-skeletal effects of OPG, RANKL and RANK

B1.2.4.4.1. Effects on cells in immune system

Recombinant RANKL significantly stimulated mouse and human dendritic cell cluster formation (Anderson et al. 1997; Wong et al. 1997a). It also increased the survival of the dendritic cells from mouse bone marrow and human peripheral blood by up-regulating the expression of Bcl-XL, an antiapoptotic protein (Wong et al. 1997a). Though it had no direct effect on B cell and T cell proliferation, it enhanced the capacity of dendritic cells to stimulate the naïve T cell growth in a mixed leukocyte system, revealing its role in the interaction between dendritic cell and T cells (Anderson et al. 1997; Wong et al. 1997a). Moreover, in the presence of TGF-β, which induced arrest of proliferation and death of the activated human T cells from peripheral blood, addition of RANKL significantly increased the number of viable activated T cells (Anderson et al. 1997). The activation of the signaling transducer by RANKL was cell-
type specific. It activated c-Jun N-terminal kinase (JNK) in T cells, but not in B cells and dendritic cells (Wong et al. 1997b). In RANKL-deficient mice, B cell and T cell differentiation and lymph node development were influenced due to the lack of RANKL (Kong et al. 1999c). These results demonstrated that RANKL is a functional molecule in lymph organogenesis, immune cell development and immune response.

B1.2.4.4.2. Other effects of OPG

OPG stimulated the proliferation of human foreskin fibroblasts in a dosage-dependent manner, consistent with the detection of the OPG receptor on these cells (Kwon et al. 1998). However, this effect was not reproducible by the other groups (Yasuda et al. 1999).

Although OPG itself did not show direct effects on the survival of cells (Hakeda et al. 1998), apoptosis was induced in the human kidney cell line 293-EBNA, which expressed the recombinant membrane-anchored OPG containing the transmembrane domain of Fas. This revealed the potential cytotoxicity of the DDH regions of OPG (Yamaguchi et al. 1998; Takahashi et al. 1999). OPG was found to bind to TNF-α. Therefore, the apoptosis induced by TNF-α was diminished by OPG (Bu et al. 2001).

Fig. 1-7. Mode of action for OPG/RANK/RANKL (Adopted from Nakagawa et al. 1998, with modification)
B1.2.4.5. Modes of action and signaling pathway

Based on the physiological effects and biochemical characteristics of OPG, RANKL and RANK, the mode of the action of these peptides on osteoclastogenesis was established (Fig. 1-7). In this system, the three molecules are involved in the signaling to osteoclasts. RANK is a membrane receptor expressed by osteoclasts or their precursors, while RANKL is a membrane-bound (or soluble) ligand expressed by osteoblasts. RANKL binds to its cognate receptor RANK and thus stimulates the development and function of osteoclasts. At the same time, OPG, a secreted peptide by osteoblasts, can also bind to RANKL and hence interrupts the interaction between RANK and RANKL. In this way, OPG acts as a down-regulator and is called a decoy receptor. In the culture systems with osteoclast precursors and osteoblasts, an osteotropic factor, such as PTH, 1,25-(OH)2D3, PGE2 or IL-11, is required to induce osteoclast formation. It is now known that these factors indirectly stimulate osteoclast formation by inducing RANKL expression by osteoblasts (Hofbauer and Heufelder 1998; Takahashi et al. 1999; Yasuda et al. 1999).

The effect of RANKL signaling to its cognate receptor RANK can be mimicked by polyclonal anti-RANK antibodies through inducing cross-linkage and clustering of the antigens (RANK). In contrast, the Fab fragments of the antibodies can only bind to the antigens without causing linkage and clustering. Therefore, the Fab fragments act as an antagonist by blocking the binding sites on the antigens (Nakagawa et al. 1998). It was found that, in the presence of M-CSF, RANKL or the polyclonal antibodies against the extracellular domain of RANK, induced osteoclastogenesis, while the Fab fragments completely blocked this process (Nakagawa et al. 1998; Hsu et al. 1999). Soluble RANK is also an antagonist for the RANK on cell surface by blocking the binding sites on RANKL. Direct administration of soluble RANK in normal mice or over-expression of soluble RANK-Fc fusion protein in the transgenic mice produced severe osteopetrosis accompanied by markedly reduced bone marrow space (Hsu et al. 1999). These results demonstrated that RANK is the sole cognate receptor for RANKL. This is different from some members of the TNF ligand family, such as TNF-α, Leukotriene-α (LT-α), LT-β and TRAIL, which utilise multiple receptors to transduce the signals (Nakagawa et al. 1998).
The signal transduction pathway downstream of RANK has been studied. It was shown that RANK, like some members from the TNFR family, interacts directly with TRAF to activate NF-κB and JNK. The binding sites for three TRAFs have been identified in the intracellular domain of RANK: between 336 and 454 aa for TRAF-6, and the C terminus for TRAF-2 and TRAF-5 (Darnay et al. 1998; Wong et al. 1998; Hsu et al. 1999). Deletion of TRAF binding sites in RANK suggests that RANK-TRAFs interaction is necessary for the activation of NF-κB, but not for the activation of JNK pathway (Darnay et al. 1998). However, the possibility of TRAF-independent NF-κB activation by RANK was also suggested by the over-expression of TRAF dominant negative proteins, which competed with TRAF for RANK (Wong et al. 1998).

B1.3. OTHER FACTORS INVOLVED IN BONE REMODELING

B1.3.1. Vitamin D

Vitamin D, which consists of a group of closely related secosteroids, is required for many biological processes including bone development and maintenance. The biologically active form of vitamin D, 1α,25 dihydroxyvitamin D₃ [1,25-(OH)₂D₃], is derived from vitamin D₃ (cholecalciferol) produced in skin from its precursor 7-dehydrocholesterol by ultraviolet irradiation. The conversion of vitamin D₃ to 1,25-(OH)₂D₃ includes two steps taking place in liver and kidney separately (Christakos 1996; Norman and Collins 1996).

The biological effect of 1,25-(OH)₂D₃ is mediated by its plasma binding protein, nuclear receptor and membrane receptor. Its effect on gene transcription is known to be mediated by the nuclear receptor, while the rapid response is postulated to be mediated by the membrane receptor via the activation of adenylate cyclase, phospholipase C and protein kinase C, and the regulation of calcium channel. The plasma binding protein functions as a carrier for vitamin D₃ and for its metabolites (Norman and Collins 1996).

The major target organs for 1,25-(OH)₂D₃ in mineral homeostasis are bone (development and remodeling), intestine (calcium absorption), kidney (calcium transport) and parathyroid glands (inhibition of PTH and PTH-related protein production) (Christakos
A predominant biological activity of 1,25-(OH)₂D₃ is to induce osteoclastogenesis in systems including bone marrow cultures, and co-cultures of osteoblastic cells and osteoclast precursors (Nakagawa et al. 1998). The receptor for 1,25-(OH)₂D₃ is present in osteoprogenitors, osteoblasts precursors and mature osteoblasts rather than in osteoclasts (Christakos 1996), and its stimulatory effect on osteoclasts is mediated by the osteoblastic factor now identified as RANKL (McSheehy and Chambers 1987; Nakagawa et al. 1998).

### B1.3.2. Sex hormones

The sex steroids involving in skeletal development and maintenance are estrogen, testosterone and, to a lesser extent, progestin (Harris et al. 1996). They play a role in the normal morphology of the skeleton, bone mineral density and strength. Sexual dimorphism of the skeleton, emerging during adolescence, is due to the impact of sex steroids. The normal closure of growth plates requires the presence of estrogen (Compston 2001). Its role in maintaining normal bone density can be seen in ovariectomized rats and monkeys, which show decreased ash weight, lower bone mineral density and increased volume of the medullary canal in tibiae (Harris et al. 1996). The insufficiency of estrogen is the major cause of osteoporosis in postmenopausal women. Therefore, estrogen replacement can prevent bone loss and reduce the risk of fracture for postmenopausal women (Compston 2001). The effect of estrogen in maintaining bone mass is likely related to the suppressed production of some cytokines such as IL-1, IL-6 and TNF-α, which are stimulators of osteoclast function (Reid 1999). Estrogen is also known to induce or increase the expression of osteoblastic genes for a number of peptides including IGF-I, TGF-β, BMP-6, type I collagen, osteocalcin, receptors for 1,25-(OH)₂D₃ and progesterone, etc. Its effect on osteoblast proliferation and alkaline phosphatase activity is conflicting (Compston 2001). Estrogen does not have direct effect on osteoclasts (Martin and Udagawa 1998; Compston 2001).
Testosterone also has positive effects on bone mineral density. Deficiency of testosterone caused by orchiectomy leads to increased bone turnover and rapid bone loss (Compston 2001). These changes can be prevented by the replacement of testosterone. Androgens promote osteoblast proliferation and differentiation (Orwoll 1996). However, further research is still required to elucidate the relationship between the age-related decrease in testosterone levels and reduced bone density in men (Compston 2001).

B1.3.3. Parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP)

PTH is synthesized in cells in the parathyroid gland. Mature PTH (1-84 aa) is produced and secreted upon the cleavage of the signal sequence (25 aa) and the "pro" region (6 aa) in the N-terminus from the primary translation product (115 aa). In the mature molecule, the region (25-34) is the principle-binding domain for its receptor, while the N-terminus (1-6) is responsible for the activation of receptor signal transduction. The N-terminus (1-34) is already fully functional (Chorev and Rosenblatt 1996).

The biological activity of PTH is mediated by two kinds of receptors: PTH/PTHrP receptor and PTH-2 receptor. PTH/PTHrP receptor is coupled to two distinct signal transduction pathways: adenylyl cyclase-cyclic AMP-protein kinase A and inositol triphosphate-cytosolic calcium-protein kinase (Chorev and Rosenblatt 1996). PTH plays a key role in the maintenance of calcium homeostasis by acting on bone to stimulate calcium release and on kidney to enhance calcium re-absorption (Fitzpatrick and Bilezikian 1996). PTH directly stimulates osteoblast proliferation, and indirectly enhances osteoclast formation by stimulating the production of RANKL by the osteoblast (Fitzpatrick and Bilezikian 1996; Inoue and Matsumoto 2000) and reciprocally inhibiting the expression of OPG (Ma et al. 2001). In addition to its effect on bone, PTH is also involved in the regulation of phosphorus metabolism and smooth muscle relaxation (Fitzpatrick and Bilezikian 1996).

PTHrP was initially identified as a causal factor of humoral hypercalcemia of malignancy in 1980s. The mRNA for PTHrP has at least three different sizes due to alternative
splicing. These mRNA encode the isoforms of 139, 141 and 173 aa with an identical sequence from the N-terminus until aa 139 (Inoue and Matsumoto 2000). The human PTHrP gene had similar intron/exon boundaries with the PTH gene and was mapped to chromosome 12p11-12, the analogous region of 11p15 where the PTH gene is located. PTH and PTHrP share 8 of the first 13 aa in the N-terminus and the portions of 13-34 aa have considerable similarity in three-dimensional configuration. These suggest that the PTH gene and PTHrP gene are derived from a common ancestral origin (Silver and Kronenberg 1996; Yang and Stewart 1996).

The specific receptor for PTHrP has yet to be identified. However, it can function by binding to the PTH/PTHrP common receptor via its N-terminal portion (1-36), and produce PTH-like effects, such as the development of hypercalcemia (Inoue and Matsumoto 2000), stimulation of bone resorption, inhibition of calcium excretion and relaxation of smooth muscle (Martin et al. 1997). PTHrP also displays effects distinct from PTH. It regulates placental calcium transport to maintain the maternal-fetal calcium gradient. This activity lies in the midregion of the molecule (Moseley and Martin 1996; Martin et al. 1997). In addition, the C-terminal fragment inhibits bone resorption by osteoclasts (Inoue and Matsumoto 2000).

**B1.3.4. Calcitonin (CT)**

Calcitonin (CT), together with estrogen and alendronate, is one of the three agents approved by the U.S. Food and Drug Administration (FDA) for treatment of osteoporosis in postmenopausal women (Cosman and Lindsay 1998). It is produced by the parafollicular cells of the thyroid gland and was initially identified as a hypocalcemic factor in the early 1960s (Wallach et al. 1990; Goldring 1996). Mature CT consists of 32 aa and is derived from a precursor of 141 aa by post-translational processing. Within the single-chain peptide, there is a disulfide bridge formed between the two cysteine residues at positions 1 and 7 of the N-terminus. The gene for CT was mapped to human chromosome 11. Amylin, and CT-gene-related peptides I and II are also products of this gene family consisting of the genes *CALC-I* to *CALC-IV* (Becker et al. 1996).
The receptor for CT was identified in a variety of cell types, tissues and organs, including osteoclasts, lymphocytes, renal tubular cells, brain, placenta, ovary and testis (Goldring 1996). CT displays a wide spectrum of biological activities, particularly in bone metabolism. It potently inhibits bone resorption by directly inhibiting osteoclast activity (Martin 1999). Therefore, it has been used as a therapeutic agent to reduce bone loss for the patients with high bone turnover, such as those with Paget’s disease. CT was found to increase the calcium and phosphate excretion in kidney, which is a principle site of CT degradation. The affects of CT in bone and kidney lead to the decrease of serum calcium levels (Becker et al. 1996). The function of CT in the central nervous system has also been documented. It affects body temperature and pain-relief. In addition, CT acts in the digestion system, regulating the secretion of gastric acid, pepsin, pancreatic amylase and pancreatic polypeptide, etc (Becker et al. 1996).

Long-term treatment with CT leads to a resistance to this hormone, a phenomenon known as "escape" with a gradual decrease in the response. The mechanism for this is not fully understood yet, but the prevalent explanations include the production of the antibodies and suppression of the receptor expression (Gennari and Camporeale 1997; Martin 1999).

B1.3.5. Summary of the effects of some osteotropic factors

There are many other factors involved in bone metabolism. Their effects are summarised in Table 1-3.

**Table 1-3.** Summary of some factors involved in bone metabolism

<table>
<thead>
<tr>
<th>Factors</th>
<th>Osteoblasts/ bone formation</th>
<th>Osteoclasts/ bone resorption</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-(OH)2D3</td>
<td>+</td>
<td>+</td>
<td>Murakami et al. 1998</td>
</tr>
<tr>
<td>adrenomedullin</td>
<td>+</td>
<td>-</td>
<td>Cornish et al. 1997a</td>
</tr>
<tr>
<td>Amylin</td>
<td>+</td>
<td>-</td>
<td>Cornish et al. 1995; Reid and Cornish 1996</td>
</tr>
<tr>
<td>ATP</td>
<td>+</td>
<td>+</td>
<td>Morrison et al. 1998</td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td>-</td>
<td>+</td>
<td>Hughes et al. 1995</td>
</tr>
<tr>
<td>Calcium</td>
<td>-</td>
<td>-</td>
<td>Kameda et al. 1998; Kanatani et al. 1999</td>
</tr>
<tr>
<td>CGRP</td>
<td>+</td>
<td>-</td>
<td>Reid and Cornish 1996</td>
</tr>
</tbody>
</table>

(To be continued)
<table>
<thead>
<tr>
<th>Factors</th>
<th>Osteoblasts/ bone formation</th>
<th>Osteoclasts/ bone resorption</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dex</td>
<td>-</td>
<td>+</td>
<td>Hughes-Fulford et al. 1992; Hofbauer et al. 1999</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>±</td>
<td>+</td>
<td>Povolny and Lee 1993; Martin and Udagawa 1998; Quinn et al. 1998</td>
</tr>
<tr>
<td>IGF-1</td>
<td>+</td>
<td>+</td>
<td>Stashenko et al. 1989; Santhanagopalan and Dixon 1999</td>
</tr>
<tr>
<td>IL-1</td>
<td>+</td>
<td></td>
<td>Roodman 1999</td>
</tr>
<tr>
<td>IL-4</td>
<td>-</td>
<td></td>
<td>Roodman 1999</td>
</tr>
<tr>
<td>IL-6</td>
<td>+</td>
<td></td>
<td>Martin and Udagawa 1998</td>
</tr>
<tr>
<td>IL-11</td>
<td>+</td>
<td></td>
<td>Girasole et al. 1994</td>
</tr>
<tr>
<td>IL-17</td>
<td>+</td>
<td></td>
<td>Udagawa et al. 1997; Kotake et al. 1999</td>
</tr>
<tr>
<td>IL-18</td>
<td>+</td>
<td>-</td>
<td>Roodman 1999; Makiishi-Shimobayashi et al. 2001</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>-</td>
<td>-</td>
<td>Nanes et al. 1990; Roodman 1999</td>
</tr>
<tr>
<td>Leptin</td>
<td>+</td>
<td>-</td>
<td>Cornish et al. 2002b; Gordeladze et al. 2002</td>
</tr>
<tr>
<td>M-CSF</td>
<td>+</td>
<td></td>
<td>Sarma and Flanagan 1996; Martin and Udagawa 1998</td>
</tr>
<tr>
<td>Nitric acid</td>
<td>-</td>
<td></td>
<td>Roodman 1999</td>
</tr>
<tr>
<td>Neomycin</td>
<td>-</td>
<td></td>
<td>Kameda et al. 1998; Kanatani et al. 1999</td>
</tr>
<tr>
<td>PGE₂</td>
<td>±</td>
<td>+</td>
<td>Pilbeam et al. 1996; Martin and Udagawa 1998</td>
</tr>
<tr>
<td>TGF-α</td>
<td>±</td>
<td>+</td>
<td>Ibbotson et al. 1986; Kukreja et al. 1993</td>
</tr>
<tr>
<td>TGF-β</td>
<td>+</td>
<td>-</td>
<td>Chenu et al. 1988; Gurlek and Kumar 2001</td>
</tr>
<tr>
<td>TNF-α and β</td>
<td>+</td>
<td>+</td>
<td>Frost et al. 1997; Roodman 1999</td>
</tr>
</tbody>
</table>

("+", promotion; "-", inhibition)
PART C SCOPE OF THE CURRENT INVESTIGATION

In the field of osteoclast biology, the most important discovery over the decades has been the delineation of the RANKL, RANK and OPG system (Yasuda et al. 1999). As reviewed in the Section B1.2 of this chapter, many studies in recent years have been focused on their roles in osteoclast development and activity, their involvement in bone disorders and their functions in immune system. However, further exploration is still required, in particular, in the areas of the structure-activity relationship and their involvement in the action of other osteotropic factors.

In the area of bone cell biology, the search for the new regulators has led to the identification of many osteotropic molecules including FGF-1 and -2 (Dunstan et al. 1999; Shimoaka et al. 2002). FGF is a family with at least 24 known members (Draper et al. 2003; Fischer et al. 2003). It is possible that other factors in this family also have an impact on bone cells. In this regard, FGF-8 is a particular candidate because of its demonstrated effects on limb bud development (Lewandoski et al. 2000). Therefore, its potential role in bone cell biology is to be explored in the current study.

Over the last decade or so, it has been clear that there are important relationships between fat mass and bone density, suggesting that some of the factors that regulate appetite and which in turn are regulated by adiposities have potential effects on bone (Reid 2002). One of these factors is leptin, which has a dramatic effect on bone cell function and bone density when centrally administered (Cornish et al. 2002b). This raises the possibility that other neuropeptides that are influenced by leptin and have effects on appetite might have an effect on bone cell activity. In this regard, the skeletal effects of α-MSH were studied.

Whereas the three lines of investigation outlined in the above paragraphs have been logical extensions of major recent developments in bone cell biology, these factors have not previously been investigated in the assays reported in this thesis and the factors may play important roles in bone cell biology. In addition, the current research scope has
been further extended to bioprospect for novel osteotropic activities within one of the richest biological fluids—milk. The investigation started from the assessment of activities in crude fractions and then went on toward the actual identification of molecules that are active on bone.

Overall, this project has studied the osteotropic effects of RANKL, OPG, FGF-8a, α-MSH, milk fractions and their components including lactoferrin, defining their novel activity and potential application. After the osteotropic activities of the above agents were demonstrated, their effects on RANKL and OPG expression was studied to look into the mechanisms by which they act on bone cells.
CHAPTER 2: MATERIALS AND METHODS

This chapter lists most of the general materials and methods used in the current research. Those used for the particular projects are described in the individual chapters.

2.1. ANIMALS AND CELL LINES

The mice (Swiss) and rats (Wistar) were supplied by the animal resources unit of the School of Medicine, University of Auckland. The osteoblastic cell line UMR-106.06 was kindly provided by Professor Jane Moseley, St. Vincents Institute for Medical Research, Melbourne, Australia. The study had the approval of the local institutional review board.

2.2. CULTURE MEDIA AND CULTURE CONDITIONS

All the media purchased as powders were dissolved in ultra-high purity water (Milli Q water) and were made up with the additional ingredients indicated below. The Milli Q water was prepared by using the Elgastat UHP unit from Elga Ltd (High Wycombe Bucks, England) and had the purity of >15-Megohm-cm resistivity at 25°C and <0.0001 AU at 254 nm. These media were sterilized by filtering through a 0.20 µm membrane (Minisart, Cat # 16534, Santorius AG, Göttingen, Germany). Unless otherwise indicated, all the media used contained 100 units/mL of penicillin and 100 µg/mL of streptomycin (Gibco, Cat # 15140-122, Life Technologies, Grand Island, NY), and all cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

* Minimum Essential Medium (MEM, Gibco, Cat # 41500-034), 0.25% sodium bicarbonate, 0.029% L-glutamine
* Medium199 (Gibco, Cat # 31100-035), 0.22% sodium bicarbonate, 0.01% L-ascorbic acid
* Minimum Essential Medium Alpha Medium (αMEM, Gibco, Cat # 12000-022), 0.22% sodium bicarbonate
* Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Cat # 12100-046), 0.22% sodium bicarbonate

* Earle’s MEM (aqueous, Gibco, Cat # 41090-036).

All the media above were the products of Life Technologies, Grand Island, NY and were purchased from Invitrogen NZ Ltd., Auckland.

### 2.3. SOLUTIONS FOR CELL CULTURE, FIXATION AND STAINING

* Phosphate buffered saline (PBS): 137 mM NaCl, 7.9 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH 7.4, sterilized by autoclaving or filtering

* Fixative for osteoclasts from bone marrow culture: The fixative was made of 0.01 M sodium citrate buffer (pH 6.0), acetone and 37% formaldehyde in the proportion of 25:65:8 (v/v). This fixative is stable at 4 °C for up to two months.

* Fixative for isolated osteoclasts in pit formation assay: 2.5% gluteraldehyde in PBS

* Pit staining solution: 1% toluidine blue in 1% di-sodium tetraborate

### 2.4. GROWTH REGULATORS

* Salmon calcitonin (sCT): Miacalcic®, Novartis International AG, Basel, Switzerland; purchased from Auckland Hospital Pharmacy

* Bovine parathyroid hormone (PTH) was kindly provided by Dr Peter Barling, School of Biological Sciences, University of Auckland.

* Rat amylin (rAmylin): rAmylin was kindly provided by Professor Gath Cooper, School of Biological Sciences, University of Auckland, or was purchased from Peptides International Inc, Louisville, KY, USA (Cat # PAM-4220-v) and from Bachem AG, Bubendorf, Switzerland.

* 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]: Roche Pharmaceuticals Ltd., Basel, Switzerland
2.5. SERA AND ENZYMES

* Bovine serum albumin (BSA): Cat # ABRE010, Immuno-chemical Products Ltd., Auckland
* Foetal bovine serum (FBS): Gibco, Cat # 10091-148 and Cat # 26140-079, Life Technologies, Grand Island, NY, USA; purchased from Invitrogen NZ Ltd., Auckland
* Normal horse serum: Invitrogen Co., Carlsbad, CA, USA
* Collagenase (Type II): Cat # C6885, Sigma Chemical Co., St. Louis, MO, USA; purchased from Sigma-Aldrich Ltd., Auckland
* Trypsin-EDTA: Gibco, Cat # 15400-054, Life Technologies, Grand Island, NY, USA; purchased from Invitrogen NZ Ltd., Auckland

2.6. SOLUTIONS AND REAGENTS FOR MOLECULAR BIOLOGY WORK

* DNA/RNA agarose gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% (v/v) glycerol
* First strand buffer (5 x) for reverse transcription: Part # Y00146, Invitrogen NZ Ltd., Auckland
* PCR buffer (10 x): Part # Y02616, Invitrogen NZ Ltd., Auckland
* TBE buffer: 90 mM H$_3$BO$_3$, 2 mM Na$_2$EDTA, 90 mM Tris-HCl (pH 8.0)
* Agarose: Gibco, Life Technologies, Grand Island, NY
* Boric acid: Cat # 76324, USB, Cleveland, Ohio
* Dithiothretol (DTT) solution (0.1 M): Part # Y00147, Invitrogen NZ Ltd., Auckland
* DNA ladders (100 bp and low mass): Invitrogen NZ Ltd., Auckland
* dNTP: Invitrogen NZ Ltd., Auckland
* Ethidium bromide (10 mg/mL): Gibco, Cat # 15585-011, Life Technologies, Grand Island, NY
* Eukaryotic 18S rRNA endogenous control for real-time PCR: Cat # 4319413E, Applied Biosystems, Foster City, CA, USA
* MgCl$_2$ solution (50 mM): Part # Y02616, Invitrogen NZ Ltd., Auckland
* QIAquick gel extraction kit: Cat # 28704, QIAGEN, Valencia, CA
* Random primers: Cat # 48190-011, Life Technologies, Grand Island, NY
* RNAlater™: Ambion, Austin, Texas
* RNase-Free DNase Set: Cat # 79254, GIAGEN, Valencia, CA
* RNA OUT™ (ribonuclease inhibitor, recombinant): Cat # 10777-019, Invitrogen NZ Ltd., Auckland
* RNase Mini Kit: Cat # 74104, QIAGEN, Valencia, CA; purchased from Biolab Scientific Ltd., Auckland
* Superscript™ reverse transcriptase: Invitrogen NZ Ltd., Auckland
* Platinum® Taq DNA polymerase: Cat # 10966-034, Invitrogen NZ Ltd., Auckland
* Taq Man® Universal PCR master mix (2 x): Cat # 4304437, Applied Biosystems, Foster City, CA, USA
* Tris: BDH Laboratory Supplies, Poole, England

2.7. OTHER REAGENTS

* 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) used for osteoblast alkaline phosphatase staining: Cat # B-565, Sigma Chemical Co., St. Louis, MO, USA
* Leukocyte acid phosphatase kit used for tartrate-resistant acid phosphatase (TRAP) staining: Cat # 387-A, Sigma Diagnostics Inc., St. Louis, MO, USA
* Substrate for alkaline phosphatase (4-methylumbelliferyl phosphate): Molecular Probes Inc., Eugene, OR, USA
* Bicinchoninic acid (BCA) protein assay kit: Cat # 23225, Pierce Chemical Co., Rockford, IL, USA; purchased from Global Science and Technology Ltd., Auckland

* Antibiotics: mix of penicillin and streptomycin (Cat # 15140-122): Gibco, Life Technologies, Grand Island, NY, USA; purchased from Invitrogen NZ Ltd., Auckland
* Isotopes: 45CaCl2 (Cat # CES3) and [methyl-3H]-thymidine (Cat # TRK120-1 mCi/5 mCi) were the products of Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, England, and were purchased from Amersham Pharmacia Biotech Ltd., Auckland.
* Scintillant (BCS): Cat # NBCS 104, Amersham International plc, Amersham, Buckinghamshire, England; purchased from Life Sciences NZ Ltd., Auckland

* Acetone: AnalR, BDH Laboratory Supplies, Poole, England
* Ascorbic acid; 2-phosphate sesquimagnesium salt, Cat # A8960, Sigma Chemical Co., St. Louis, MO, USA
* L-ascorbic acid (free acid): Cat # A4544, Sigma Chemical Co., St. Louis, MO, USA; purchased from Sigma-Aldrich Ltd., Auckland
* Diethyl ether: AnalR, BDH Laboratory Supplies, Poole, England
* Ethylenediaminetetra-acetic acid disodium salt (EDTA-Na2): AnalR, BDH Laboratory Supplies, Poole, England
* Ethanol (absolute): AnalR, BDH Laboratory Supplies, Poole, England
* Halothane: NZ Pharmacology Ltd., Christchurch
* Potassium chloride: Sigma Chemical Co., St. Louis, MO, USA; purchased from Sigma-Aldrich Ltd., Auckland
* Potassium phosphate monobasic (anhydrous): Sigma Chemical Co., St. Louis, MO, USA; purchased from Sigma-Aldrich Ltd., Auckland
* di-Sodium tetraborate: BDH Laboratory Supplies, Poole, England
* Sodium bicarbonate: Sigma Chemical Co., St. Louis, MO, USA; purchased from Sigma-Aldrich Ltd., Auckland
* Sodium phosphate dibasic (anhydrous): Sigma Chemical Co., St. Louis, MO, USA; purchased from Sigma-Aldrich Ltd., Auckland
* Thiomerasol: purchased from Auckland Hospital Pharmacy
* Toluidine blue: BDH Laboratory Supplies, Poole, England
* Trichloroacetic acid (TCA): AnalR, BDH Laboratory Supplies, Poole, England
* Tween 20: BDH Laboratory Supplies, Poole, England

2.8. EQUIPMENT

* Balances: Mettler H20T and AC 100 balances, Mettler Instrument AG., Zürich, Switzerland; CAHN electrobalance, model 4100, Ventron Instruments Co., Cerritos, CA, USA
* ELISA reader: Wallace 1420 Multiplate reader, PerkinElmer Life Sciences/Wallac Oy, Turku, Finland
* Gel documentation system: Bio-Rad Laboratories, Inc., Hercules, CA, USA
* Homogenizer: Arthur H. Thomas Co., Philadelphia, PA, USA
* Incubators: Model 3546 CO$_2$ incubator, Forma Scientific Inc., Parker, CO, USA; Heraeus HERAcell CO$_2$ incubator, Kendro Laboratory Products, Hanau, Germany
* Laminating hoods: Model CF 43S laminating hoods, Clemco, Australia; Model BH143 laminating hood, Gelman Sciences, Australia
* Low speed saw: Isomet™, Buehler Ltd., Lake Bluff, IL, USA
* Micro-centrifuge: Heraeus Biofuge Pico, Kendro Laboratory Products, Hanau, Germany
* Microscopes: Confocal Laser Scanning Microscope: TCS 4D, Leica, Wetzlar, Germany; Olympus BX 50, Olympus BH-2 and Olympus CK40, Olympus Optical Co. Ltd., Tokyo, Japan;
* Milli Q water unit: Elgastate UHP, Elga Ltd., High Wycombe Bucks, England
* PCR machine: Mastercycler, Eppendorf, Hamburg, Germany
* pH meter: Denver Instrument Co., Arvada, CO, USA
* Pipettes and multi-pipettes: Eppendorf, Hamburg, Germany
* Real-time PCR machine: ABI PRISM® 7700 Sequence Detection System, Applied Biosystems, Foster City, CA, USA
* Scintillation counters: LKB 1217 Rackbeta liquid scintillation counter, and 1450 Microbeta Trilux liquid scintillation and luminescence counter, Wallac Oy, Turku, Finland
* Sonicator: Wave energy systems, model BS132-6, Newtown, PA, USA
* Ultra-low temperature freezer: MDF-U71V, Sanyo, Japan
* UV photometer: BioPhotometer, Eppendorf, Hambury, Germany

2.9. OTHER MATERIALS

* 35 mm and 100 mm sterile Petri dishes: Falcon, Becton Dickinson and Co., Franklin Lakes, NJ, USA
* 6-well, 24-well and 96-well sterile plates: Greiner Labortechnik, Frickenhausen, Germany; purchased from Raylab NZ Ltd., Auckland

* 12-well and 48-well sterile plates: Corning Inc., New York, USA

* Chamber slides: LAB-TEK, Cat # 17745, Nalge Nunc International, Naperville, IL, USA; purchased from Invitrogen NZ Ltd., Auckland

* Sterile tissue culture flasks (75 cm²), Cat # COR430641, Corning Inc., New York, USA

* ELISA plates: opaque high protein binding LIA plates, Greiner Labortechnik, Frickenhausen, Germany

* MicroAmp® 96-well reaction plates for real-time PCR: Cat # N801-0560, Applied Biosystems, Foster City, CA, USA

* Optical adhesive covers for real-time PCR: Cat # 4311971, Applied Biosystems, Foster City, CA, USA

* Scintillation vials (5 mL, Cat # WLL 1200-421) for 1450 Microbeta Wallac Trilux liquid scintillation and luminescence counter: purchased from Biolab Scientific Ltd., Auckland

* Scintillation vials (6 mL) for LKB 1217 Rackbeta liquid scintillation counter: purchased from Global Science and Technology Ltd., Auckland

* Glass scintillation vials: Epsom Glass Industries Ltd., Epsom, England

* 15 mL sterile centrifuge tubes: Cellstar, Cat # 188.271, Greiner Labortechnik, Frickenhausen, Germany or Sarstedt, Cat # 62.554, 502, Artiengesellschaft & Co., Nümbrecht, Germany

* 50 mL sterile centrifuge tubes: Falcon, Cat # 35-2070, Becton Dickinson and Co., Franklin Lakes, NJ, USA; purchased from Baxter Healthcare Ltd., Auckland

* 120 mL sterile containers: Oxford, Cat # 207026, purchased from Medic Corporation Ltd., Auckland

* 250 mL sterile containers: Cat # LBS 32254G, purchased from Biolab Scientific Ltd., Auckland

* 1 mL, 10 mL and 30 mL sterile syringes: Becton Dickinson and Co., Franklin Lakes, NJ, USA

* 0.2 µm sterile filters: Minisart, Cat # 16534, Santorius AG., Göttingen, Germany
2.10. MOUSE BONE MARROW CULTURE AND HISTOCHEMICAL STAINING OF OSTEOCLASTS

Bone marrow was obtained from the long bones of normal Swiss male mice aged 4-6 weeks. According to the experimental procedures described previously (Cornish et al. 2001b), the mice were sacrificed by cervical dislocation while under halothane (NZ Pharmacology Ltd., Christchurch) anaesthesia. The femurs and tibiae were aseptically removed and dissected free of adherent soft tissues. The marrow cavity was flushed with αMEM (Gibco, Life Technologies, Grand Island, NY) through a sterile 23G needle and the marrow cells were collected in a 50 mL centrifuge tube. After centrifugation at 1200 rpm for 2 minutes, the cell pellet was suspended in 15 mL of αMEM containing 10% FBS (Gibco, Cat # 26140-079, Grand Island, NY). The marrow cells were incubated in two 100 mm Petri dishes (7.5 mL/dish) for 2 hours and then the non-adherent cells were collected by spinning again at 1200 rpm for 2 minutes.

The cells were re-suspended in αMEM containing 15% FBS and seeded onto 48-well plates (Costar, Corning Inc., New York) at the density of $10^6$ cells/mL (0.5 mL/well, day 0). The cultures were fed 0.5 mL of fresh medium on day 2 and then replaced with 0.5 mL of fresh medium on day 4. Test factors and 1,25(OH)_{2}D_{3} (Roche Pharmaceuticals Ltd., Basel, Switzerland, at the final concentration of $10^{-8}$M) were added on days 0, 2 and 4 unless otherwise indicated [Negative controls remained in the absence of 1,25(OH)_{2}D_{3}].

On day 7, the cells were fixed with citrate-acetone-formaldehyde (25:65:8, vol/vol) for 30 seconds and then stained for TRAP by using the leukocyte acid phosphatase kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instruction. TRAP-positive cells with three or more nuclei were counted as osteoclast-like cells under an Olympus CK 40 inverted microscope.

2.11. MOUSE CALVARIAL ORGAN CULTURE

This model was used to measure bone resorption and $[^{3}H]$-thymidine incorporation based on the protocol described previously (Wilson et al. 1981; Reid et al. 1990). One- to
three-day-old Swiss mice were injected subcutaneously with $^{45}$CaCl$_2$ (3 μCi/mice, Amersham Pharmacia Biotech UK Ltd.) 3 days before dissection. Then the hemicalvariae consisting of the frontal and parietal bones were dissected out aseptically along the sutures and free of surrounding soft tissues.

The bone was cultured on metal mesh grids in a 35 mm plastic Petri dish (Falcon, Becton Dickinson and Co., Franklin Lakes, NJ) containing 3 mL of Medium 199 (Gibco, Life Technologies, Grand Island, NY) plus 0.1% BSA (Immuno-chemical Products Ltd., Auckland). After pre-incubation for 24 hours, the medium was completely replaced and the peptide or vehicle was added at the same time. After 48 hours of incubation, an aliquot of 300 μL of medium was counted for $^{45}$Ca, by using a LKB 1217 Rackbeta liquid scintillation counter (Wallac Oy, Turku, Finland), to determine the amount of calcium released from the bone. At this stage, $[^3]$H-thymidine (Amersham Pharmacia Biotech UK Ltd., 2 μCi/bone, in 500 μL) was added to the medium and the incubation was prolonged for additional 4 hours. At the end of incubation, the bone was washed with Medium 199 and placed in 1 mL of 5% trichloroacetic acid (TCA, BDH Laboratory Supplies, Poole, England) followed by storage at 4°C overnight to extract the $^{45}$Ca remaining in the bone. Then, an aliquot of 100 μL of TCA was counted for $^{45}$Ca.

To determine the $[^3]$H-thymidine incorporation, the calvaria was dehydrated by treatment with acetone for 1.5 hours followed by ether for another 1.5 hours, and then left to dry at room temperature for about 2 hours. Bone weight was measured by using a CAHN electrobalance (model 4100, Ventron Instruments Co., Cerritos, CA). Then the bone was placed in a glass scintillation vial (Epsom Glass Industries Ltd., Epsom, England) containing 500 μL of 1 M KOH and incubated at 80°C for about 15 minutes. Following the addition of 500 μL of 1 M HCl, to neutralise the solution of the dissolved bone, 10 mL of scintillant (Amersham International plc, Amersham, Buckinghamshire, England) was added, and counted for $[^3]$H using the counter mentioned above.

Bone resorption was defined as the percentage of $^{45}$Ca in medium over total $^{45}$Ca. $[^3]$H-thymidine incorporation was expressed as total DPM of dissolved bone/bone weight (μg).
2.12. OSTEOLAST PREPARATION AND PROLIFERATION ASSAY

Osteoblast preparation: The osteoblast preparation and proliferation assay were as previously described (Cornish et al. 1998b). To isolate the osteoblasts, the calvariae of fetal Wistar rats were dissected aseptically and the central portions of the frontal and parietal bones free of periosteum, suture and surrounding soft tissues were collected. The bones were washed twice (15 minutes each time) with PBS containing 3 mM EDTA-Na₂ (pH 7.4) at 37°C in a shaking water bath followed by washing once with PBS (pH 7.4) only for 15 minutes under the same conditions. Then the bones were digested with 1 mg/mL type II collagenase (Sigma Chemical Co., St. Louis, MO) dissolved in PBS at 37°C for 7 minutes. The supernatant was discarded and the precipitate was further digested twice with 2 mg/mL of type II collagenase at 37°C for 30 minutes each time. At this stage, the supernatant was retained and centrifuged at 140 g for 45 seconds. The cell pellet was washed with MEM (Gibco, Life Technologies, Grand Island, NY) containing 10% FBS (Gibco, Cat # 10091-148). Finally, the cells were suspended in DMEM (Gibco) containing 10% FBS, seeded in a 75 cm² flask (Corning Inc., New York) at the density of 10⁵ cells/mL and cultured for one week. The purity of this osteoblast preparation was over 95% based on the staining for alkaline phosphatase with BCIP/NBT alkaline phosphatase substrate (Sigma Chemical Co., St. Louis, MO).

Proliferation assay: Semi-confluent osteoblast cultures from the above steps were washed twice with PBS and then freed from the culture surface by treatment with 0.005% trypsin (Gibco, Life Technologies, Grand Island, NY) at 37°C for ~5 minutes. The cells were harvested by spinning at 1,200 rpm for 45 seconds. After being washed with MEM containing 5% FBS, the cells suspended in the same medium were seeded onto 24-well plates (Cellstar, Greiner Labortechnik, Frickenhausen, Germany) at the density of 5 x 10⁴ cells/mL (0.5 mL/well) and incubated for 24 hours. Then the medium was tipped off and replaced with MEM (1 mL/well) containing 0.1% BSA (Immuno-chemical Products Ltd., Auckland). After another 24 hours of culture, the medium was completely replaced and test drug or vehicle was added. In 24 hours, [³H]-thymidine (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire) was added (0.5 μCi/50 μL/well) and
the cells were further incubated for 2 hours. At the end of incubation, the medium was removed and the cells were washed once with MEM (1 mL/well). The plates were treated with 10% TCA (1 mL/well) and stored at 4 °C for at least 2 hours. Then TCA was tipped off and the plates were rinsed twice with ethanol: ether (3:1, 1 mL/well). When the solvents were completely evaporated (about 2 hours in the fume-hood), an aliquot of 200 μL of 2 M KOH was added to each of the wells, and the plates wrapped with gladwrap were incubated at 55 °C for 30 minutes. Finally, the plates were neutralised with 1 M HCl (400 μL/well) and an aliquot of 300 μL of the solution was counted for [³H] by using a Microbeta Wallac Trilux liquid scintillation and luminescence counter (model 1450, Wallac Oy, Turku, Finland).

2.13. MATURE OSTEOCLAST ACTIVITY ASSAY

**Bone slice preparation:** Bovine cortical bone or ivory was cut to 4.5 x 4.5 mm slices with the thickness of about 0.3 mm by using a low speed saw (Isomet™, Buehler Ltd., Lake Bluff, IL). The slices were sonicated for about 5 minutes to remove dirt on the surface and stored in 70% ethanol. When used, the sterile slices were washed twice with PBS and equilibrated with culture medium made of 72 mL of Earle’s MEM (Gibco, Life Technologies, Grand Island, NY), 8 mL of FBS (Gibco, Cat # 10091-148), 0.8 mL of penicillin and streptomycin mix (Gibco, Cat # 15140-122) and 72 μL of 12 M HCl.

**Osteoclast isolation and culture:** Osteoclasts were isolated from the long bones of 1-day-old Wistar rats according to the methods from Dr. Tim Arnett’s Laboratory (University College of London, UK) and Burgess et al (Burgess et al. 1999). The separation of osteoclasts from non-osteoclastic cells was based on the different rates of settling of the cells to the bone surface (Hakeda et al. 1998). To isolate the osteoclasts, rat long bones free of surrounding soft tissues were split longitudinally and then minced with a homogenizer (Arthur H. Thomas Co., Philadelphia, PA) and chopped with a scalpel in the culture medium indicated above. Then, the cell suspension (6 mL in total) was loaded onto the bone slices (170 μL/slice) in a 96-well plate (Cellstar, Greiner Labortechnik, Frickenhausen, Germany). After incubation for 25 minutes to allow the attachment of the cells, the bone slices were rinsed vigorously in PBS and then in culture.
medium to remove the less adherent non-osteoclastic cells. Subsequently, the bone slices with enriched osteoclast population were transferred to a 12-well plate (Costar, Corning Inc., New York) containing 2.5 mL/well of culture medium (4 slices/well). After incubation for 1 hour, the cells were treated with drug or vehicle and continuously incubated.

Osteoclast and pit staining and counting: After incubation for 24 hours, the cells on bone slices were fixed with 2.5% gluteraldehyde (in PBS) for 2 minutes followed by washing twice with distilled water. Then the cells were subjected to TRAP staining with the leukocyte acid phosphatase kit (Cat # 387-A, Sigma Diagnostics Inc., St. Louis, MO) according to the manufacturer's instruction. The TRAP-positive cells with three or more nuclei were counted as osteoclasts under an inverted microscope (Olympus CK 40). After counting, the cells were removed by gentle brushing and the bone slices were stained for pits with 1% toluidine blue (in 1% di-sodium tetraborate) for 30 seconds. To count the pits formed by the osteoclasts, the bone slices were examined under a reflection-light microscope (Olympus BH-2). The bone-resorbing activity of the osteoclasts was expressed as the number of pits produced by per osteoclast.

2.14. RNA EXTRACTION, RT-PCR AND REAL-TIME PCR

Cell culture and RNA preparation: The rat brain (about 30 mg/sample) that was used for RNA preparation was collected and stored for 2-3 days at 4 °C in RNAlater™ (Ambion, Austin, Texas). Osteoblasts were seeded at 1.25 x 10⁶ cells in 2.5 mL media/well in 12 well plates, and bone marrow cells were seeded at 4 x 10⁶ cells in 3 mL media/well in 6 well plates. The culture conditions and drug treatments of osteoblasts and bone marrow cells were as described in osteoblast proliferation assay (Section 2.12) and osteoclast formation assay (Section 2.10), respectively. Cells were harvested at the indicated time points and RNA was extracted by using RNeasy Mini Kit (QIAGEN, Valencia, CA) and RNease-Free DNase Set (QIAGEN, Valencia, CA) according to the manufacturers’ protocols.
RT-PCR: First-strand cDNA was synthesized from 2 μg of total RNA in a reaction volume of 20 μL by random priming and by using Superscript™ reverse transcriptase (Invitrogen NZ Ltd., Auckland) according to the manufacturer’s instruction. Unless otherwise stated, 2.5 μL of cDNA was subjected to PCR with reaction profile of 94°C (2 minutes), 34 cycles of 94°C (30 seconds)/58°C (30 seconds)/72°C (1 minute) and 72°C for 5 minutes as a final extension step. PCR was carried out with Platinum® Tap DNA polymerase (Invitrogen NZ Ltd., Auckland), 1.5 mM MgCl₂ and the appropriate primers at a final concentration of 1 μM each, in a total volume of 25 μL. An aliquot of 5 or 10 μL from the PCR reaction was electrophoresed on a 1% agarose gel.

PCR primers: The sequences of the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rat β-actin and rat FGF-8 primers are listed below.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequences</th>
<th>Amplicons (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>forward: 5’ CATCA TCTCC GCCCC TTCCG 3’ reverse: 5’ CCTGC TTCAC CACCT TCTTG 3’</td>
<td>437</td>
</tr>
<tr>
<td>β-actin</td>
<td>forward: 5’ TACAA CCTCC TTGCA GCTCC 3’ reverse: 5’ GGATC TTCAT GAGGT AGTCT 3’</td>
<td>630</td>
</tr>
<tr>
<td>FGF-8</td>
<td>forward: 5’ ATGGC AGAAG ACGGA GACC 3’ reverse: 5’ GTGAA GGGCG GGTAG TTGAG 3’</td>
<td>374</td>
</tr>
</tbody>
</table>

Quantitative real-time PCR: Real-time PCR reactions were conducted in triplicates in 96-well optical reaction plates (Applied Biosystems, Foster City, CA) in a total volume of 25 μL/well containing 12.5 μL of Taq Man® Universal PCR master mix (2x, Applied Biosystems), 1.25 μL of VIC lapelled 18S rRNA endogenous control (Applied Biosystems), 1.25 μL of FAM lapelled OPG or RANKL primer-probe mix, 5 μL of Milli Q H₂O and 5 μL of cDNA sample. The mouse OPG and RANKL primer-probe sets were purchased as Assay-on-Demand service from Applied Biosystems. Multiplex PCR was performed by using ABI PRISM® 7700 Sequence Detection System (Applied Biosystems) with reaction profile of 50°C (2 minutes), 95°C (10 minutes), 40 cycles of 95°C (15 seconds)/60°C (1 minute).
2.15. AGAROSE GEL ELECTROPHORESIS

Horizontal agarose gel electrophoresis (1.0% agarose in TBE buffer) was carried out according to Sambrook et al (Sambrook and Russell 2001) to separate DNA/RNA fragments and access DNA/RNA concentrations in samples. DNA/RNA samples were mixed with 0.2-0.3 volume of DNA/RNA agarose gel loading buffer before being loaded onto the gel. Power was set to 10-15 V/cm. After electrophoresis, gels were stained with ethidium bromide (0.5 µg/mL) for ~30 minutes and bands visualised in 320 nm UV light by using the Gel Documentation System (Rio-Rad Laboratories, Inc., CA). Molecular weight and quantitation ladders (100 bp/low mass, Invitrogen NZ Ltd., Auckland) were run concurrently along with the DNA/RNA samples.

2.16. STATISTICAL ANALYSIS

All the data presented are from the experiments repeated 2-3 times in order to obtain consistent results. Data analysis was performed using Student’s t-test or one-way analysis of variance with post hoc examination of significant main effects using the method of Tukey or Dunnett. All tests were 2-tailed and a 5% significance level was maintained throughout these analyses.
CHAPTER 3: REGULATION OF OSTEOCLAST DIFFERENTIATION AND ACTIVITY BY RANKL AND OPG

3.1. INTRODUCTION

Bone is a dynamic tissue that undergoes constant remodeling involving the resorption of old bone by osteoclasts and formation of new bone by osteoblasts. Normally, these processes are under accurate control through the local and systemic factors acting on the development and function of the bone cells. These factors include 1,25-(OH)_{2}D_{3}, PGE_{2}, IL-1, PTH and M-CSF, etc (Murakami et al. 1998; Rodan 1998).

Osteoclasts are known to differentiate from hematopoietic precursor cells present in bone marrow, spleen and peripheral blood (Suda et al. 1996). The development of osteoclasts from their precursors usually requires the direct contact with supporting osteoblasts, which originate from pluripotent (multipotential) mesenchymal stem cells (Aubin and Liu 1996). This led to the hypothesis that membrane-associated factor(s) might be involved in this process. This hypothesis was proven in late 90's with the discovery of a trio of peptides, OPG, RANKL and RANK (Martin and Udagawa 1998). RANKL was cloned independently in 1997 by Anderson et al in human, and by Wong et al in human and mouse. It consists of 317 and 316 amino acids in human and mouse respectively (Anderson et al. 1997; Wong et al. 1997b). More recently, the rat RANKL (rRANKL) gene was also cloned. This peptide consists of 318 amino acids, and shares 84% and 96% identity in amino acid sequence with the human RANKL (hRANKL) and murine RANKL (muRANKL) respectively (Xu et al. 2000). RANKL is usually membrane-bound and contains an extracellular portion present on the surface of osteoblastic cells. In addition to osteoblasts, it was also identified on prehypertrophic and hypertrophic chondrocytes, mesenchymal cells of the periosteum (Kartsogiannis et al. 1999), T cells (Kong et al. 1999b) and synovial cells (Romas et al. 2000). RANKL was found to stimulate osteoclast formation and function through its cognate receptor RANK, which is also a membrane-bound protein present on osteoclasts and their precursors. However, the
interaction between RANKL and RANK can be inhibited by the decoy receptor OPG, a soluble protein produced by osteoblasts, and hence the signaling transduction from osteoblasts/RANKL to osteoclasts/RANK is blocked (Yasuda et al. 1999).

The effects of RANKL and OPG on bone remodeling have been demonstrated in both in vivo and in vitro systems. Mice deficient in OPG exhibited severe osteoporosis with loss of trabecular bone and dramatic decreases in bone strength (Mizuno et al. 1998). On the other hand, over-expression of OPG in transgenic mice resulted in profound osteopetrosis and a decrease in osteoclast differentiation (Simonet et al. 1997). Systematic treatment of OPG in normal mice led to significantly increased bone mineral density and bone volume, and decreased osteoclast number (Simonet et al. 1997; Yasuda et al. 1998a). In contrast, severe osteopetrosis was seen in RANKL knockout (opgt<sup>−/−</sup>) mice, in which osteoclastogenesis was blocked and tooth eruption was inhibited (Kong et al. 1999c).

In in vitro assays, the effects of OPG and RANKL on osteoclast development have been well established, such as in cultures of bone marrow cells (Tsuda et al. 1997; Lacey et al. 1998), spleen cells (Simonet et al. 1997; Yasuda et al. 1998a), peripheral blood monocytes (Shalhoub et al. 1999), a mouse macrophage cell line RAW<sub>264</sub>.7 (Xu et al. 2000) and long bones (Kwon et al. 1998). RANKL in combination with M-CSF was sufficient to induce osteoclast formation from mouse spleen cells, human peripheral blood monocytes and a mouse macrophage-like osteoclast progenitor cell line C7, without the presence of supporting osteoblastic stromal cells (Quinn et al. 1998; Yasuda et al. 1998b). However, these peptides have not been examined previously in calvarial organ culture. Since calvarial bone contains minimal bone marrow cells, bone resorption mainly reflects the activity of mature osteoclasts and is independent of the proliferation of osteoclast progenitors (Pfeilschifter et al. 1988). Furthermore, calvarial organ culture enables the study of bone resorption occurring in a unique microenvironment, which is close to that of the in vivo system, compared to cell culture systems.

Thus, many studies have established the importance of OPG and RANKL in regulating osteoclast formation, but little is known of their effects on mature osteoclast activity,
particularly for OPG. There are only two reports about OPG's effect on bone-resorbing activity of mature osteoclasts (Hakeda et al. 1998; Kwon et al. 1998). Due to the difficulty in obtaining pure osteoclast preparations, more work is needed to determine whether OPG influences the activity of mature osteoclasts.

This research project studies the effects of RANKL and OPG on osteoclastogenesis from bone marrow cultures, on bone resorption in calvarial organ cultures and on mature osteoclast activity in isolated osteoclast cultures. Particular interest was given to rRANKL and its different truncates, since they were cloned more recently and had not been well tested in any of the above three culture systems previously.

The research aims in this project were: (1) to compare the effects of RANKL and OPG in different assay systems; (2) to investigate the effect of newly cloned factor rRANKL; (3) to study the activity-structure relationship to determine the essential sequences of rRANKL for its biological activity.

3.2. MATERIALS AND METHODS

3.2.1. Solutions

* Alkaline phosphatase buffer: 0.1 M bicarbonate/carbonate, 0.1% thiomersol
* Antibody buffer: 45 mL PBS-T, 2.25 g Blotto (non-fat dried milk), 0.9 mL normal horse serum
* Coating buffer: PBS (pH 9.95) containing 0.2% Thiomersol, 0.42% NaHCO₃, 0.8% Na₂HPO₄ and 0.01% NaCl
* ELISA assay buffer: 0.5 M Tris (pH 7.5), 0.8% NaCl, 0.5% BSA and 0.5% Tween 20
* ELISA wash buffer: 0.5 M Tris (pH 7.5), 0.8% NaCl and 0.5% Tween 20
* PBS-T: PBS (pH 7.4) containing 0.1% Tween 20

3.2.2. Peptides and immuno-reagents

* Human OPG (hOPG) dimer and muRANKL: These peptides were kindly provided by Dr Colin Dunstan from the Amgen Inc, Thousand Oaks, CA, USA. The full-length
OPG cDNA linked to human IgG1 Fc region was cloned into the eukaryotic expression vector pDSRα and expressed in CHO cell lines. The secreted hOPG (aa22-201)-Fc protein was purified from the CHO cell-conditioned media (Simonet et al. 1997; Burgess et al. 1999). muRANKL(158-316) was expressed in E. coli and purified by anion and cation-exchange chromatography (Lacey et al. 1998; Burgess et al. 1999).

* hRANKL: Cat # CRR100B, Cell Sciences, Inc., Norwood, MA, USA
* rRANKL: Recombinant rRANKL was kindly provided by Dr Ming-hao Zheng from the University of Western Australia. The rRANKL cDNA encoding aa160-318, aa239-318 or aa246-318 of the TNF-like core region was cloned into the bacterial expression vectors pGEX-3X and pGEX-2T in correct frame with glutathione S-transferase (GST) tag. The fusion proteins were expressed in bacterial strain Top 10.’
* Biotinylated rabbit anti-goat IgG: Jackson Immunoresearch Laboratories, West Grove, PA, USA.
* Monoclonal mouse anti-hRANKL antibody (capture antibody): Cat # MAB626, R & D systems Inc., Minneapolis, MN, USA
* Polyclonal goat anti-hRANKL antibody, Cell Sciences Inc., Norwood, MA, USA
* Streptavidin-alkaline phosphatase complex: Dako A/S and Cytomation Inc., Glostrup, Denmark

3.2.3. Measurement of RANKL by ELISA

An indirect sandwich ELISA method was employed in this project based on the protocol from Dr. Jeff Keelan of the Liggins Institute, Auckland. Firstly, A 96-well opaque high protein binding LIA plate (Greiner Labortecnik, Frickenhausen, Germany) was coated with the capturing monoclonal mouse anti-hRANKL antibody (R & D systems Inc.) diluted to 1 μg/mL with the coating buffer by incubation at 4°C overnight (100 μL/well). The coating solution was then tipped off and the non-specific binding sites were blocked by the treatment of 2% BSA (Immuno-Chemical Products Ltd., Auckland) (in PBS, 200 μL/well) at room temperature for 1 hour with shaking followed by washing. In this experiment, all the washes were conducted by using the ELISA wash buffer and Microwell system washer (model 430, Organon Teknika, Belgium). Unless otherwise indicated, the washer was set to Wash Program 5 with 4 washes. After the addition of the
ELISA assay buffer (50 μL/well) containing 4.76% normal horse serum (Invitrogen Co.), an aliquot of 100 μL of sample or standard hRANKL (Cell Sciences, Inc.) diluted with the ELISA assay buffer (78-5000 pg/mL) was added to the wells and incubated at 4°C overnight. Then the plate was washed and the polyclonal goat anti-hRANKL antibody (Cell Sciences, Inc.) diluted to 1 μg/mL with the antibody buffer was added to the plate (100 μL/well) followed by incubation at room temperature for 2 hours. The plate was washed again and incubated at room temperature for 1 hour with the biotinylated rabbit anti-goat IgG (Jackson Immunoresearch Laboratories) diluted with the antibody buffer (1:5000, 100 μL/well). The free antibody was removed by washing. Then the streptavidin-alkaline phosphatase complex (Dako A/S and Cytomation Inc.) diluted with the ELISA assay buffer (1:2000) was added (100 μL/well), and the plate was incubated at room temperature for 30 minutes. After the plate was washed 6 times (using Wash Program 6), the substrate for alkaline phosphatase (4-methylumbelliferyl phosphate, Molecular Probes Inc., Eugene, OR), diluted with the alkaline phosphatase buffer (1:100), was added to the plate (100 μL/well). Finally, the plate was incubated at room temperature for approximately 150 minutes with shaking, and the resulting fluorescence was measured by using a Wallace 1420 Multiplate reader (PerkinElmer Life Sciences/Wallac Oy).

3.3. RESEARCH RESULTS

3.3.1. Effect in mouse calvarial organ cultures

To assess the effects of RANKL and OPG in the calvarial organ cultures, bone resorption was measured over a 48-hour period and [³H]-thymidine incorporation was measured over the last 4 hours of the incubation period. In this assay, muRANKL greatly stimulated the release of pre-labeled ⁴⁵Ca from the bone (Fig. 3-1). At the concentration range tested, a significant effect was observed at concentrations of 10 ng/mL and greater. A maximum increase in bone resorption, approximately 100% higher than the control, was reached at the concentrations of 50 and 100 ng/mL. At the same time, [³H]-thymidine incorporation into the calvariae, which reflects the rate of DNA synthesis and cell proliferation, was also increased in a dose-dependent manner (Fig 3-1).
Fig. 3-1. muRANKL stimulates $^{45}$Ca release (a) and $[^3]$H-thymidine incorporation (b) in mouse calvarial organ cultures. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "**" ($P \leq 0.01$).

Fig. 3-2. hOPG inhibits $^{45}$Ca release (a) and $[^3]$H-thymidine incorporation (b) in mouse calvarial organ cultures. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "*" ($P \leq 0.05$) and "**" ($P \leq 0.01$).
Fig. 3-3. Antagonistic effect of hOPG and muRANKL on $^{45}$Ca release in mouse calvarial organ cultures. Both OPG and muRANKL were added at the same time and cultures were continued for 48 hours. Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "*" ($P<0.05$).

In contrast to muRANKL, hOPG inhibited both $^{45}$Ca release and $[^3]$H-thymidine incorporation by about 35% at a concentration of 50 ng/mL. Similar to muRANKL,
maximal effects were produced at this concentration (Fig. 3-2). Since hOPG and muRANKL showed opposite effect in this culture system, their antagonistic action was tested. The results showed that the inhibitory effect on $^{45}$Ca release by hOPG at maximal concentration (50 ng/mL) was reversed by the addition of muRANKL (Fig. 3-3).

The more recently cloned rRANKL was also tested in this calvarial organ culture. The recombinant rRANKL(160-318), which contains the full TNF-like core region and is comparable to the homogeneous sequence of the recombinant muRANKL(158-318) used in this project, significantly stimulated $^{45}$Ca release at concentrations of 100 ng/mL and greater (Fig. 3-4). However, compared to muRANKL, rRANKL seemed to be less potent. PTH is a more potent stimulator of bone resorption in this culture system, presumably through endogenous increases in RANKL.

![Fig. 3-5.](image)

**Fig. 3-5.** muRANKL (a) and hOPG (b) have no effect on the proliferation of rat primary osteoblasts in culture. Data are presented as the mean ± SE, $n=6$ /group.

In these experiments, the effects of RANKL and OPG on bone resorption were so obvious that the difference between control and treatment could even be seen by the relative hardness of the bones at the end of cultures. Bones with increased resorption by RANKL were softer, while those with decreased resorption by OPG were more rigid.
Fig. 3-6. Effects of muRANKL on osteoclast number (a) and degree of multinucleation (b) in mouse bone marrow cultures. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=8/group. The osteoclast number and nucleus number were originally scored as osteoclasts/well and nuclei/osteoclast respectively. Significant differences from control are indicated with “*” (P<0.05) and “**” (P<0.01).

Fig. 3-7. Effect of rRANKL on osteoclast formation from mouse bone marrow cultures. Data are presented as the mean ± SE, n=8/group. Significant differences from control are indicated with “**” (P<0.01).
3.3.2. No effect in osteoblast cultures

The above results (Fig. 3-1 and 3-2) showed that muRANKL and hOPG not only affected $^{45}$Ca release, but also affected $[^3]$H-thymidine incorporation in calvarial organ cultures. This indicates that the peptides may be affecting the osteoblasts, which are thought to be the major contributing cell type for $[^3]$H-thymidine incorporation in these cultures (Cornish et al. 1997b). To look at the direct action on osteoblasts, both muRANKL and hOPG were tested in the cultures of primary osteoblasts prepared from fetal rat calvariae. However, in this highly pure population of osteoblasts, neither muRANKL nor hOPG at 10, 50 and 100 ng/mL showed any detectable effect (Fig. 3-5).

![Fig. 3-8. Effect of hOPG on osteoclast formation from mouse bone marrow cultures. Data are presented as the mean ± SE, n=8/group. Significant differences from control are indicated with "**" (P≤0.01).]

3.3.3. Effect on osteoclastogenesis from mouse bone marrow cultures

To compare the effects of the peptides in the calvarial organ culture, the potency of the peptides, muRANKL, rRANKL and hOPG was tested in their regulation of osteoclast formation in mouse bone marrow cultures. The results showed that these peptides were more potent in regulating osteoclast formation from mouse bone marrow cultures than in regulating bone resorption (predominantly mature osteoclast activity) in mouse calvarial organ cultures. muRANKL at 1 ng/mL and 50 ng/mL enhanced the osteoclast-like cell number by 2.4 and 5-fold respectively. No further stimulation was seen when the
concentration was increased to 100 ng/mL (Fig 3-6a). Salmon calcitonin (sCT) was used in this study as a positive control. While the number of osteoclast-like cells was increased by muRANKL, the average nucleus number in the osteoclast-like cells was also significantly increased by about 1.5-fold at concentrations of 50 and 100 ng/mL (Fig 3-6b). rRANKL(160-318) enhanced osteoclast-like cells formation by 2- and 3.8-fold at concentrations of 5 and 50 ng/mL respectively (Fig 3-7), again more potent than its effect in calvarial organ cultures (Fig. 3-4).

In contrast, OPG, the decoy receptor for RANKL, strongly inhibited osteoclastogenesis from bone marrow cells. It reduced the osteoclast-like cell number by over 90% at a concentration of 1 ng/mL. When the concentrations were increased to 10 ng/mL and greater, the osteoclast-like cell formation was almost completely blocked (Fig. 3-8).

![Graph](image)

**Fig. 3-9.** Stimulatory effect of muRANKL on mature osteoclast activity. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=8/group. Significant differences from control are indicated with “*” (P≤0.05) and “**” (P≤0.01).
Fig. 3-10. Effects of rRANKL and muRANKL on mature osteoclast activity. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=8/group. Significant differences from control are indicated with "***" (P<0.01).

3.3.4. Effects in mature osteoclast assays

Since bone resorption from calvarial organ cultures mainly reflects the activity of mature osteoclasts, the modulated bone resorption in the calvarial bones (Fig. 3-1 to 3-4) is suggestive of the direct action of RANKL and OPG on the mature osteoclasts. To support this hypothesis, the isolated osteoclast assays were employed.

3.3.4.1. Effect of muRANKL

With the treatment of muRANKL at concentrations of 1, 10 and 50 ng/mL, the osteoclast activity (expressed as pits/osteoclast) was increased by about 1.7-, 3.5- and 4.0-fold respectively. In contrast, sCT at 10−9 M significantly inhibited the isolated osteoclast
activity. In this experiment, no significant difference in osteoclast number was seen between control and muRANKL treatment, indicating that no osteoclast formation occurred in this culture system (Fig 3-9).

3.3.4.2. Effect of rRANKL

Similar to muRANKL, rRANKL(160-318) stimulated isolated osteoclast activity without affecting osteoclastogenesis. Fig. 3-10 shows that pit formation was significantly elevated upon the treatment of rRANKL at 100 ng/mL, but not at 10 ng/mL. The stimulatory effect of rRANKL at 100 ng/mL was close to that by muRANKL at 10 ng/mL, indicating that muRANKL was still more potent than this preparation of rRANKL as was seen in the mouse calvarial organ cultures and mouse bone marrow cultures.

3.3.4.3. Antagonistic effect of rRANKL truncates

A previous study showed that the truncation of RANKL in the TNF-like core region greatly reduced the osteoclast inductivity (Xu et al. 2000). However, the interaction between the truncated molecules and the molecule containing the full TNF-like core region has not been investigated. Therefore, rRANKL(239-318) and rRANKL(246-318) truncates (Fig. 1-6) were tested in combination with rRANKL(160-318) in the isolated osteoclast model.

As shown in Fig. 3-11 and 3-12, N-terminal deletion up to 239 and 246 in the rRANKL sequence completely eliminated their effect on mature osteoclast activity measured by pit formation on bone slices.

To investigate the effect of the truncated molecules on the fully functional rRANKL(160-318), the isolated osteoclasts were co-treated with both of the molecules. The results showed that rRANKL(239-318) was not antagonistic to rRANKL(160-318) even when rRANKL(239-318) was added at a higher concentration than rRANKL(160-318) (Fig. 3-11). In contrast, rRANKL(246-318) at 100 ng/mL tended to antagonise the effect of
rRANKL(160-318) at the same concentration (Fig. 3-12a), whereas at higher concentrations, rRANKL(246-318) partially blocked (25%) the effect of rRANKL(160-318) (Fig. 3-12b).

**Fig. 3-11.** Effect of co-treatment with rRANKL(239-318) and rRANKL(160-318) on mature osteoclast activity. rRANKL(239-318) was added 30 minutes prior to the addition of rRANKL(160-318). The experiments were conducted separately with combinations of lower (a) and higher (b) concentrations of rRANKL(239-318) and rRANKL(160-318). The isolated osteoclasts were cultured in the medium with (+) or without (-) the factors indicated. Data are presented as the mean ± SE, n=8/group. Significant differences from control are indicated with "**" (P≤0.01).
Fig. 3-12. Effect of co-treatment with rRANKL(246-318) and rRANKL(160-318) on mature osteoclast activity. rRANKL(246-318) was added 30 minutes prior to the addition of rRANKL(160-318). The experiments were conducted separately with combinations of lower (a) and higher (b) concentrations of rRANKL(246-318) and rRANKL(160-318). The isolated osteoclasts were cultured in the medium with (+) or without (-) the factors indicated. Data are presented as the mean ± SE, n=8/group. Significant differences from control and from rRANKL (160-318) alone are indicated with "**" (P≤0.01) and "#" (P≤0.05) respectively.

3.3.4. Effect of hOPG

Based on the generally recognized mode of action, OPG only interact with RANKL. However, it is uncertain whether any other molecule on osteoclasts can interact with
OPG. Therefore, hOPG was tested in this isolated osteoclast assay system. It was found that hOPG strongly inhibited pit formation at concentrations of 10 ng/mL and greater (Fig. 3-13). To find out if the effect of hOPG was still through the interaction with endogenous RANKL produced by the contaminated osteoblasts, the culture medium was then subjected to ELISA for RANKL. The result showed that no detectable RANKL was present in the samples tested.

Fig. 3-13. Effect of hOPG on mature osteoclast activity. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=8/group. Significant differences from control are indicated with "***" (P≤0.01).

3.3.5. Concentrations required for significant effect in different models

By comparing the results above, it can be seen that the concentrations of the peptides required for significant effects vary greatly between different culture systems. Overall, the concentrations needed in bone marrow cultures are much lower (10- to 50-fold lower) than in calvarial organ cultures and in isolated mature osteoclast cultures. To compare the difference in these models, the lowest concentrations required for the significant effects are summarised in Table 3-1.
Table 3-1. Comparison of the minimal concentration (ng/mL) required for the significant effects produced in different culture systems

<table>
<thead>
<tr>
<th></th>
<th>Calvariae</th>
<th>Bone marrow</th>
<th>Isolated osteoclasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>hOPG</td>
<td>50</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>muRANKL</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>rRANKL</td>
<td>100</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

3.4. DISCUSSION

3.4.1. Effect on mature osteoclasts

The recruitment of new osteoclasts and the activation of mature osteoclasts are the two major processes governing the rate of bone resorption. Previous reports on the effects of RANKL and OPG on bone resorption have focused predominantly on the former process. Therefore, in this project, RANKL and OPG were examined by using two models of mature osteoclast function: calvarial organ culture and isolated osteoclast culture.

Calvariae contain little bone marrow, thus few osteoclast precursors are present. It was reported that hydroxyurea, a DNA synthesis inhibitor, did not inhibit the rate of bone resorption in neonatal mouse calvariae (Pfeilschifter et al. 1988). This indicates that bone resorption in calvariae is independent of cell proliferation, which is a major process leading to osteoclast recruitment and formation. In this research project, novel observations have been made showing that RANKL and OPG exhibit potent effects on $^{45}$Ca release from the calvarial bones. Based on the above features of calvarial bones, it is reasonable to believe that the effect of RANKL on bone resorption in this model was likely through its direct action on the mature osteoclasts rather than through the action on osteoclast precursors to develop into mature osteoclasts. Consistently, RANKL was found to stimulate pit formation in the isolated osteoclast assays. This provides further evidence for the direct effect of RANKL on the activity of the calvarial mature osteoclasts. OPG significantly inhibited bone resorption in calvarial organ cultures and this could be through the blockade of endogenous RANKL function. However, since it has been found to inhibit pit formation in isolated osteoclast assays in a previous study.
(Hakeda et al. 1998) and the current investigation, it is likely that OPG also directly regulated the activity of the mature osteoclasts in the calvarial bones.

In addition to the direct effect on the functional activity of mature osteoclasts, the impact on mature osteoclast viability might have also contributed to the altered rates of calvarial bone resorption by RANKL and OPG. It was previously reported that RANKL alone (Jimi et al. 1999) or in combination with M-CSF (Lacey et al. 2000) elevated the survival of osteoclasts generated from mouse bone marrow cultures. Administration of OPG in mice resulted in over 90% reduction of osteoclast number within 48 hours due to apoptosis (Lacey et al. 2000). Other groups also found that the survival of osteoclasts was impaired by the addition of OPG (Murakami et al. 1998), but enhanced by the addition of RANKL (Udagawa et al. 1999; Hofbauer et al. 2000) or M-CSF (Udagawa et al. 1999). Therefore, it is most likely in the calvarial organ cultures that two mechanisms are involved in the modulation of bone resorption by RANKL and OPG: through regulation of mature osteoclast activity and through the effect on mature osteoclast survival.

In the isolated osteoclast cultures in this project, no change in osteoclast number was observed upon the treatment with RANKL. This indicates that neither osteoclastogenesis nor mature osteoclast survival was affected by the peptide. In this mature osteoclast-enriched preparation, contamination of osteoclast precursors might not be completely avoided. However, they did not seem to contribute to the pit resorption by becoming functional osteoclasts. This was likely due to the unfavorable incubation conditions. Firstly, the incubation duration was so short (24 hours) that osteoclastogenesis was unlikely to complete. Secondly, the low pH (~7.0) medium only favored the activity of osteoclasts, but not osteoclast development (Dr Julian Quinn, University of Melbourne, personnel communication). Lastly, it was demonstrated that RANKL alone was not sufficient to induce osteoclast formation from the hematopoietic monocytes without the addition of M-CSF (Quinn et al. 1998; Shalhoub et al. 1999). In this isolated osteoclast assay, no M-CSF was added. Based on these situations and the fact of the unchanged osteoclast number, the possibility of osteoclastogenesis in this assay system could be excluded. RANKL was found to enhance the formation of the F-actin ring, the
cytoskeletal structure correlated with bone resorption activity (Burgess et al. 1999). Therefore, its effect on pit formation is most likely through stimulating the function of the mature osteoclasts.

The direct effect of OPG on mature osteoclasts has not been well investigated and the results have been controversial. Previous reports showed that OPG affected mature osteoclast activity only through the interaction with RANKL. Its direct action on mature osteoclasts was excluded (Lacey et al. 1998; Burgess et al. 1999). However, Kwon et al (Kwon et al. 1998) found that OPG inhibited bone resorption in the crude isolated osteoclast cultures and in fetal mouse long bone cultures. Moreover, Hakeda et al (Hakeda et al. 1998) reported that OPG directly inhibited mature osteoclast activity with the identification of an OPG binding protein on the surface of the osteoclasts. In their isolated osteoclast preparation, no RANKL mRNA was detected by means of Northern blots. In this project, the data from the isolated osteoclast assays with hOPG was consistent with the results from Kwon et al and Hakeda et al. Further to the previous reports, the presence of RANKL protein in the medium was not detected by a sensitive ELISA method in this study. This provides further evidence, which excludes the possibility of the interaction between exogenous OPG and endogenous RANKL, and supports the direct impact of OPG on mature osteoclasts. However, since it is very difficult to purify osteoclasts to a satisfactory degree, the existence of very trace amounts of endogenous RANKL produced by the contaminated osteoblasts still could not be completely excluded. In addition, it is uncertain whether mature osteoclasts themselves can express RANKL or not (Kartsogiannis et al. 1999; Myers et al. 1999). Therefore, further study, particularly with the isolated osteoclasts from RANKL-null animals, will be required to address this question.

3.4.2. Effect on osteoblast proliferation

Previously, it was reported that OPG stimulated the proliferation of human foreskin fibroblasts (Kwon et al. 1998), and that RANKL stimulated the proliferation of osteoblasts (Teitelbaum and Ross 2003). However, the data from the current study, where the peptides were investigated in primary osteoblasts, do not support the previous
observations and show that neither muRANKL nor hOPG is effective in regulating the growth of osteoblasts in culture.

Since muRANKL and hOPG have no direct effect on osteoblasts, it could be speculated that the changed rates of \(^{3}H\)-thymidine incorporation in the calvariae were the indirect effect of the peptides on osteoblasts. It has been well documented that bone matrix contains various kinds of osteoblast-stimulating factors such as IGF-1 and -II, and TGF-\(\beta\). While bone matrix is resorbed by osteoclasts, these factors are released, and then stimulate osteoblast proliferation and bone formation to balance the bone lost (Howard et al. 1981; Mundy 1999a). This process has been recognized to contribute to the coupling of bone resorption and formation. Therefore, the changed rates of \(^{3}H\)-thymidine incorporation by RANKL and OPG in the calvarial bones could be the secondary events. RANKL stimulated bone resorption and led to higher levels of the factors released and osteoblast proliferation, while OPG slowed down these processes.

3.4.3. Blocking effect of rRANKL truncates

Deletion experiments showed that the sequences up to amino acid 239 and 246 were critical for the biological function of rRANKL. Truncation up to these positions resulted in dramatic loss of the original activity on bone resorption compared to rRANKL(160-318) with full TNF-like core region. Consistently, the results from a co-laboratory in the University of Western Australia showed that rRANKL(239-318) and rRANKL(246-318) had decreased levels of interaction with the receptor RANK in the in vitro binding assays, and had reduced ability to induce NF-\(\kappa\)B activation in the luciferase reporter gene assays (Tan et al. 2001). The loss of the function due to deletions has revealed the structural determinants for the biological activity. Structural analysis has showed that the crystallised ectodomain of muRANKL has displayed four unique surface loops, which are associated with the formation of homotrimer. Within the ectodomain, several residues have been proposed to be functionally important based on their genetic conservation. These residues include Lys-180 in the AA" loop, Gln-236 in the \(\beta\)D strand and Ile-248 in the DE loop. All three of these residues are conserved in mouse, human and rat RANKL (Lam et al. 2001; Ito et al. 2002).
rRANKL(246-318), the AA" loop containing Lys-180, CD loop and Gln-236 were deleted. This might affect the trimeric formation of RANKL and their interaction with the receptor RANK, and thus impaired the biological function.

In this study, the truncate rRANKL(246-318) is inactive on osteoclast stimulation. However, it was found to significantly block the pit formation stimulated by rRANKL(160-318). It seems likely that this antagonistic effect is due to the interference of the truncate to the interaction between rRANKL(160-318) and its receptor RANK. It was shown that rRANKL(246-318) could still bind to the receptor RANK, though at reduced levels (Tan et al. 2001). This binding ability will partially compete with the binding sites for rRANKL(160-318) and diminish its activity. Similarly, some studies have indicated that peptide sequences (mimotopes), which mimic conformational epitopes of ligand-receptor interactions, are able to block the biological activities of cytokines such as TNF-α (Partidos et al. 1997; Partidos and Steward 2002). The antagonistic effect demonstrated in this study has suggested that small molecule mimetics to attenuate RANKL’s function and hence ameliorate the relative osteopenic disorders of bone are likely.

3.4.4. Effective concentrations of the peptides in different models

The concentrations required for significant effects of the peptides differed between the different culture systems (calvarial organ cultures, bone marrow cultures and isolated osteoclast cultures) with the lowest effective concentration seen in bone marrow cultures. Similarly, another group also reported that about 10-fold higher concentration of OPG was required to inhibit bone resorption in mature osteoclast assays and long bone organ cultures than to inhibit osteoclast formation from bone marrow cells (Kwon et al. 1998).

The different effective concentrations in these culture systems might be due to the difference in the microenvironment and due to the different effects (i.e., on osteoclast formation or activity) of the peptides. Unlike bone marrow and isolated osteoclast cultures that investigate, respectively, osteoclast differentiation and activity only, in calvarial bone organ cultures, bone resorption as well as bone formation takes place.
(Gronowicz and Raisz 1996). In addition, the roles of the peptides in the bone marrow culture is on the formation of osteoclasts from their precursors, while those in the isolated osteoclast and calvarial organ cultures are predominantly, if not completely, on the regulation of mature osteoclast activity. This suggests that the peptides are more effective in modulating the osteoclast development from the precursors than in modulating the activity of the existing mature osteoclasts.

3.5. SUMMARY AND CONCLUSIONS OF CHAPTER 3

RANKL and OPG were tested for the first time in calvarial organ cultures. Both muRANKL and rRANKL potently stimulated, whilst hOPG potently inhibited bone resorption in this culture system, reflecting predominantly effects on mature osteoclast activity.

muRANKL and rRANKL directly stimulated isolated mature osteoclast activity, supporting their direct interaction with mature osteoclasts.

The osteoclast formation from mouse bone marrow cultures was strongly stimulated by muRANKL and rRANKL, and inhibited by hOPG. The effective concentrations were much lower than those in calvarial organ cultures and isolated mature osteoclast cultures.

muRANKL and hOPG had no direct effect on primary osteoblast proliferation. Therefore, the altered rates of cell proliferation by these peptides in calvarial organ cultures were likely due to secondary events.

The N-terminal amino acid sequence up to 239 and 246 in rRANKL was critical for the biological activity of regulating bone resorption.

The inactive rRANKL(246-318) was an antagonist for the active rRANKL(160-318), revealing a new potential approach to inhibit bone resorption via blocking the signaling pathway of functional RANKL.
The inhibitory effect of hOPG in the isolated osteoclast assay system was suggestive of its direct interaction with mature osteoclasts. However, further work is still required to confirm if any interaction with RANKL is involved.
CHAPTER 4: EFFECT OF FIBROBLAST GROWTH FACTOR-8a (FGF-8a) ON BONE CELL GROWTH, DEVELOPMENT AND FUNCTION

4.1. INTRODUCTION

Fibroblast growth factors (FGFs) were initially identified in 1970s from extracts of bovine brain and were named based on their mitogenic activity on fibroblasts (Szebenyi and Fallon 1999). The first two members identified were designated as acidic FGF (aFGF, now also called FGF-1) and basic FGF (bFGF, now also called FGF-2) according to their isoelectric points (Galzie et al. 1997). In recent years, great progress has been made with the identification of at least 24 members in the FGF family (Anonymous 2000; Yamashita et al. 2000; Draper et al. 2003; Fischer et al. 2003). These molecules are now described by the acronym FGF followed by a numerical designation. Though the name was used by convention, it does not mean that all the members in this family have mitogenic activity, e.g. FGF-7, which does not stimulate fibroblast proliferation. In fact, the criteria for defining the members of this family are based on the molecular structure (a highly homologous core region), and their strong binding affinity for heparin and heparan-like glycosaminoglycans (Powers et al. 2000). FGFs have been found to play important roles in normal development and wound healing. In addition, their involvement in a wide range of pathological conditions, such as tumor development and metastasis, has also been established (Galzie et al. 1997).

4.1.1. Molecular and biochemical aspects of FGFs

4.1.1.1. Structural and biochemical features

Each of the FGF members contains a conserved core region consisting of about 120 amino acids and sharing 22-66% identity in amino acid sequence (Szebenyi and Fallon 1999). This core region is responsible for the receptor binding and heparin binding (Szebenyi and Fallon 1999). The FGFs are single chain peptides comprised of varying
amino acid number (155 to 267 for FGF-1 to -9) (Galzie et al. 1997). Some FGFs (such as FGF-3 to -8 and -10) contain consensus hydrophobic signal sequences at their N-terminals for secretion through the endoplasmic reticulum (ER)-Golgi pathway, while FGF-1, -2 and -11 to -14 have no consensus secretory signal sequences. The secretion of FGFs lacking a signal sequence is considered to take place via ER-Golgi-independent mechanisms such as mechanical damage and heat-shock. FGF-9 has no classical leader sequences, but has an internal hydrophobic domain, which likely serves as an internal signal sequence and has been suggested to facilitate the insertion of the molecule into the ER membrane for secretion (Hurley and Florkiewicz 1996; Szebenyi and Fallon 1999).

It was found that the binding of FGF-2 to heparin resulted in conformational change and greatly increased thermal stability of FGF-2 (Prestrelski et al. 1992). This property is also useful in extracting and purifying the FGFs (Shing et al. 1984).

4.1.1.2. FGF genes (fgfs) and FGF-8 isoforms

Each of the FGFs is encoded by a distinct gene, whose chromosomal localization in human has been identified. All the FGF genes (fgfs) have exon/intron organisation, but the number of exons/introns is variable. For example, fgf-1 to -6 and fgf-15 have three exons while fgf-13 has 5 exons (Hurley and Florkiewicz 1996; Szebenyi and Fallon 1999).

The FGF-8 gene, fgf-8, is the most divergent member in the FGF family. The murine fgf-8 consists of at least six exons, named exons 1A, 1B, 1C, 1D, 2 and 3. Alternative splicing of the mRNA in the first 4 exons gives rise to 8 potential isoforms, 7 of which have already been identified. These isoforms differ structurally at the N-terminus of the mature peptides (Fig. 4-1) (MacArthur et al. 1995b). They also display different binding affinities to the FGF receptors (MacArthur et al. 1995b; Blunt et al. 1997) and different cell-transforming activities ((MacArthur et al. 1995a). Unlike murine FGF-8, human FGF-8 has only 4 isoforms due to the marked sequence divergence leading to an interrupted reading frame in exon 1B (Gemel et al. 1996).
Fig. 4-1. Schematic diagram showing the murine FGF-8 gene and the eight isoforms. This gene consists of six exons indicated as 1A, 1B, 1C, 1D, 2 and 3.
- □--exon sequences common to all isoforms; ■--exon sequences for part of some isoforms; ■--exon for the shared signal peptide; □--non-translated exons (Adopted from Blunt et al. 1997).

4.1.1.3. FGF receptors (FGFRs)

The effects of FGFs are produced through the signaling pathway with their cell-surface receptors associated with tyrosine phosphorylation (Coughlin et al. 1988). So far, four types of receptors for FGFs, named FGFR1, FGFR2, FGFR3 and FGFR4, have been identified (Powers et al. 2000). Each member of the FGF family interacts with one or
more specific receptors (Szebenyi and Fallon 1999). The expression of all four FGFRs has been identified in osteoblasts, while only FGFR1 expression was detected in isolated osteoclasts and the mouse macrophage-like cell line C7, which can differentiate into osteoclasts in the presence of stimulating factors (Chikazu et al. 2000; Chikazu et al. 2001). In addition, a number of other studies have detected the presence of FGFRs in skeletal tissue (Hurley and Florkiewicz 1996). For example, the expression of FGFR1, FGFR2 and FGFR3 were detected in growth plates of developing long bones and calvarial bones in human fetus (Delezoide et al. 1998), in neonatal rat cartilage and isolated chondrocytes (Hamada et al. 1999; Molteni et al. 1999). FGFR4 was highly expressed in newborn mouse calvarial bones (Cool et al. 2002).

4.1.1.4. Expression of FGF-8

FGF-8, a secreted factor with a basic isoelectric point, was initially isolated from the androgen-dependent mouse mammary carcinoma cell line, Shionogi carcinoma-3. Therefore, it is also called androgen-induced growth factor (AIGF) (Tanaka et al. 1992).

FGF-8 is expressed preferentially in the earlier stages of embryogenesis. In the embryos, it is expressed in the developing brains (Crossley et al. 1996a; Maruoka et al. 1998; Xu et al. 1999), limbs (Heikinheimo et al. 1994; Crossley et al. 1996b), skeleton (Xu et al. 1999), heart (Crossley and Martin 1995; Reifers et al. 2000), lung (Wu et al. 1997), teeth (Kettunen and Thesleff 1998) and renal system (Crossley and Martin 1995). Its expression in adult tissues is restricted and at lower levels. In some reports, in adult murine tissues, FGF-8 was only detected in the gonadal tissue (Lorenzi et al. 1995; MacArthur et al. 1995a; MacArthur et al. 1995c), whereas, other groups found that it was also expressed in the prostate, brain, heart, lung and kidney (Ghosh et al. 1996; Schmitt et al. 1996; Cancilla et al. 2001). The expression in the adult rat brain, heart, lung and kidney could still be detected upon castration (Schmitt et al. 1996). Circulating concentrations of FGF-8 remain unknown.

FGF-8 was also found in a number of carcinoma cells and tissues including human ovarian tumors and human ovarian-cancer cell lines (Valve et al. 2000), human prostate
4.1.2. The biological activities of FGF-8

4.1.2.1. In the patterning of limbs

The apical ectodermal ridge is critical for normal limb development. Removal of the ectodermal ridge in chick embryo resulted in truncated limbs (Lewandoski et al. 2000). It was suggested that signaling of FGF-8 in this area plays an important role in limb development. Firstly, FGF-8 seems to be the only FGF expressed throughout the ectodermal ridge. Secondly, blockade of FGF-8 function resulted in a substantial reduction in limb-bud size, a delay expression of sonic hedgehog (Shh), a signaling molecule known to be important for limb development, and hypoplasia or aplasia of specific skeletal elements (Lewandoski et al. 2000). Moreover, FGF-8 can substitute for the ectodermal ridge to maintain Shh expression, and outgrowth and patterning of the developing limbs (Vogel et al. 1996; Lewandoski et al. 2000). Application of FGF-8 resulted in the formation of ectopic limbs in chick embryos (Crossley et al. 1996b; Vogel et al. 1996; Szébenyi and Fallon 1999). These results indicate that FGF-8 may be a key signaling molecule for limb initiation, outgrowth and development.

4.1.2.2. In the patterning of brains

FGF-8-soaked beads placed in the caudal forebrain of the chick embryo induced the formation of an ectopic midbrain and the expression of the genes normally specific to mid/hindbrain (Crossley et al. 1996a). FGF-8b-soaked beads transformed regions of the rostral mouse brain into a hindbrain fate and regulated the expression of the midbrain/hindbrain genes in the caudal forebrain explants (Liu et al. 1999).

4.1.2.3. In cell proliferation and transformation

FGF-8 stimulated the proliferation of avian chondrocytes in vitro. Its mitogenic potency was similar to FGF-6, but lower than FGF-2, FGF-4 and FGF-9 in this assay system.
Stable transfection of NIH3T3 cells with FGF-8b cDNA led to marked morphological transformation. The transfected cells could rapidly induce tumorigenicity in the nude mice. In contrast, the transformation activity of FGF-8a, FGF-8c and FGF-8e was much lower and the transfected cells were only weakly tumorigenic in the nude mice (MacArthur et al. 1995a; Ghosh et al. 1996). The transforming effect could also be produced by the addition of the recombinant FGF-8 isoforms to the culture medium at 10 nM in the presence of 3 μg/ml heparin (MacArthur et al. 1995a). These results suggest that these FGF-8 isoforms may have different functions in vivo (MacArthur et al. 1995a; Ghosh et al. 1996).

4.1.2.4. Other activities

FGF-8 was also found to be involved in the development of other organs. Zebrafish embryos with mutation in fgf8 could not establish normal circulation, suggesting the role of FGF-8 in the development of cardio system (Reifers et al. 2000). In addition, application of FGF-8 induced both lens formation and lens fiber differentiation in chick (Vogel-Hopker et al. 2000). In the transgenic mouse embryos specifically expressing FGF-8 in the lens, changes in ocular development and formation of microphthalmic eyes were seen due to the induction of lens epithelial cells to undergo premature fiber differentiation (Lovicu and Overbeek 1998).

4.1.3. The bases for the current research and research goals

The effects of FGFs on cell proliferation and differentiation have been well documented. Among them, FGF-1 and 2 have been studied extensively in bone, but the results have been variable, and the underlying mechanism is not clear. Among the FGF family members, FGF-8 is most homologous to FGF-17 and FGF-18 with 63.7% and 56.8% identity, respectively, at the amino acid level (Maruoka et al. 1998). FGF-8 has been suggested to be involved in the development of the fetal skeleton based on its ability to induce ectopic limb formation in chick embryos (Crossley et al. 1996b; Szebenyi and Fallon 1999) and its expression in the developing skeleton, an expression pattern similar to FGF-17 and FGF-18 (Xu et al. 1999; Ohbayashi et al. 2002). FGF-18 was also found
to stimulate the proliferation of mouse cultured osteoblasts and chondrocytes, induce osteoclast formation from mouse bone marrow cultures and stimulate mature osteoclast activity (Shimoaka et al. 2002), while FGF-8 has not previously been studied in bone cells.

In a mouse fibroblast-derived cell line C3H10T1/2, FGF-8 stimulated the expression of Cjab1, a well-characterized osteoblast differentiation factor (Zhou et al. 2000). Recently, colleagues in the Department of Anatomy at Auckland University found that FGF-8a stimulated the differentiation of chondrocytes and the expression of the gap junction protein connexin 43 (Cx43) in cultured mesenchymal cells from the chick limb buds (Lin et al. 2000). Cx43 has been found to be involved in the ossification and osteoblast function (Lecanda et al. 2000). These results are suggestive of the potential role of FGF-8 on the skeleton.

Based on the previous results, this project investigated the effect of FGF-8 on osteoblast and osteoclast development and function. Its expression in osteoblasts was also examined by RT-PCR.

4.2. MATERIALS AND METHODS

4.2.1. Reagents

FGF-8a was kindly provided by Dr Jun-sheng Lin, Department of Anatomy, University of Auckland. Murine FGF-8a cDNA without a signal sequence was cloned into the bacterial expression vector pET28a(+) (Novagen Inc., Madison, WI, USA). The recombinant FGF-8a with a histidine tag at its N-terminal was expressed in a E.coli strain BL21(DE3) (Novagen Inc.).

Monoclonal mouse anti-Cx34 antibodies specific to the cytoplasmic loop (amino acid sequence 131-142) were kindly provided by Dr. David Becker, University College of London, UK.
Secondary fluorescence antibodies (goat anti-mouse, Alexa 568) were from Molecular Probes, Oregon, USA.

4.2.2. DNA sequencing

The DNA band of interest was excised from the gel and the DNA was extracted by using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). DNA sequencing was carried out using an ABI PRISM® 3100 sequencer (Applied Biosystems, Foster City, CA) operated by Kristine Boxen in the School of Biological Sciences, University of Auckland, based on the dideoxy-mediated chain-termination method (Sanger et al. 1977) with dye labeling. An amount of 100 ng of double-stranded DNA template was used for each sequencing.

4.2.3. Immunostaining of Cx43

Osteoblast culture and drug treatment methodology were the same as stated in Chapter 2.12 except culture in chamber slides. The immunostaining was based on the protocol previously described (Makarenkova et al. 1997). At the end of culture, medium was tipped off and cells washed with PBS were fixed onto the slides by the treatment of 4% paraformaldehyde (in PBS) for 10 minutes followed by two washes with PBS (5 minutes each). Then the cells were treated with 0.01% Triton 100 for 5 minutes and washed twice with PBS (5 minutes each). Non-specific binding sites were blocked by 10% FBS for 1 hour. After 3 washes with PBS (15 minutes each), the slides were incubated with monoclonal mouse anti-Cx43 antibodies (50 μL/well, 1:200 diluted with PBS) at 4°C overnight. Following the removal of the free antibodies and 3 washes with PBS (15 minutes each), secondary fluorescence antibodies (50 μL/well, 1:200 diluted goat anti-mouse Alexa 568, Molecular Probes) were added and left at room temperature for 1 hour in darkness. After 3 washes with PBS (15 minutes each) and addition of Citifluor antifade medium (Alltech Associates, Inc., Auckland), the slides were mounted and sealed with nail polish. The fluorescence images were visualized with TCS 4D Confocal Laser Scanning Microscope (Leica).
4.2.4. Nodule formation assay

Rat osteoblast preparation was as described in Chapter 2.12. Nodule formation assay was based on Bellows et al.'s protocol with modification (Bellows et al. 1986). Rat primary osteoblasts in α-MEM containing 15% FBS and 50 μg/mL ascorbic-2-phosphate were seeded onto 35-mm dishes at a total volume of 3 mL/dish and at a final population of 3.5 x 10⁴ cells/dish. Test factors and β-glycerophosphate (at final concentration of 10 mM) were added at the stage when the cultured cells reached confluency. Media and test factors are changed twice a week. After culture for 21 days, the cells were stained with Vonkossa. The nodule area in the image was measured by the program TAS (version 2.05, Steve Paxton, 1999).

4.3. RESEARCH RESULTS

4.3.1. Effect of FGF-8a on osteoblast proliferation

To determine the involvement of FGF-8a in bone metabolism, its effect on primary osteoblast proliferation was firstly investigated by measuring [³H]-thymidine incorporation. The result showed that FGF-8a dose-dependently stimulated osteoblast proliferation with significant effect seen at concentrations of 25 ng/mL (≈ 0.96 x 10⁻⁹ M) and above. When the concentration was increased to 500 ng/mL, the cell proliferation was increased by approximately 2.2-fold (Fig. 4-2).

The cells directly isolated from the animal and used in Fig. 4-2 have well-recognized phenotype of osteoblasts. However, since it is very difficult to make them pure enough to completely exclude the influence of other types of cells, an osteoblastic cell line UMR-106 was used to further confirm the mitogenic activity of FGF-8a. Consistently, FGF-8a also stimulated the proliferation of osteoblastic UMR-106 cells with a significant effect seen at 25 ng/mL. At the concentration of 100 ng/mL, the proliferation rate was elevated by about 30% (Fig. 4-3).
Fig. 4-2. Stimulatory effect of FGF-8a on rat primary osteoblast proliferation. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=6/group. Significant differences from control are indicated with "**" ($P<0.01$).

Fig. 4-3. Stimulatory effect of FGF-8a on the proliferation of rat osteoblastic cell line UMR-106. Data are presented as the mean ± SE, n=6/group. Significant differences from control are indicated with "**" ($P<0.01$).
4.3.2. Connexin 43 (Cx43) expression in osteoblasts

Gap junctions are the intercellular channels, which allow the passage of small molecules and communication between adjacent cells. In osteoblasts and osteoclasts, connexin 43 (Cx43) is the major protein to constitute the gap junctions (Ilvesaro et al. 2000; Lecanda et al. 2000). It has been documented that Cx43 is involved in osteoblast proliferation and differentiation (Shiokawasawada et al. 1997; Lecanda et al. 2000; Gramsch et al. 2001). Therefore, the relationship between Cx43 expression and the osteoblast proliferation enhanced by FGF-8a was investigated.

The expression of Cx43 was examined by immuno-staining and confocal microscopy. The fluorescent signal for Cx43 was clearly seen along the borders of the adjacent osteoblasts cultured on the chamber slides. However, the fluorescent density showed that treatment with FGF-8a (100 ng/mL) for 24 hours had no obvious effect on the expression of Cx43 in rat primary osteoblasts (Fig. 4-4). This is different from the observation in chick mesenchymal cell cultures, where FGF-8a markedly stimulated Cx43 expression within the same duration of treatment (Lin et al. 2000).

![Confocal image](image.png)

**Fig. 4-4.** Confocal image (400 x 400) of connexin 43 expression in rat primary osteoblasts. The treatment of FGF-8a (100 ng/mL) was for 24 hours.
4.3.3. Effect on nodule formation

To look at the effect of FGF-8a on osteoblast differentiation, long-term bone nodule cultures of rat primary osteoblasts were employed. In this assay, FGF-8a was added when the cell cultures were confluent, which minimizes the effect on cell proliferation. Fig. 4-5 shows that FGF-8a at 25 and 100 ng/ml significantly reduced nodule formation by 11.8 and 13.2% respectively.

Fig. 4-5. Effect of FGF-8a on nodule formation from rat osteoblast cultures. Data are presented as the mean ± SE, n=6-8/group. Significant differences from control are indicated with * (P≤0.05).

4.3.4. Osteoclastogenesis in mouse bone marrow cultures

The effect of FGF-8a on osteoclastogenesis induced by 1,25(OH)₂D₃ was investigated in mouse bone marrow cultures. In this assay, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) after seven days of culture, and the TRAP-positive cells with three or more nuclei were counted as osteoclast-like cells. The result from this assay showed that FGF-8a moderately reduced the number of osteoclasts with a significant effect seen at concentrations of 0.05 to 25 ng/mL (approximately 5 X 10⁻¹² to 10⁻⁹ M). At the maximal concentration of 25 ng/mL, the osteoclast number was reduced by about 13% (Fig. 4-6). In contrast, a significant effect of FGF-8a on osteoblast proliferation could only be produced at 25 ng/mL or greater (Fig. 4-2 and 4-3).
Fig. 4-6. Inhibitory effect of FGF-8a on osteoclast formation from mouse bone marrow cultures. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=8/group. Significant differences from control are indicated with "*" (P<0.05) and "**" (P<0.01).

Since RANKL and OPG play a key role in osteoclast development, their expression upon FGF-8a treatment was assessed by real-time PCR. Fig. 4-7 shows that FGF-8a had no significant effect on the individual expression of RANKL or OPG in mouse bone marrow cells, but significantly increased the RANKL/OPG ratio on day 5 of culture. This indicates that the expression levels of these peptides were not in favor of the inhibitory effect of FGF-8a on osteoclast formation, thus this data indicate that the RANKL/OPG system does not likely explain the decrease in osteoclastogenesis.

4.3.5. Effect of FGF-8a on osteoclast activity

In calvarial organ culture, the pre-labeled $^{45}$Ca release and $[^3]$H-thymidine incorporation measured bone resorption and cell proliferation, respectively. Fig. 4-8 shows that FGF-8a at concentrations of 1 to 100 ng/mL had no significant effect on either $^{45}$Ca release or $[^3]$H-thymidine incorporation in mouse calvarial organ cultures. Here, rat amylin at 5 X $10^{-8}$ M was included as a positive control.
It was reported that FGF-2 increased pit formation by directly stimulating mature osteoclast activity (Chikazu et al. 2000; Kawaguchi et al. 2000). To test if FGF-8a can resemble the effect of FGF-2, isolated mature osteoclast culture was used. The result showed that the pit formation on bone slices was not altered by FGF-8a at 1 to 200 ng/mL (Fig. 4-9), demonstrating that FGF-8a was different from FGF-2 and had no direct interaction with the mature osteoclasts.

**Fig. 4-7.** Effects of FGF-8a on RANKL and OPG expression in mouse bone marrow cells. FGF-8a or vehicle was added on days 0, 2 and 4, and the cells were harvested for mRNA preparation on the days indicated. RANKL and OPG expression were measured by multiplex real-time PCR with 18 S rRNA as internal control. Data are presented as the mean ± SE, n=3/group. Significant difference from control is indicated with “*” (P≤0.05).
Fig. 4-8. FGF-8a has no effect on $^{45}$Ca release (a) and $[^3]$H-thymidine incorporation (b) in mouse calvarial organ cultures. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "***" ($P<0.01$).

Fig. 4-9. FGF-8a has no effect on rat mature osteoclast activity. Data are presented as the mean ± SE, n=8/group.

4.3.6. FGF-8 expression in osteoblasts

FGF-8 has been shown to be expressed preferentially in the tissues of developing embryos and, to a lesser extent, in some tissues of the adult animals (Section 4.1.1.4). However, its expression in osteoblasts was previously unknown. To address this question, studies were conducted by using RT-PCR.
In this study, the total RNA was extracted from the cells and the tissues indicated, and the RNA was then subjected to RT-PCR. Since some potential isoforms of FGF-8 share the same coding regions and have similar size in the nucleotide sequence (Blunt et al. 1997), it is impossible to distinguish all of them by PCR. Therefore, the primers for PCR were designed to be common to all of the 8 potential isoforms by using the rat FGF-8 cDNA sequence (Accession: AB079113). The predicted size of the amplicon is 374 bp.

**Fig. 4-10.** Detection of FGF-8 expression in fetal rat brains, cultured UMR-106 cells and rat primary osteoblasts (OB) by RT-PCR. The upper panels (a) show the 374 bp amplicon (FGF-8) by RT-PCR with 35 cycles; The middle panels (b) show the equivalent expression of β-actin detected by RT-PCR with 27 cycles; The lower panels (c) show the equivalent amount of total RNA used in RT-PCT.

The gel resolution showed that RT-PCR of the RNA from the fetal rat brain, cultured rat primary osteoblasts or an osteoblastic cell line UMR-106 generated a band with the size expected (Fig. 4-10). To confirm its identity, the DNA in the gel was extracted and analyzed by sequencing. The result showed that the sequence of this RT-PCR product was identical to the FGF-8 cDNA sequence from the Genebank (Accession: AB079113),
demonstrating the expression of FGF-8 in these cells. The semi-quantitative RT-PCR also revealed the markedly different levels of FGF-8 expression in the above cells. As seen in Fig. 4-10, FGF-8 expression in the primary rat osteoblasts was obvious, but at much lower levels than those in the fetal rat brain or in UMR-106 cells.

4.4. DISCUSSION

While the role of FGF-8 on fetal organ development has been well documented (Crossley et al. 1996a; Crossley et al. 1996b; Vogel et al. 1996; Liu et al. 1999; Lewandoski et al. 2000), its role in bone cell biology had not been previously investigated. The results from this study demonstrated, for the first time, that FGF-8a potently stimulated the proliferation of osteoblastic cells and mildly decreased bone nodule formation in long-term cultures. In addition, osteoclast formation from mouse bone marrow cultures was dose-dependently inhibited by this molecule. Its involvement in bone cell physiology was further supported by the detection of its expression in the cultured osteoblasts.

4.4.1. Effect on osteoblast proliferation and differentiation

Among the FGF family members, FGF-1 and FGF-2 has been most widely studied. Their mitogenic effect on osteoblasts has been well documented in fetal rat calvarial organ cultures (Canalis et al. 1988; Canalis and Lian 1988; Shen et al. 1989; Hurley et al. 1992), cultured osteoblasts derived from calvaria (Rodan et al. 1989; Debiasis et al. 1998), and cultured osteoblastic cell lines ST2 and MC3T3-E1 (Jimi et al. 1996; Shimoaka et al. 2002). FGF-18 was also found to stimulate the proliferation of osteoblasts in culture (Shimoaka et al. 2002). However, FGF-1 (Shen et al. 1989; Tang et al. 1996), FGF-2 (Rodan et al. 1989; Mansukhani et al. 2000) and FGF-18 (Shimoaka et al. 2002) were found to inhibit nodule formation, mineralization and the expression of alkaline phosphatase, an osteoblast differentiation marker. The results from this study showed that FGF-8 closely resembled the effects of FGF-1, FGF-2 and FGF-18 on osteoblast proliferation and differentiation.
It is known that all four types of FGFR are expressed in osteoblasts (Chikazu et al. 2000; Powers et al. 2000; Chikazu et al. 2001; Cool et al. 2002). While it is generally agreed that FGF-8 preferentially interacts with FGFR4 and, to lesser extent, with FGFR3 and FGFR2 (Szebenyi and Fallon 1999; Powers et al. 2000), it is likely that FGFR4, and possibly FGFR3 and FGFR2 are the signaling mediators through which FGF-8a affects osteoblast proliferation and differentiation. However, at this stage, it is unknown whether the effect of FGF-8a on osteoblast proliferation and differentiation is mediated by the same FGFR or by the specific individual FGFR. To specify this, further studies are still required.

While exogenous FGF-8 was demonstrated to stimulate osteoblast proliferation, the expression of FGF-8 was further detected by RT-PCR in cultured primary osteoblasts and osteoblastic UMR-106 cells. This provided the evidence for the first time that FGF-8 may play a physiological role on osteoblasts via an autocrine mechanism.

The importance of Cx43 in bone cells has been revealed based on the evidence including: over-expression of Cx43 in osteoblastic UMR-106 cells increased cell proliferation, calcium content of extracellular matrix and nodule formation (Gramsch et al. 2001); whereas deficiency of Cx43 resulted in a delay in ossification of the developing skeleton in embryos and reduced differentiation of osteoblasts in culture (Lecanda et al. 2000). FGF-8a had previously been shown to stimulate Cx43 expression in developing limb buds (Lin et al. 2000). Based on these lines of evidence, the expression of Cx43 under FGF-8a treatment was investigated. However, from fluorescent density measurements using confocal microscopy, no difference in Cx43 expression was seen between the control and the FGF-8a treatment, indicating that this is not a function of FGF-8a in mature osteoblasts.

FGF-8a exhibited potent mitogenic activity on osteoblasts derived from rat calvariae, however, it had no effect on [3H]-thymidine incorporation in cultured calvarial bones. This indicates that the mitogenic activity of FGF-8a is restricted by the microenvironment of the calvarial bones. At present, the underlying mechanism leading to this phenomenon is still unknown. The elucidation of this will be an interesting research topic.
4.4.2. Effects on osteoclast formation and functioning

FGF-8a moderately inhibited osteoclast formation from mouse bone marrow cultures. However, RANKL and OPG expression was not accordingly decreased or increased, respectively, by FGF-8a at concentrations effective on osteoclast formation. This indicated that FGF-8a modulated osteoclast formation via a RANKL/OPG-independent pathway.

FGF-8a showed no effect on $^{45}$Ca release in calvarial organ cultures, in which bone resorption mainly reflects action of mature osteoclasts since there is very little bone marrow present. The ineffectiveness of FGF-8a in this assay indicated the lack of action on mature osteoclasts in these bones. This presumption was further supported by the isolated osteoclast assay, in which FGF-8a did not affect absorption pit formation either. In contrast, it has been documented that FGF-2 stimulated the resorption in both calvarial organ cultures and isolated osteoclast cultures (Kawaguchi et al. 1995; Chikazu et al. 2000; Kawaguchi et al. 2000). This further suggests the correlation of the effects between the two models, and indicates the difference between FGF-8 and FGF-2 in term of their modes of action.

The above observation implied the absence of the functioning receptor for FGF-8 in the osteoclasts. It has been documented that the only type of FGFR expressed in osteoclasts and their precursors is FGFR1 (Chikazu et al. 2000; Chikazu et al. 2001). At present, it is still uncertain as to whether FGF-8 interacts with FGFR1 or not. While it has been reported that FGF-8 has almost no specific interaction with FGFR1 (MacArthur et al. 1995b; Blunt et al. 1997; Chellaiah et al. 1999; Szebenyi and Fallon 1999), some evidence showed that FGF-8 bound to and activated this type of FGFR (Sato et al. 1993; Koga et al. 1995; Galzie et al. 1997; Valve et al. 2001). In comparison with FGF-2, which interacts with FGFR1 and shows direct effect on osteoclasts (Kawaguchi et al. 1995; Szebenyi and Fallon 1999; Chikazu et al. 2000; Kawaguchi et al. 2000), the results from the calvarial organ cultures and isolated osteoclast assays from this study do not support the proposed interaction between FGFR1 and FGF-8.
4.5. SUMMARY AND CONCLUSIONS OF CHAPTER 4

The results from this study demonstrated for the first time that FGF-8 was active in bone cells. It potently stimulated osteoblast proliferation, whilst mildly inhibiting bone nodule formation. In addition, FGF-8a moderately inhibited osteoclast formation in vitro via a RANKL/OPG-independent pathway.

The expression of FGF-8 was detected in the osteoblastic cell line UMR-106 (at higher levels) and in primary rat osteoblasts (at lower levels). Therefore, it is possible that FGF-8 plays a physiological role in bone metabolism via, at least in part, an autocrine mechanism.

FGF-8a had no effect on bone resorption in calvarial organ cultures and isolated osteoclast cultures, indicating that it had no direct interaction with mature osteoclasts.

To confirm whether the in vitro effects of FGF-8a translate into similar in vivo effects, it is the intention of our laboratory to further test FGF-8a in a systemic model.
CHAPTER 5: α-MELANOCYTE STIMULATING HORMONE (α-MSH) IS A NOVEL REGULATOR OF BONE

5.1. INTRODUCTION

The study of the role of pituitary gland on pigmentation led to the identification of α-melanocyte stimulating hormone (α-MSH) in the 1950s (Lerner 1993). However, its physiological role is not confined to this. It has been documented that this peptide is also active in energy homeostasis (Cone 1999) and is a down-regulator of body weight (McMinn et al. 2000). Meanwhile, studies found that body weight is positively correlated to bone mass (Felson et al. 1993) and that reduction of overweight is accompanied by the loss of bone mineral density (Fogelholm et al. 2001). Based on the relationship between body weight and bone mineral density, it need to be addressed whether the effect of α-MSH on body weight is associated with bone metabolism. In addition, α-MSH has been found to be correlated to the levels of other osteotrophic factors such as leptin (Fehm et al. 2001; Cornish et al. 2002b), insulin (Shimizu et al. 1995; Fehm et al. 2001), amylin (Cornish et al. 1995) and preptin (Cornish et al. 2002a). This enhances the possible impact of α-MSH on skeleton and bone cell biology.

5.1.1. Biochemistry, molecular biology and production of α-MSH

α-MSH is a 13-amino acid peptide derived from the proteolytic cleavage of a 32-kD propeptide called pro-opiomelanocortin (POMC). In addition to α-MSH, POMC also gives rise to β-MSH, γ-MSH, adrenocorticotropin (ACTH) and β-endorphin (Krude and Gruters 2000). The gene for POMC in the human was mapped to chromosome 2p21 (Pritchard et al. 2002). As shown in Fig. 5-1, it consists of three exons and two introns. Exon 1 corresponds to the 5' non-coding region. Exon 2 corresponds to part of the 5' non-coding region and encodes the signal sequence of the pro-POMC. Exon 3 encodes all the five peptides above and the 3'-untranslated mRNA (Takahashi et al. 1983; Slominski et al. 2000). These encrypted peptides in POMC are generated upon the
sequential cleavage by prohormone convertase 1 and 2, which belong to the family of serine proteases (Pritchard et al. 2002).

Fig. 5-1. The genomic structure of the gene encoding proopiomelanocortin (POMC), which is the precursor of the five known peptides including α-MSH. The number indicates the nucleotide position. The shaded boxes indicate the coding sequence for POMC (Adopted from Krude and Gruters 2000, with modification).

The expression of POMC was detected in the pituitary gland, brain, skin, immune system and gastrointestinal tract. In rodents, its expression is down-regulated by food restriction (Cone 1999) and up-regulated by high-fat feeding (Ziotopoulou et al. 2000). It is suggested that this transcriptional control is at least in part mediated via leptin (Krude and Gruters 2000; Pritchard et al. 2002), a factor recently shown to have effects on bone mass when administered into the central nervous system (Ducy et al. 2000), or locally (Cornish et al. 2003). The average circulating α-MSH concentration in human is $21.30 \pm 0.63$ pg/mL and is generally stable over time. However, its levels decrease rapidly in infants after birth and continue to fall with age (TEM 11:304, 00@). It was found that the plasma levels of α-MSH were significantly higher in obese men and were positively correlated to the volume of visceral fat of the obese subjects. Though it is suggested that the adipose tissue may be a potential site of α-MSH production, it is still unclear by what mechanism the circulating concentrations of α-MSH were elevated in the obese humans (Katsuki et al. 2000).
5.1.2. Biological activities of α-MSH

Except for β-MSH whose physiological function is still uncertain, POMC-derived peptides, α-MSH, γ-MSH, ACTH and β-endorphin, are known to have exclusive or overlapping biological activities. The biological activities of α-MSH, γ-MSH and ACTH are mediated by five receptors: melanocortin-1 receptor (MC1-R) to MC5-R, while the pain sensation function of β-endorphin is mediated by another set of molecules called opioid receptors (Krude and Gruters 2000).

5.1.2.1. Skin and hair pigmentation

The determinant for skin and hair colour is the ratio of the two melanins called eumelanin (brown-black) and phaeomelanin (red-yellow), which are synthesized by melanocytes (Graham et al. 1997). A major effect of α-MSH is on the synthesis of the pigments. In the hair follicular melanocytes of yellow mice and in human epidermal melanocytes, α-MSH preferentially stimulates eumelanin synthesis, which confers black coat colour to skin and hair (Thody 1999). In humans, α-MSH increased pigmentation in skin, particularly in sun-exposed areas. Since ultraviolet (UV)-radiation is capable of enhancing α-MSH production and MC1-R expression in melanocytes, it is suggested that α-MSH may play a central role in mediating the cutaneous melanogenesis effect of UV-radiation (Chakraborty et al. 1999; Scholzen et al. 1999).

The role of α-MSH on melanogenesis is mediated exclusively by the cAMP-coupled receptor MC1-R, which in turn activates tyrosinase. Among the five subtypes of receptors, MC1-R is the only receptor expressed in melanocytes and has highest affinity for α-MSH, which is also expressed in melanocytes. Therefore, an autocrine mechanism is involved in the cutaneous effect of α-MSH (Thody 1999; Catania et al. 2000a).

α-MSH also stimulates the dendricity and proliferation of melanocytes (Hunt et al. 1994; Abdel-Malek et al. 1995), and protects melanocytes from oxidative damage (Thody 1999).
5.1.2.2. Food intake and body weight

α-MSH and agouti peptide act, as agonist and antagonist respectively, at MC4-R. Blockade of α-MSH signaling by the ectopic expression of agouti peptide within the nervous system (agouti peptide is normally expressed in skin) resulted in the obesity syndrome in mouse. This led to the discovery of the key role of α-MSH in the central regulation of appetite and body weight (Fan et al. 1997). In addition, targeted disruption of MC4-R also resulted in obesity in mice (Huszar et al. 1997), further demonstrating the role of α-MSH in reducing body weight through MC4-R signaling.

Other evidence to support this include the following observations and studies: The frameshift mutations (a 4-bp deletion and a 4-bp insertion in the coding region) in human MC4-R were found to be associated with a dominant form of obesity (Vaisse et al. 1998; Yeo et al. 1998); Chronic α-MSH infusion into the third cerebral ventricle of rats for 6 days significantly reduced food intake and body weight (McMinn et al. 2000); Ventricular injections of synthetic MC4-R-specific agonists melanotan II reduced food intake in rats, while injections of MC4-R antagonists, SHU9119, had the opposite effect (Cabeza de Vaca et al. 2002; Yang et al. 2002).

It seems to be likely that α-MSH also regulates energy homeostasis via MC3-R signaling, since MC3-R knock-out mice show increased fat mass and reduced lean body mass (Butler et al. 2000; Chen et al. 2000).

5.1.2.3. Anti-inflammation and anti-infection

It has been documented that α-MSH is involved in host defense. Both α-MSH (1-13) and its C-terminal tripeptide KPV, α-MSH (11-13), were found to reduce the viability of two representative infectious agents: the gram-positive bacterium Staphylococcus aureus and the yeast Candida albicans. In C. albicans, these peptides also inhibited the formation of germ tubes, a morphologic structure necessary for the infection process (Catania et al. 2000b). The changes in circulating and local concentrations of α-MSH in
response to infection and diseases also suggest its role in anti-infection. For example, higher levels of α-MSH were observed in the synovial fluid from the patients with rheumatoid arthritis (Catania and Lipton 1994); Circulating α-MSH was increased in HIV-positive patients and treatment with this peptide inhibited HIV replication in the infected cells (Catania et al. 1998; Barcellini et al. 2000).

5.1.2.4. Cardiovascular effect

While γ-MSH has been demonstrated to have a predominant and marked role in cardiovascular system, the effect of α-MSH in this system has also been reported (Versteeg et al. 1998). Intracerebroventricular administration of α-MSH significantly increased the lumbar sympathetic nerve activity and the mean arterial pressure in rats (Dunbar and Lu 1999; Lu et al. 2000). Intravenous injections of α-MSH in rats improved survival from fatal hypovolemic shock and resuscitation from ventilation interruption (prolonged asphyxia) primarily due to the cardiac function of α-MSH (Bertolini et al. 1986; Guarini et al. 1997).

Contrary to the above pressor and tachycardiac effects, injection of α-MSH into the medullary dorsal-vagal complex and nucleus tractus solitarius of rats caused hypotension and bradycardia, and attenuated the pressor effect of dynorphin 1-9, respectively (Carter and Lightman 1987; Li et al. 1996).

Although both MC3-R and MC4-R have been suggested to be the mediators of the cardiovascular effect of α-MSH (Li et al. 1996), it is generally agreed that this effect is mediated by MC3-R, which both α-MSH and γ-MSH interact with (Versteeg et al. 1998; Krude and Gruters 2000).

5.1.3. The bases for current research proposal and research goals

Body weight is an important determinant of bone mineral density (Reid et al. 1992a; Felson et al. 1993; Ravn et al. 1999) and is one of the most important risk factors for
osteoporotic fractures (Williams et al. 1982; Kiel et al. 1987; Ensrud et al. 1997). The two major components of body weight, fat mass and lean mass, probably each contribute to these relationships, but in a number of studies fat mass has been shown to have a substantial independent effect on both bone density (Reid et al. 1992a; Reid et al. 1994; Ravn et al. 1999; Pluijm et al. 2001) and fracture rates (Schott et al. 1998; Lau et al. 2000). The effects of fat mass on skeletal load may contribute to this relationship, though they do not explain it in non-weight-bearing sites (Reid et al. 1992a). Similarly, estrogen production in the adipocyte may contribute to these relationships in postmenopausal women, but it does not explain the relationship between fat mass and bone density before the menopause (Reid et al. 1992b). It is therefore of interest to assess the skeletal impact of hormonal factors that either regulate fat mass or are influenced by it. For these reasons there has been a recent focus of attention on the roles of insulin, amylin, leptin and preptin on skeletal metabolism (Reid 2002). However, there are a number of other hormones that could contribute to these relationships, one of which is α-MSH.

α-MSH acts via the melanocortin receptors, and as mentioned above, it has been reported that humans deficient in MC4-R have markedly increased bone mass (Farooqi et al. 2000). Furthermore, it has recently been noted that the MC4-R is present in an osteoblast-like cell line, UMR 106 (Dumont et al. 2001), raising the possibility that α-MSH may act directly on the skeleton.

The present studies investigate the involvement of α-MSH in bone metabolism by assessing the effects of α-MSH on cells of the osteoblast and osteoclast lineages, and assess the effect of its systemic administration to intact adult mice. Its effect on bone cells was further studied by looking at its effect on the expression of osteoblast differentiation markers (alkaline phosphatase, osteocalcin and collagen I) and osteoclast formation regulators (OPG and RANKL).

5.2. MATERIALS AND METHODS

5.2.1. Peptide

α-MSH was kindly provided by Dr Kathy Mountjoy, Department of Paediatrics, University of Auckland.
5.2.2. Assessment of expression of osteoblast differentiation markers

Primary rat osteoblasts were plated in MEM containing 10% FBS, at 1x10⁶ cells/75 cm² flask. The next day (day 1), the media were changed to αMEM containing 15% FBS, 5 μg/mL L-ascorbic acid 2 phosphate and 10 mM β-glycerophosphate, and one-half of the flasks were supplemented with 10⁻⁸ M α-MSH. The media were changed to fresh media with or without α-MSH on days 4 and 7. On days 4, 7 and 10, one flask treated with α-MSH and one control culture were harvested. Unless otherwise indicated, RNA extraction and semi-quantitative RT-PCR were performed as described in Chapter 2.14. PCR products were analysed after 28, 30 and 35 cycles for alkaline phosphatase, 35 cycles for osteocalcin, and 20, 25 and 30 cycles for collagen I α1. The PCR experiments were repeated three times with similar results using the following primers:

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequences</th>
<th>Amplicons (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>forward: 5' CCCAA AGGCT TCTTC TTG 3'</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>reverse: 5' CCTGG TAGTT GTTGT GAGCA 3'</td>
<td></td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>forward: 5' CTCTC TGCTC ACTCT GCTGG 3'</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>reverse: 5' AAGCC GATGT GGTCG GC 3'</td>
<td></td>
</tr>
<tr>
<td>Collagen I α1</td>
<td>forward: 5' GTGGT CAGGC TGTTG TGATG 3'</td>
<td>565</td>
</tr>
<tr>
<td></td>
<td>reverse: 5' GACCA CGGAC GCCAT CTT 3'</td>
<td></td>
</tr>
</tbody>
</table>

5.2.3. Osteoblast, osteoclast and chondrocyte cultures

Osteoblast preparation and culture was as described in Chapter 2.12. Osteoclast formation from mouse bone marrow culture and rat isolated osteoclast activity assay were as described in Chapter 2.10 and 2.13, respectively.

Chondrocytes were isolated by removing cartilage (full-depth slices) from the tibial and femoral surfaces of adult dogs under aseptic conditions. Slices were placed in DMEM (Gibco, Life Technologies, Grand Island, NY) containing 5% FBS (v/v) and chopped
finely with a scalpel blade. Tissue was removed and incubated at 37°C with pronase (0.8% w/v for 90 minutes) followed by collagenase (0.1% w/v for 18 hours) to complete the digestion. The cells were isolated from the digest by centrifugation (10 minutes at 1300 rpm), re-suspended in DMEM containing 5% FBS, passed through a nylon mesh screen of 90 μm pore size to remove any undigested fragments, and recentrifuged. The cells were washed twice, re-suspended in the same media and seeded into a 75 cm² flask containing DMEM-10% FBS and 5 μg/mL ascorbic acid. The cells were incubated under 5% CO₂/95% air at 37°C. Confluence was reached by day 7, at which time the cells were subcultured. After trypsinization using trypsin-EDTA (0.05%/0.53 mM), the cells were rinsed in DMEM containing 5% FBS and re-suspended in fresh medium, then seeded into 24-well plates (5x10⁴ cells/mL, 0.5 mL/well). Measurement of cell numbers and thymidine incorporation were performed in growth-arrested cell populations as for the osteoblast-like cell cultures.

5.2.4. Systemic study
5.2.4.1. Experimental design

Two groups of 20 sexually mature male Swiss mice aged between 40 and 50 days and weighing 25-36 g were given daily subcutaneous injections (4.5 μg of α-MSH in 50 μL of water, or water alone) in the loose skin at the nape of the neck for 5 days/week over 4 consecutive weeks. This dose was chosen because the same molar doses of amylin and adrenomedullin in this model produce substantial effects on bone turnover and bone area (Cornish et al. 1998a; Cornish et al. 2001a), and these peptides have similar effects on osteoblast proliferation. Animals were housed in a room maintained at 20°C on 12-hour light/dark cycles. They were fed diet 86 rodent pellets (New Zealand Stockfeed Ltd) ad libitum throughout the experiment. Each animal’s weight was recorded at the beginning and end of the experiment.

5.2.4.2. Histomorphometry

Histomorphometric analyses were carried out in the proximal tibia. The tibiae were dissected free of adherent tissue, and bone lengths were recorded by measuring the
distance between the proximal and the distal epiphyses with an electronic micrometer (Digimatic Calipers, Mitutoyo, Japan). Tibiae were then processed as previously described (Cornish et al. 2000). Briefly, bones were fixed in 10% phosphate-buffered formalin for 24 hours and then dehydrated in a graded series of ethanol solutions and embedded undecalcified in methyl methacrylate resin. Tibiae were sectioned longitudinally through the frontal plane. Sections (4 μm thick) were cut using a Leitz rotary microtome and a tungsten-carbide knife, then mounted on gelatin-coated slides and air-dried. They were stained with Goldner’s tri-chrome and examined using an Olympus BX 50 microscope, which was attached to an Osteomeasure Image Analyzer.

Tibial histomorphometric analyses were made from three adjacent sections one third of the way through the anterior/posterior depth of the proximal tibiae. All trabecular bone tissue in the secondary spongiosa was quantified for bone volume in each section using a 10 x objective, and parameters were derived using the formulae of Parfitt et al (Parfitt et al. 1983). Parameters of bone formation and resorption were measured using a 20 x objective in all trabecular bone tissue in the secondary spongiosa in the second of the three adjacent sections. Cell numbers were expressed as per unit of bone area. Cortical width was measured on both sides of the tibial shaft 2.5 mm below the epiphyseal growth plate. Epiphyseal growth plate thickness was measured at three sites evenly spaced along its length. All measurements were made by one operator who was blinded to the treatment group of each bone.

5.2.4.3. Phase-contrast X-ray imaging

X-ray microimaging was performed at CSIRO Manufacturing Science and Technology, Clayton, Victoria, Australia. This method has been described previously (Wilkens et al. 1996). Excised tibiae from the animals were fixed and stored in 70% ethanol. The tibiae were prepared for imaging by orienting the bones in a brass clamp to enable mounting in the X-ray beam. These images were obtained using a microfocus X-ray source operated at 30 kV and with a 4-μm source size. Samples were mounted at distance R1=10 cm from the X-ray source. An imaging plate was placed at distance R2=190 cm from the sample, resulting in an experimental magnification of x 20. Images were collected with
Fuji imaging plates scanned using a BAS5000 Phosphorimager (Fuji, Japan). As described previously, imaging plates are particularly suited to the quantitative in-line phase-contrast X-ray technique because of their large linear dynamic range (Gureyev et al. 2000). The features of the images were quantified using MCID Version 6 Elite software (Berthold, Melbourne, Australia). The trabecular region was outlined, and the circumference and length of the trabecular area were determined. A “form factor”, indicative of the shape of the trabecular area, was also calculated.

5.2.4.4. Fat mass estimations

Fat mass estimations were made from measurements of the animals’ body densities calculated from water displacement. Immediately after they were killed, the mice were submerged head-first to the base of the tail into a 250 mL measuring cylinder containing 150 mL of water, and the displacement volume was recorded. The fraction of body weight that was fat mass was calculated using a modification of the Siri equation for use in rodents (Muscaritoli et al. 1993). The coefficient of variation for repeated measures of fat mass is 7%.

5.3. RESEARCH RESULTS

5.3.1. Primary osteoblasts and chondrocytes

α-MSH dose-dependently stimulated thymidine incorporation into primary cultures of fetal rat osteoblasts, indicating a stimulation of DNA synthesis. This was accompanied by an increase in cell number after 24 hours of culture. Both these effects were seen with α-MSH at concentrations of 10^{-9} M and greater (Fig. 5-2).

The effect of α-MSH on osteoblast differentiation was assessed by measuring the expression levels of alkaline phosphatase, collagen I and osteocalcin in control and α-MSH-treated osteoblast cultures. Primary osteoblasts were treated with 10^{-8} M α-MSH, RNA was extracted after 4, 7 and 10 days of treatment, and semi-quantitative RT-PCR was used to compare the expression levels of the osteoblast differentiation markers. Using this semi-quantitative system, no significant differences were seen between the
treatment and the control groups. Alkaline phosphatase and collagen I were expressed at high levels at all the time points studied, while osteocalcin was only visible on day 10, both in the treatment and the control cultures.

α-MSH had a similar effect on the proliferation of primary cultures of canine chondrocytes. Both thymidine incorporation and cell number were increased (Fig. 5-3).

![Graphs showing effect of α-MSH on cell number and thymidine incorporation](image)

**Fig. 5-2.** Effect of α-MSH on cell number (a) and [³H]-thymidine incorporation (b) in primary cultures of fetal rat osteoblasts. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=6/group. Significant differences from control are indicated with "*" (P < 0.05) and "**" (P < 0.01).

### 5.3.2. Osteoclast effects

The effect of α-MSH on the development of osteoclasts was studied in cultures of mouse bone marrow (Fig. 5-4a). The number of osteoclasts present after 7 days of culture was doubled by α-MSH at 10⁻⁷ M. In contrast, α-MSH did not affect the activity of mature osteoclasts. This was assessed from the number of resorption pits formed on bovine bone exposed to osteoclasts prepared from neonatal rats (Fig. 5-4b). These results were confirmed in studies of a second model of mature osteoclast function, the neonatal mouse calvaria (Fig. 5-4c). Because there is virtually no bone marrow in this tissue, these
explants reflect mature osteoclast function, and again showed no effect of α-MSH treatment.

Since α-MSH markedly increased osteoclast number from mouse bone marrow cultures, it is of interest to know if this effect is mediated by RANKL and OPG. Real-time PCR result shows that treatment with α-MSH at $10^{-9}$ and $10^{-8}$ M on days 0, 2 and 4 significantly elevated the ratio of RANKL/OPG on day 5 of mouse bone marrow culture (Fig. 5-5). The elevated ratio of RANKL/OPG levels was consistent with the increased osteoclast number resulted from the treatment with α-MSH.

![Graph](image)

**Fig. 5-3.** Effect of α-MSH on cell number (a) and [³H]-thymidine incorporation (b) in primary cultures of canine chondrocytes. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=6/group. Significant difference from control is indicated with "*" (P < 0.05).

### 5.3.3. Systemic administration of α-MSH

Normal adult male mice were treated systemically with α-MSH or vehicle over a 4-week period. Histomorphometry of the proximal tibiae indicated that bone turnover was increased in the α-MSH-treated animals (osteoblast number/μm²: control 20.1 ± 1.5, α-MSH 28.6 ± 1.7, P < 0.01; osteoclast number/μm²: control 0.26 ± 0.02, α-MSH 0.29 ± 0.03, P > 0.05). However, trabecular bone volume was decreased by almost a quarter in the animals receiving α-MSH (Fig. 5-6). This resulted from a decrease in trabecular
number and an increase in trabecular separation, rather than any change in trabecular thickness (control 24.5 ± 1.3 μm, α-MSH 25.1 ± 1.1 μm, P = 0.74). There was no difference in cortical width between the groups (control 0.19 ± 0.01 mm, α-MSH 0.19 ± 0.01 mm) and growth plate thickness was also comparable in the two groups (control 0.087 ± 0.004 mm, α-MSH 0.087 ± 0.004 mm).

**Fig. 5-4.** Stimulation of α-MSH on osteoclast formation from mouse bone marrow cultures (a) without significant effect on mature osteoclast activity as assessed in isolated osteoclast cultures (b) and calvarial organ cultures (c). Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "***" (P ≤ 0.01).

The results of the histomorphometric analysis of trabecular structure were confirmed by phase-contrast X-ray images of the tibiae. **Fig. 5-7** shows images of 3 tibiae from the control group and 3 from the α-MSH-treated group. Simple inspection indicates that the extent of trabecular bone is diminished following α-MSH treatment. The ‘form factor’ of
the trabecular zone was unchanged. These results indicate that α-MSH caused a reduction in trabecular bone mass without changing the shape of the bones.

Weight gains during the study were not significantly different in the two groups – weight increased in the course of the study from $27.5 \pm 0.8 \text{g}$ to $33.6 \pm 0.8 \text{g}$ in animals treated with vehicle, and from $27.4 \pm 0.8 \text{g}$ to $33.2 \pm 0.5 \text{g}$ in those receiving α-MSH. However, the percent fat mass of the animals at the end of the experiment was lower in the α-MSH-treated group ($8.7 \pm 0.6\%$) compared with control ($10.2 \pm 0.5\%$, $P = 0.05$). Tibial lengths were not different between the groups (control $18.6 \pm 0.1 \text{mm}$, α-MSH $18.7 \pm 0.1 \text{mm}$).

![Graphs showing the effect of α-MSH on RANKL and OPG expression in mouse bone marrow cultures. α-MSH was added on days 0, 2, and 4. Data are presented as the mean ± SE, $n=3$/group. Significant differences ($P<0.05$) between control and $10^{-9} \text{M}$ α-MSH, and between control and $10^{-8} \text{M}$ α-MSH are indicated with "*" and "#", respectively.](image)

Fig. 5-5. Real-time PCR results showing the effect of α-MSH on RANKL and OPG expression in mouse bone marrow cultures. α-MSH was added on days 0, 2, and 4. Data are presented as the mean ± SE, $n=3$/group. Significant differences ($P<0.05$) between control and $10^{-9} \text{M}$ α-MSH, and between control and $10^{-8} \text{M}$ α-MSH are indicated with "*" and "#", respectively.
Fig. 5-6. Effect of daily administration of $\alpha$-MSH (4.5 $\mu$g/mouse/time, 20 times over 4 weeks) on trabecular bone volume, trabecular number and trabecular separation in the proximal tibiae of normal adult male mice. Data are presented as the mean $\pm$ SE, $n=5-8$/group. Significant differences from control are indicated with "*" ($P<0.05$) and "**" ($P<0.01$).

Fig. 5-7. Effect of systemic administration of $\alpha$-MSH on trabecular bones. Phase-contrast x-ray images of the trabecular region of tibiae. The methodology for $\alpha$-MSH treatment and x-ray image were described in Section 5.2.4.
5.4. DISCUSSION

5.4.1. Effects of α-MSH on skeletal cells

The present study is the first to address the direct effects of α-MSH on skeletal cells. It demonstrates that this peptide increased the proliferation of osteoblasts without affecting their differentiation. It was also mitogenic to chondrocytes. While it stimulated the development of osteoclasts from their precursors in bone marrow, it was also found to increase RANKL/OPG ratio, suggesting that a RANKL/OPG pathway was involved in the action of this peptide on osteoclast precursors. However, α-MSH did not act directly on the mature osteoclast, either when studied in isolated cell culture or when assessed in an organ culture model lacking osteoclast precursors.

Though α-MSH is a neuropeptide, it does have direct actions on cells outside the central nervous system (Boston 2000). As its name implies, it regulates pigment production in melanocytes (Mountjoy et al. 1992; Robbins et al. 1993) and there is also evidence of direct actions of α-MSH on adipocytes [where it promotes lipolysis (Boston and Cone 1996)], and the pancreatic β-cell [where it decreases insulin secretion (Shimizu et al. 1995)]. Its adipocyte effects are particularly relevant to the present study, since osteoblasts and adipocytes share a common precursor in the bone marrow stromal cell. The concentrations of α-MSH found to be effective in skeletal cells in the present studies are comparable to those which are active in *in vitro* studies of adipocytes (Boston and Cone 1996) and pancreatic β-cells (Shimizu et al. 1995), and are consistent with the EC₅₀ for cAMP production stimulated by α-MSH via either MC1-R, MC3-R or MC4-R (~10⁻⁹ M) in transfected cell lines (Mountjoy et al. 1992; Gantz et al. 1993a; Gantz et al. 1993b; Mountjoy et al. 1994). The lowest active concentrations of α-MSH in osteoblasts and chondrocytes, are comparable to the circulating concentrations in humans (Katsuki et al. 2001; Nam et al. 2001) and rats (Usategui et al. 1976), suggesting that these direct actions in bone may be physiologically relevant.
5.4.2. The mechanisms of α-MSH action on the balance of bone turnover

The results of systemic administration of α-MSH to adult mice are consistent with the increased osteoblast and osteoclast development in vitro, which may lead to increased bone turnover. In vivo, this was significant with respect to the histomorphometric assessment of osteoblast numbers but not those of osteoclasts, possibly reflecting the inaccuracy of the assessment of bone resorption indices from static histomorphometry. However, the balance of these effects was towards bone loss, resulting in a 22% decrease in trabecular bone volume in the animals receiving α-MSH, and similar changes in the radiographic indices of trabecular mass. This may indicate that, in vivo, the stimulatory effects of α-MSH on osteoclastogenesis predominate over its actions on osteoblast development, though the histomorphometry does not provide clear evidence of this. A second explanation is that the direct effects of α-MSH on bone cells are modulated by its effects on other tissues, such as adipose tissue, the pancreatic β-cell, or the central nervous system. These possibilities will now be discussed.

As reviewed above, fat mass and bone mass have frequently been found to be positively related to one another (Reid et al. 1992a; Reid et al. 1992b; Pluijm et al. 2001), so the lower fat mass of the α-MSH-treated animals is likely to contribute to the negative effect of α-MSH on bone mass. This may have been contributed to, in part, by the lower circulating leptin levels following treatment with α-MSH (Fehm et al. 2001; Cornish et al. 2002b). The effect of α-MSH on fat mass is well established: from studies of α-MSH administration in humans (Fehm et al. 2001), from studies in mice (Huszar et al. 1997) and humans (Gu et al. 1999; Farooqi et al. 2000) lacking a functioning MC4-R, from findings in mice and humans deficient in the α-MSH precursor peptide (POMC) (Krude et al. 1998; Yaswen et al. 1999), and in the mouse which over-expresses agouti, an antagonist of the MC4-R (Huszar et al. 1997). The weight regulatory actions of centrally-administered α-MSH are the product of appetite suppression and increased metabolism resulting from α-MSH effects in the hypothalamus (Butler et al. 2001), though there may also be some contribution from its direct lipolytic effects on adipocytes.
(Boston and Cone 1996). When the peptide is administered peripherally, as in the present studies, effects on fat mass presumably result from the same two mechanisms of action, though the central effect must be considerably less.

α-MSH has been shown to directly inhibit secretion of insulin from the pancreatic β-cell (Shimizu et al. 1995), and decreased circulating insulin concentrations have also been reported in humans treated systemically with α-MSH analogs (Fehm et al. 2001). In vivo, insulin concentrations correlate closely with bone density (Reid et al. 1993; Stolk et al. 1996). This relationship may have a number of mechanisms. Insulin interacts directly with osteoblasts, stimulating cell proliferation in vitro and in vivo (Hickman and McElduff 1989; Pun et al. 1989; Cornish et al. 1996). Also, insulin inhibits sex hormone binding globulin production in the liver, thereby increasing free concentrations of sex hormones (Gafny et al. 1994; Loukovaara et al. 1995). Furthermore, insulin is co-secreted with other bone-active factors, such as amylin, a direct stimulator of osteoblast growth and an inhibitor of osteoclasts (Cornish et al. 1995), and preptin, a fragment of pro-IGF-2 (Buchanan et al. 2001) that is also a potent osteoblast growth factor (Cornish et al. 2002a). Thus, a cascade of bone anabolic factors comes from the pancreatic β-cell and will be reduced by α-MSH treatment. Blockade of this pathway may contribute to the bone loss seen in the in vivo study.

Systemically administered α-MSH is most likely primarily acting through peripheral melanocortin receptors. However, a central mechanism of action cannot be completely ruled out, since there is evidence that it may cross the blood-brain barrier to a small extent (De Rotte et al. 1980). The concept of a centrally acting agent impacting on bone was firstly introduced by Ducy et al (Ducy et al. 2000), who demonstrated that the intracerebroventricular injection of the adipocyte hormone, leptin, resulted in trabecular bone loss similar to that observed in the present in vivo study. The present findings may contribute to an explanation of the Ducy’s work, since α-MSH is an important mediator of the central effects of leptin. Leptin action in the hypothalamus stimulates α-MSH release from neurons of the arcuate nucleus (Seeley et al. 1997) resulting in appetite suppression and weight loss, and leading to effects mediated by the autonomic nervous system. These include the regulation of insulin secretion, which is decreased by as much
as 80% by central α-MSH administration (Fan et al. 2000). This effect is abrogated by sympathetic blockade with phentolamine (Fan et al. 2000), confirming that it is mediated by the autonomic nervous system. Thus, any central effects of α-MSH in this model may act in concert with its direct effects on the pancreatic β-cell to decrease bone mass.

5.4.3. Skeletal effects of α-MSH in different models

Takeda et al have recently provided significant new information regarding the relationship between the bone effects of leptin and α-MSH (Takeda et al. 2002). They reported that the intracerebroventricular administration of an α-MSH analog to ob/ob mice does not affect bone mass; that the yellow agouti mouse, which is resistant to α-MSH, has a normal bone mass, and shows the same bone response to leptin as wild-type mice; and that mice deficient in MC4-R have normal bone mass. However, the latter finding directly contradicts what has been found in humans with this deficiency, in whom bone mass is markedly increased (Farooqi et al. 2000). This discrepancy could be related to differences between species in the role of α-MSH, differences in the gene mutation, effects of the mutations on skeletal size, or differences in techniques for assessment of bone mass (histomorphometry of trabecular bone in the vertebral body in the mouse studies vs. whole body dual-energy X-ray absorptiometry in the humans). Confirmation of the results of Takeda et al will be important to clarify understanding in this area. In the meantime, the present data tend to support the suggestion from the Farooqi study (Farooqi et al. 2000), that α-MSH does have an important effect on bone mass.

There is a striking parallel in the present data between the effects of α-MSH on chondrocytes and osteoblasts. α-MSH is a potent mitogen for both cell types in vitro, but there is no evidence of this effect in vivo. In the case of chondrocytes, this is reflected in the lack of effect of α-MSH on the width of the growth plate or on tibial growth. This contrasts with our previous work in this mouse model, in which factors stimulating chondrocyte proliferation to a degree comparable to that seen with α-MSH, have also increased growth plate width and tibial length in vivo (Cornish et al. 1998a; Cornish et al. 1998b).
2001a). It is likely that the catabolic effects of systemically administered α-MSH, also account for this dissociation of its *in vitro* and *in vivo* effects on cartilage.

This is not the first study of the effects of systemic α-MSH administration on bone. Stenstrom et al showed that α-MSH administration for 20 days increased cortical width in hypophysectomized rats (Stenstrom et al. 1979). In contrast, Aspenberg et al found no effect in similar studies in normal rats (Aspenberg et al. 1985), implying that the Stenstrom’s result was specific to hormone-deficient animals. Thus these results agree with those of the present study, which also found no effect on cortical bone in normal animals. Variable effects have also been seen in an ACTH analog, which has been shown to either stimulate or depress osteoblast proliferation *in vivo*, depending on dose and time of day (Walker et al. 1985). The variability of these effects probably reflects an interaction with endogenous ACTH release, which itself shows a marked diurnal rhythm. Referred to the situation in ACTH, which is derived from the proteolytic cleavage of the same pro-peptide as α-MSH, interference with secretion of endogenous peptides is another mechanism by which the effects of α-MSH on bone may be modified *in vivo*.

### 5.5. SUMMARY AND CONCLUSIONS OF CHAPTER 5

α-MSH at $10^{-9}$ M or greater significantly stimulated rat primary osteoblast proliferation without affecting the differentiation of these cells. It was also mitogenic to canine chondrocytes in culture. These results indicate that α-MSH acts directly on both osteoblasts and chondrocytes.

In mouse bone marrow cultures, increased osteoclast formation upon α-MSH treatment was associated with the increased ratio of RANKL/OPG, suggesting the involvement of RANKL/OPG in this process. α-MSH did not affect the activity of isolated mature osteoclasts.

*In vivo*, administration of α-MSH led to increased bone turnover and reduced trabecular bone volume. The reduction in trabecular bone volume may be mediated, to some extent,
by the effects of α-MSH to decrease fat mass and to diminish the secretion of anabolic hormones from the pancreatic β-cell. Therefore, if α-MSH analogs were to be developed for the treatment of obesity, then monitoring of their effects on bone would be an important safety consideration.
CHAPTER 6: EFFECTS OF MILK COMPONENTS ON BONE CELL DEVELOPMENT AND ACTIVITY

6.1. INTRODUCTION

6.1.1. The composition of milk

Milk is well recognized as an important source of nutrition, particularly for neonates. It consists of various components, which are protein (13-34 g/L), fat (saturated and unsaturated, ~40 g/L), carbohydrate (46-70 g/L, mainly lactose), minerals/major elements (potassium, calcium, phosphorus, chloride, sodium and magnesium, totally ~5.4 g/L) and trace elements (iron, copper, manganese, zinc and iodine). Among these elements, potassium (1.55 g/L) and calcium (1.24 g/L) are most abundant in milk. In addition, milk also contains vitamins (A, C, D, E, K, B1, B2, niacin/niacinamide, B6, B12, folic acid, pantothenic acid/calcium pantothenate and biotin), peptide and steroid hormones, and some minor components including organic acids (such as acetate and citrate) and urea (Cockburn 1983; Peaker and Faulkner 1983; Shah 2000).

Milk proteins fall into two categories: casein and whey protein, which account for about 80% and 20% of the total milk proteins respectively. The components of casein are α-casein (αs1 and αs2, 13.0 g/L), β-casein (9.3 g/L), γ-casein and κ-casein (3.3 g/L). The major components of whey protein and their concentrations in bovine milk include: β-lactoglobulin (3.2 g/L, absent from human milk), α-lactalbumin (1.2 g/L), glycomacropeptide (1.2 g/L), proteose-peptone (1.2 g/L), immunoglobulins (0.7 g/L), serum albumin (0.4 g/L), lactoferrin (0.1 g/L), lactoperoxidase (0.03 g/L; very low in human milk) and lysozyme (0.0004 g/L; 0.1 g/L in human milk) (Thomas 1983; Shah 2000; van Hooijdonk et al. 2000).

Milk fat is a mix of various lipids. The majority of lipids in milk are triacylglycerols as well as, in smaller amount, diacylglycerols and monoacylglycerols. In addition, milk lipids also include phospholipids, glycolipids, steroids (mainly cholesterol), and other minor constituents such as waxes, alcanols, carotinoids, vitamins and lipoproteins. Fatty
acids are the major components of lipids. The average content of the saturated fatty acids, which are mainly those with hydrocarbon chains of 4 to 16 carbons, is about 60% of the total milk fatty acids. The unsaturated fatty acids are mainly oleic acid (20.1-20.8%), linoleic acid (1.2-2.0%) and α-linolenic acid (0.5-0.7%) (Molkentin 1999; Molkentin 2000).

6.1.2. The biological activities of milk components

Milk and colostrum exhibit various physiological and biochemical activities including antitumorigenesis (Gill and Cross 2000), immunomodulation (Gill et al. 2000; Korhonen et al. 2000a), regulation of blood pressure (Yamamoto and Takano 1999; Groziak and Miller 2000), reduction of the risk for cardiovascular diseases (Pfeuffer and Schrezenmeir 2000), anti-infection by microorganisms (van Hooijdonk et al. 2000) and antioxidation (Lindmark-Mansson and Akesson 2000), etc.

The anti-microbial activities of milk components are specific and non-specific. The specific activity is conferred by the immunoglobulins in milk while the non-specific activity, which might act synergistically with the specific activity, is conferred by other components, predominantly lactoferrin and lactoperoxidase (Korhonen et al. 2000a; van Hooijdonk et al. 2000).

6.1.2.1. Immunoglobulins

Bovine milk contains three major classes of immunoglobulins, IgG, IgM and IgA. In the first colostrum, the concentration of total immunoglobulins is very high (40-200 mg/mL) and then decreases to 0.7-1.0 mg/mL within a few days (Korhonen et al. 2000b). Milk, particularly colostrum, provides protection to the newborn, whose immune system is still immature (Shah 2000).

In animal models, studies with immune milk from hyperimmunised cows or purified immunoglobulins from colostrum or immune milk have shown that they were effective in
treating diarrhea caused by rotavirus (Castrucci et al. 1988; Lecce et al. 1991) and enterotoxigenic Escherichia coli (Snodgrass et al. 1982; Moon and Bunn 1993). In humans, treatment with the immunoglobulins from hyperimmune bovine milk also produced a protective effect against microbial infection caused by enterotoxigenic E. coli (Freedman et al. 1998), rotavirus (Ebina et al. 1985), Helicobacter pylori (the major aetiological agent of active chronic gastritis and peptic ulcer disease) (Thomas et al. 1993; Casswall et al. 1998) and other pathogenic agents such as oral pathogens streptococci (Filler et al. 1991) and the enteric protozoan parasite Cryptosporidium parvum (Greenberg and Cello 1996; Okhuysen et al. 1998).

The studies showed that the immunoglobulins were relatively resistant to proteolysis and intestinal absorption of immunoglobulins can occur in neonates (Korhonen et al. 2000b; Shah 2000).

6.1.2.2. Lactoferrin and lactoperoxidase

Both lactoferrin and lactoperoxidase have a non-specific anti-infection function and enable the host to resist a broader spectrum of microorganisms. They were found to be highly resistant to proteolysis. This property allows them to function in the gastrointestinal tract. Even after partial hydrolysis, a derived peptide containing the N-terminal region of lactoferrin still displays antimicrobial activity (van Hooijdonk et al. 2000).

Two mechanisms of action have been proposed for the antimicrobial activity of lactoferrin. Its bacteriostatic activity seems to be attributable to its high affinity for Fe$^{3+}$, which results in a hypoferraemic state, while its bactericidal activity is achieved by its direct interaction with bacterial membrane and subsequent alteration in membrane permeability through dispersion of lipopolysaccharides (Naidu and Arnold 1977). Lactoferrin exists not only in milk, but also elsewhere in the body. Upon infection, inflammation, tumor development and iron overload, endogenous lactoferrin is released from its pool in polymorphonuclear neutrophils (Levay and Viljoen 1995). Its function against inflammation seems to be related to its regulatory role in the production of TNF-
α and IL-6 (Machnicki et al. 1993). Other reported biological activities of lactoferrin are antioxidation, immunomodulation and anticarcinogenesis (Shah 2000).

Lactoperoxidase exerts its antimicrobial activity by working with its co-factors, thiocyanate (SCN⁻) and H₂O₂. In the presence of H₂O₂, the oxidation of SCN⁻ by the catalysis of lactoperoxidase gives rise to an intermediate product with antimicrobial activity. Usually, the supply of SCN⁻ from tissues and secretions from mammals are sufficient while the availability of H₂O₂ is limited. Therefore, to facilitate the functioning of lactoperoxidase, peroxidogenic enzymes such as glucose oxidase are usually added to the system (Shah 2000; van Hooijdonk et al. 2000).

In addition to the major contributors lactoferrin and lactoperoxidase, lysozyme also takes part in the non-specific defense system in milk, particularly in human milk, where its concentration is much higher than in bovine milk (van Hooijdonk et al. 2000).

**6.1.2.3. Bioactive peptides derived from milk proteins**

Some bioactive factors are encrypted in the milk proteins. For example, caseins, which only display the role as ion carriers (Ca, PO₄, Fe, Zn and Cu) in their intact forms, can serve as the precursors of bioactive peptides upon proteolysis digestion *in vitro* and in the gastrointestinal tract *in vivo* (Shah 2000). In addition to caseins, bioactive peptides can also be derived from other milk proteins. Morphiceptin (derived from β-casein), β-casomorphin (derived from β-casein), α-lactorphin (derived from α-lactalbumin) and β-lactophin (derived from β-lactoglobulin) are found to possess pharmacological similarities to opium (morphine) and are opioid agonists. In contrast, lactoferroxins (derived from lactoferrin) and casoxins (derived from κ-casein) are found to behave as opioid antagonists. Digestion of α- or β-casein could also give rise to casokinins, casein phosphopeptides and immunopeptides (Shah 2000). Casokinins exhibit antihypertensive activity by inhibiting the production of the octapeptide angiotensin II (Shah 2000). Casein phosphopeptides were found to be effective in toothpaste as anticariogenic additives to prevent enamel demineralization (Reynolds 1998). Casein-derived immunopeptides can stimulate the host defense system against phages and bacteria such
as *Klebsiella pneumoniae* (Migliore-Samour et al. 1989). Many other peptides derived from milk proteins are active in immunomodulation by regulating the proliferation and function of lymphocytes and the formation of antibodies (Gill et al. 2000).

### 6.1.2.4. Milk fats

In bovine milk, about 75 wt% of bioactive substances are milk fats, among which *trans* fatty acids, some saturated fatty acids with 4 and 10 to 12 carbons, essential fatty acids linoleic acid and *α*-linolenic acid, conjugated linoleic acids and some phospholipids have attracted most interest. In milk, fatty acids and their metabolites are the major components that confer milk fats bioactivity (Molkentin 1999; Molkentin 2000).

*Trans* fatty acids in milk are mainly *trans*-octadecenoic acid. They have been found to be associated with premature atherosclerosis and coronary heart disease likely due to its effect on low-density lipoproteins (LDL)/high-density lipoproteins (HDL) ratio (increasing the unfavorable LDL level and decreasing the favorable HDL level). *Trans* fatty acids were also found to inhibit the synthesis of prostaglandin by impairing the desaturation and chain elongation of some essential fatty acids (Molkentin 2000). In contrast, the monounsaturated fatty acid, oleic acid, and the polyunsaturated fatty acid, linoleic acid, decrease the level of LDL and are regarded as antiatherogenic. The oxidation products of polyunsaturated fatty acids or their metabolites exhibited effects on mutagenesis, immunomodulation, blood pressure and coagulation (Molkentin 1999). Conjugated linoleic acids displayed anticarcinogenic properties possibly due to their inhibitory effects on oxidation, nucleotide synthesis and cell proliferation. The short-chain saturated fatty acid butyric acid was reported to regulate the expression of oncogenes and suppressor genes, to inhibit tumor development and to modulate the proliferation and differentiation of neoplastic cells (Molkentin 2000).

### 6.1.2.5. Milk components and bone cells

The effects of milk proteins on bone metabolism have not been thoroughly investigated. The Snow Brand Milk Product Co. Ltd, Japan, reported that ovariectomized rats fed...
fractionated whey proteins on low calcium diets had increased bone strength, lower serum alkaline phosphatase levels and higher femoral content of total amino acids, proline and hydroxyproline, the typical amino acids of collagen. These results suggested that the fractionated whey proteins contained active components, which affected bone metabolism and bone collagen synthesis (Takada et al. 1997a; Takada et al. 1997c). These activities were later found to be in the basic protein fraction (Kato et al. 2000). Further experiments showed that humans who orally took milk basic protein had increased bone mineral density and decreased levels of bone resorption markers: urinary deoxypyridinoline and urinary cross-linked N-telopeptides of type-I collagen (Aoe et al. 2001; Toba et al. 2001). Oral administration of the milk basic protein was found to prevent bone loss in aged ovariectomized rats. Consistently, it was found that the milk basic protein inhibited pit formation in an isolated rabbit osteoclast assay (Toba et al. 2000), inhibited osteoclast formation from mouse spleen cells (Takada et al. 1997b) and stimulated the proliferation and differentiation of osteoblastic MC3T3-E1 cells (Takada et al. 1996).

In support of the above effect of oral administration of the milk basic protein, its stability and transportability were examined. The results showed that the active components in the milk basic protein fraction were resistant to both heat (75-90°C for 10 min) and pepsin/pancreatin digestion. It was also found that the active components were able to pass through the wall of the gut-sacs made of rat small intestine (Takada et al. 1996; Takada et al. 1997b).

Three active components were then purified from the above Snow Brand milk basic protein fraction. One of them was cystatin, which was purified from whey protein and was found to suppress bone resorption by osteoclasts. Based on the report that cystatin is an inhibitor of the protease cathepsin, which is secreted by osteoclasts and resorbs bone, it was suggested that the inhibitory effect of whey protein on bone resorption resulted from the inhibition of cathepsin by cystatin (Toba et al. 2000; Aoe et al. 2001). The second one was a 10 kD component isolated from the milk basic protein fraction and was found to be identical to bovine high mobility group (HMG) protein-1. HMG proteins are nonhistone components of chromatin and considered to be implicated in DNA replication. This HMG-like protein from milk was found to stimulate osteoblast
proliferation and differentiation (Takada et al. 1996; Yamamura et al. 1999). The amino acid sequence revealed that the third isolated component was very similar to the 17 kD kininogen fragment 1.2. This component exhibited specific mitogenic activity for osteoblasts (MC3T3-E1), but not for fibroblasts BALB/3T3. Kininogen fragment 1.2, together with bradykinin, is derived from the digestion of kininogen by its specific protease kallikrein. Kininogen, a known factor in blood coagulation, was detected in bovine milk, and kallikrein was detected in the bovine mammary gland. So it is likely that kininogen is cleaved by kallikrein in mammary gland and the digested products are then secreted into milk (Yamamura et al. 2000). Bradykinin, the other digestion product of kininogen, was reported to stimulate bone resorption in neonatal mouse calvaria (Gustafson and Lerner 1984).

6.1.2.6. Other bioactive components

In addition to the bioactive components above, bovine or human milk also contains various hormones and growth factors. Some of those involved in bone metabolism include type I colony stimulating factor (Sinha and Yunis 1983), PTHrP (Rouffet and Barlet 1995), CT, FGFs, granulocyte-macrophage colony stimulating factor, insulin, insulin-like growth factors, leptin, prostaglandins and TGF (Francis et al. 1995; Donnet-Hughes et al. 2000).

6.1.3. The bases for the current research and research goals

Although many known hormones and growth factors related to bone metabolism have been detected in milk, it is believed that further bioprospecting of milk for bone-active factors is worthwhile. In addition, the functional components identified by the Japanese research group from the Snow Brand Milk Product Co. Ltd were all from the basic protein fractions. Little documentation exists on the activity of milk acidic fractions.

In the current research, basic and acidic fractions have been screened for their activities on bone cells by using a number of model systems: mouse calvarial organ cultures, rat osteoblast proliferation assays and rat isolated osteoclast assays. Further fractionation has led to the identification of new osteotropic components in milk, which are being investigated for their potential roles as nutraceuticals and pharmaceuticals.
6.2. MATERIALS AND METHODS

All the milk fractions and components tested in this study were provided by the laboratory of New Zealand Dairy Research Institute (recently renamed: Fonterra Research Centre), Palmerston North.

6.2.1. Milk basic fractions

Milk basic and acidic fractions were split by cation and anion exchange chromatography. Milk basic fraction (MBF-1) was a 0.35 M NaCl eluate of skim milk run through a SP-Sepharose column at pH 6.8, while MBF-2 was a 1 M NaCl eluate of mineral acid whey protein concentrate (WPC) run through a SP-Sepharose column at pH 7.0. The collections were ultrafiltered (MWCO 3000 Daltons) to lower the volume, exhaustively dialysed (Spectrapor MWCO 2000 Daltons) and finally lyophilised.

Lactoferrin (about 80 kD), which forms part of basic protein fractions, was prepared from skim milk by passing through a cation exchanger (SP-Sepharose) and selective elution by 1 M NaCl followed by ultrafiltration (MWCO 30 000 Daltons). The purity of the lactoferrin preparation was over 95%.

6.2.2. Milk acidic fractions

Milk acidic fraction (MAF)-1 and MAF-2 were prepared from a cheese WPC and a mineral acid WPC, respectively. The WPCs were separated by anion exchange chromatography with resin Q-Sepharose at pH 4.5. The fractions eluted by 1 M NaCl were collected and then subjected to concentration, dialysis and lyophilises.

Components from the acidic fraction used in this study: Three major components of MAF-1 were further investigated. They are proteose peptone component 5 (PP5, large peptide fragment of β-casein), osteopontin and glycomacropeptide (GMP).

Peptic hydrolysate-1 and -2 are the hydrolysate of MAF-1 and -2 respectively.
6.3. RESEARCH RESULTS

6.3.1. Basic fractions MBF-1 and MBF-2

To search the active components in milk, the crude fractions MBF-1 and MBF-2 were firstly assessed in rat osteoblast proliferation assay, in which both MBF-1 and MBF-2 were potently mitogenic with significant effects seen at the concentration of 1 µg/mL for both of the fractions. At the concentration of 100 µg/mL, MBF-1 and MBF-2 increased [3H]-thymidine incorporation by about 5- and 3-fold, respectively (Fig. 6-1).

Then the fractions MBF-1 and MBF-2 were subjected to HPLC resolution, which identified a number of components including lactoribonuclease, lactoperoxidase, lactogenin, angiogenin, lactoferrin, folate binding protein and β-lactoglobulin. It is noted that lactoferrin was the predominant component in both of the fractions, particularly in MBF-2 (Fig. 6-2), suggesting that lactoferrin may be a major molecule contributing to the above activities. Thus, lactoferrin was picked up for further investigation in the following study.

Fig. 6-1. MBF-1 (a) and MBF-2 (b) stimulate rat osteoblast proliferation. Data are expressed as the mean ± SE, n=6/group. Significant differences from control are indicated with "**" (P≤0.01).
**Fig. 6-2.** HPLC profiles showing the compositions of MBF-1 (a) and MBF-2 (b) (Kindly provided by Dr. Kate Palmano from the laboratory of New Zealand Dairy Research Institute, Palmerston North).

**Fig. 6-3.** Lactoferrin stimulates rat osteoblast proliferation.

Data are presented as the mean ± SE, n=6/group. Significant differences from control are indicated with "***" (P≤0.01).
Fig. 6-4. Lactoferrin inhibits osteoclast formation from mouse bone marrow cultures. Data are presented as the mean ± SE, n=8-10/group. Significant differences from control are indicated with "**" (P≤0.01).

Fig. 6-5. Effect of lactoferrin on $^{45}$Ca release (a) and $[^3H]$-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "***" (P≤0.01).

6.3.2. Effects of lactoferrin

Interestingly, lactoferrin was found to be effective on bone cells in vitro. In a rat primary osteoblast proliferation assay, a potent mitogenic effect was seen with lactoferrin at 1 μg/mL or greater. At the concentration of 10 μg/mL, cell proliferation was increased by
about 6-fold (Fig. 6-3). In contrast, it was inhibitory to osteoclastogenesis from mouse bone marrow cultures with significant effect seen at 1 μg/mL or greater (Fig. 6-4). In calvarial organ cultures, lactoferrin significantly stimulated [³H]-thymidine incorporation at 10 μg/mL or greater while no significant effect on ⁴⁵Ca release was seen within the concentrations tested (1-100 μg/mL). This indicated that lactoferrin stimulated cell (osteoblast) proliferation without affecting bone resorption (Fig. 6-5). Lactoferrin did not alter the activity of the isolated mature osteoclasts at concentrations of 1 to 100 μg/mL, indicating the lack of direct interaction between lactoferrin and osteoclasts (Fig. 6-6).

While lactoferrin potently inhibited osteoclastogenesis, its effects on OPG and RANKL expression were studied by using real-time PCR. Upon lactoferrin treatment, both RANKL and OPG expression in mouse bone marrow cells were decreased at almost the same proportion (Fig. 6-7). This could not explain its inhibitory effect on osteoclast formation.

![Fig. 6-6. Lactoferrin has no effect on rat isolated osteoclast activity. Data are presented as the mean ± SE, n=8/group.](image)

### 6.3.3. Acidic fractions MAF-1 and MAF-2

Two acidic fractions, which in this thesis are referred as milk acidic fractions (MAF)-1 and -2, were firstly screened for their effects on bone resorption in mouse calvarial organ cultures. Fig. 6-8 shows that MAF-1 had an inhibitory effect on bone resorption and [³H]-thymidine incorporation. In contrast, MAF-2 was a stimulator in this culture.
system. At 100 µg/mL, it increased $^{45}$Ca release and $[^3]$H-thymidine incorporation by 77% and 73%, respectively (Fig. 6-9). In osteoblast proliferation assays, both MAF-1 and MAF-2 significantly increased $[^3]$H-thymidine incorporation (Fig. 6-10). In isolated osteoclast assays, MAF-2 stimulated the bone resorption activity while MAF-1 had no significant effect (Fig. 6-11). Therefore, MAF-2 appears to be an up-regulator of bone turn-over by stimulating osteoblast proliferation and osteoclast activity. In contrast, MAF-1 seems to be anabolic to bone based on its mitogenic effect on osteoblasts and either inhibitory or non-effective on osteoclast activity depending on the model system.

![Graphs](image)

**Fig. 6-7.** Real-time PCR results showing the effect of lactoferrin on RANKL and OPG expression in mouse bone marrow cultures. Lactoferrin was added on days 0, 2 and 4. Data are presented as the mean ± SE, n=3/group. Significant differences between control treatment are indicated with "*" ($P \leq 0.05$) and "**" ($P \leq 0.01$), respectively.
Fig. 6-8. Effect of MAF-1 on $^{45}$Ca release (a) and $[^3H]$-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "*" (P<0.05) and "**" (P<0.01).

Fig. 6-9. Effect of MAF-2 on $^{45}$Ca release (a) and $[^3H]$-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "*" (P<0.05) and "**" (P<0.01).
Fig. 6-10. MAF-1 (a) and MAF-2 (b) stimulate rat osteoblast proliferation. Data are expressed as ratios of treatment to control and presented as the mean ± SE, n=6/group. Significant differences from control are indicated with "*" (P<0.05) and "**" (P<0.01).

Fig. 6-11. Effect of MAF-1 (a) and MAF-2 (b) on rat mature osteoclast activity. Data are presented as the mean ± SE, n=8/group. Significant differences from control are indicated with "**" (P<0.01).
Fig. 6-12. Effect of proteose peptone component 5 (PP5) on \(^{45}\)Ca release (a) and \(^{3}H\)-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "*" (P<0.05) and "**" (P<0.01).

Fig. 6-13. Effect of osteopontin on \(^{45}\)Ca release (a) and \(^{3}H\)-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "*" (P<0.05) and "**" (P<0.01).
6.3.4. Effects of the components from MAF-1

As MAF-1 demonstrated an attractive feature of inhibiting bone resorption, efforts have been made to identify the active components in this acidic fraction. So far, three components have been identified by our co-laboratory in the New Zealand Dairy Research Institute, Palmerston North. They were PP5, osteopontin and GMP.

These components were again tested in mouse calvarial organ cultures. Both PP5 and osteopontin were stimulatory to $^{45}$Ca release and [$^{3}$H]-thymidine incorporation (Fig. 6-12 and 6-13). In contrast, GMP did not produce a significant effect (Fig. 6-14). The anabolic factor is yet to be identified from this MAF-1 that is inhibitory to bone resorption. This is an ongoing project in our laboratory.

Fig. 6-14. Glycomacropeptide (GMP) has no effect on $^{45}$Ca release (a) and [$^{3}$H]-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, n=5-8/group. Significant differences from control are indicated with "*" ($P<0.05$) and "**" ($P<0.01$).

6.3.5. Hydrolysates of MAF-1 and MAF-2

Milk proteins are degraded by proteases in the digestion system when taken orally. Thus it was important to investigate the effect of the hydrolysates of the milk acidic fractions,
MAF-1 and MAF-2. Here, the peptic hydrolysates of MAF-1 and MAF-2 were tested for their activities on bone metabolism using calvarial organ culture.

**Fig. 6-15.** Effect of peptic hydrolysate-1 on $^{45}$Ca release (a) and $[^3]$H-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, $n=5-8$ /group. Significant differences from control are indicated with "*" ($P<0.05$) and "**" ($P<0.01$).

**Fig. 6-16.** Effect of peptic hydrolysate-2 on $^{45}$Ca release (a) and $[^3]$H-thymidine incorporation (b) in mouse calvarial organ cultures. Data are presented as the mean ± SE, $n=5-8$ /group. Significant differences from control are indicated with "*" ($P<0.05$) and "**" ($P<0.01$).
The peptic hydrolysate-1 (derived from MAF-1) and -2 (derived from MAF-2) were found to significantly inhibit $^{45}$Ca release at 100 $\mu$g/mL. In addition, peptic hydrolysate-1 and -2 significantly inhibited $[^3H]$-thymidine incorporation at concentrations of 100 and 10 $\mu$g/mL respectively (Fig. 6-15 and 6-16). Thus peptic hydrolysis of both MAF-1 and MAF-2 resulted in fractions that interestingly demonstrated inhibitory activity on bone resorption. These findings are consistent with some preliminary results obtained from in vivo studies conducted at Massey University (data not shown).

6.4. DISCUSSION

6.4.1. Lactoferrin—a novel osteotropic factor in milk

Previous studies found that a milk basic fraction prepared by Snow Brand Milk Co. contained anabolic activity to bone and three causative candidates have been identified from them: cystatin (Toba et al. 2000; Aoe et al. 2001), a 10-kD high mobility group (HMG) protein-1 (Takada et al. 1996; Yamamura et al. 1999) and a 17-kD kininogen fragment 1.2 (Yamamura et al. 2000). In this project undertaken as part of the thesis work, a number of components in further basic fractions MBF-1 and MBF-2 have been identified according to the HPLC resolution. Among these components, lactoferrin has been demonstrated to increase osteoblast proliferation and inhibit osteoclastogenesis in vitro. The lactoferrin used in this study was from the skim milk with the purity over 95%. Recently, similar osteotropic effects have been obtained in our laboratory with the recombinant bovine lactoferrin expressed in rice plants. Lactoferrin has been previously recognized as an anti-inflammatory and anti-microbial molecule (Irvay and Viljoen 1995). Its mitogenic effect on osteoblasts has not been previously documented. While this project was in progress, a research group reported that lactoferrin inhibited osteoclast formation from human osteoclast precursors via RANKL/OPG-independent mechanism. It was also found to inhibit rabbit isolated osteoclast activity at 10 $\mu$g/mL or greater after culturing for 3 days (Lorget et al. 2002). The present results of RANKL and OPG expression measured by real-time PCR support the RANKL/OPG-independent mechanism by which lactoferrin affects osteoclast formation. The results from the current study found that lactoferrin did not affect mature osteoclast activity as seen in
calvarial organ cultures and isolated osteoclast assays, indicating the lack of its direct interaction with osteoclasts.

Our laboratory is currently studying the mode of action of lactoferrin and its interaction with other known osteotropic factors.

6.4.2. Osteotropic activity in milk acidic fractions

Most milk proteins such as casein and major whey proteins (α-lactalbumin, β-lactoglobulin and bovine serum albumin) are acidic. However, almost all protein growth factors identified in milk have basic isoelectric points (Francis et al. 1995; Kato et al. 2000). In addition, the milk osteotropic activities revealed by previous researchers were all from the basic fractions (Kato et al. 2000; Toba et al. 2000). Therefore, it is worthwhile to explore active factors in milk acidic fractions. The results from this project demonstrated that milk acidic fractions also contained osteotropic activity as seen in MAF-1 and MAF-2.

MAF-1 showed moderate inhibitory effect on bone resorption in calvarial organ cultures. However, the tested components (PP5 and osteopontin) from MAF-1 exhibited stimulatory effect on $^{45}$Ca release in the same assay system and GMP did not affect bone resorption. This means that the molecule(s) responsible for the interesting anti-resorptive activity seen in MAF-1 are yet to be identified, and this work is ongoing.

6.4.3. Activity derived from hydrolysis of milk fractions

Previous studies found that some bioactive factors have been derived from milk proteins upon hydrolysis as reviewed in Section 6.1.2.3. Interestingly, current research found that the peptic hydrolysate of MAF-2 was inhibitory to bone resorption, though MAF-2 itself showed stimulatory effects in both calvarial organ cultures and isolated osteoclast cultures. This indicates that hydrolysis of MAF-2 by the protease gave rise to new product(s), which are anabolic to bone metabolism. It is likely that this hydrolysis event can also take place in the gastrointestinal tract when milk is taken orally. If the derived
products can be absorbed and transported by the intestine, as seen in the milk basic protein (Takada et al. 1996; Takada et al. 1997b), oral administration of MAF-2 may be effective on bone metabolism and this is worthy of further investigation.

Due to the limited scope of this project, the compositions of most of the fractions tested in this project have not been thoroughly analyzed, and the profile of the active components in them is still unknown. Milk contains many known hormones and growth factors, some of which are involved in bone metabolism (Francis et al. 1995; Rouffet and Barlet 1995; Donnet-Hughes et al. 2000), but undoubtedly many yet remain unidentified.

6.5. SUMMARY AND CONCLUSIONS OF CHAPTER 6

Milk basic fractions MBF-1 and MBF-2 were highly mitogenic to osteoblasts in culture.

It was found for the first time that lactoferrin potently stimulated the proliferation of rat osteoblasts in vitro. It also inhibited osteoclast formation from mouse bone marrow cultures via a RANKL/OPG-independent pathway. In contrast, it did not affect the activity of isolated osteoclasts, indicating the lack of the direct interaction between this molecule and osteoclasts.

Both MAF-1 and MAF-2, the milk acidic fractions, were found to be mitogenic to osteoblasts in culture. In calvarial organ cultures, MAF-1 was inhibitory, but MAF-2 was stimulatory to bone resorption. MAF-2 also stimulated isolated osteoclast activity while MAF-1 did not. It appears that MAF-2 is a general mitogen of bone-turnover, whereas MAF-1 is being further investigated for potential inhibitory effects on osteoclasts.

PP5 and osteopontin, components from MAF-1, stimulated both 45Ca release and [3H]-thymidine incorporation in mouse calvarial organ cultures. GMP did not affect bone resorption. The components conferring MAF-1 inhibitory activity on bone resorption are yet to be identified.

Peptic hydrolysis of MAF-2 gave rise to new inhibitory activity on 45Ca release and [3H]-thymidine incorporation in calvarial organ cultures.
CHAPTER 7: CONCLUDING DISCUSSION AND SUMMARY

7.1. INVOLVEMENT OF RANKL/OPG SIGNALING

Since the understanding of the critical role of RANKL/OPG in regulating osteoclast differentiation and functioning, various osteotropic and osteolytic agents known to have an impact on osteoclast and bone resorption have been found to exert their effects through the mediation of RANKL/OPG. For examples, osteoclast stimulators, PTH, 1,25-(OH)2D3, PGE2 and IL-11, act on osteoblasts and result in increased production of RANKL (Hofbauer and Heufelder 1998; Takahashi et al. 1999; Yasuda et al. 1999) and a reciprocal decrease in OPG expression (Murakami et al. 1998; Nagai and Sato 1999). In contrast, some osteoclast inhibitors are found to increase OPG expression and/or decrease RANKL production as seen in TGF-β (Murakami et al. 1998; Thirunavukkarasu et al. 2001) and calcium (Kostenuik and Shalhoub 2001). In addition, the activated T cells (Horwood et al. 1999; Kong et al. 1999b), prostate and breast cancer cells (Chikatsu et al. 2000; Zhang et al. 2001a) are capable of inducing osteoclast formation through their ability to produce RANKL, suggesting that some pathologic conditions result in osteolytic bone resorption through this molecule. These lines of evidence indicate that RANKL and OPG are the key mediators through which the osteotropic agents and pathologic conditions affect osteoclasts and bone metabolism.

In the present study, α-MSH-stimulated osteoclastogenesis was likely, at least in part, to act through a mechanism involving RANKL/OPG signaling since α-MSH elevated the ratio of RANKL/OPG. In contrast, another two novel bone-active agents, FGF-8a and lactoferrin, which were studied in the current research program and were found to inhibit osteoclastogenesis, had no corresponding impact on the expression levels of RANKL or OPG. This indicates that the osteoclastic effect of FGF-8a and lactoferrin is RANKL/OPG-independent and has provided more evidence for RANKL/OPG-independent mechanisms, evidence of which is becoming apparent in the last couple of
years from studies of other osteotropic agents, such as TNF-α, IL-1 and lipopolysaccharide (Suda et al. 2001; Kobayashi and Takahashi 2003), etc.

Kobayashi et al reported that TNF-α, in the presence of M-CSF, induced osteoclast formation from mouse bone marrow cultures. This induction was not blocked by OPG or the Fab fragment of anti-RANK antibody. Furthermore, experiments using osteoclast precursors from TNF receptor 1- and 2-deficient mice and using antibodies against these receptors demonstrated the critical role of TNF-α in osteoclastogenesis (Kobayashi et al. 2000). Similarly, osteoclast formation induced by TNF-α in human CD14+ macrophage cultures was blocked by the antibodies against the TNF receptors, but not by OPG or RANK (Sabokbar et al. 2003). IL-1 has also been demonstrated to induce osteoclast formation via a RANKL/OPG-independent mechanism (Suda et al. 2001). It was found that TNF-α and IL-1 had an impact on mature isolated osteoclasts, either stimulating actin ring formation (Fuller et al. 2002) or activity (Udagawa 2002). This further indicates that these factors directly acted on osteoclasts without the mediation of RANKL. Lipopolysaccharide, a cell component of Gram-negative bacteria, was also found to induce osteoclast formation even in the presence of OPG and IL-1 receptor antagonists. This indicated that its osteoclastogenic activity was independent of RANKL and and IL-1 (Jiang et al. 2002; Suda et al. 2002). Consistent with the RANKL/OPG-independent mechanism observed from the current study, lactoferrin was also reported to inhibit osteoclast formation from the human CD14+ cell cultures, in which RANKL and OPG mRNA was not detected (Lorget et al. 2002).

Based on the results from the current research and previous studies, it can be seen that multiple mechanisms exist in the regulation of osteoclast formation and functioning. While current data indicate the RANKL/OPG-independent mechanism by which FGF-8a and lactoferrin affect osteoclast formation, the modes of their action are still unknown and this is worthy of further investigation.
7.2. ABOUT THE RESEARCH MODELS

In the current research, most of the assays were conducted in *in vitro* models. These research models have been demonstrated to be useful tools in predicting activities of bone-active factors *in vivo*. In brief, the calvarial organ cultures provide the microenvironment close to that in the *in vivo* situation, and bone resorption from these cultures predominantly reflects the activity of mature osteoclasts. Osteoblast proliferation and nodule formation assays measure the direct effect of test factors on the growth and differentiation of osteoblasts, respectively. Bone marrow cultures provide a model of osteoclastogenesis while isolated osteoclast assays reflect the direct action of test factors on mature osteoclast activity.

Long bone cultures have been used by others to assess osteoclast activity (Kwon et al. 1998), however, this model does not separate osteoclast development from mature osteoclast activity as demonstrated when hydroxyurea, a DNA synthesis inhibitor, also inhibited bone resorption (Pfeilschifter et al. 1988). In contrast, hydroxyurea did not affect bone resorption from calvarial organ culture (Pfeilschifter et al. 1988).

To examine the direct effect of test factors on osteoclast resorption, isolated osteoclast cultures on bone slices have been used in the current study and many other laboratories (Fuller et al. 1998; Lacey et al. 1998; Chikazu et al. 2000; Lorget et al. 2002). In this system, osteoclasts have been enriched, but the existence of other cell populations is still visible. However, since the contaminated cells are in low numbers, the interference by cells other than osteoclasts is minimised (Fuller et al. 1998; Hakeda et al. 1998; Burgess et al. 1999). Osteoclasts are relatively rare cells and are difficult to isolate at high purity in large quantity (Hentunen et al. 1999). This problem has been an obstacle in studying osteoclasts and has sometimes led to the questioning of results, for example, the expression of RANKL in this cell type (Kartsogiannis et al. 1999; Myers et al. 1999). Highly pure osteoclast preparations will greatly facilitate the investigation on bone metabolism and provide more convincing evidence. In future studies, this may be possible by adopting the methodologies including flow cytometry (Benito et al. 2002),
7.3. BONE METABOLISM IN RELATION TO OTHER PHYSIOLOGICAL PROCESSES

Present knowledge has shown that bone metabolism is related to other physiological processes such as energy homeostasis (Felson et al. 1993; Khosla et al. 1996; Fogelholm et al. 2001) and immune response (Lorenzo 2000; Grcevic et al. 2001). This means that bone metabolism is not an isolated event and is under the modulation of other physiological processes. So far, common mediators between immune and bone regulation (Lorenzo 2000; Grcevic et al. 2001), and between body mass and bone metabolism (Cornish et al. 2002b; Reid 2002) have been demonstrated or proposed. However, to fully understand these interacting events and to elucidate the mechanisms of bone disorders, further study on novel osteotropic factors is still required.

7.3.1. Body mass and bone metabolism

The investigation of α-MSH in the current study was initiated by the suggested relationship between body mass and bone mass (Reid et al. 1992a; Felson et al. 1993; Marcus et al. 1994; Ravn et al. 1999). It has been recorded that obesity prevents bone loss and reduces the rate of osteoporotic fractures (Reid et al. 1992a; Valtuena Martinez 2002). A number of mechanisms have been proposed for this protective effect of obesity on skeleton. This includes the mechanical loading on skeleton, increased secretion of anabolic factors from adipose tissue (such as estrogens and leptin) and from pancreatic β-cell (such as insulin, amylin, and preptin) (Khosla 2002; Reid 2002; Cornish 2003). It has been found that α-MSH has a role in down-regulating food intake and hence body weight. The evidence for this includes the induction of obesity upon blockade of α-MSH signaling by expression of α-MSH antagonist (agouti peptide) (Fan et al. 1997) and by targeted disruption of α-MSH receptor (Huszar et al. 1997). However, it was previously unknown if the effect of α-MSH on food intake and body weight will result in alteration...
in bone metabolism. The current study found that α-MSH enhanced osteoclast formation from bone marrow cultures in vitro, and increased bone turnover and reduce trabecular bone volume in vivo. This indicates that α-MSH is another mediator between body weight and bone density and provides a further explanation for the linkage between the two events. However, the available knowledge at present is still not enough to fully elucidate the association between body mass and skeletal states (Reid 2002). It is likely that more mediators are involved in these processes and future study towards this is needed.

7.3.2. Immune system and bone system

The genetical and functional linkage between immune and bone systems has been well documented (Lorenzo 2000; Grcevic et al. 2001). Cytologically, both B lymphocytes and osteoclasts share the common progenitors, and the commitment and differentiation of B lymphocytes or osteoclasts from the progenitors are under the control of the transcription factor Pax5 (Nutt et al. 1999; Lorenzo et al. 2000). In addition, the two systems are also linked via the cytokines produced by immune cells and bone cells (Grcevic et al. 2001). The first lymphokine found to affect bone resorption was termed osteoclast-activating factor (OAF) (Horton et al. 1972), which was later identified as IL-1 (Dewhirst et al. 1985). Subsequently, a variety of cytokines involved in immune response have been found to be effective on bone. These cytokines includes TNF, IL-4, IL-6, IL-11, IL-13, IL-15, IL-17, IL-18, IFN-γ, TGF-β, prostaglandins (Kong et al. 2000; Lorenzo 2000; Grcevic et al. 2001) and M-CSF (Weitzmann et al. 2001). The understanding of the relationship between the two systems was greatly advanced in recent years following the discovery of RANKL, a key osteoclastogenic factor initially identified by the research groups working independently on bone system (Lacey et al. 1998; Yasuda et al. 1998b) and immune system (Anderson et al. 1997; Wong et al. 1997b). RANKL is expressed not only by osteoblasts, but also by activated T lymphocytes. Therefore, activated T lymphocytes can also support osteoclast formation without the presence of osteoblasts.
(Horwood et al. 1999; Takahashi et al. 1999; Weitzmann et al. 2001). In addition to the osteotropic effect, RANKL also stimulates dendritic cell function and survival, and enhances dendritic cell-mediated T lymphocyte proliferation and lymph-node organogenesis (Anderson et al. 1997; Wong et al. 1997a; Kong et al. 2000). On the grounds of the above knowledge, RANKL is a notable bridging molecule between immune and bone systems, and its role in mediating the effect of inflammatory disease on bone metabolism has been revealed. Available data show that increased RANKL expression by local T lymphocytes activated by inflammation has contributed to the bone erosion and joint destruction as seen in rheumatoid arthritis (Kong et al. 1999b; Rehman and Lane 2001) and psoriatic arthritis (Ritchlin et al. 2003).

As stated above, activated T lymphocytes express RANKL and are capable of supporting osteoclast formation. However, since OPG at saturating concentrations failed to completely block the osteoclastogenesis induced by activated T lymphocytes, an unknown RANKL-independent mechanism seems to be involved in this osteoclastogenesis system (Weitzmann et al. 2001). It is likely that the unidentified factor(s) from T lymphocytes has contributed to this process, and this requires further investigation.

In addition to RANKL, lactoferrin may also be a candidate mediator between immune and skeleton systems. It has been well documented that lactoferrin is involved in immune response based on the evidence including: its expression by polymorphonuclear neutrophils (Lonnerdal and Iyer 1995) and increased release of this molecule from the neutrophils in inflammatory conditions (Levay and Viljoen 1995); its effect on maturation of B and T lymphocytes (Dhennin-Duthille et al. 2000) and the presence of lactoferrin receptor in immune cells (Mincheva-Nilsson et al. 1997). In comparison to the knowledge of lactoferrin’s role in immune system, its role on bone metabolism had been almost previously unknown (Lorget et al. 2002). Therefore, this molecule was tested in the current research and was found to be a novel anabolic factor on bone.
According to the above knowledge and the results from the current study, one more linkage between immune and bone systems via lactoferrin is newly established.

7.4. THE SIGNALING BETWEEN OSTEOBLASTS AND OSTEOCLASTS

The communication between osteoblasts and osteoclasts is a key mechanism to modulate bone metabolism and maintain the balance of bone resorption and bone formation. The signaling from osteoblasts to osteoclasts has been well established. It has been documented that osteoclast development and functioning are under the control of the many factors produced by osteoblasts. The most important ones are OPG and RANKL, which regulate osteoclast differentiation and directly modulate mature osteoclast activity (Tsuda et al. 1997; Hakeda et al. 1998; Lacey et al. 1998; Burgess et al. 1999; Wani et al. 1999)(also the results from the current study). Other factors such as 1,25-(OH)$_2$D$_3$, PGE$_2$, PTH and IL-11 regulate osteoclast formation via stimulation of RANKL production by osteoblasts (Nakagawa et al. 1998; Takahashi et al. 1999).

The impact of osteoclasts on osteoblasts is noted in the current study. In calvarial organ cultures, the effects of the test factors on $^{45}$Ca release were usually associated with the alteration in $[^3H]$-thymidine incorporation. An earlier publication from our group showed that increased DNA synthesis in this model is associated with increases in the osteoblast lineage of cells (Cornish et al. 1997b). Present data show that a higher rate of bone resorption in this model is usually associated with a higher rate of osteoblast proliferation, implying the coupling of catabolic and anabolic processes. The knowledge available at present to explain this is the presence of some mitogenic factors (such as IGF-1, IGF-II, TGF-β and FGFs) in bone matrix (Howard et al. 1981; Hauschka et al. 1986; Globus et al. 1989; Mundy 1999a). Upon bone resorption, these factors are released, and in turn act on osteoblasts and boost the anabolic process to balance the catabolic process.

In contrast, the signaling from osteoclasts to osteoblasts is much less documented. In addition to the above indirect pathway, it is still unknown if any other mechanism has
also contributed to the "feed-back" control from bone resorption to bone formation. Since the balance between bone resorption and formation is so important to maintain normal bone physiology, it is likely that osteoclasts will signal to osteoblasts in response to the stimulation from osteoblasts. To address this question, further study will be required to investigate the factors, which are expressed by osteoclasts and are active on bone formation.

7.5. THERAPEUTIC STUDY FOR BONE DISORDERS

The normal structure and function of bone require the delicate coupling of bone resorption and bone formation, which are the functions of osteoclasts and osteoblasts, respectively. The excess of bone resorption over bone formation will lead to osteoporosis, which affects a large number of elderly people (Mundy 1999b). The therapeutic approaches for such bone disorders depend on drugs, which inhibit osteoclast formation and activity, and/or enhance bone formation by osteoblasts.

So far, some drugs and hormones have been developed as the remedies for bone resorption disorders through their inhibitory effects on osteoclast development and function. However, the application of these therapeutic agents has been limited by their efficiency, their ability to rebuild bone that is lost as well as their side effects (Bell 2003). For example, treatment with calcitonin for long period results in lack of response by osteoclasts, a phenomenon called "calcitonin escape". This is partly due to the down-regulation of calcitonin receptor expression caused by calcitonin (Roodman 1996). Continuous application of estrogen over 5 years increases the risk of cardiovascular disease and breast cancer (Nelson et al. 2002). Raloxifene, a selective estrogen receptor modulator, is associated with increased incidence of thromboembolic disease while it has been shown to increase bone density (Seeman 2001). Bisphosphonates administration is potentially associated with microdamage accumulation of bone as the consequence of suppressed bone turnover, as is seen in dog studies (Mashiba et al. 2001; Bell 2003). Due to the limitation of the drugs available at present, the development of novel remedies has been a research focus. As a result of the effort, a breakthrough was achieved when
RANKL/OPG/RANK system was identified (Takahashi et al. 1999). At present, OPG is under investigation for clinical application. It was shown that a single subcutaneous injection of OPG reduced bone resorption over a 6-week period in postmenopausal women (Bekker et al. 2001). It is envisaged that OPG is a highly promising agent for treating osteoporosis and other diseases associated with bone destruction. However, potential therapeutic limitations for OPG should be considered, due to its big molecular size and the role of RANKL (the ligand for OPG) in the immune system. The application of OPG may result in an immune response or interfere with the functioning of immune system (Rodan and Martin 2000).

While the therapeutic strategy through suppression of bone resorption by osteoclasts has been widely studied and well developed, it has been pointed out that "far less attention has been paid to promoting bone formation with, for example, growth factors or hormones" (Rodan and Martin 2000). Recently, PTH was approved by the U.S. Food and Drug Administration (FDA) as the first therapies for stimulating bone formation (Sayegh and Stubblefield 2002; Srivastava and Deal 2002). However, it was found to have side effects of causing hypercalcemia (Morley et al. 2001) and inducing tumor in rats if used for long-term (Rhee et al. 2004). Therefore, there is still a need to develop new remedy for treating osteoporosis through stimulating bone formation. In consideration of this, the current research interest has focused not only on the effect of the factors on osteoclasts but also on the effect on osteoblasts. It was found that FGF-8a, α-MSH, lactoferrin and some milk fractions were stimulatory to osteoblast proliferation, suggesting the potential therapeutic application for osteoporosis via the modulation of bone formation by osteoblasts. Certainly, these are preliminary results and further study is still required.

7.6. SUMMARY AND FUTURE DIRECTION

Aiming at the novel activity on bone metabolism, the current research has studied RANKL/OPG, FGF-8a, α-MSH and milk fractions and has resulted with the following interesting data: (1) Truncated rRANKL [rRANKL(246-318)] was found to lose the original stimulatory activity on osteoclast and become antagonizing to the stimulatory
effect of rRANKL(160-318) in a mature osteoclast activity assay; (2) FGF-8a was effective on both osteoclasts and osteoblasts in the assays *in vitro*. The expression of FGF-8 in osteoblasts suggests an autocrine mechanism to regulate osteoblast development; (3) The net stimulatory effects of α-MSH on both osteoblasts and osteoclasts resulted in the reduction of trabecular bone volume. This is consistent with its negative role on energy homeostasis, which is associated with bone mass as previously suggested; (4) Milk basic and acidic fractions displayed activities on bone cells. Lactoferrin, a component from milk basic fraction, was found to be anabolic to bone; (5) While RANKL and OPG are critical regulators of osteoclast development and functioning, RANKL/OPG-independent mechanisms have been revealed.

From the current research results, further investigation is still needed in the following areas: (1) More RANKL truncates at different positions need be tested to find out the best fragment in terms of blocking the original RANKL activity on osteoclasts; (2) To further demonstrate the direct interaction between OPG and mature osteoclasts, the interference of endogenous RANKL should be excluded. Therefore, further study should be conducted with a model with interrupted RANKL gene; (3) As FGF-8a displays potent effects on bone cells *in vitro*, further study using an *in vivo* model is required to assess its role on bone metabolism. In addition, the interaction between FGF-8 and other osteotropic regulators, such as androgen and estrogen, would be an interesting topic; (4) The current study on milk fractions reports preliminary results only. While lactoferrin has been identified as a novel osteotropic factor in milk, other active components in milk fractions are yet to be further investigated; (5) Finally, the current research has revealed the RANKL/OPG-independent pathway in regulating osteoclast development as seen in FGF-8a and lactoferrin. The elucidation of the underlining mechanisms will contribute significantly to bone cell biology.
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