# Mechanisms of Skeletal Disease Mediated by Haematological Malignancies

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## Errata

- Table of Contents, 1.1 and Page 2, section 1.1: "GIBE" should be "GIVE"
- Page VI, BMPs: "marphogenetic" should be "morphogenetic"
- Page 3, line 10: ">3/dl" should be ">3g/dl"
- Page 4, 12 lines up: "lyphocyte" should be "lymphocyte"
- Page 9, line 5-6: "a calcified connective tissue matrix (hydroxyapatite)" should be "a calcified (highly-substituted hydroxyapatite) connective tissue matrix"
- Page 9, 14 lines up: "In the normal physiology" should be "In normal physiology"
- Page 11, 1st paragraph: "Osteoblasts are derived from the stromal or mesenchymal cell system and represent…" should be "Osteoblast lineage is derived from the stromal or mesenchymal cell system and represented by…"
- Page 15, 15 lines up: "ostolytic" should be "osteolytic"
- Page 16, 14 lines up: "cycloocygenase" should be "cyclooxygenase"
- Page 18, 7 lines up: "macrophage" should be "macrophages"
- Page 20, line 13: "an" should be "and"
- Page 35, line 2: "localises to in" should be "localises in"
- Page 40, 13 lines up: "Collin, 2001 #511" should be "Collin-Osdoby *et al.*, 2001"; 3 lines up "Le Brun, 1999 # 765" should be "Le Brun et al., 1999"
- Page 43, line 2: "…one of major…" should be "…one of the major…"
- Page 48, 6 lines up: "normal healthy," should be "normal healthy donors,"
- Page 74, line 4 "WST-1" should be "WST-1{4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate}".
- Page 84, 7 lines up: "It has reported…" should be "It has been reported…"
- Page 85, 3 lines up: "OB-like cell" should be "OB-like cells"
- Page 86, line 13: "It has shown that ..." should be "It has been shown that…"
- Page 143, insert reference "Le Brun, G., Aubin, P., Soliman, H., Ropiquet, F., Villette, J. M., Berthon, P., Creminon, C., Cussenot, O., Fiet, J. (1999). Upregulation of endothelin 1 and its precursor by IL-1beta, TNF-alpha, and TGF-beta in the PC3 human prostate cancer cell line. Cytokine 11(2), 157-62."
- Figure 3.3 legend, 4 lines up: "(A-D)" should be "(A-D for d1, 3, 5 and 7, respectively)"
- Figure 3.4 legend, add "NT: no treatment" after the last sentence
- Figure 3.5 legend, 3 lines up: "IL-1β (A), TNF-α (B), COX2 (D)" should be "IL-1β (B), TNF-α (C), COX2 (F)"

## DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any other university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

BEIQING PAN

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## ABSTRACT

Multiple Myeloma (MM) is a B cell malignancy, characterised by the presence of a monoclonal population of plasma cells (PCs), which localise to sites in the bone marrow (BM) close to the endosteal surface. Like a number of other cancers, MM PCs mediate massive destruction of the axial skeleton by recruiting large numbers of activated bone resorbing osteoclasts (OCs) to the bone surface. In addition to the heightened resorption seen in these patients, recent studies suggest that MM PCs also mediate a suppression of osteoblastic bone formation, further shifting the normal balance of bone formation and resorption in favour of resorption. The studies presented in this thesis were designed to further our understanding of the underlying molecular and cellular mechanisms responsible for skeletal complications associated with MM. Specifically, it deals with the molecular and biological effects of two potent MM-derived factors, interleukin-1β (IL-1β) and endothelin-1 (ET-1) on bone-derived osteoblast (OB)-like cells. Furthermore, it details findings which suggest that mesenchymal stem cells and their osteoprogenitor progeny, express the potent osteoclastogenic molecule, receptor activator of nuclear factor-κB ligand (RANKL).

Studies outlined in Chapter 3, show that in the early phase of MM, the pro-inflammatory cytokine, IL-1β, which is produced by myeloma cells or OBs in response to MM cells, may serve to increase the number of immature OB-like cells (osteoprogenitors). Using an established *in vitro* model of OB differentiation, these studies showed that immature OB-like cells with a greater proliferative index, expressed higher levels of the cell surface-expressed stromal progenitor antigen, identified by the monoclonal antibody (mAb) STRO-1. Importantly, the expression of STRO-1 was positively associated with high levels of transmembrane (TM) RANKL protein expression by human OB-like cells, indicating that an IL-1β-mediated increase in STRO-$1^+$ osteoprogenitors was also accompanied by a significant increase in RANKL protein expression. RANKL represents the ultimate common mediator of humoral signals that regulate osteoclastogenesis, and in association with its physiological antagonist, osteoprotegrin (OPG), ultimately determines if OC formation will occur. The presence of myeloma cells, which are capable of sequestering and degrading OPG in the BM microenvironment, may also serve to decrease the level of OPG relative to the local RANKL concentration. This would result in a microenvironment more suited to supporting OC formation. In addition, our studies show that the STRO-$1^+$ osteoprogenitor cells secrete higher levels of the potent myeloma cell growth factor, IL-6,

when compared with STRO-1$^-$ cells. These studies therefore indicate that the phenotypic change of OB-like cells in response to MM-derived IL-1β would provide a BM microenvironment, which would be favourable for both myeloma and OC cell growth, thereby facilitating disease progression and osteolysis.

Studies outlined in Chapter 4 indicate that stromal stem cells, isolated from BM, express high levels of RANKL protein. These studies therefore suggest that stromal stem cells, in addition to giving rise to all the mesenchymal lineages, may also serve to facilitate OC recruitment and development.

ET-1 is a 21 amino acid vasoactive peptide that has been implicated in many malignancies, including breast, colon, prostate, ovary and lung cancers. Studies presented in Chapter 5, show for the first time, that MM patients express high BM plasma levels of ET-1 protein when compared with patients with the pre-myeloma disorder, MGUS, or normal age matched controls. The increased plasma level of ET-1 maybe, in part, due to the increased secretion of ET-1 by OBs in response to myeloma cells. Furthermore, the increased plasma level of ET-1 may also be due to the decreased expression of the non-signalling form of the $ET_BR$ receptor, $ET_BR\Delta5$, by OBs. This would serve to decrease the clearance of active ET-1 and result in increased effects on myeloma bone marrow mononuclear cells *via* their over-expression of $ET_AR$. In addition, our *in vitro* and *in vivo* studies suggest that ET-1 decreases bone formation by inhibiting the differentiation of OB-like cells.

In conclusion, this study further supports the concept that the underlying mechanisms of MM-mediated osteolytic bone disease are attributed to the increased osteoclastic bone resorption caused by the over-expression of OC activating factors secreted by myeloma cells and/or OBs in response to myeloma cell stimulation. Myeloma cells themselves secrete RANKL, ET-1 and OC activating factors, which results in the inhibition of OB differentiation. The immature OBs and stromal precursors produce high levels of RANKL and IL-6 and provide a favourable environment for OC and plasma cell growth. In combination, these factors create an ideal environment for both MM disease progression and osteolytic bone disease.

## ABBREVIATIONS

| | |
|---|---|
| $1,25\text{-}(OH)_2D_3$ | 1 alpha, 25-dihydroxyvitamin $D_3$ |
| 7-AAD | 7-aminoactinomycin |
| $A_{450}$ | Absorbance at 450 nm |
| α-MEM | Alpha modification of minimum essential medium |
| α-SMA | α-smooth muscle actin |
| ALP/AP | Alkaline phosphatase |
| ASC-2P | L-ascorbic acid 2-phosphate |
| ATP | Adenosine triphosphate |
| bFGF | Basic fibroblast growth factor |
| BM | Bone marrow |
| BMMNCs | Bone marrow mononuclear cells |
| BMSCs | Bone marrow stromal cells |
| BMSSCs | bone marrow stromal stem cells |
| BMT | Bone marrow transplant |
| BMP(s) | Bone marphogenic protein(s) |
| bp | Base pair(s) |
| BSP | Bone sialoprotein |
| BSA | Bovine serum albumin |
| CAM | Cell adhesion molecule |
| CAM-DR | CAM - mediated drug resistance |
| cAMP | Cyclic adenosine monophosphate |
| CD | Cluster of differentiation |
| cDNA | Complimentary DNA |
| CFSE | Carboxyfluorescein diacetate succinimidyl ester |
| CFU-F | Colony forming unit-fibroblast |
| cm | Centimetre |
| COLI | Collagen type I |
| COX 2 | Cycloocygenase 2 |
| CSF | Colony stimulating factor |
| d1, d3,d5,d7 | Day1, day3, day5 and day7 |
| D1 | Division 1, 2,3… |
| DAG | 1,2-diacylglycerol |

| | |
|---|---|
| DEPC | Diethylpyrocarbonate |
| DEX | Dexamethasone sodium phosphate |
| DMEM | Dulbecco' modified eagles medium |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| DNAse I | Deoxyribonuclease I |
| ECE-1 | Endothelin converting enzyme-1 |
| ECGS | Endothelial cell growth supplements |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assays |
| ET-1 | Endothelin-1 |
| $ET_AR$ | Endothelin-1 A receptor |
| $ET_BR$ | Endothelin-1 B receptor |
| $ET_BR\Delta5$ | Endothelin-1 B receptor splice variant, $ET_BR$ delta 5 |
| ES/BS | Eroded surface/trabecular bone surface |
| FACS | Fluorescence activated cell sorting |
| FCS | Foetal calf serum |
| FGF | fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| FN | Fibronectin |
| G-CSF | Granulocyte-CSF |
| GM-CSF | granulocyte/macrophage-CSF |
| GPCR(s) | G-protein-coupled receptor(s) |
| h | Human |
| HA/TCP | Hydroxyapatite/tricalcium phosphate ceramic particles |
| HBSC | Human bone stromal cell |
| HBSS | Hank's balanced salt solution |
| H-CAM | Hyaluronic-cullular adhesion molecule, CD44 |
| H&E | Haematoxylin and eosin |
| HGF | Hepatocyte growth factor |
| HHF | HBSS supplemented with 5% FCS |
| HLA | Human Leucocyte Antigen |
| HSCR | Hirshchsprung's disease |

| | |
|---|---|
| hr(s) | Hour(s) |
| HUVECs | Human umbilical vein endothelial cells |
| i.u. | International units |
| ICAM | Intercellular adhesion molecule, CD54 |
| Ig | Immunoglobulin |
| $IgG_1$, $IgG_{2a}$, $IgG_{2b}$ | Immunoglobulin gamma-1, gamma-2a and gamma-2b isotype |
| Ig M | Immunoglobulin M |
| IGF | Insulin-like growth facotrs |
| IL-1,3,6 | Interleukin-1, 3, 6 |
| IL-1Ra | Interleukin-1 receptor antagonist |
| $IP_3$ | Inositol triphosphate |
| IMDM | Iscove's modified Dulbecco's medium |
| IMVS | Institute of Medical and Veterinary Science |
| kDa | Kilodalton |
| LB | Luria-Bertani |
| LFA | Leukocyte function-related antigen |
| Lin | Lineage |
| LPS | Lipopolysaccaride |
| M | Molar |
| mAb | Monoclonal antibody |
| MACS | Magnetic activated cell sorting |
| MAPK/ERK | Mitogen-activated protein kinase /extracellular signal-regulated kinase |
| MCS | Multiple cloning site |
| M-CSF | Macrophage colony-stimulating factor |
| 2-ME | 2-Mercaptoethanol |
| MFI | Mean fluorescence intensity |
| MGUS | Monoclonal gammopathies of unknown significance |
| mg | Milli gram |
| mIgG, mIgM | Mouse immunoglobulin G or M |
| min | Minute |
| ml, mm, mM | Milli litre, Milli metre, Milli Molar |
| MM | Multiple Myeloma |
| MMP | Matrix metalloproteinase |

| | |
|---|---|
| MNC | Multinuclear cell |
| mRNA | Messenger RNA |
| MSCs | mesenchymal stem cells |
| N-CAM | Neural cell adhesion molecule, CD56 |
| ng | Nano gram |
| nm | Nano metre |
| NHBC | Normal human bone cells |
| NO | Nitric oxide |
| NOD | Normal osteoblast donor |
| NP-40 | Nonidet P40 |
| NT | No treatment |
| OAFs | Osteoclast-activating factors |
| OB(s) | Osteoblast(s) |
| OC(s) | Osteoclast(s) |
| Oc.S/BS | Osteoclast surface/trabecular bone surface |
| OCN | Osteocalcin |
| ON | Osteonectin |
| O/N | Overnight |
| OP | Osteopontin |
| OPG | Osteoprotegerin |
| PB | Peripheral blood |
| PBMNCs | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered saline |
| PCs | Plasma cells |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factors |
| PE | Phycoerythrin |
| $PGE_2$ | Prostaglandin $E_2$ |
| P.I. | Proliferation index |
| $PIP_2$ | Phosphatidylinositol |
| PKB | Protein kinase B |
| PLC | Phospholipase C |
| POEMS | Acronym of polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy and skin changes |

| | |
|---|---|
| PSA | Prostate-specific antigen |
| PsA | Psoriatic arthritis |
| PSCT | Peripheral stem cell transplant |
| PTHrp | Parathyroid hormone related-protein |
| PTP | Protein tyrosine phosphotase |
| RANK(L) | Receptor activator of nuclear factor-κB (ligand) |
| RNA | Ribonucleic acid |
| RPMI-1640 | Rosewell Park Memorial Institute Medium 1640 |
| RT | Room temperature |
| RT-PCR | Reverse transcription- polymerase chain reaction |
| SDM | Serum deprived medium |
| SEM | Standard error of mean |
| TACE | TNF-alpha converting enzyme |
| TEMED | N, N, N' N'-tetramethyl-ethylenediamine |
| TGF-β | Transforming growth factor β |
| TIMP | Tissue inhibitors of metalloproteinases |
| TM | Transmembrane |
| TNF-α | Tumour necrosis factor-α |
| TNF-β | Tumour necrosis factor-β or lymphotoxin |
| TRAIL/Apo2L | TNF-related apoptosis-inducing ligand |
| 2-ME | 2-Mercaptoethanol |
| UV | Ultra violet |
| µl, µg, µm, µM | Microliter, Micro gram, Micro metre, Micro Molar |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VECs | Vascular endothelial cells |
| VEGF | Vascular endothelial growth factor |
| v/v | Volume per volume |
| VLA | Very late acting antigen |
| VSMCs | Vascular smooth muscle cells |
| w/v | Weight per volume |

## PUBLICATIONS

1. **Pan B,** Farrugia NA, To LB, Green J, Lynch K, Findlay MD, Zannettino ACW. The Nitrogen-Containing Bisphosphonate, Zoledronic Acid, Influences RANKL Expression in Human Osteoblast-Like Cells by Activating TNF-alpha Converting Enzyme (TACE). *J Bone Miner Res.* 2004 Jan; 19(1):147-154.

2. **Pan B,** To LB , Farrugia NA, Findlay MD. , Green J, Evdokiou A, Lynch K, Atkins JG, Zannettino ACW. The Nitrogen-Containing Bisphosphonate, Zoledronic Acid Increases Mineralization of Human Bone-Derived Cells *in vitro. Bone.* 2004 Jan;34(1):112-23.

3. Zannettino ACW, **Pan B,** Farrugia NA, Atkins JG, To LB. Identification and Clinical Relevance of Receptor Activator of Nuclear Factor κB Ligand Expression of Myeloma Cells. *Cancer Research.* 2004 Jan; 64: 774-775.

4. Farrugia NA, Atkins GJ, To LB, **Pan B**, Horvath N, Kostakis P, Findlay DM, Bardy P, Zannettino ACW. RANKL Expression by Human Myeloma Cells Mediates Osteoclast Formation *in vitro* and Correlates with Bone Destruction *in vivo*. *Cancer Research.* 2003 Sept; 63:5438-5445.

5. Atkins GJ, Kostakis P, **Pan B**, Farrugia A, Gronthos S, Evdokiou A, Harrison K, Findlay DM, Zannettino AC. RANKL Expression Is Related to the Differentiation State of Human Osteoblasts. *J Bone Miner Res.* 2003 Jun;18(6):1088-98.

6. **Pan B**, To LB, Farrugia NA, Kostakis P, Atkins GJ, Zannettino ACW. Multiple Myeloma-Derived Pro-Inflammatory Cytokines Enhance Osteoclastogenesis by Increasing the Number of RANKL-Expressing STRO-1 Positive Osteoprogenitor Cells. *Blood.* 2003 Nov; 102(11): #5207.

# CHAPTER 1 GENERAL INTRODUCTION

Skeletal disease occurs in a number of medical conditions such as postmenopausal osteoporosis, primary hyperparathyoidism, Paget's disease, and bone metastases of malignant disease. Among the haematological malignancies, multiple myeloma (MM) and POEMS syndrome exhibit bone lesions which present as discrete osteolytic, osteoblastic, or a mixture of both types of lesions. Although these bone disorders are common and cause considerable morbidity, little is known of the molecular and cellular mechanisms responsible for the dysfunctional bone remodelling. Throughout life, the adult skeleton is constantly remodelled to maintain skeletal integrity and this process is accomplished by the coordinated actions of the bone resorbing multinucleated osteoclasts (OCs) and the bone forming osteoblasts (OBs). In pathological bone disease, the normal equilibrium of bone remodelling becomes uncoupled and the imbalance between the amount of bone resorption and bone formation results in these different types of bone lesions.

MM is an incurable B cell malignancy, characterised by the presence of a monoclonal population of end-stage B cells (plasma cells), a monoclonal immunoglobulin (Ig) protein (M-, or para-protein) and "punched out" lytic bone lesions. Osteolytic bone destruction, a major cause of morbidity and mortality, is a striking clinical feature for approximately 80% of patients with MM. It is generally accepted that the osteolysis is related to the accumulation of the myeloma cell clone within the bone marrow (BM), in close vicinity to the bone trabeculae. While the underlying mechanisms responsible for the bone lesions in MM remain to be fully elucidated, studies show that it is due to increased osteoclastic resorption, induced by the factors produced by the myeloma cells or the marrow microenvironment in response to the tumour cells (Michigami *et al.*, 2000; Roodman, 1997). These factors include osteoclast-activating factors (OAFs) tumour necrosis factor-α (TNF-α), TNF-β (lymphotoxin), interleukin-1β (IL-1β), IL-6, macrophage colony-stimulating factor (M-CSF), parathyroid hormone related protein (PTHrP) (Costes *et al.*, 1998; Filella *et al.*, 1996; Pfeilschifter *et al.*, 1989), and receptor activator of nuclear factor-κB ligand (RANKL) (Farrugia *et al.*, 2003; Heider *et al.*, 2003).

Unlike patients with MM, patients with POEMS syndrome, a paraneoplastic syndrome of plasma cell dyscrasia, present with osteoblastic/osteosclerotic bone lesions. In spite of disparate bone pathologies, namely osteolytic and osteoblastic bone lesions, both diseases

are representative of plasma cell dyscrasia. Although a number of cytokines have been implicated in the aetiology of these diseases, few studies have explored what factors of bone microenvironment are capable of mediating these two seemingly disparate skeletal diseases. Therefore, a comprehensive investigation of the factors which are involved in these two types of diseases, may reveal factors that are deterministic with respect to the type of bone lesion which result.

In this project, we have examined the role of a number of factors including IL-1β, RANKL and endothelin-1 (ET-1), with respect to their effects on OB phenotype and function. The clarification of the role of these factors in bone turnover may reveal the underlying mechanisms of physiological bone remodelling and pathological bone disease.

## 1.1 HAEMATOLOGICAL MALIGNANCIES WHICH GIBE RISE TO BONE LESIONS

Haematological malignancies frequently involve the skeleton. Both osteolytic and osteoblastic lesions can occur in patients with lymphomas, particularly Hodgkin's disease, MM and POEMS syndrome. The majority of patients with MM display osteolytic lesions. Although there may be a mixture of discrete osteolytic and diffuse osteopenic lesions in some patients, more than 95% of patients exhitbit some type of bone loss. In contrast, a paraneoplastic syndrome of plasma cell dyscrasia, POEMS syndrome, displays sclerotic/osteoblastic bone lesions. Although the cellular mechanisms responsible for these bone lesions are unclear, it is proposed that the local cytokines secreted by tumour cells, or by the marrow microenvironment in response to the tumour cells, are responsible for the osteolytic or osteoblastic bone lesions. MM and POEMS provide us two models to explore these underlying mechanisms.

### 1.1.1 Osteolytic Bone Disease Mediated by Multiple Myeloma

#### 1.1.1.1 General Description

Multiple myeloma (MM) is a differentiated B cell neoplasm characterized by marrow expansion of a malignant plasma cell clone, resulting in the gradual replacement of the haemopoietic matrix and dissemination of osteolytic lesions as nests of tumor cells. A major clinical feature of MM is the development of osteolytic bone lesions characterized by the presence of bone pain, hypercalcemia, and pathologic fracture.

MM is a rare cancer with an elusive aetiology. It has higher incidence among Negroids than Caucasians. MM accounts for 2.9% of all malignancies in blacks and 1.0% in whites, with similar male to female ratio 1.5:1. To date, epidemiological investigation has been unable to determine the reasons for these apparent racial differences. The median age of diagnosis is 69 and 71 years for males and females, respectively. The average rates of annual age-specific incidence increases sharply with age and is independent of race and gender (Pottern *et al.*, 1996).

The diagnosis of MM depends upon the demonstration of increased numbers of marrow plasma cells (>10%) which present as atypical and immature forms (Figure 1.1), the presence of monoclonal paraprotein (M-protein, >3/dl) in serum (Figure 1.2), Bence-Jones protein in urine, and osteolytic skeletal lesions (Figure 1.3). The stage of MM is generally assessed by the Durie and Salmon staging system which was developed in 1975. In this system, MM patients can be divided into three stages based on plasma cell number, haemoglobin value, serum calcium level, bone lesion number, M-protein value and urine light chain M-component on electrophoresis (Woodruff *et al.*, 1979). Besides this traditional staging system, MM can be classified into distinct stages according to gene expression profiles linked to late-stage B-cell differentiation using recently developed microarray techniques (Zhan *et al.*, 2003). The prognosis for MM is highly dependent upon the stage of disease and various other factors, including the serum β2 microglobulin and albumin levels at the time of diagnosis.

Monoclonal gammopathy of uncertain significance (MGUS) often precedes MM and is characterised by the presence of M-protein without evidence of MM, macroglobulinemia, amyloidosis, or a related plasma cell proliferative disorder. Approximately 17% of MGUS patients will develop a lymphoplasmacytic disease at 10 years and 33% at 20 years (Kyle *et al.*, 2003).

Although various combination chemotherapy regimens have been developed, no definitively curative approach has been identified for MM. High-dose chemotherapy regimens with bone marrow transplant (BMT) or peripheral stem cell transplant (PSCT) have been attempted to improve outcomes (Kaufman *et al.*, 2004). However, to date no consensus has been arrived as to the best way of managing MM.

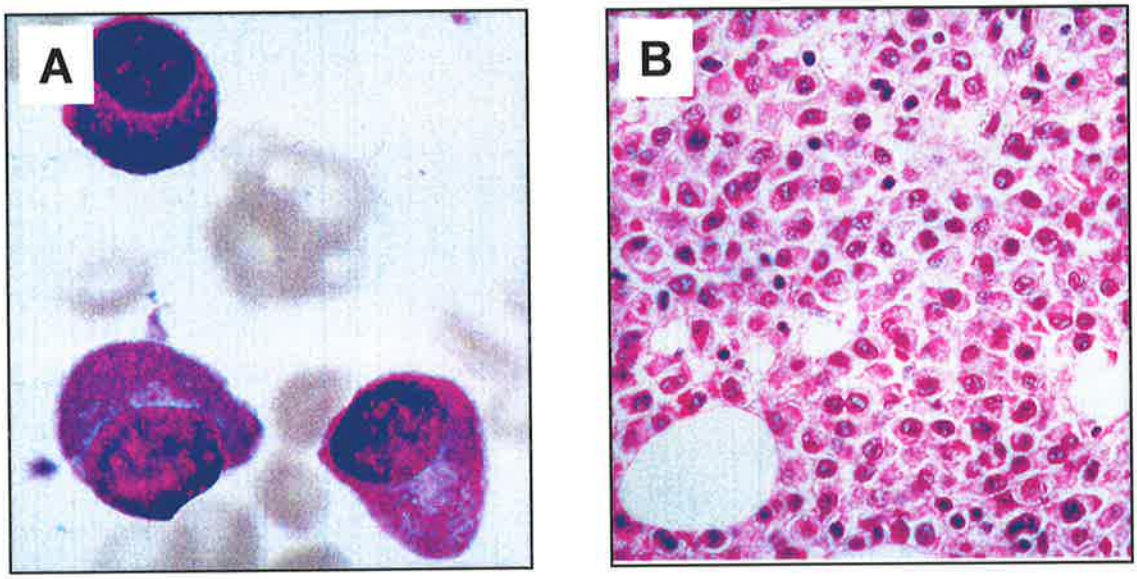



Figure 1.1. Myeloma plasma cells. (A) A smear of a bone marrow aspirate showing abnormal plasma cells. (B) Trans-iliac bone marrow trephine biopsy illustrating almost complete replacement of normal haemopoietic tissue with abnormal plasma cells.

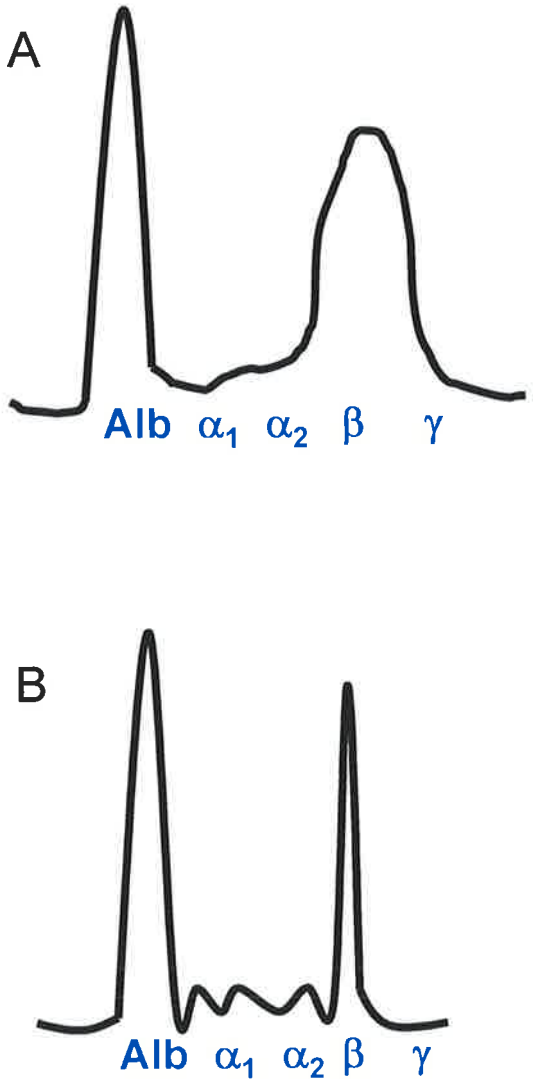



Figure 1.2. Densitometric appearance of serum protein following electrophoresis. (A) Broad polyclonal peak represents the summation of thousands of physiologic monoclonal peaks in patients with chronic infection. (B) Narrow homogeneous peak situated in the midst of the γ globulin region signifies a monoclonal M component. Modified from Jandl, J.H. (Jandl, 1996).

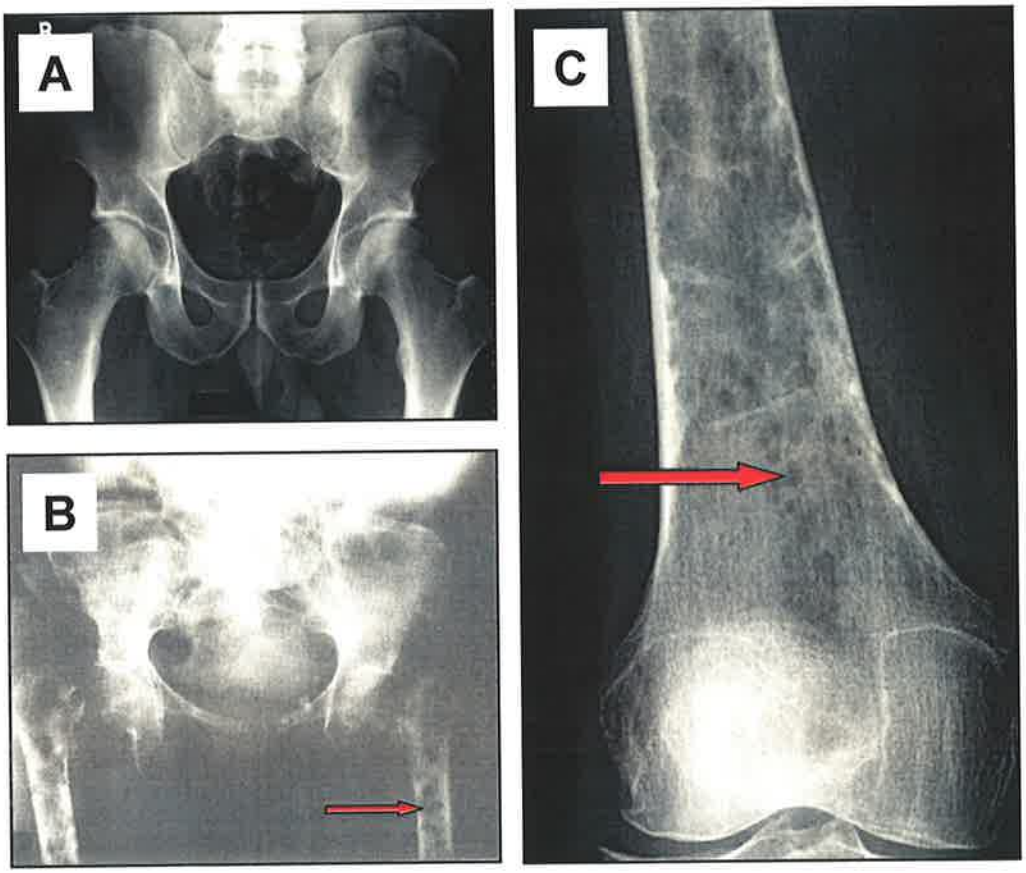



Figure 1.3. Radiograph of the pelvic girdle and femora. (A) Normal radiograph of the pelvic girdle and proximal femora. (B) and (C) The typical multiple "punched out" osteolytic lesions are presented in pelvic girdle and proximal femora (B) and distal femora (C).

### 1.1.1.2 Pathophysiology of MM

Normal plasma cell development initially occurs in lymphoid tissues, a site of antigen presentation. The earliest morphologically distinct Ig-producing cell is the plasmablast, which is capable of rapid cell proliferation. Cells with intermediate or transitional plasma cell characteristics retain their mitotic potential while continuously developing into mature plamsa cells. These oligoclonal or polyclonal Ig-producing plasma cells relocate to various lymphoid tissues, including germinal centre of the spleen, lamina propria of the gut, and follicles of lymph nodes (Potter, 1996).

In contrast to the normal plasma development, the myeloma tumor cells grow in the bone marrow. Myeloma cells are found in the close vicinity of bone trabeculae, suggesting a strong relationship between tumour cells and their environment. MM tumors arise from primitive clonogenic cells, possibly pre-B cells or even pluripotential stem cells which possess an ability to selectively home to the bone marrow (BM). Upon reaching the growth conducive BM microenvironment, the pre-B cells cease circulating and reside within the BM (Epstein *et al.*, 1990; Grogan *et al.*, 1987). The myeloma cells are then induced to express additional adhesive molecules, including hyaluronic acid-cellular adhesion molecule (H-CAM, CD44), intercellular adhesion molecule-1 (ICAM-1, CD54), neural cell adhesion molecule (N-CAM, CD56) and lyphocyte function-related antigen (LFA-1). Fibronectin receptor $\alpha4\beta1$ is predominant adhesion receptor involved in the homing and localization of myeloma cells to the BM microenvironment (Barker *et al.*, 1992; Jensen *et al.*, 1993). Studies show that myeloma cell lines and patient-derived myeloma cells express $\alpha4\beta1$ and $\alpha5\beta1$ (Damiano *et al.*, 1999), whilst fibronectin and vascular cell adhesion molecule (VCAM-1), are expressed on the surface of MM stromal monolayers (Faid *et al.*, 1996; Lokhorst *et al.*, 1994). Antibodies to VCAM-1 (Kim *et al.*, 1994; Robledo *et al.*, 1998), $\alpha4$ and $\beta1$ have been reported to inhibit the adhesion of myeloma cells to intact MM stroma (Lokhorst *et al.*, 1994; Uchiyama *et al.*, 1992; Uchiyama *et al.*, 1993; van Riet *et al.*, 1994). Recent studies suggest that there is considerable interdependence between malignant myeloma cells and BM stromal cells present within the bone environment, through the interaction of different adhesion receptors with their ligands.

The BM microenviroment is a site of synthesis of numerous cytokines which are important in the proliferation and/or survival of myeloma cells (Bataille *et al.*, 1996). In addition, myeloma cells themselves can produce a number of cytokines and local growth factors, including IL-1β, lymphotoxin and M-CSF. The adhesion of myeloma cells to MM stroma triggers synthesis of a number of cytokines from both tumour and stromal cells in response to co-adhesion and α4β1 ligation (Uchiyama *et al.*, 1993), such as IL-6, to stimulate the proliferation and differentiation of malignant plasma cells (Caligaris-Cappio *et al.*, 1991; Klein *et al.*, 1995).

It has been shown that MM patients with advanced aggressive disease, secrete significantly higher levels of IL-6, IL-1β, TNF-α, TNF-β, and PTHrP (Bataille *et al.*, 1992; Caligaris-Cappio *et al.*, 1992; Filella *et al.*, 1996; Lichtenstein *et al.*, 1989). In addition to the growth stimulating effect on myeloma cells, cytokines, such as IL-1β and IL-6, are considered potent osteoclast activating factors (OAFs) (Bataille *et al.*, 1992; Caligaris-Cappio *et al.*, 1992) which can stimulate osteoclastogenesis *in vitro* and *in vivo*.

#### 1.1.1.3 Osteolytic Bone Disease

Extensive bone disease is a hallmark of MM and contributes to most of the debilitating symptoms associated with this disease (Roodman, 1995). Multiple myeloma induced osteolytic bone lesions or osteoporosis involves the axial skeleton and the long bones. It causes intractable bone pain and other distressing symptoms such as pathological fractures after trivial injury, spinal cord compression, and perturbation in calcium homeostasis. Approximately 80% of patients present with pain of varying intensity, often in the lower back or ribs. Sudden severe pain is often a sign of fracture or collapse of a vertebral body. At diagnosis, approximately 50% of patient have vertebral fractures, and approximately 30% will also develop pathological fractures of the long bones, ribs and sternum with advanced disease (Lahtinen *et al.*, 1992). The majority of patients with lytic bone lesions have diffuse MM infiltration within the marrow. Net osteolysis in MM patients causes a continuing loss of calcium from the skeleton. At presentation, approximately 30% of patients exhibit elevated serum calcium levels that may cause dehydration and uraemia. The significant morbidity and mortality associated with the MM-mediated osteolysis therefore necessitates effective therapies to inhibit bone loss. To achieve this goal, it is of importance to better understand the biological mechanisms involved in the pathogenesis of MM-induced bone disease.

It is generally considered that the underlying mechanism of osteolysis in MM patients is as a result of increased osteoclastic bone resorption. This is consistent with the histologic findings in myeloma bone disease in which enhanced and uncontrolled osteoclastic bone resorption is often seen adjacent to areas of plasma cell infiltrates (Figure 1.4). Interestingly, both OCs and OBs are present in increased numbers in early stage of MM (Bataille *et al.*, 1991; Karadag *et al.*, 2000b; Wong *et al.*, 2000), but their specific functions (bone resorption and formation) are targeted differently by tumor cells. Loss of bone in MM is explained by increased OC activity and inadequate bone regeneration, due to the inhibition of OB function, as there is no evidence of new bone formation within the skeleton lesions or in their vicinity.

In contrast to the osteolytic bone lesion seen in patients with osteolytic MM, very rare patients develop osteosclerotic MM, which is a plasma cell dyscrasia characterised by sclerotic bone lesions and progressive demyelinating polyneuropathy (Lacy *et al.*, 1997). The clinical management of POEMS syndrome is similar to osteosclerotic MM and is described below.

### 1.1.2 Osteoblastic Bone Disease Mediated by POEMS

#### 1.1.2.1 General Description

In 1980, the acronym POEMS syndrome was coined to describe a rare multissystem disorder characterised by polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes (Bardwick *et al.*, 1980). Polyneuropathy associated with POEMS is characterised by mixed sensorimotor changes, which are caused by a mixture of axonal degeneration and segmental demyelination. In biopsies from patients with POEMS syndrome, diffuse M protein deposits are found in the endoneurial space, particularly in the subperineurial area, without deposit in the axons (Adams *et al.*, 1998). Organomegaly usually presents as hepatosplenomegaly and lymphoadenopathy, revealing pathological angiofollicular hyperplasia. Endocrinopathies include gonadal dysfunction, glucose intolerance, hyperprolactinaemia, hypothyroidism, and hypoparathyroidism (Cabezas-Agricola *et al.*, 1996). Monoclonal gammopathy is found in the majority of patients with POEMS syndrome, with Ig G (41%) and Ig A (39%) heavy chains, and lambda light chains. Skin changes consist of hyperpigmentation, plethora, scleroderma-like thickening, hypertrichosis, clubbing, and Raynaud's phenomenon. In addition, additional features have

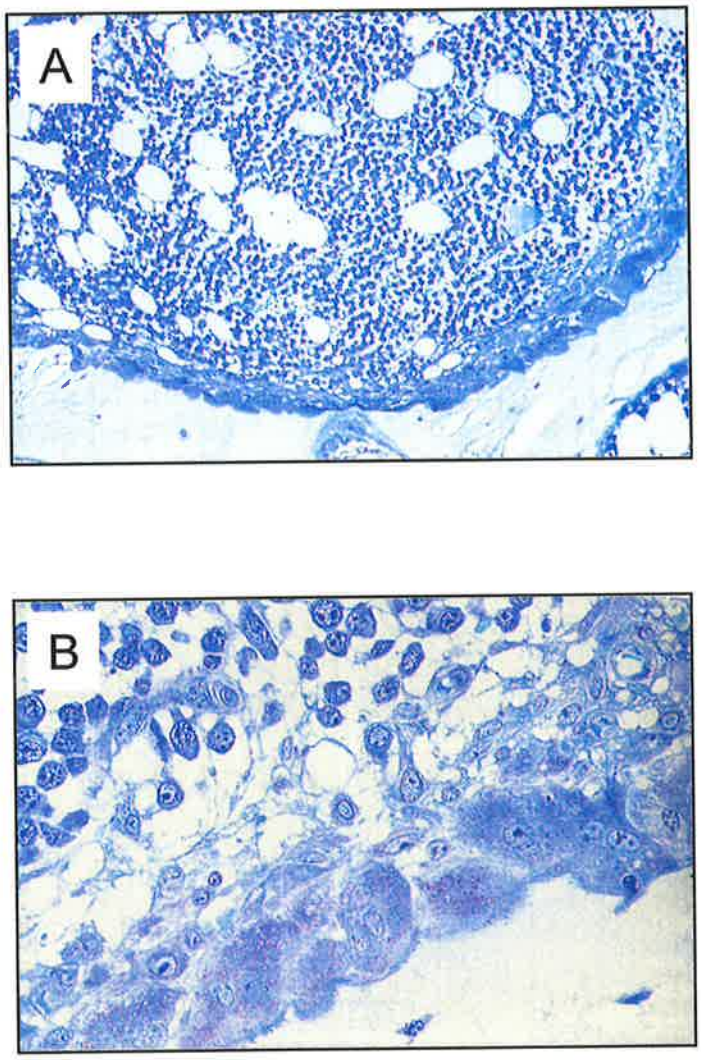



Figure 1.4. Osteoclastic bone resorption in multiple myeloma. (A) trabecular surface with scalloped edge lined by a layer of osteoclasts together with a dense myelomatous infiltration (Giemsa stain; × 100). (B) at higher magnification, scalloped trabecular surface filled with active osteoclasts and separated from myelomatous infiltration by a broad zone of connective tissue (Giemsa stain; × 400). Adapted from Bartl, R. (Bartl *et al.*, 1996).

been described in patients with POEMS, including necrotizing vasculitis in the adrenal gland (Shibata *et al.*, 2000), pulmonary hypertension (Lesprit *et al.*, 1998), inflammatory and fibrotic myopathy (Goebels *et al.*, 2000).

POEMS syndrome is usually associated with plasma cell dyscrasia, mainly of osteosclerotic myeloma and solitary plasmocytoma, in which patients rarely have symptoms of typical myeloma such as bone pain, hypercalcemia, anemia, or renal insufficiency. Although some patients present with mixed lytic and sclerotic changes (Hogan *et al.*, 2001), most patients present with multiple and widespread nodular sclerotic lesions (Figure 1.5).

### 1.1.2.2 Pathophysiology of POEMS Syndrome

The aetiology and pathophysiology of POEMS syndrome remain unclear. The success of chemotherapeutic approaches based on traditional therapy for multiple myeloma supports the hypothesis that POEMS syndrome is a paraneoplastic syndrome of plasma cell dyscrasia. Long term MP (melphalan, prednisolone) chemotherapy improves the symptoms of POEMS syndrome, even in the absence of osteoblastic lesion (Kuwabara *et al.*, 1997). In patients with a solitary plasmacytoma, external-beam radiation therapy with or without surgical excision may lead to long term regression of the neuropathy.

It is proposed that POEMS is a paraneoplastic phenomenon mediated by aberrant cytokines or angiogenic factors secreted by neoplastic plasma cells. The strong correlation between the high serum levels of IL-6, IL-1β , and TNF-α and disease activity has been reported (Goebels *et al.*, 2000; Lesprit *et al.*, 1998). Furthermore, patients with POEMS syndrome have higher levels of IL-6 and TNF-α than patients with classic MM. It also has been noted that vascular endothelial growth factor (VEGF) serum levels in patients with POEMS were markedly elevated, which may be an important mediator in the evolution of vasculitic lesions (Shibata *et al.*, 2000; Soubrier *et al.*, 1997; Soubrier *et al.*, 1998), and POEMS syndrome-associated pulmonary hypertension (Lesprit *et al.*, 1998). VEGF is known to increase the release of matrix metalloproteinases (MMP) in both endothelial cells and vascular smooth cells *in vitro*. Both VEGF and MMP are considered important mediators of angiogenesis and neovascularization. Moreover, MMP are involved in various immune-mediated demyelinating neuropathies. In support of these data, serum circulating levels of MMP-1,-2,-3,-9, and tissue inhibitors of metalloproteinases (TIMP) are increased in POEMS syndrome (Michizono *et al.*, 2001).

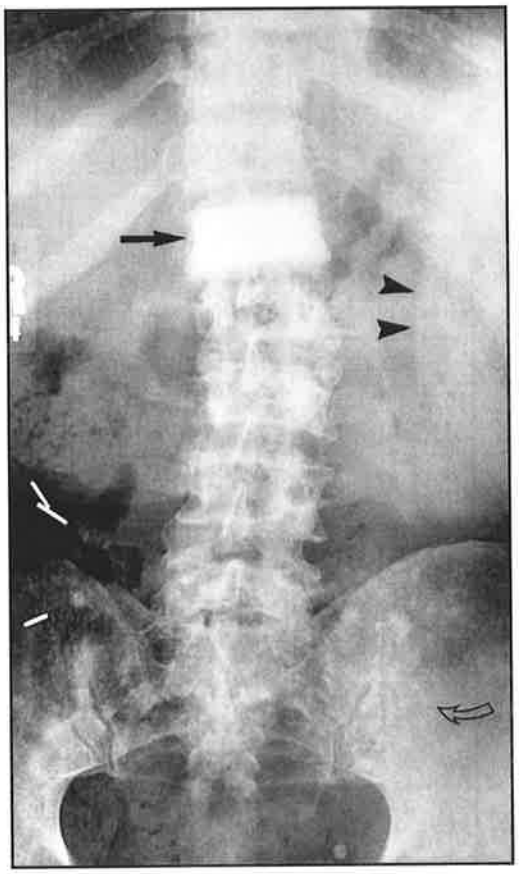


Figure 1.5. Radiograph of vertebrae and pelvic girdle from a patient with POEMS syndrome. The ivory osteoblastic bone lesion is highlighted on lumbar vertebrae (L1, black arrow) and multiple and widespread nodular sclerotic bone lesion are highlighted on pelvic girdle (indicated by open arrows). Adapted from Chung, J.B. (Chung *et al.*, 2002).

#### 1.1.2.3 Osteoblastic Bone Disease

Sclerotic bone lesions, either solitary or multiple, occur in most POEMS syndrome patients. A study by Dispenzieri *et al* (2003) showed that 97% patients with POEMS syndrome displayed abnormal findings on bone radiography (Dispenzieri *et al.*, 2003). Among this, 47% patients have osteosclerotic lesions only, 51% patients have mixed sclerotic and lytic bone lesions, and only 2% patients have lytic bone lesions alone (Dispenzieri *et al.*, 2003). In addition, the lytic lesions tend to have a scelrotic rim (Dispenzieri *et al.*, 2003). Although other studies have reported lower percentage of bone involvement, the sclerotic lesions are a consistent feature (Nakanishi *et al.*, 1984; Soubrier *et al.*, 1994).

Although the study by Dispenzieri *et al* (2003) suggested that the prognosis of patients with POEMS syndrome was independent of the number of bone lesions (Dispenzieri *et al.*, 2003), Soubrier *et al* (1994) showed that the prognosis was significantly better for patients with solitary bone lesion than those with multiple lesions (Soubrier *et al.*, 1994). In addition, response to therapy was found to be better in patients with radiated dominant bone lesions (Dispenzieri *et al.*, 2003).

Although the presence of osteosclerotic bone lesions in patients with POEMS syndrome was described more than 3 decades ago, the underlying mechanisms responsible for this disease remain to be determined. Interestingly, high serum levels of OAFs, including IL-6, IL-1β and TNF-α have been reported in patients with POEMS syndrome (Goebels *et al.*, 2000; Lesprit *et al.*, 1998). This observation appears contradictory, given our present understanding of the role of these factors in bone physiology. The section below (Section 1.2) reviews the current literatures regarding the molecular basis of bone formation and resorption.

## 1.2 BONE PHYSIOLOGY

Bone formation occurs throughout human development and life to maintain structural integrity. This bone formation occurs by two fundamental processes: endochondral ossification and intramembranous ossification. Furthermore, the shape and structure of formed bone is continually renewed and modified by two processes, modelling and remodelling. Modelling, which occurs principally during growth, results in a change in the

shape of the skeleton. Bone remodelling represents the predominant form of bone turnover in the adult human skeleton and prevents the accumulation of micro-damage and allows the skeleton to respond appropriately to changes in mechanical loading.

### 1.2.1 Physiological Bone Remodelling

Bone is a highly organised structure comprised of a calcified connective tissue matrix (hydroxyapatite) formed by OBs, bone lining cells and osteocytes. Although trabecular bone represents 20% of the skeletal mass, it accounts for up to 80% of the bone turnover. In contrast, cortical bone represents 80% of the skeleton but only accounts for 20% of the bone turnover. The integrity of the adult skeleton is maintained by normal bone remodelling process, which is mediated by the highly coordinated activities of two cell types, namely the bone resorbing OCs and the bone forming OBs (Parfitt, 1984). Osteoclastic bone resorption is preceded by a cascade of events that commences with the activation of haemopoietic stem cells and is followed by proliferation, differentiation, chemotaxis, attachment, and subsequent terminal differentiation and fusion of the OC precursors into functioning multinucleated OCs. Following activation, OCs erode and form a cavity (lacuna) on the bone surface, a process which lasts for approximately two weeks. After completion, the OCs apoptose and OBs subsequently differentiate and form new bone at the same site. Newly formed sites remain quiescent, normally for 2-3 years, until a new activation stimulus event occurs at this site. In the normal physiology, the amount of bone formation in the remodelling process is equal to the amount of bone resorption.

### 1.2.2 Bone Resorption

#### 1.2.2.1 Osteoclasts

Osteoclasts are seen frequently at active sites of bone remodelling, such as the metaphyses of growing bones or adjacent to collections of tumour cells. Osteoclasts are characterised histochemically by high levels of tartrate-resistant acid phosphatase (TRAP) activity and by their expression of cell surface receptors for calcitonin and vitronectin (Suda *et al.*, 1997). Usually the large multinucleated OCs are from 20 to 150 μm and contain 2 to 50 nuclei. They have primary lysosomes, numerous and pleomorphic mitochondria, and a specific area of the cell membrane that forms adjacent to the bone surface, known as the ruffled border. This area of the cell membrane comprises folds and invaginations, which facilitates intimate contact with bone resorption sites and the formation of a resorption pit (also known as the Howship's lacuna or resorption bay). The ruffled border is surrounded

by a clear zone, which contains actin filaments, and appears to anchor the ruffled border area to the bone surface undergoing resorption. This structure may be critical to the polarisation of the OC, which occurs immediately when the cell is activated prior to undergoing resorption. In addition, OCs have an $H^+$-ATPase proton pump that is responsible for creation of the fundamental acidic microenvironment to bone resorption (Mundy, 1995).

The recruitment, activation and apoptosis of OCs are modulated by a series of hormones and cytokines. The three main hormones modulating bone resorption are parathyroid hormone (PTH), 1 alpha, 25-dihydroxyvitamin D3 ($1,25\text{-}(OH)_2D_3$), and calcitonin. Furthermore, other important factors, such as TNF-$\alpha$, IL-1$\beta$, IL-6, PTHrP, and RANKL and its decoy receptor OPG are also involved in regulating the formation and function of OCs, as discussed below.

### 1.2.2.2 Osteoclast Stem Cells

Osteoclasts are derived from pluripotent precursors of the monocyte/macrophage lineage in the BM where they are in physical contact with stromal cells. OC precursors express CD11b, CD14, CD51/CD61 ($\alpha v\beta 3$) and RANK (Shalhoub *et al.*, 2000). The development of OCs is comprised of several stages, including recruitment, proliferation, differentiation of the progenitors into mononuclear OCs, and fusion of mononuclear OCs to form activated multinucleated OCs capable of resorbing bone. Experimental data suggest that OBs and their progeny (bone lining cells) are required for *in vitro* maturation of macrophages into OCs (Udagawa *et al.*, 1990). Interactions may occur by either direct cell-cell contact or by the production of soluble mediators that are critical for all stages of OC development. It has demonstrated that OBs express two essential molecules, macrophage colony-stimulating factor (M-CSF) and RANKL. M-CSF binds to its receptor, c-Fms, expressed on early OC precursors, providing signals crucial for survival and proliferation of monocytic progenitors (Udagawa *et al.*, 1990). Using the mononucleated fraction of peripheral blood of adults as a source of precursors, terminally differentiated OCs capable of extensive resorption pit formation can be generated *in vitro* either in the presence of OBs or essential mediators M-CSF and RANKL (Azuma *et al.*, 2000; Fujikawa *et al.*, 1996; Lacey *et al.*, 1998; Matayoshi *et al.*, 1996). The detailed role of RANKL in osteoclastogenesis is discussed in the Section 1.3.2.

### 1.2.3 Bone Formation

Osteoblasts are derived from the stromal or mesenchymal cell system and represent a heterogenous population of cells, including mature OB, bone lining cells, which cover bone surfaces, and osteocytes, which are buried within bone and communicate with each other *via* the canalicular system. The functions each of these cell populations are different and will be considered separately.

#### 1.2.3.1 Osteoblast Cells

Mature OBs line the bone surface, and have a single eccentric nucleus and a well-developed endoplasmic reticulum and Golgi apparatus (Mundy, 1995). They are identified morphologically by their cuboidal appearance and their association with newly synthesized mineralized bone matrix (hydroxyapatite) at sites of active bone formation. Mineralisation of bone matrix may be mediated, in part, by subcellular particles known as matrix vesicles, which are generated from the OB cytoplasm and are enriched in alkaline phosphatase. Bone matrix comprises 90% of collagen and about 10% of various non-collagenous proteins (Robey, 1996). Osteoblasts are normally involved in bone formation by producing both collagenous and noncollagenous proteins. Osteoblasts also synthesise other bone matrix constituents that may be important in the mineralisation process such as phospholipids and proteoglycans. Therefore, committed osteogenic cells are characterised histologically by the synthesis of certain bone-associated matrix proteins, such as collagen type I (COLI), osteocalcin (OCN), bone sialoprotein (BSP), osteonectin (ON), and osteopontin (OP). Expression of the membrane-bound ectoenzyme alkaline phosphotase (ALP) is one of the earliest, and most widely accepted indicators that a cell has initiated a program of osteogenic differentiation. ALP plays an essential role in bone mineralisation and is frequently used by bone cell biologists as a marker of cells with an OB phenotype, and by clinicians as a serum marker of OB activity (Martin *et al.*, 1997).

Studies on human bone have shown that cells of the OB lineage express a range of integrin $\alpha$ and $\beta$ subunits both *in situ* and in culture (Clover *et al.*, 1992; Gronthos *et al.*, 1997; Grzesik *et al.*, 1994; Saito *et al.*, 1994). $\beta$1 integrins appear to be the predominant adhesion receptor subfamily used by stromal precursor cells to adhere to matrix glycoproteins commonly found in the BM microenvironment and bone surfaces (Gronthos *et al.*, 2001). In addition, the ability of human BM stromal cells (BMSC) to initiate matrix mineralisation has been found to be significantly diminished in the presence of a functional

blocking monoclonal antibody to the β1 integrin subunit, demonstrating a role for this integrin subfamily during bone formation. While studies suggest that the resorption of bone by OC may be mediated through integrins such as αvβ3 and αvβ5 (McHugh *et al.*, 2000), it appears that OB development and bone formation may be more dependent on β1 integrin interactions (Zimmerman *et al.*, 2000).

### 1.2.3.2 Osteoblast Stem Cells

The fact that the bone remodelling process continues throughout adult life, and both OBs and OCs have a limited functional lifespan, points to the existence of precursor cell populations with the potential for extensive proliferation and differentiation. The osteogenic cell lineage belongs to the stromal fibroblastic system of the BM, which includes other stromal tissue, such as cartilage, smooth muscle, and fat (Beresford, 1989; Owen *et al.*, 1988). Although there is heterogeneity within the OB lineage with regard to their proliferative capacity and functional state, these cells have a common stromal cell precursor capable of multilinage differentiation (Liu *et al.*, 1997).

Osteoblasts differentiate from mesenchymal progenitors through the regulatory action of cell-cell and cell-matrix interactions and by the actions of growth factors produced locally or present in the circulation. Although the factors involved in controlling the differentiation of precursors into cells of the OB lineage have not yet been fully determined, a number of growth regulatory factors have been identified as reviewed by Mundy (Mundy, 1995). Among them, transforming growth factor β (TGF β), bone morphogenic proteins (BMP), platelet-derived growth factors (PDGF) and the insulin-like growth factors (IGF) secreted by OBs and stored in the bone matrix are considered important (Hauschka *et al.*, 1986). However, their individual roles *in vivo* are not yet clear.

The exact location of BM stromal precursor cells in the marrow spaces is still a matter of conjecture. Histological evidence suggests that multipotential stromal progenitors may reside in the soft fibrous tissue of the marrow stroma. The osteogenic potential of the marrow stroma is attributed to the presence of a small population of highly adherent, clonogenic cells, termed colony-forming units fibroblastic (CFU-F) (Owen *et al.*, 1988). A monoclonal antibody (mAb) STRO-1 has been described which recognises a trypsin-resistant cell surface antigen present on the osteoprogenitor stem cell subpopulation of BM cells (Simmons *et al.*, 1991). BM mononuclear cells sorted on the basis of STRO-1

expression are capable of establishing an adherent stromal layer *in vitro*, consisting of a number of phenotypically distinct stromal cell types, including fibroblasts, smooth muscle cells, adipocytes, and OBs (Simmons *et al.*, 1991). In addition, it has been demonstrated that the STRO-$1^+$ population of human BM cells is capable of osteogenic differentiation and that osteoprogenitors are almost exclusively present within the STRO-$1^+$ population (Gronthos *et al.*, 1994). The factors required for the expansion of the marrow CFU-F population *in vitro* have also been characterised, including L-ascorbate, the glucocorticoid dexamethasone, PDGF-BB and epidermal growth factor (EGF) (Gronthos *et al.*, 1995).

Using two independent cell surface markers, STRO-1 and ALP, cells of the OB lineage at different stages of maturation have also been characterised (Gronthos *et al.*, 1999; Stewart *et al.*, 1999). The bone /liver/kidney isoform of ALP is broadly used as a general marker of committed OB, and STRO-1 is used as a marker representiative of a preosteoprogenitor phenotype. Gronthos *et al* (1999) showed that the majority of normal human bone cells (NHBCs), which express the STRO-$1^-$/$ALP^+$ and STRO-$1^-$/$ALP^-$ phenotypes, appear to represent fully differentiated OB. In contrast, the cells expressing STRO-$1^+$/$ALP^+$ phenotype are considered to be representative of osteoprogenitors, and cells which express STRO-1 antigen alone representative of stromal precursors (Gronthos *et al.*, 1999). These findings are based on the expression of a comprehensive array of bone-associated matrix proteins and the ability of each subpopulation to form a mineralised bone matrix *in vitro*.

### 1.2.3.3 Osteocytes

Each OB can have one of three fates: the majority of OB die by apoptosis, those remaining transform to lining cells on the bone surface or become osteocytes (Jilka *et al.*, 1998). During the course of the bone formation process, some of the OBs cease synthesising matrix, become embedded within bone and communicate with each other *via* the canalicular system and are referred to as osteocytes. An osteocytes have two possible fates: it may remain in the bone until it is replaced by remodeling, or it may die by apoptosis, leaving an empty lacuna before the bone is remodeled.

Osteocyte density is positively related to the proportion of osteoid surface covered by OBs and is inversely related to the proportion of OBs that undergo apoptosis. Osteocytes can inhibit bone remodeling and the loss of inhibitory effect depends not only on the

absolute decrease of osteocyte number but also on the relative decline in osteocyte density (Qiu *et al.*, 2002). Osteocyte density reflects the balance between the mean initial density when the bone is formed and the mean natural lifespan, reflecting the timing of death by apoptosis (Qiu *et al.*, 2002). Accumulating evidence suggest that osteocyte apoptosis is induced by fatigue microdamage of bone and osteocyte apoptosis provides an important signal to activate osteoclastic bone resorption (Verborgt *et al.*, 2000). However, the mechanisms initiating osteocyte apoptosis are currently unknown.

#### 1.2.3.4 Lining Cells

When the OBs are not in the process of forming bone, they become flat quiescent cells referred to as resting OBs or lining cells. Although it is well known that lining cells and active OBs form a membrane to cover the surface of the bone tissue, the role of these cells remains to be determined.

## 1.3 FACTORS INVOLVED IN BONE LESIONS

As previously described, the bone integrity of mammals is maintained by normal processes of bone remodelling. Any factors that affect either bone formation or bone resorption, or both, are likely to cause bone lesions. In MM, the increased osteoclastic resorption induced by factors produced by the myeloma cells or the marrow microenvironment in response to the tumour cells is primarily responsible for the bone lesion in MM (Michigami *et al.*, 2000; Roodman, 1997). These factors include an important TNF ligand family member RANKL (Farrugia *et al.*, 2003; Giuliani *et al.*, 2001; Pearse *et al.*, 2001) and well-known OAFs, such as IL-6, TNF-α, TNF-β, PTHrP and M-CSF (Costes *et al.*, 1998; Filella *et al.*, 1996; Pfeilschifter *et al.*, 1989). Notably, most studies have focused on the OC promoting effects of these cytokines. A more comprehensive understanding of the role of these factors on the activity and function of OBs is necessary as bone formation represents an additional aspect in the normal equilibrium of bone remodelling.

Compared with MM, the cellular mechanisms responsible for osteoblastic bone lesions in POEMS syndrome are largely unknown. In an attempt to better understand the nature of these bone lesions, it is important to review the putative mechanisms responsible for the bone metastases of solid tumour. Bone metastases have been widely investigated to identify the process of tumour metastases to bone and the mechanisms by which solid tumours

ultimately affect bone cell function. Moreover, it also provides an approach to define the important cytokines regulating bone turnover.

When solid tumours metastasise to the skeleton, a variety of bone lesions may result, including discrete osteolytic lesions, osteoblastic lesions, diffuse osteopenia, or a combination of all of the above. The destructive or osteolytic lesion is the most common lesion which is characterised by a marked increase in OC formation and OC activity, with blunt or absent subsequent osteoblastic response. Less commonly, solid tumour causes an increase in OB activity and results in an osteoblastic lesion characterised by the formation of new bone around the tumour cell deposits. This may occur without obvious previous osteoclastic resorption and the newly formed bone may lay down directly on trabecular bone surfaces without a preceding resorptive episode (Charhon *et al.*, 1983). It may also be associated with prior resorption at the same site with relatively exaggerated formation phase. Prostate carcinoma is the most common tumour that causes this response. Shimazaki *et al* (1992) analysed untreated bone lesions of prostate cancer and found these lesions could be classified into five types: osteoblastic (15%), mixed, but mainly osteoblastic (31%), mixed, but mainly osteolytic (17%), ostolytic (10%), and undetermined with a positive bone scan (27%) (Shimazaki *et al.*, 1992). The natural course of bone lesions showed a tendency to change from the osteolytic to osteoblastic type, which appeared as curative changes. In contrast, the relapse was often accompanied by an increase of the osteolytic type lesion (Shimazaki *et al.*, 1992).

The mechanisms responsible for the osteoblastic metastases of prostate carcinoma are unknown but are of great interest. Infiltrating prostate cancer cells are believed to cause a sclerotic reaction and impaired mineralization. It is likely that the bone formation in osteoblastic metastases is stimulated by soluble factors produced by the metastasising cancer cells (Charhon *et al.*, 1983). In prostate carcinoma, a number of factors have been suggested as potential mediators, including endothelin-1 (ET-1), TGF-β, bone morphogenic protein (BMP), fibroblast growth factor (FGF), plasminogen activator sequence and prostate-specific antigen (PSA). These findings provide us with a number of candidate molecules which may also be involved in MM- and POEMS syndrome-mediated bone lesions. The factors of interest in this study are reviewed below.

### 1.3.1 Osteoclast Activating Factors (OAFs)

#### 1.3.1.1 Interleukin-1β

Interleukin-1 (IL-1) is a prototypic "multifunctional" cytokine. There are two IL-1 agonistic proteins, IL-1α and IL-1β. IL-1α accounts for about 10%, and IL-1β for 90% of IL-1 protein made by lipopolysaccharide (LPS)–activated human monocytes. IL-1β is active only as secreted mature product, whereas IL-1α is active as a precursor or membrane-associated molecule (reviewed in (Dinarello, 1996)).

Two IL-1 receptors type I and type II have been identified and they are related members of the immunoglobulin superfamily. Type I IL-1R is widely expressed and is the signaling receptor. In contrast, type II is a related molecule but only has a short cytoplasmic domain (20 amino acids) and act as a decoy receptor for IL-1β. There is one antagonistic protein, the IL-1 receptor antagonist (IL-1Ra). IL-1Ra binds to the IL-1 receptor type I without transmitting an activation signal and, thus, represents a physiological inhibitor of IL-1 activity (reviewed in (Dinarello, 1996)).

IL-1 secreted by myeloma BM cells has been found to be significantly greater than that found in similarly processed BM cells from control individuals (Filella *et al.*, 1996; Lichtenstein *et al.*, 1989; Yamamoto *et al.*, 1989). IL-1 induces cycloocygenase 2 (COX2), an important enzyme involved in prostaglandin $E_2$ ($PGE_2$) synthesis. IL-1 has been shown to be mainly responsible for IL-6 production in the tumoral BM environment of patients with MM *via* a $PGE_2$ synthesis pathway (Costes *et al.*, 1998; Lu *et al.*, 1995). Inhibition of $PGE_2$ synthesis using an IL-1Ra and indomethacin, an inhibitor of COX which can block $PGE_2$ synthesis, has been found to inhibit myeloma cell proliferation by reducing IL-1-induced endogenous IL-6 production *in vitro* and *in vivo* (Costes *et al.*, 1998). In addition, IL-1β is also a principal bone resorption agent present in the supernatants of myeloma cell cultures that enhances destructive bone lesions in the patients with MM (Cozzolino *et al.*, 1989; Yamamoto *et al.*, 1989). The bone resorbing activity and the levels of IL-1β in these culture supernatant are highly correlated (Torcia *et al.*, 1996). This bone-resorbing activity can be neutralised by antibodies directed against IL-1β (Cozzolino *et al.*, 1989; Kawano *et al.*, 1989a). Thus, IL-1 can promote the secretion of IL-6, and both IL-1 and IL-6 (see below) enhance the bone lytic lesions in MM.

### 1.3.1.2 Interleukin-6

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by a variety of cells. It binds to two forms of IL-6R, a membrane bound form and a truncated soluble form. The binding of IL-6 and IL-6R can trigger the association of a non-ligand binding 130-kD signal-transducing molecule, gp130 (Taga *et al.*, 1989). The ubiquitous expression of gp130 in all organs partially reveals the pleiotropic function of IL-6 (Saito *et al.*, 1992).

IL-6 is a critical growth factor for normal B-cell growth and development and induces the final maturation of B cells into antibody-producing cells (Hirano *et al.*, 1986). In addition, IL-6 represents a major growth factor for myleoma cells and can augment myeloma cell growth, enhance cell survival and confer drug resistance (Klein *et al.*, 1995; Nilsson *et al.*, 1990; Ohtake *et al.*, 1990). It is well accepted that IL-6 promotes myeloma cell growth *via* an autocrine and paracrine mechanisms (Kawano *et al.*, 1988; Klein *et al.*, 1989). The high levels of both IL-6 (Filella *et al.*, 1996) and soluble IL-6R (Galibert *et al.*, 1998) has been detected in patients with MM.

Although the origin of IL-6 is controversial, some primary plasma cells have been shown to produce, or can be induced to produce IL-6 (Jourdan *et al.*, 1991; Ohtake *et al.*, 1990). IL-6 is thought to mediate autocrine growth of human myeloma cells by modulating IL-6 receptor expression and IL-6 signalling in myeloma cells (Hirano *et al.*, 1990; Jernberg-Wiklund *et al.*, 1992; Kang *et al.*, 1996). Autocrine IL-6 production reflects a highly malignant phenotype of myeloma cells, and together with deregulated apoptosis, may induce expansion of selective IL-$6^+$ myeloma cells resistant to spontaneous and drug-induced cell death (Frassanito *et al.*, 2001). The development of MM is a multistep transformation process in which several oncogenic events result in the selection and malignant expansion of a single IL-$6^+$ clone (Hallek *et al.*, 1998). De Hon *et al* (1994) has demonstrated the importance of the IL-6/IL-6R interaction in MM. They showed that the growth of MM cell line XG-1 could be inhibited by the addition of mutant form of IL-6 which displayed a 5-fold enhanced affinity for IL-6R, but was incapable of signalling through the IL-6R (de Hon *et al.*, 1994).

In addition to myeloma cell-derived IL-6, some studies show that IL-6 is produced by stromal components grown from myeloma BM, suggesting a paracrine regulation of tumour growth. These studies imply that the proliferation of myeloma cells is dependent upon close

contact with stromal cells (Lichtenstein *et al.*, 1989; Lokhorst *et al.*, 1994). In accord with this, it has been demonstrated that myeloma cell lines upregulate IL-6 secretion in osteoblastic cell lines (Barille *et al.*, 1995) and primary human OBs (Karadag *et al.*, 2000a) through cell-to-cell contact, and down-regulate the level of osteocalcin production (Barille *et al.*, 1995). Studies in which myeloma cells and OBs are physically separated by transwell devices have revealed direct cell-to-cell contact contribute significantly to the induction of IL-6 (Karadag *et al.*, 2000a).

Furthermore, IL-6 and related cytokines are involved in bone resorption. It is likely that IL-6R is shed from the surface of myeloma cells and binds to IL-6 released by the bone marrow cells. It is this IL-6/IL-6R complex that may then be able to associate with gp 130 on the surface of OC precursors to promote their recruitment and differentiation (Karadag *et al.*, 2000b; Kurihara *et al.*, 1990; Tamura *et al.*, 1993). Although IL-6 can stimulate OC-like cell formation, the role of IL-6 in osteoclastic bone resorption is complex and remains unclear.

#### 1.3.1.3 Tumour Necrosis Factor-α

Tumour necrosis factor (TNF) family members are type II transmembrane proteins with extracellular C-terminal, a transmembrane region and a cytoplasmic region. There are a number of TNF-related cytokines which have been molecularly defined including TNF-α (cachectin) and TNF-β (lymphotoxin) which share 30% homologous at the amino acid level (reviewed in (Vilcek *et al.*, 1991)). Both TNF-α and TNF-β react with the TNF receptors (TNFR) p55 type I and p75 type II. Members of the TNFR family are type I membrane glycoproteins with conserved features and are expressed on virtually all cells tested (reviewed in (Vilcek *et al.*, 1991)).

TNF-α is mainly secreted by macrophage in response to factors such as oxidized LDL, acetylated LDL, physically damaged extracellular matrix, or bacterial infection. It can be cleaved by a metalloprotease-disintegrin TNF-α-converting enzyme (TACE), and both surface and shed forms are active. TNF-α produces pleiotropic effects on cells that can be attributed to its ability to activate a variety of genes in a multitude of target cells. Moreover, TNF-α is one of the major inflammatory cytokines and is involved in a number of diseases including osteolytic bone disease mediated by MM (reviewed in (Vilcek *et al.*, 1991)).

TNF-α is found at higher levels in supernatants from BM cultures of MM patients (Filella *et al.*, 1996; Lichtenstein *et al.*, 1989) and is capable of markedly stimulating OC-like multinucleated cell formation in human marrow cultures (Pfeilschifter *et al.*, 1989). Interestingly, TNF-α has a more potent effect than IL-6 in stimulating the growth of primary myeloma cultures suggesting that TNF-α in some myeloma patients may have growth-promoting effects equal to that of IL-6 (Borset *et al.*, 1994). The role of TNF-β in myeloma bone disease has been downplayed by recent studies which have failed to find significant differences in the levels of this cytokine in supernatants derived from fresh BM plasma cultures from myeloma patients and normal controls. In addition, antibodies to TNF-β do not reduce the bone resorbing activity of fresh BM plasma from myeloma patients (Kawano *et al.*, 1989b).

In conclusion, IL-1, IL-6 and TNF-α act synergistically and interdependently to enhance the lytic bone lesions in MM. They have shown interactive induction as well as autoamplification of their gene expression (Linkhart *et al.*, 1991). IL-1β and TNF-α can induce IL-6 mRNA and protein expression through the $PGE_2$ synthesis pathway in the MCT3-E1 mouse osteoblastic cell line, and primary OB-like cells derived from foetal mouse calvaria and human (Costes *et al.*, 1998; Lu *et al.*, 1995; Thomas *et al.*, 1998). These findings suggest that the circulating level of IL-6, not IL-1β, may provide a more accurate assessment of the severity of bone disease.

#### 1.3.1.4 Parathyroid Hormone Related Protein

Parathyroid hormone related protein (PTHrP) is a widely expressed hormone that is a major mediator of malignancy-associated hypercalcemia. It possesses 70% homology to parathyroid hormone (PTH). The binding of PTHrP to the PTH receptor activates cyclic adenosine monophosphate (cAMP) and enhances osteoclastic bone resorption. The elevated PTHrP can be measured in the plasma of patients with myeloma (Horiuchi *et al.*, 1997; Schneider *et al.*, 1998). PTHrP released by myeloma cells acts as a paracrine factor by binding to the PTH receptor expressed by OC to produce local bone resorption. When secreted in large amounts by malignant cells, PTHrP may act in a humoral manner to cause systemic hypercalcemia (Firkin *et al.*, 1996). Thus, PTHrP might be an important contributor to the skeletal complications in MM.

Although several OAFs have been implicated in the enhanced OC activity, the precise role of any of these factors in osteolytic bone disease in patients with MM has not been clearly demonstrated *in vivo*. Recently, a new cytokine system RANKL/RANK/OPG has been identified and provides us with a novel paradigm as to the mechanisms of osteoclastogenesis and bone resorption in physiological bone remodelling and MM mediated bone lesions.

### 1.3.2 RANKL/RANK/OPG System

#### 1.3.2.1 General Description

Studies have shown that the RANK signalling pathway is the ultimate common mediator of humoral signals that regulate osteoclastogenesis including OC differentiation and activation (Anderson *et al.*, 1997; Lacey *et al.*, 1998). Numerous studies demonstrate that RANKL provides an essential signal to OC progenitors through the membrane-anchored receptor RANK, an thereby initiating osteoclastogenic signal transduction (Anderson *et al.*, 1997; Hofbauer *et al.*, 2000; Hofbauer *et al.*, 2001; Hsu *et al.*, 1999; Yasuda *et al.*, 1999; Yasuda *et al.*, 1998b). This signalling pathway can be blocked by a naturally occurring decoy receptor for RANKL, OPG, by disrupting the interaction between RANKL and RANK (Yasuda *et al.*, 1998a; Yasuda *et al.*, 1999).

Receptor activator of nuclear factor-κB ligand (RANKL) is a member of the TNF ligand family, variously termed TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), or OC differentiation factor (ODF) (Anderson *et al.*, 1997; Lacey *et al.*, 1998). It is expressed on the surface of BM OB/stromal cells, chondrocytes, mesenchymal cells of the periosteum, OCs, some endothelial cells and activated T cells (Anderson *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998b). RANKL is a type II membrane-anchored polypeptide, which is released from the plasma membrane by TACE (Lum *et al.*, 1999; Pan *et al.*, 2004a) resulting in a 45-kDa transmembrane (TM) protein and a 31-kDa product of metalloproteinase cleavage (Figure 1.6). Studies show that cell-to-cell interaction is essential for OC formation in the co-culture system, suggesting a pivotal role for the membrane-bound RANKL in osteoclastogenesis (Udagawa *et al.*, 1990). However, soluble RANKL (sRANKL) has been shown to illicit murine and human OC differentiation when co-stimulated with M-CSF *in vitro* (Quinn *et al.*, 1998; Udagawa *et al.*, 1999), suggesting that RANKL-mediated signals can be transduced not only by cell-to-cell contact

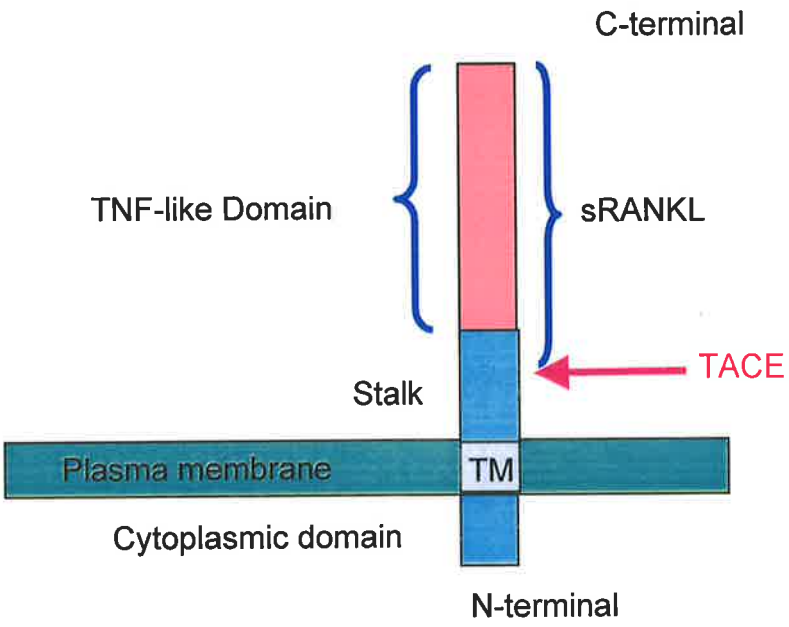



Figure 1.6. Schematic representation of the structure of RANKL. RANKL is a type II membrane-anchored polypeptide, which includes a TNF-like extracellular domain, transmembrane domain and a cytoplasmic domain. Soluble RANKL (sRANKL) is released from the plasma membrane by the metalloprotease-disintegrin TNF-converting enzyme (TACE).

between OB/stromal cells and OC progenitors, but also by the cleaved or soluble form of RANKL in a paracrine manner. The cleaved-form of RANKL may play a role in pathological bone resorption mediated by abnormal osteoclastogenesis. RANKL-deficient mice show severe osteopetrosis, defects in tooth eruption, and a complete lack of OCs as a result of the inability of OBs to support osteoclastogenesis (Kong *et al.*, 1999a; Kong *et al.*, 1999b). Taken together, these results demonstrate that RANKL is an essential OC differentiation factor *in vivo* and *in vitro*.

Receptor activator of nuclear factor-κB (RANK) is a member of the TNF receptor family and represents the membrane-associated counter receptor for RANKL (Yasuda *et al.*, 1999). Although expressed by other cells, RANK appears to be mainly expressed by OC precursor cells. RANK is capable of initiating osteoclastogenic signal transduction after ligation with RANKL or anti-RANKL agonist antibodies (Anderson *et al.*, 1997; Hsu *et al.*, 1999). RANK deficient mice show a profound defect in bone resorption and bone remodelling, resulting from an apparent block in OC differentiation and an intrinsic defect in OC function (Dougall *et al.*, 1999; Li *et al.*, 2000). Severe osteopetrosis has been observed in transgenic mice expressing a soluble RANK-Fc fusion protein which binds with high affinity to RANKL and blocks OC differentiation and activation *in vivo* (Hsu *et al.*, 1999).

Osteoprotegerin (OPG), also termed osteoclastogenesis inhibitory factor (OCIF), is a heparin-binding basic glycoprotein (Figurer 1.7). OPG is a secreted member of the TNF receptor family, containing four cysteine-rich domains and two regions which exhibit homology to death domains (Simonet *et al.*, 1997; Yamaguchi *et al.*, 1998). It has been isolated as both a monomer and a disulfide-linked homodimer, with a molecular weight of 55 kD and 110 kD, respectively (Simonet *et al.*, 1997). OPG is a key factor acting as a negative regulator of osteoclastogenesis. OPG inhibits osteoclastogenesis by blocking cell-to-cell signalling between stromal cells and OC progenitors (Yasuda *et al.*, 1998a). It acts as a naturally occurring decoy receptor for RANKL by disrupting the interaction between RANKL and RANK (Yasuda *et al.*, 1999). Targeted ablation of OPG in knockout mice leads to severe osteoporosis (Bucay *et al.*, 1998). A mouse co-culture system using calvarial OB and BM cells prepared from OPG-deficient mice revealed that OPG produced by OB/stromal cells is a physiologically important regulator in OC differentiation and function (Udagawa *et al.*, 2000). Conversely, the over-expression of OPG in transgenic mice results in severe osteopetrosis (Kong *et al.*, 1999a; Kong *et al.*, 1999b; Simonet *et al.*, 1997).

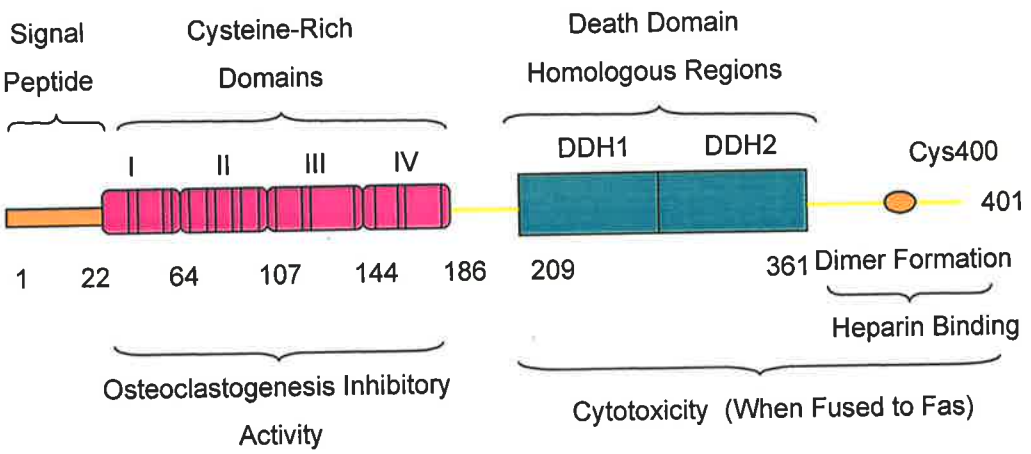



Figure 1.7. Schematic representation of the functional domains of human OPG. OPG is a member of the TNF receptor family, containing four cysteine-rich domains and two regions which exhibit homology to death domains. OPG is a heparin-binding basic glycoprotein and it exists as both a monomer and disulfide-linked homodimer. OPG is a negative regulator of osteoclastogenesis. Adapted from Yasuda, H. (Yasuda *et al.,* 1999).

### 1.3.2.2 RANKL/OPG Ratio and Bone Lesion

Increased RANKL/OPG ratio has been implicated in the pathogenesis of postmenopausal osteoporosis, hyperthyroidism, Paget's disease, bone loss associated with rheumatoid arthritis, periodontal disease, bone tumours and bone metastases (reviewed in (Hofbauer *et al.*, 2000; Hofbauer *et al.*, 2001)).

In patients with metabolic bone disease, the eroded surface (ES/BS, percentage of trabecular bone surface) and OC surface (Oc.S/BS, percentage of trabecular bone surface) are positively associated with RANKL/OPG mRNA ratio (Fazzalari *et al.*, 2001; Siggelkow *et al.*, 2003). In Psoriatic arthritis (PsA), an inflammatory joint disease where bone resorption is coupled with adjacent new bone formation, RANKL expression has been found to be dramatically upregulated in the synovial lining layer (Ritchlin *et al.*, 2003). The elevated RANKL expression in the joint is thought to be induced by increased secretion of TNF-α by PBMCs (Ritchlin *et al.*, 2003). In contrast, mice over-expressing thrombopoietin display osteosclerosis predominantly *via* an up-regulation of OPG in host stromal cells leading to disruption of osteoclastogenesis (Chagraoui *et al.*, 2003).

Thus, OC formation may be determined principally by the relative ratio of RANKL to OPG in the BM microenvironment. Alterations in this ratio may be a major cause of bone loss in many osteolytic neoplasms including MM.

### 1.3.2.3 Increased RANKL/OPG Ratio in MM

It has been demonstrated that myeloma cells affect the RANKL/OPG ratio in the BM environment by stimulating bone marrow-residing stromal cells to over express RANKL through direct cell-to-cell contact (Giuliani *et al.*, 2001). In marrow infiltrated by MM, RANKL has been found in stromal and activated T cells at the interface of MM with normal marrow elements (Pearse *et al.*, 2001). *In vitro*, RANKL expression by stroma is induced by coculture with myeloma cell lines but not by addition of cell line-conditioned media (Giuliani *et al.*, 2001; Pearse *et al.*, 2001). The immunohistochemical observations suggest that nodular infiltration of myeloma cells is correlated with a higher number of RANKL positive stromal cells and an increased bone resorption (Giuliani *et al.*, 2001). Furthermore, fresh myeloma cells (as defined by their high expression of the CD38 cell surface molecule) isolated from patients with MM, are able to induce OC formation and bone resorption in co-

culture system in the absence of OB (Farrugia *et al.*, 2003). These observations confirm that the RANKL/OPG system is involved in the pathogenesis of MM induced osteolysis.

In addition to elevated RANKL expression, patients with MM have been found to have OPG serum levels 18% lower than that of control subjects (Lipton *et al.*, 2002; Seidel *et al.*, 2001; Standal *et al.*, 2002). Moreover, constitutive stromal expression of OPG is found to be reduced by coculture with myeloma cell lines (Giuliani *et al.*, 2001; Pearse *et al.*, 2001).

Therefore, alterations in the relative ratio of RANKL to OPG in the BM microenvironment may be a major cause of bone loss in MM-mediated osteolytic bone disease. Recombinant OPG may be a candidate to treat MM-induced bone lesions. Recent studies have shown that recombinant OPG inhibits bone resorption in murine model of MM (Croucher *et al.*, 2001) and patients with MM or breast carcinoma (Body *et al.*, 2003; Vanderkerken *et al.*, 2003).

#### 1.3.2.4 The Regulation of RANKL/OPG Ratio in MM

Because the RANKL/OPG ratio is an essential determinant of osteoclastogenesis, any agent that affects either RANKL or OPG, is likely to indirectly regulate OC activity. It has been demonstrated that a number of osteotropic agents including 1,25-$(OH)_2D_3$, PTH, IL-11, and $PGE_2$ can upregulate RANKL expression to increase the ratio of RANKL to OPG, and stimulate OB-induced pit-forming activity of OCs (Horwood *et al.*, 1998; Udagawa *et al.*, 1999). Several MM-associated OAFs, including IL-1β, IL-6 and IL-11, produced by either malignant plasma cells or stromal cells following interaction between the two cell types, have been shown to induce stromal expression of RANKL and inhibit transcription of OPG *in vitro*. In addition, Dai and colleagues (2000) reported that alcohol ingestion induced osteoclastogenesis and bone loss was mediated through IL-6-induced RANKL upregulation (Dai *et al.*, 2000). However, data from others suggest that IL-1β and TNF-α, but not IL-6, can stimulate RANKL gene expression in human osteoblastic cells (Hofbauer *et al.*, 1999). These findings suggest that IL-6 may use RANKL-dependent and –independent pathways to stimulate osteoclastogenesis.

Beside the hormones and cytokines described above, other molecules implicated in MM may be involved in the regulation of RANKL/OPG ratio in MM-mediated bone lesion. These molecules include tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, also called Apo2L), syndecan-1 (CD138) and hepatocyte growth factor (HGF).

TRAIL is a member of a superfamily of cell death-inducing ligands that also includes TNF-α and Fas ligand (FasL or CD95L). TRAIL induces apoptosis of malignant cells by interacting with a complex system of cell surface receptors, including the two death-signalling receptors DR4 (also known as TRAIL-R1) and DR5 (TRAIL-R2) (Pan *et al.*, 1997; Sheridan *et al.*, 1997). These 2 receptors transmit a pro-apoptotic signal *via* interaction of their intracellular death domains with adaptor proteins, which are not well characterised. The death domains of these adaptors constitutively bind to caspases, initiating a cascade of proteolytic activation of downstream caspases and apoptosis (Schulze-Osthoff *et al.*, 1998). Additional antagonist decoy receptors have also been described and include DcR1/TRID/TRAIL-R3 and DcR2/TRAIL-4. These receptors have an extracellular TRAIL-binding domain and a transmembrane domain, but lack an intracellular death signalling domain (Pan *et al.*, 1997; Sheridan *et al.*, 1997). Thus, ectopic expression of DcR1 and DcR2 protects mammalian cells from TRAIL-induced apoptosis and inhibits TRAIL cytotoxicity (Degli-Esposti *et al.*, 1997; Sheridan *et al.*, 1997). Interestingly, it has been subsequently found that OPG represents a fifth TRAIL receptor (Emery *et al.*, 1998). OPG-Fc binds TRAIL with an affinity of 3.0 nM, which is marginally weaker than the interaction of DcR1-Fc or DR5-Fc with TRAIL. It has been reported that OPG could inhibit TRAIL-induced apoptosis of Jurkat cells and myeloma cells(Shipman *et al.*, 2003), whilst in contrast, TRAIL was found to block the anti-osteoclastogenic activity of OPG (Emery *et al.*, 1998), suggesting OPG and TRAIL may mediate potential cross-regulation.

Syndecan-1 is a heparin sulfate bearing integral membrane proteoglycan that is expressed and actively shed from the surface of most myeloma cells (Dhodapkar *et al.*, 1998; Saunders *et al.*, 1989). Syndecan acts as a matrix receptor on human myeloma cells by binding type I collagen *via* heparin sulfate chains, and it is thought to participate in regulating myeloma cell adhesion to the BM stromal cell matrix (Ridley *et al.*, 1993). Therefore, syndecan-1 may play a dual role in inhibiting the metastasis of tumour cells by promoting cell adhesion to the extracellular matrix and suppressing the proteolytic activity

needed for invasion (Kaushal *et al.*, 1999; Liebersbach *et al.*, 1994). It has been found that elevated level of syndecan-1 in serum is a strong and independent negative prognostic factor for patients with myeloma (Seidel *et al.*, 2000b). However, some studies show that syndecan-1 can induce apoptosis and inhibit the growth of myeloma tumour cells (Dhodapkar *et al.*, 1998). In addition, syndecan-1 was found to increase OB development and inhibited OC formation in murine BM (Dhodapkar *et al.*, 1998). Since syndecan-1 can bind with heparin-binding proteins, such as OPG or hepatocyte growth factor, it has been suggested that it may also regulate the biological activity of heparin-binding cytokines (Borset *et al.*, 2000). These data indicate that soluble syndecan-1 may participate in the pathology of myeloma induced osteolysis by modulating RANKL/OPG ratio within the BM.

HGF is a cytokine overproduced by myeloma cells and has negative prognostic implications in patients with MM (Borset *et al.*, 1999). The biological significance of HGF over-expression in multiple myeloma is likely to include effects on bone turnover and angiogenesis (Borset *et al.*, 1999). HGF exists in a complex with soluble syndecan-1. Washing myeloma cells with purified soluble syndecan-1 was found to effectively displace HGF from the cell surface, suggesting that soluble syndecan-1 could act as a carrier for HGF *in vivo* (Seidel *et al.*, 2000a). HGF is also a heparin-binding cytokine, which may compete with OPG to bind to syndecan-1, and indirectly affect RANKL/OPG ratio in MM. The relationship between HGF, OPG, and syndecan-1 and the effects of HGF on bone turnover remain to be fully elucidated.

In summary, RANKL and OPG represent a key agonist and antagonist cytokine system in regulating MM-induced osteolysis. Other molecules such as IL-6, IL-1β, TNF-α, PTHrP and syndecan-1 may in part act *via* the RANKL/RANK/OPG system, thereby regulating bone turnover in MM patients (Figure 1.8).

Although numerous studies examing the mechanisms of MM-mediated lytic bone disease have been described, little is known about underlying mechanisms responsible for osteosclerotic bone lesion in patients with POEMS syndrome. As described previously, some potential mediators such as ET-1 have been implicated in prostate carcinoma with osteoblastic metastases, suggesting it may have potential effects in osteosclerotic bone lesion in patients with POEMS syndrome. Using microarray analysis, recent studies from our laboratory (Zannettino AC & Gronthos S, unpublished data) suggest that BMMNCs

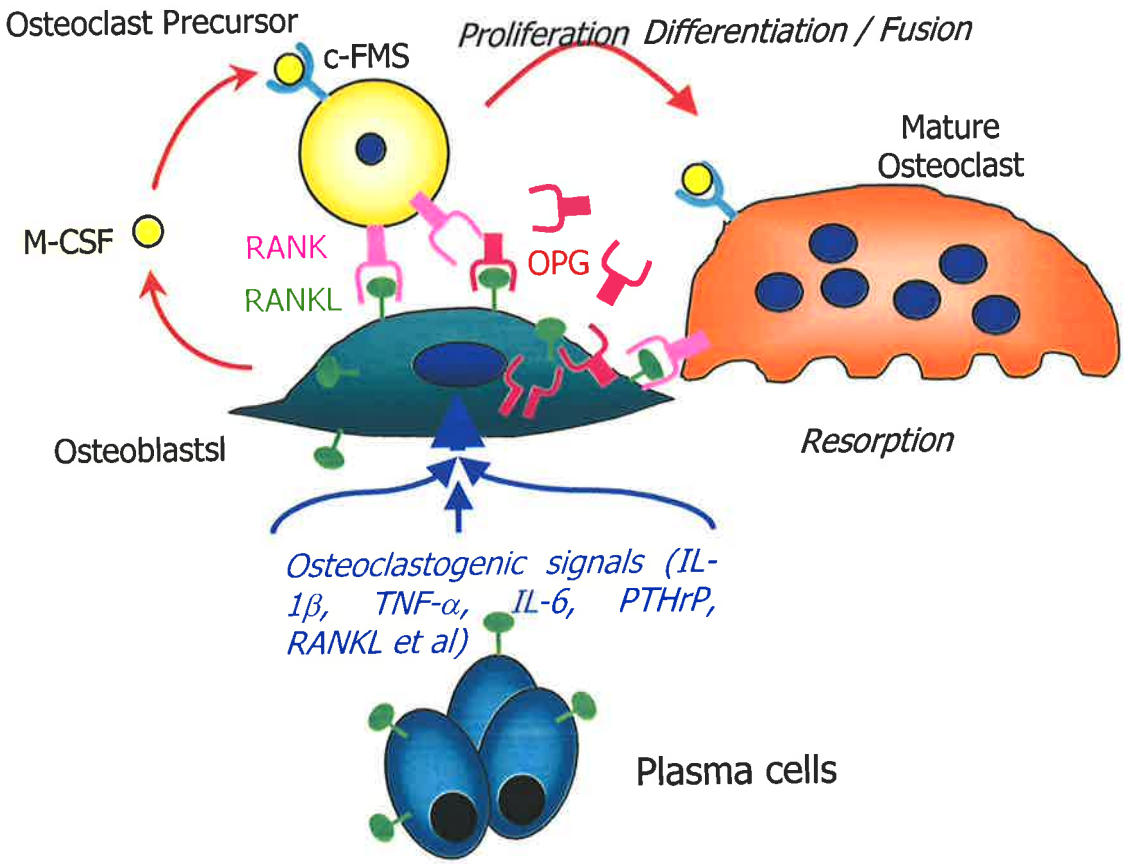



Figure 1.8. The relationship between plasma cells, osteoblasts and osteoclasts in patients with MM. Myeloma plasma cells secrete osteoclast activating factors, such as IL-6, IL-1β, TNF-α, PTHrP, which induce stromal expression of RANKL and inhibit transcription of OPG. In addition, plasma cells also produce RANKL. Thus, the increased RANKL to OPG ratio in patients with MM results in the development of osteolytic bone lesions.

derived from patients with MM express higher levels of ET-1. As the role of ET-1 in pathological bone will be examined in this thesis, the current understanding of ET-1 is reviewed in the next section.

### 1.3.3 Endothelin-1 (ET-1)

#### 1.3.3.1 ET-1 Structure

ET-1 was first isolated from porcine aortic endothelial cells and consists of 21 amino acid residues with two sets of intrachain disulfide linkages (Yanagisawa *et al.*, 1988). ET-1 belongs to the endothelin family which is composed of three structurally related isoforms, namely ET-1, ET-2 and ET-3, encoded by separated genes located on chromosome 6, 1 and 20, respectively (Arinami *et al.*, 1991; Inoue *et al.*, 1989a). Mammalian endothelins share a high degree of sequence and structural similarity to the sarafotoxin (SRT) family of 21 residue peptide toxins from the snake *Atractaspis engaddensis* (Kloog *et al.*, 1988). The ET-1 gene encodes a 212 (human)/203(porcine) amino acid precursor peptide, preproendothelin-1, which is cleaved by a furin convertase to form proendothelin-1 or big ET-1 (Yanagisawa *et al.*, 1988). This 38-residue (human) or 39-residue (porcine) inactive intermediate, big ET-1, is further cleaved by endothelin converting enzyme-1 (ECE-1) giving rise to active ET-1 (Yanagisawa *et al.*, 1988) (Figure 1.9). Human and porcine endothelin have identical amino acid sequence (Bloch *et al.*, 1989). In contrast, rat and human endothelin differ in 6 of 21 residues (Bloch *et al.*, 1989).

#### 1.3.3.2 Endothelin Converting Enzyme-1 (ECE-1)

The proteolytic cleavage of $Trp^{21}$ and $Val^{22}$(human and porcine)/$Ile^{22}$(rat) bond of big ET-1 by ECE-1 is critical to the function of ET-1, with the precursors having negligible biologic activity. Three isoforms of endothelin converting enzyme have been identified to date, termed ECE-1, ECE-2 and ECE-3 (Hasegawa *et al.*, 1998). They belong to the type II membrane-bound zinc metalloprotease family and are partially inhibited by an inhibitor of neutral endopeptidase 24.11 (NEP) phosphoramidon (Ahn *et al.*, 1992). Although ECE-1 (located in chromosome 1p36.1) (Matsuoka *et al.*, 1996) and ECE-2 (located in chromosome 3q28-q29) (Lorenzo *et al.*, 2001) are encoded by two different genes, their sequences are 59% identical. However, ECE-1 is the most efficient, physiologically relevant enzyme among the three isopeptides because of its wide action and expression. It has been shown that ECE-1 is a unique, highly glycosylated neutral metalloprotease with a single transmembrane domain near the N-terminus (Shimada *et al.*, 1994; Takahashi *et al.*, 1993).

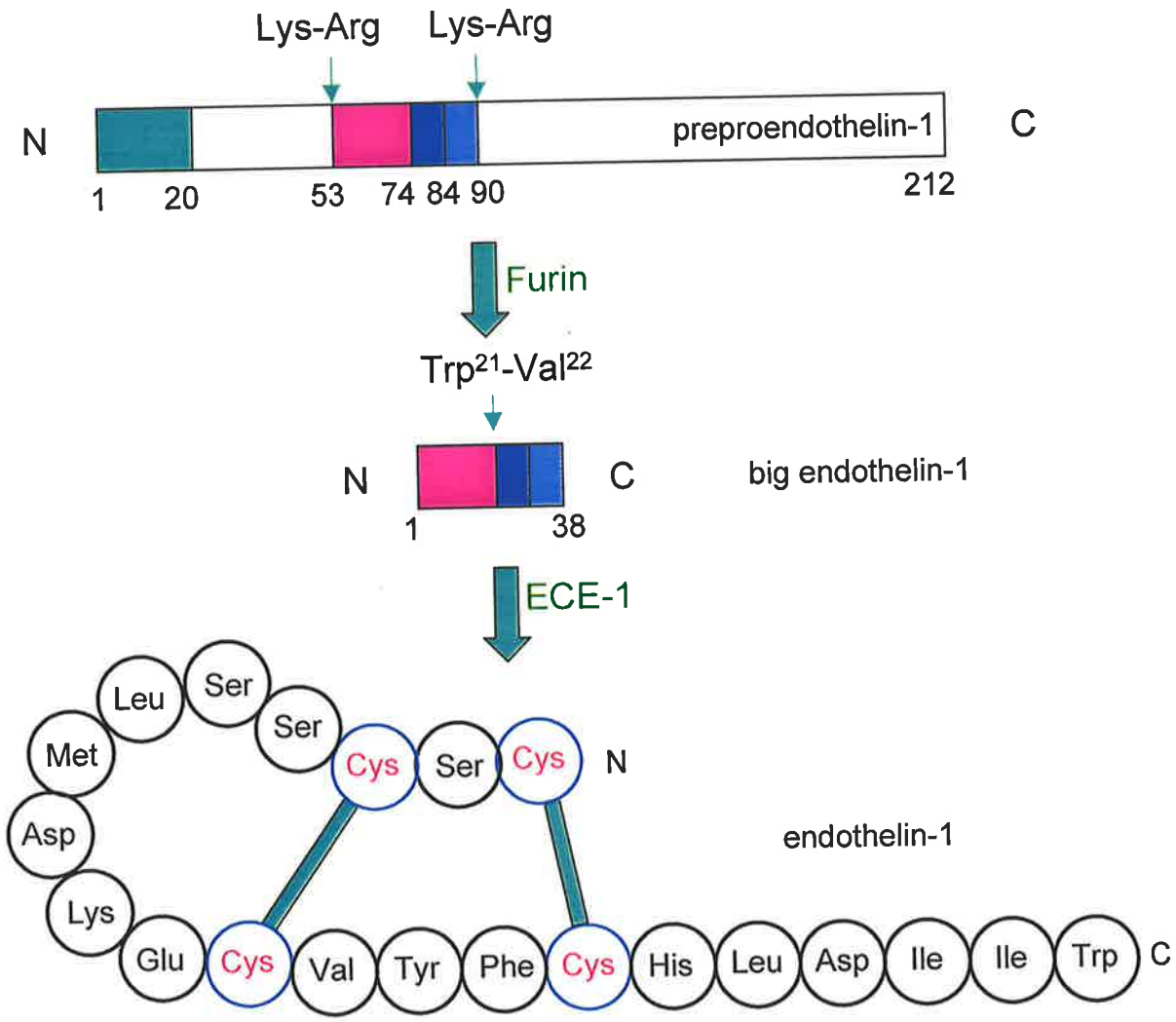



Figure 1.9. Pathway of human endothelin-1 (ET-1) biosynthesis. The putative secretory signal sequence and the endothelin sequence are represented by the coloured boxes. A 38-amino acid residue intermediate, or "big endothelin-1", is generated from preproendothelin-1 following cleavage by the furin convertase. Mature endothelin-1 is then produced through further cleavage by an endothelin converting enzyme-1 (ECE-1). Adapted from Yanagisawa, M. (Yanagisawa et al., 1988).

It is a bona fide activating protease for both big ET-1 and ET-3 *in vivo* (Yanagisawa *et al.*, 1998), whilst the specificity of the ECE-1 for the different isoforms of big ET-1 differs as follows: big ET-1 > big ET-2 > big ET-3. The generation of an ECE-1 knock out mouse demonstrated the importance of ECE-1 in the regulation of ET-1 activity. A targeted null mutation in the mouse ECE-1 gene ($ECE^{-/-}$ term embryos) resulted in a phenotype virtually identical to $ET\text{-}1^{-/-}$ and $ET_A^{-/-}$ (one of ET-1 receptors) mice, suggesting impaired ET-1/$ET_A$-mediated signaling without ECE-1 activity in $ECE\text{-}1^{-/-}$ mice (Yanagisawa *et al.*, 1998).

There are four isoforms of ECE-1, namely ECE-1a, ECE-1b, ECE-1c and ECE-1d (Schweizer *et al.*, 1997; Shimada *et al.*, 1995; Valdenaire *et al.*, 1999; Valdenaire *et al.*, 1995), generated by alternate use of four distinct promoters located upstream of a single gene (located on chromosome 1p36) (Schweizer *et al.*, 1997; Valdenaire *et al.*, 1999). Therefore, ECE-1 isoforms differ only in their N-terminal regions, which accounts for their respective subcellular distribution. ECE-1b is predominantly intracellular (Schweizer *et al.*, 1997), while ECE-1a, ECE-1c and ECE-1d are found in human at the plasma membrane with their catalytic domain oriented towards the outside of cells (Ahmed *et al.*, 2000); (Schweizer *et al.*, 1997). In spite of their distinct N-terminus, all four isoforms of ECE-1 are found to cleave the three big endothelins, but with a clear preference for big ET-1. In addition, the active site of the enzyme is located in its C-terminal part, which is common to all four isoforms (Schweizer *et al.*, 1997; Valdenaire *et al.*, 1999) (Figure 1.10).

### 1.3.3.3 ET-1 Receptors

ET-1 is hydrophilic and unable to cross the plasma membrane. Thus, it has to bind to specific cell-surface receptors to regulate its effect. There are at least two distinct, widely expressed receptor subtypes of the rhodopsin-like guanine-nucleotide-binding (G) protein-coupled heptahelical receptors, endothelin A (Arai *et al.*, 1990; Lin *et al.*, 1991) and B receptors (Sakurai *et al.*, 1990). Furthermore, two additional receptors have been cloned and characterized from *Xenopus laevis* dermal melanophores ($ET_CR$) (Karne *et al.*, 1993) and heart ($ET_{AX}$) (Kumar *et al.*, 1994).

Human $ET_AR$ ($hET_AR$) gene consists of eight exons and seven introns spanning more than 40 kilobases (kb) and is located on human chromosome 4 (4q28) (Hosoda *et al.*, 1992). In contrast, human $ET_BR$ (h $ET_BR$) gene spans 24 kb and consists of seven exons interspersed by six introns and is assigned to human chromosome 13 (Arai *et al.*, 1993).


Figure 1.10. N-terminal amino acid sequences of ECE-1 isoforms. Blue text highlights the identical sequence in all four isoforms. Underlined red text indicate common sequences in ECE-1b, ECE-1c and ECE-1d. Adapted from Lee, M.E. (Lee *et al.*, 1990).

Compared to $hET_AR$, $hET_BR$ gene lacks an intron corresponding to the intron of $hET_AR$ gene that is located in the 5'-noncoding region. The intron 1-6 of $hET_BR$ gene is located at equivalent position to the intron 2-7 of $hET_AR$ gene (Arai *et al.*, 1993; Hosoda *et al.*, 1992) (Figure 1.11). Although, $hET_AR$ consists of 427 amino acids (Hosoda *et al.*, 1991) and $hET_BR$ consists of 442 amino acids (49 kDa), the overall identity between two proteins is 55%. Furthermore, the transmembrane domains and the cytoplasmic loops are highly conserved between $hET_AR$ and $hET_BR$, but the N-terminal extracellular domain exhibits a divergence in amino acid sequence and in length (80 residues for $hET_AR$ versus 101 residues for $hET_BR$).

An $ET_BR$ splice variant has been identified from a human placental cDNA library (Elshourbagy *et al.*, 1996). This 436 amino acid long polypeptide has a putative molecular mass of approximately 48-kDa and shares 91% identity to the human $ET_BR$. Another novel transcript of $hET_BR$ gene, $ET_BR\Delta5$, is derived from splicing and/or RNA editing of the primary $ET_BR$ transcript in which the entire fifth exon (134bp) is removed. The shortened $ET_BR$ transcript has the same amino acid sequence as that of $ET_BR$ up to the proximal region of the third intracellular protein loop and then a completely different peptide sequence of 19 amino acids which is followed by an early stop codon (Tanoue *et al.*, 2002) (Figure 1.12). The deduced polypeptide sequence consists of 336 amio acid residues with a calculated molecular mass of ~37 kDa (Tanoue *et al.*, 2002). However, the function of this splice variant is unknown.

Molecular weight estimation of the endothelin receptors by affinity labeling and gel filtration has revealed the presence of high and low molecular weight species in various tissues. The lower molecular weight species (34 kDa, major) is an $NH_2$ terminally truncated form of the higher 52-kDa (minor) species (Hagiwara *et al.*, 1991; Kozuka *et al.*, 1991; Schvartz *et al.*, 1991). It has lost 79 amino acids in the $NH_2$-terminal region by proteolytic cleavage between the Ala-79 and Gly-80. In bovine $ET_B$, the initial 26 amino acid residues constitute a signal sequence, and the proteolytic cleavage site is located near the $NH_2$-terminal end of the first transmembrane domain (Saito *et al.*, 1991). Interestingly, the 34 kDa form is fully active and the two forms are indistinguishable in their affinities for endothelins, suggesting the ligand binding activity of the endothelin receptor resides within the 34-kDa domain (Kozuka *et al.*, 1991). $ET_AR$ binds selectively ET-1 and ET-2 but not ET-3 at physiological concentration (Arai *et al.*, 1990), whereas the $ET_BR$ binds all the three

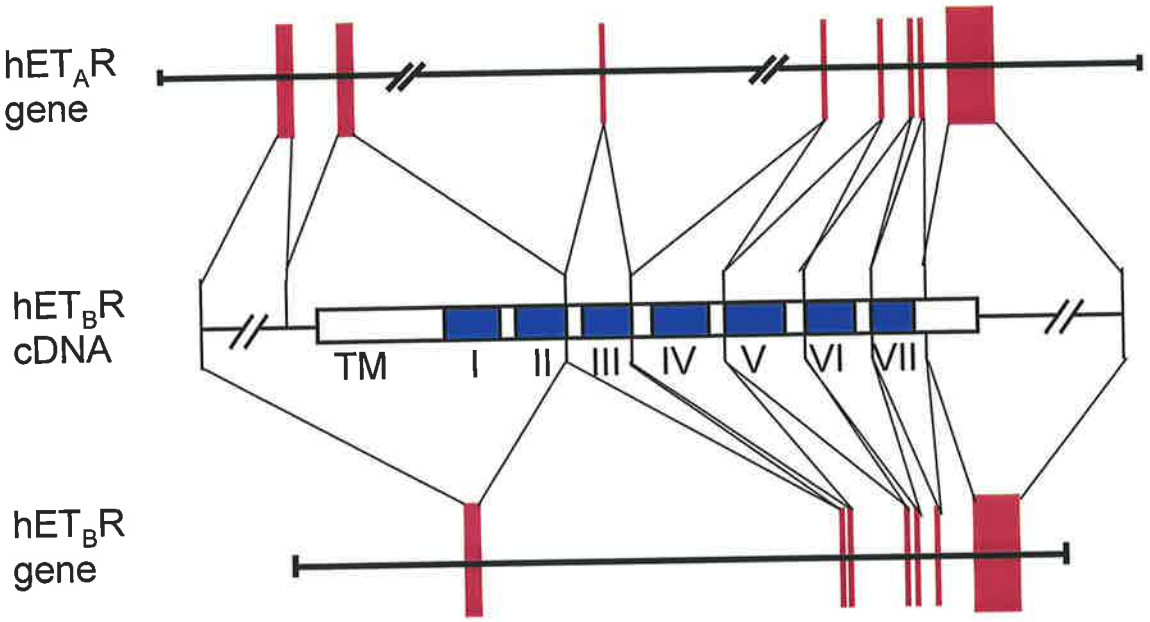



Figure 1.11. Structural organization of the hET$_B$R gene and the hET$_A$R gene. The gene locus is represented by a solid black bar, and exons are shown as dark red rectangles at the top (the hET$_A$R gene) and the bottom (the hET$_B$R gene). The structure of the hET$_B$R cDNA is presented in the middle panel. Transmembrane domains (TM I-VII) and other domains are indicted as blue boxes and open boxes, respectively. Exons in the genomic DNA and their corresponding regions of the cDNA are connected by solid lines. Adapted from Arai, H. (Arai *et al.*, 1993).

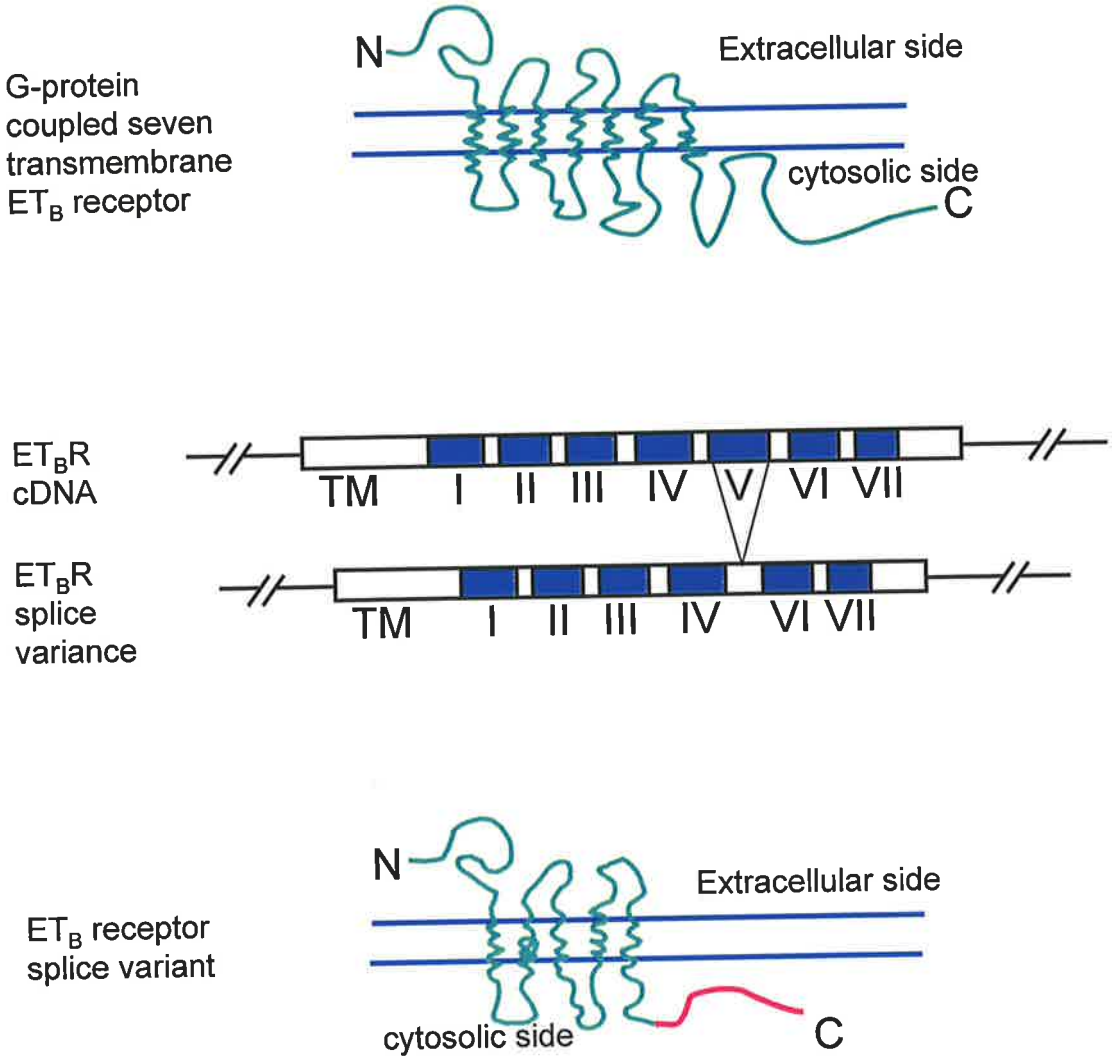



Figure 1.12. Generation of the splice variant, $ET_BR\Delta5$. The shortened $ET_BR$ transcript has the same amino acid sequence as that of $ET_BR$ up to the proximal region of the third intracellular protein loop (green line) and then a completely different peptide sequence of 19 amino acids (red line) which was followed by an early termination codon.

ligands with equal high affinity (Sakurai *et al.*, 1990) (Figure 1.13). While $ET_CR$ displays high affinity for ET-3 compared to ET-1, $ET_{AX}$ receptors display extremely weak affinity for BQ123 ($ET_AR$ selective antagonist) as well as sarafotoxin S6c which is a highly selective $ET_BR$ agonist (Williams *et al.*, 1991).

$ET_AR$ and $ET_BR$ belong to the seven transmembrane-spanning G-protein-coupled receptors (GPCRs) with an extracellular N terminus and a cytoplasmic C terminus (Arai *et al.*, 1990). The GPCRs are activated by the binding of small molecular weight ligand to the sites within the hydrophobic core of GPCRs formed by the N terminus and extracellular hydrophilic loops joining the transmembrane domains (reviewed in (Gether *et al.*, 1998)). Following activation, GPCRs convert extracellular signals into intracellular signals by activating heterotrimetric G-proteins.

G proteins consist of three subunits: the α-subunit which carries the guanine-nucleotide binding site, and the β- and γ-subunits which form a tightly bound dimer. Twenty-seven α-subunit genes have been cloned, and on the basis of sequence similarities they can be divided into four families (Gαs, Gαi/o, Gαq, and Gα12). Gα12 subfamily consists of Gα12 and Gα13 that share 67% identity in amino acid. Gα13 is ubiquitous expressed on all cells, suggesting that Gα13 might play a role in basic cellular function. In addition to the numerous α-subunits, five G protein β subunits and thirteen G protein γ subunits also have been identified (reviewed in (Sadee *et al.*, 2001)), suggesting GPCRs are diverse signal transduction system in eukaryotic cells. Upon activation by ligands, GPCRs are converted into the active conformation and are able to complex with and activate G-proteins. Although the third inner loop sequences of many GPCRs, particularly those near the sixth transmembrane domain, appear to be critical in coupling to Gq and Gi, the inner loop 1, 2 and the C-terminus have also been reported to contribute G protein coupling (reviewed in (Sadee *et al.*, 2001)). Inactive G-proteins compose a GDP-bound α-subunit associated with the Gβγ-dimer. The activated GPCRs function as GDP/GTP exchange factors and promote the release of GDP and the binding of GTP to the α-subunits. This leads to dissociation of the α-subunit and the Gβγ-dimer, each capable of regulating the activity of a variety of effector enzymes and ion channels to modulate cellular signalling pathway (reviewed in (Bunemann *et al.*, 1999)). The main pathways include the regulation of adenylyl cyclases and cAMP phosphodiesterases, phospholipase C (PLC) pathways, and

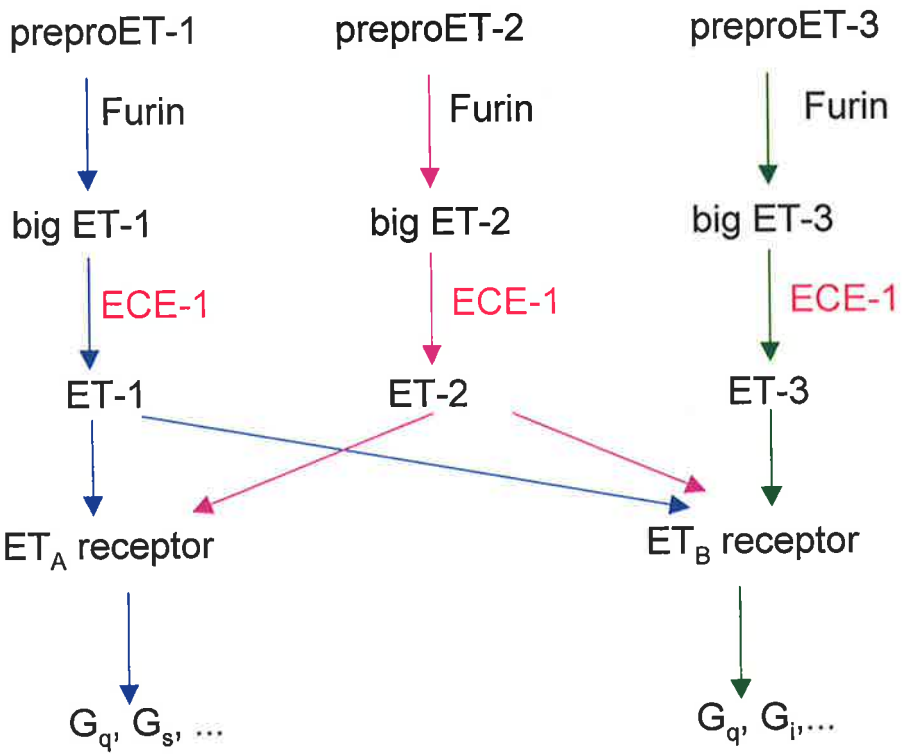



Figure 1.13. Endothelin pathway. Big endothelins are first produced from the three prepro-precursors through proteolytic processing catalysed by furin or similar serin protease(s). ECE-1 and ECE-2 are two known membrane-bound metalloproteases that can cleave the biologically inactive big endothelins into the 21-amino-acid, active endothelins. Mature endothelins act on the two known G protein-coupled endothelin receptors, $ET_AR$ and $ET_BR$. Adapted from Yanagisawa, H. (Yanagisawa *et al*, 1998).

regulation of ion channel activity. Importantly, the G-protein activation process is reversed by production of Gα/GDP and its reassociation with Gβγ to form the inactive heterotrimer because of the inherent GTPase activity of the Gα subunit, (Figure 1.14).

### 1.3.3.4 The Expression and Function of ET-1 and Its Receptors

ET-1 has been found in epithelial, mesangial, leukocyte, macrophages, smooth muscle cells, cardiomyocytes, neuronal and glial cells (reviewed in (Luscher *et al.*, 2000)). Although ET-1 mRNA is widely expressed in human tissues *in situ* (Nunez *et al.*, 1990), ET-1 protein does not seem to accumulate in secretory granules within the endothelial cells (Nakamura *et al.*, 1990). However, stimuli such as hypoxia, ischemia, or shear stress induce the transcription of endothelin-1 mRNA and the synthesis and secretion of endothelin-1 within minutes (reviewed in (Noll *et al.*, 1996)). The intracellular half-life of the preproendothelin-1 mRNA is approximately 15 minutes (Inoue *et al.*, 1989b) and the plasma half-life of ET-1 is approximately 4-7 minutes and cleared mostly by pulmonary circulation (de Nucci *et al.*, 1988). Thus, mature ET-1 acts only locally, but big ET-1 can act as a long-distance carrier of the biological action of ET-1 *in vivo*.

$ET_AR$ is expressed in the highest amounts in the heart and lung, and is also present in the brain and many other peripheral tissues in lesser amounts (Arai *et al.*, 1990; Elshourbagy *et al.*, 1993). In the vascular wall, $ET_A$ is expressed mainly in smooth muscle cells (Hosoda *et al.*, 1991). In contrast, $ET_BR$ is not expressed in vascular smooth muscle cells (Sakurai *et al.*, 1990), but in endothelial cells where it is localised along the apical plasma membrane of endothelin cells (Kayashima *et al.*, 1999). Like $ET_AR$, $hET_BR$ mRNA is expressed in many tissues with the highest level in the cerebral cortex and cerebellum, and with high levels in the placenta, lung and liver (Elshourbagy *et al.*, 1993; Ogawa *et al.*, 1991).

ET-1 exerts its physiological effect *via* $ET_AR$ and/or $ET_BR$. The differences in tissue-specific expression of the two receptors contribute to the different actions of the three endothelins. The $ET_AR$ subtype mediates cell proliferation and vasoconstriction. Although $ET_BR$ also has mitogenic action in some types of cells, it mainly mediates vasodilation *via* releasing nitric oxide (NO) (Verhaar *et al.*, 1998), and plays an important role in the clearance of circulating ET-1 (Fukuroda *et al.*, 1994). One of the more striking examples of ET-1 function is revealed in mice in which the $ET_BR$ gene has been disrupted. It results in ananglionic megacolon associated with coat colour spoting, resembling human

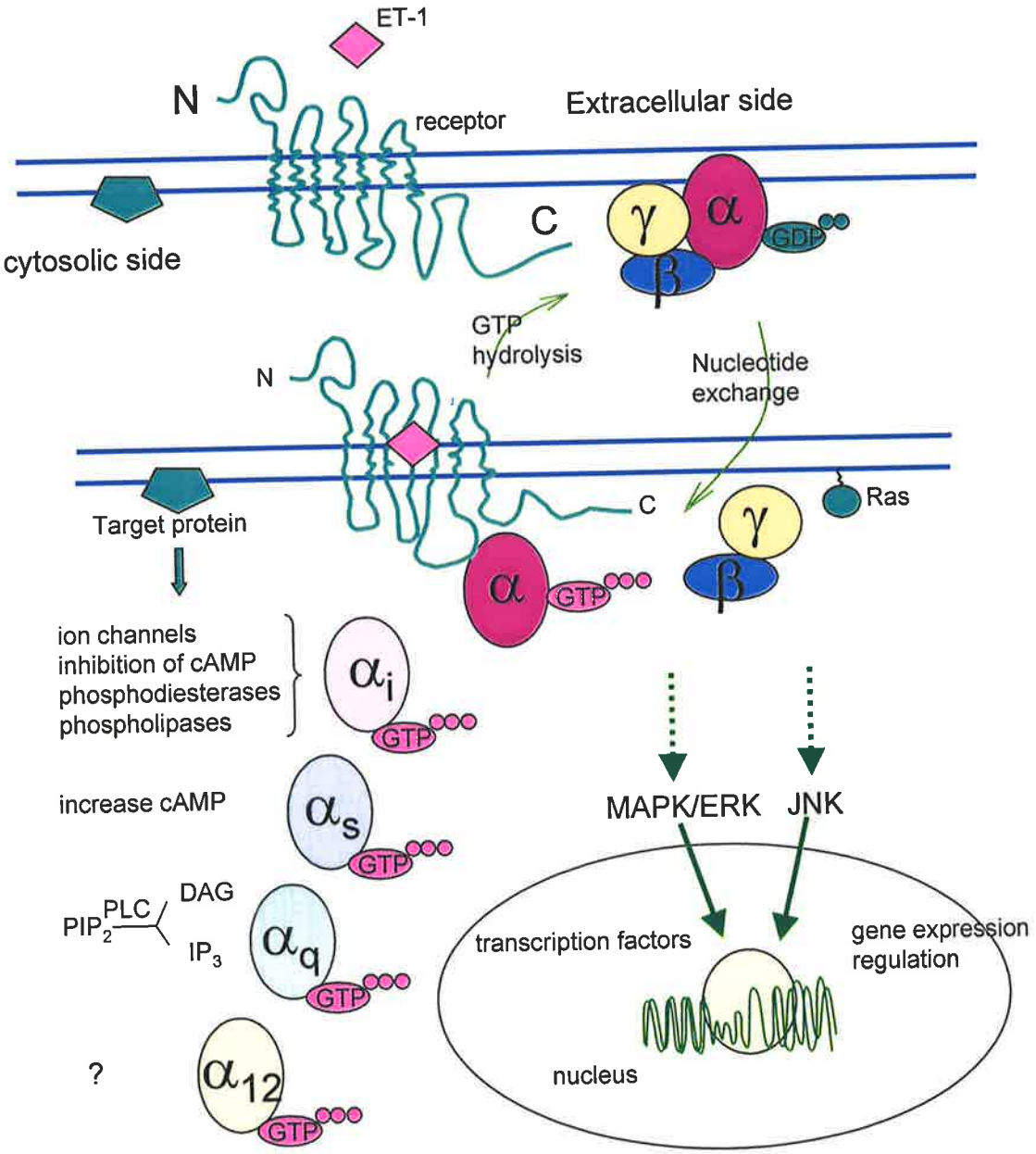



Figure 1.14. Diversity of the G protein-coupled receptor signal transduction system. Inactive G-proteins compose a GDP-bound α-subunit associated with the Gβγ-dimer. The activated GPCRs function as GDP/GTP exchange factors and promote the release of GDP and the binding of GTP to the α-subunits. This leads to dissociation of the α-subunit and the Gβγ-dimer, each capable of regulating the activity of adenylyl cyclases and cAMP phosphodiesterases, phospholipase C, and ion channels modulate cellular signalling pathway. The activation process is reversed by the reassociation of Gβγ with Gα/GDP.

PLC, phospholipase C; $PIP_2$, phosphatidylinositol; DAG, diacylglycerol; $IP_3$, inositol trisphosphate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun-NH(2)-terminal kinase. Modified from Gutkind, J.S. (Gutkind, 1998).

Hirshchsprung's disease (HSCR), indicating an essential role for $ET_BR$ in the development of two neural crest-derived cell lineages, myenteric ganglion neurons and epidermal melanocytes (Hosoda *et al.*, 1994). Although all three endothelin isopeptides can bind and activate $ET_BR$ equally, the signal conveyed by ET-3 *via* $ET_BR$ is specifically required in the development of two neural crest-derived cell lineages, the trunk neural crest-derived epidermal melanocytes and the vagal neural crest-derived enteric ganglion neurons (Baynash *et al.*, 1994).

$ET_AR$ regulates adenyl cyclase *via* Gαs in smooth muscle cells ($ET_AR$) (Aramori *et al.*, 1992) which involves the second inner loop of $ET_AR$ for coupling. It also has been shown that $ET_AR$ induces stress fiber formation *via* Gα12 in fibroblast cells (Gohla *et al.*, 1999). In contrast, $ET_BR$ regulates adenyl cyclase *via* Gαi in endothelial cells (Aramori *et al.*, 1992) which involves the third inner loop of $ET_BR$ for Gαi coupling. In addition, $ET_BR$ has been shown to couple to Gα13 in human HEK-293 cells (Gohla *et al.*, 1999) which requires the three residues (Met 128-Arg129-Asn130) in the first inner loop of $ET_BR$ for specific coupling to the Gα13 proteins (Liu *et al.*, 2003).

The ET-1 induced mitogenic action *in vitro* has been well characterised. It has been observed in several cell types, including human carcinoma cells (reviewed in (Grant *et al.*, 2003)), vascular smooth muscle cells (Komuro *et al.*, 1988; Sugitani *et al.*, 2001), and mouse embryonic Swiss 3T3 fibroblasts (Takuwa *et al.*, 1989a), rat glomerular mesangial cells (Simonson *et al.*, 1989), and airway smooth muscle cells (Noveral *et al.*, 1992. Interestingly, palmitoylation of ET receptors is an absolute requirement for ET-1-promoted mitogenic activity in various cell types whereas the C-terminal portion distal of the palmitoylation sites is dispensable {Cramer, 2001 #670). Like other GPCRs, the binding of ET-1 with $ET_AR$ and $ET_BR$ results in the dissociation of the receptor linked G-protein subunits, which may activate different signal transduction pathways in various target cells to promote cell growth.

Previous studies show that the PLC pathway is the most common pathway involved in mitogenic effect of ET-1. Both $ET_AR$ and $ET_BR$ can couple to Gαq proteins, resulting in the activation of PLC, hydrolysis of phosphatidylinositol ($PIP_2$), and mobilization of the two second messengers inositol triphosphate ($IP_3$) and 1,2-diacylglycerol (DAG). These second

messengers then lead to a biphasic increases in the intracellular free $Ca^{2+}$ concentration (Hirata *et al.*, 1988; Takuwa *et al.*, 1989a; Takuwa *et al.*, 1990). The activation of intact Gαq/PLC signaling cascade is necessary for endothelin induced activation of mitogen-activated protein kinase [MAPK/ERK (for extracellular signal-regulated kinase)] signaling cascade (Cramer *et al.*, 2001). The MAPK/ERK signaling cascade induces a rapid and transient increase of expression of transcriptional factors (proto-oncogenes, such as c-myc, c-fos, erg-1, etc) which result in mitogenic effects. Another mechanism of MAPK/ERK activation involves Gαi-coupled receptors where ligand stimulation drives the dissociation of G-protein βγ-subunits and triggers Ras-mediated MAPK activation. Alternatively, endothelin-mediated stimulation of the Ras pathway in Rat1 fibroblast may involve autophosphorylation of epidermal growth factor receptor (EGFR), which in turn activates the ERK/MAPK pathways *via* canonical routes (Daub *et al.*, 1996) (Figure 1.14).

The ET-1 mediated mitogenic effect also involves activation of prostaglandins and certain ion channels. ET-1 stimulates arachidonic acid release from phosphatidylinositol phosphate through $ET_AR$, putatively *via* phospholipase $A_2$ and phospholipase D activation (Kawanabe *et al.*, 2003; Kozawa *et al.*, 1997; Wu-Wong *et al.*, 1996), and promotes $PGE_2$ synthesis which increases cell proliferation (Baylink *et al.*, 1996). ET-1 activates Na+/H+ exchange in rat glomerular mesangial cells, causing cytosolic alkalinization (Simonson *et al.*, 1989), which is thought to be a necessary response for the $G_0/G_1$ transition and mitogenesis (L'Allemain *et al.*, 1984; Moolenaar, 1986; Schuldiner *et al.*, 1982). The increased Na/H antiporter activity has also been observed in OKP cells (a clonal opossum kidney cell line) by binding of ET-1 to $ET_BR$, which is mediated in part by increasing $[Ca^{2+}]$ and Ca-calmodulin kinase (Chu *et al.*, 1996). Thus, ET-1 functions as an autocrine/paracrine growth factor through various signal pathways in different types of cells.

### 1.3.3.5 ET-1 and Pathology

Endothelins are not only involved in a wide spectrum of normal physiological situations, such as embryonic development (Grant *et al.*, 2003) and gastrointestinal and endocrine function (Calogero *et al.*, 1998; Kanyicska *et al.*, 1998), but have also been implicated in numerous pathological conditions. These include hypertension (Bogoyevitch *et al.*, 1994), acute cardiac infarction (Dupuis *et al.*, 1998), cardiac failure (Zolk *et al.*, 1999), fibrosing connective tissue disease systemic sclerosis (scleroderma) (Shi-Wen *et al.*, 2001), pulmonary fibrosis (Mutsaers *et al.*, 1998), carcinogenesis (Grant *et al.*, 2003). Many

of these pathological conditions are associated with the role of ET-1 as an autocrine/paracrine growth factor. For example, ET-1 stimulates MAPK signaling cascade that eventually targets transcriptional factors in rat cardiac myocytes, may be involved in the initiation of the hypertropic response (Bogoyevitch *et al.*, 1994). To support this notion, the upregulated expression of $ET_AR$ mRNA has been observed in heart failure myocardial tissue (Zolk *et al.*, 1999), which may mediate the myocardial and coronary effects of ET-1. Whilst the increased tissue ET-1 concentration also has been detected in heart failure (Zolk *et al.*, 1999), this may due to a decreased cardiac $ET_BR$-mediated local sequestration and clearance of ET-1 (Brunner *et al.*, 1996). In addition to cardiac disease, a role for ET-1 as an autocrine/paracrine growth factor has been observed in carcinogenesis, angiogenesis, fibrosis and bone metabolism.

Interests in the role of ET-1 in cancer have grown over the last decade. It has been shown that ET-1 is produced by several human cancer cells, including breast (Kojima *et al.*, 1995; Yamashita *et al.*, 1991), colon (Asham *et al.*, 2001; Simpson *et al.*, 2000), cervix (Shichiri *et al.*, 1991), larynx (Shichiri *et al.*, 1991), endometrium (Pekonen *et al.*, 1992), prostate (de Matteis *et al.*, 2001; Grant *et al.*, 1997), ovary (Salani *et al.*, 2000a) and lung (Ahmed *et al.*, 2000; Bagnato *et al.*, 1999). An increased secretion of ET-1 is associated with increased expression of TNF-α and ECE-1α, and is intrinsically involved in the hyperpigmentation mechanism of seborrhoeic keratosis, which is a benign epidermal tumour with increased pigmentation (Manaka *et al.*, 2001). Moreover, increased ET-1 plasma levels have been found in patients with colorectal liver metastasis (Shankar *et al.*, 1998). Furthermore, the ectoenzyme ECE-1c is the most commonly expressed isoform in lung cancer and normal bronchial epithelial cells, suggesting preproendothelin produced by tumor cells could be activated by expressed ECE-1c, causing autocrine and/or parapcrine growth stimulation of surrounding cells *via* $ET_AR$ (Ahmed *et al.*, 2000). Besides the elevated ET-1 expression, increased $ET_AR$ expression in maligant tissue has been demonstrated using immunohistochemistry and /or autoradiography in several cancer types including colorectal (Asham *et al.*, 2001; Simpson *et al.*, 2000), ovarian (Bagnato *et al.*, 1999) and prostate tumours (Nelson *et al.*, 1996). Normal prostatic epithelial cells express ET-1, $ET_AR$ and $ET_BR$ mRNA, and normal prostatic stromal cells only express ET-1 receptors with predominant expression of $ET_BR$ (Grant *et al.*, 1997). However, the $ET_AR$ becomes prevalent in both primary tumours and their metastases (Nelson *et al.*, 1996). A plausible mechanism for reduced $ET_BR$ expression in prostate malignant tissue may due to the relative

hypermethylation of the $ET_BR$ gene that correlates with transcriptional down-regulation (Nelson *et al.*, 1997).

In addition to the elevated expression of ET-1 and $ET_AR$ expression in many types of malignant tissues, *in vitro* experiments have shown that exogenous and endogenous ET-1 directly stimulates proliferation of epithelial cell lines HeLa and Hep-2 cells (Shichiri *et al.*, 1991), human ovarian and colon cancer cell lines (Kusuhara *et al.*, 1990), and human melanoma cells (*via* $ET_BR$)(Kikuchi *et al.*, 1996).

Besides mitogenic effects in tumour growth, ET-1 is also implicated in cell apoptosis. ET-1 can serve as a survival factor for endothelial cells, firoblasts, smooth muscle cells, colon carcinoma, glioblastoma cells and ovarian carcinoma cells (Del Bufalo *et al.*, 2002; Eberl *et al.*, 2000; Shichiri *et al.*, 1997; Shichiri *et al.*, 1998; Shichiri *et al.*, 2000). ET-1 exerts its anti-apoptotic action by activation of the MAPK pathway in serum deprivation-induced apoptosis of endothelial and vascular smooth muscle cells *via* $ET_BR$ and $ET_AR$, respectively. Similarly, ET-1 exerts its anti-apoptotic action in c-myc-induced apoptosis of rat fibroblasts *via* $ET_AR$ (Shichiri *et al.*, 1997; Shichiri *et al.*, 1998; Shichiri *et al.*, 2000). Growing evidence indicate that the serine/threonine protein kinase Akt, or protein kinase B (PKB), is a key regulation of cell survival. ET-1 can activate Akt/PKB through $PI_3$-kinase pathway to protect rat colon cancer cell lines against FasL-induced apoptosis (Eberl *et al.*, 2000) and protect ovarian carcinoma cells against paclitaxel-induced apoptosis (Del Bufalo *et al.*, 2002).

### 1.3.3.6 ET-1 and Bone Cells

In view of the abundance of endothelial cells in bone marrow and the proximity of these cells to OBs and OCs on the bone surface, it is conceivable that ET-1 plays an important role in the control of bone metabolism.

Studies examining ET-1 null mice suggest that ET-1 and $ET_AR$ are involved in the formation of bone and cartilage that are derived from the branchial arches which originate from neural crest-derived ectomesenchymal cells (Kurihara *et al.*, 1994). The severe hypoplasia in facial bone observed in both ET-1 (Kurihara *et al.*, 1994) and $ET_AR$ (Clouthier *et al.*, 1998; Clouthier *et al.*, 2000) knockout mice suggests that ET-1 may modulate osteogenic cells through $ET_AR$ but not through $ET_BR$. It has been demonstrated that ET-1

localises to in young osteocytes, OBs, OCs and vascular endothelial cells in the vascular-rich bone metaphyseal region in rat (Sasaki *et al.*, 1993). It also has been found that $ET_AR$ mRNA is strongly expressed in osteogenic cells along craniofacial bones in mice (Kitano *et al.*, 1998). *In situ* hybridization studies indicate that ET-1 is found in the human fetal endothelial cells of the oral cavity and both $ET_AR$ and $ET_BR$ are detected in the human fetal developing mandibles, with the $ET_AR$ subtype five times more concentrated than $ET_BR$ subtype (Barni *et al.*, 1995). This suggests that ET-1 participates in an epithelial mesenchymal paracrine loop important for craniofacial morphogenesis during human ontogeny (Barni *et al.*, 1995). However, the cellular expression of osteonectin (ON) and osteopontin (OP) mRNAs in osteogenic cells of ET-$1^{-/-}$ homozygous mice was not suppressed, suggesting that ET-1 may regulate proliferation and migration of osteogenic cells in the maxillofacial region, rather than modulating the expression of bone matrix proteins (Kitano *et al.*, 1998).

The impaired facial bone formation in ET-$1^{-/-}$ homozygous mice suggests that OBs may be the biological targets for ET-1. Indeed, ET-1 stimulates human osteoblastic cell proliferation in a time- and dose-dependent manner (Kasperk *et al.*, 1997; Takuwa *et al.*, 1990; Tatrai *et al.*, 1992) by increasing the turnover of inositol phosphate in OBs (Hagiwara *et al.*, 1996; Takuwa *et al.*, 1989b). Consistent with this, glucocorticoids enhance the mitogenic effect of ET-1 on human OBs by increasing plasma ET-1 level and also upregulating the gene expression of $ET_AR$ in human osteoblastic cells (Borcsok *et al.*, 1998). In addition, 1, 25$(OH)_2D_3$, which can be produced locally by bone cells, increases $ET_AR$ mRNA expression in human OB cells, corresponding to an increased effect of ET-1 on osteoblastic proliferation and function (Kasperk *et al.*, 1997). However, the effects of ET-1 on OB function are controversial. Some studies show that ET-1 reduces cellular alkaline phosphatase activity in a dose-dependent manner in osteoblastic cells (Hagiwara *et al.*, 1996; Takuwa *et al.*, 1990; Takuwa *et al.*, 1989b). In addition, other studies show that ET-1 also inhibits the expression of mRNA for OCN, a well accepted marker of the maturation of OBs (Hiruma *et al.*, 1998b). Furthermore, ET-1 was found to inhibit the mineralization of MC3T3-E1 cells through the $ET_AR$ (Hiruma *et al.*, 1998a). However, other studies suggest that ET-1 increase the markers of differentiated osteoblastic function, such as ALP and type-I collagen (Kasperk *et al.*, 1997; Tatrai *et al.*, 1992). In addition, ET-1 has also been found to enhance mRNA expression of osteocalcin and osteopontin in the osteoblastic-like cells lines *via* the $ET_AR$ (Nambi *et al.*, 1995).

In spite of the controversy surrounding the *in vitro* findings for the function of ET-1 on OBs, animal studies have shown that ET-1 may be the major factor that mediates sclerotic bone lesions in prostate and breast cancer with bone metastases. The WISH human tumor cell line, derived from amnion (Hela contaminant), was transfected with an ET-1 over-expression cDNA construct to assess its effects on mouse bone formation following inoculation into the mouse tibia. Injection of these cells into mice resulted in significantly more new bone formation when compared with controls (Nelson *et al.*, 1999). In addition, breast cancer cell line ZR-75-1 was found to stimulate OB proliferation and new bone formation in a dose-dependent manner, and this effect was blocked by $ET_AR$ antagonist, BQ-123 (Guise *et al.*, 2003). Compared with MDA-MB-231, which had no effect on new bone formation in mice, ZR-75-1 produced only ET-1 in excess, suggesting tumour-produced ET-1 cause osteoblastic metastases *via* $ET_AR$ (Guise *et al.*, 2003). Furthermore, ET-1 may also induce bone formation by enhancing the effect of other OB-stimulatory factors, such as BMP-7 (Kitten *et al.*, 2001). Importantly, the paracrine effects of tumor-produced ET-1 on bone cells may predominantly provide a favoured growth environment for tumor cells in bone.

ET-1 also regulates bone metabolism by affecting osteoclastic bone resorption. The secretion of ET-1 from prostate cancer cell lines is enhanced when co-cultured on bone slices. The increased ET-1 results in the blockade of osteoclastic bone resorption (Chiao *et al.*, 2000). Consistent with this study, Alam and colleagues have shown that ET-1 causes a significant dose-dependent inhibition of osteoclastic bone resorption at nanomolar concentrations (2.5-8 nM) through inhibition of OC motility but not of acid phosphatase secretion (Alam *et al.*, 1992). However, there is no elevation of $[Ca^{2+}]_i$ in OCs in response to ET-1, suggesting a different intracellular transduction mechanism (Alam *et al.*, 1992). It seems clear that control of OC function, which is attributed exclusively to OB-produced cytokines in association with circulating calcitonin and ambient extracellular calcium, is likely to be influenced by NO produced by marrow endothelial cells. NO acts directly on OCs to produce a major shape change associated with strong inhibition of bone resorption which is not mediated by cGMP pathway (MacIntyre *et al.*, 1991).

### 1.3.3.7 Application of ET-1 in Clinic

Bosentan (Krum *et al.*, 1998) and TAK-044 (Fleisch *et al.*, 2000; Haynes *et al.*, 1996), two dural ET receptor antagonists, have been shown to decrease the blood pressure in essential hypertension and pulmonary arterial hypertension, and have been approved for clinical use (Cheng, 2003). In addition, TAK-044 can reduce the occurrence of delayed cerebral ischemic events in patients who have suffered subarachnoid hemorrhage (Shaw *et al.*, 2000).

Because of the $ET_AR$ mediated autocrine/paracrine mitogenic effects of ET-1 in human malignancy, the $ET_AR$ antaganist, atrasentan (ABT-627), has been used in the treatment of several different types of cancers, including prostate cancer (Carducci *et al.*, 2003), ovarian carcinoma (Rosano *et al.*, 2003) and cervical carcinoma (Bagnato *et al.*, 2002). It seems that atrasentan has an impact on disease progression and morbidity. Moreover, it suppresses the prostate cancer induced bone remodelling (Nelson *et al.*, 2003). Therefore, it is valuable to investigate whether myeloma cells express ET-1 and what the function of ET-1 is in MM- and POEMS syndrome-mediated bone lesions.

Besides ET-1, TGF-β and BMPs have been implicated in osteoblastic metastases of prostate carcinoma. TGF-β plays a key role in OB differentiation based on the extracellular milieu and the differentiation stage of the cells. TGF-β stimulates proliferation and early OB differentiation (Bonewald *et al.*, 1994). However, it inhibits terminal OB differentiation and continued exposure to TGF-β1 impairs bone cell differentiation and the formation of mineralised nodules (Mundy, 1997). TGF-β also stimulates the OC-forming potential of peripheral blood haemopoietic precursors (Massey *et al.*, 2001) and enhances OC differentiation (Sells Galvin *et al.*, 1999). BMPs belong to the TGF-β superfamily and exhibit chondrogenic/osteogenic activity (Mundy, 1995). BMP-2 is important for OB differentiation from uncommitted progenitors both *in vivo* and *in vitro* (Mundy, 1995). Transfection of the tumour cells with antisense constructs of the BMP-3 gene reduces their capacity to stimulate an osteoblastic response *in vivo* (Harris *et al.*, 1992). However, the precise role of TGF-β and BMPs in pathological bone microenvironment requires further investigation.

## 1.4 THE ROLE OF ANGIOGENESIS IN MM

As described previously, through direct interaction with myeloma cells, OCs are thought to play a role in the propagation of myeloma cells in the BM miroenvironment, whilst OBs are essential for the survival and proliferation of human myeloma cells *in vitro*. Moreover, growing evidence suggest that MM patients with progressive disease display increased angiogenesis compared to patients with non-active MM and MGUS (Vacca *et al.*, 1994). In addition, the increased angiogenesis is negatively correlated with survival rate of MM (Pruneri *et al.*, 2002). Therefore, it is interesting to examine the roles of factors, which are involved in MM-mediated bone lesions, with respect to angiogenesis in MM.

### 1.4.1 Angiogenesis and Tumour Growth

Blood vessel formation during embryonic development includes two processes: vasculogenesis (the differentiation of endothelial cells from progenitor cells) and angiogenesis. Angiogenesis is a fundamental step in a variety of physiological and pathological conditions including wound healing, embryonic development, endochondral bone formation, chronic inflammation, and tumor growth. Angiogenesis is a multistep process that involves migration and proliferation of endothelial cells, remodelling of the extracellular matrix, and functional maturation of the newly assembled vessels (Folkman *et al.*, 1987; Folkman *et al.*, 1992). To invade through the basement membrane, endothelial cells must degrade various constituents of the interstitial stroma and basement membrane. Matrix metalloproteinase-2 (MMP-2) is a major extracellular matrix proteolytic enzyme and is secreted when endothelial sprouting takes place, thus enhancing endothelial cells to migrate across the matrix. Maturation of the vascular system involves the recruitment of perivascular supporting cells that do not bear cell specific markers, but which do express α-smooth muscle actin (α-SMA).

Tumor enlargement requires blood vessel growth that originates with the sprouting of new capillaries from preexisting blood vessels. In the absence of access to an adequate vasculature, tumor cells become necrotic and/or apoptotic. Tumors may exist as microscopic in situ lesions for years in the absence of their own microcirculation. For tumor progression and metastasis, an angiogenic switch permits rapid growth and invasion. The angiogenic switch is a discrete genetic event that results in a change in the local equilibrium between pro- and anti-angiogenic regulators, which are produced by tumor cells as well as by the surrounding stromal cells and infiltrating leukocytes (Folkman, 1986). The important

pro-angiogenic regulators include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) which can induce proliferation, migration and morphogenesis of endothelial cells. In addition, recent evidence suggests that IL-1β, RANKL and ET-1 belong to pro-angiogenic regulators as described below.

### 1.4.2 Angiogenesis and Bone Remodelling

New blood vessel formation is required for endochondral bone formation by serving as a conduit that allows a variety of cells essential for bone morphogenesis (endothelial cells, chondroclasts, and OBs) to migrate into the growth plate. The ingrowth of blood vessel in the epiphyseal growth zone and in fracture callus formation precedes bone formation. The concept of cooperative interaction of vascular endothelial cells and bone cells in bone formation is supported by the observation that endothelial cells greatly enhance the bone-forming function of OBs when co-implanted subcutaneously into rats (Villanueva *et al.*, 1990). It is possible that endothelial cells implanted with calvarial cells can indirectly induce bone formation by promoting rapid neovascularizton and increasing the oxygen tension around the bone forming site (Villanueva *et al.*, 1990).

However, vascular endothelial cells in bone are also thought to have significant roles on pathological bone resorption such as osteolytic bone metastasis and hypercalcemia because bone resorption is often seen where blood vessels are abundant. Histological studies of bone metastasis revealed that most metastatic foci occur near the epiphysial plate where microvasculatures are abundant and that OCs are formed around blood vessels of metastatic tumors (Winding *et al.*, 2000).

### 1.4.3 IL-1β and Angiogenesis

IL-1 increases tumour invasiveness and metastasis. IL-1 expression at the site of tumour enhances the expression of adhesion molecules on endothelial and malignant cells and facilitates the invasion of malignant cells into the circulation and their dissemination to remote tissues. IL-1β accounts for the ability of tumour cells to initiate and complete the processes of angiogenesis, as manifested by the inability of tumour cells derived from IL-1β knockout mice to recruit a vascular network in Matrigel plugs (Voronov *et al.*, 2003). IL-1β has been found to induce VEGF production (Margetts *et al.*, 2002) through selectively inducing the expression of hypoxia-inducible factor-1, a dominant transcription factor for

VEGF (Jackson *et al.*, 1997; Maxwell *et al.*, 1997). However, using the rabbit cornea assay, IL-1β has been found to be a potent inhibitor of neoangiogenesis induced by recombinant bFGF (Cozzolino *et al.*, 1990). IL-1 is produced by endothelial cells and functions as a negative autocrine regulator of endothelial growth by binding to the cell surface (Cozzolino *et al.*, 1990).

### 1.4.4 RANKL and Angiogenesis

The ability of human myeloma cells to bind endothelial cells in the bone marrow microenvironment suggests that interaction between these two cell types may be of pathological significance in the homing of myeloma cells to the bone and MM-induced bone lesion.

OC precursors reside in BM and can circulate in the peripheral blood. When migrating to sites of bone resorption from BM or peripheral blood, OC precursors encounter vascular endothelial cell (VEC). During and following their transmigration across the VEC of blood vessels, OC precursors may be activated by molecules displayed on the vascular surface. It has been found that TNF-α- and IL-1β-stimulated endothelium increase the recruitment of OC precursors (Formigli *et al.*, 1995; McGowan *et al.*, 2001), which may be mediated by upregulation of RANKL expression on the endothelium cell surface {Collin-Osdoby, 2001 #511). In addition, RANKL can stimulate capillary-like tube formation in primary cultured human umbilical vein endothelial cells (HUVECs) through the Src-PLC-$Ca^{2+}$ signalling cascade in endothelial cells (Kim *et al.*, 2002b), suggesting RANKL has an important role in neovessel formation in MM. As described previously, plasma cells and OBs from MM secrete both RANKL and OAFs, such as TNF-α and IL-1β, suggesting a potential impact of increased microvasculature on bone damage in MM.

### 1.4.5 ET-1 and Angiogenesis

ET-1 can be upregulated by various stimuli including hypoxia (Kourembanas *et al.*, 1991), growth factors and inflammatory cytokines such as TGF-β, IL-1β, and TNF-α (Saleh *et al.*, 1997, Le Brun, 1999 #765) produced by cancer cells and/or surrounding tissue. Studies show that the presence of ET-1 correlates with tumor vascularity and malignancy in well-vascularized brain tumors (Stiles *et al.*, 1997) and colorectal cancer (Egidy *et al.*,

2000), suggesting that ET-1 is another pro-angiogenic regulator. Human colon stroma surrounding the cancer has highly vascularized regions with higher concentrations of ppET-1 and ECE-1 than the stroma in normal tissue (Egidy *et al.*, 2000). Moreover, $ET_BR$ is almost undetectable in normal colon mucosa, but is abundant in all vascularized areas of the cancer stroma (Egidy *et al.*, 2000), and $ET_AR$ expression is localized in carcinoma cells as well as in vascular smooth muscle cells (Salani *et al.*, 2000a).

ET-1 can act directly on endothelial cells *via* the $ET_BR$ to modulates different stages of neovascularization, including proliferation, migration, invasion, protease production and morphogenesis, and also stimulates neovascularization *in vivo*. ET-1 promotes HUVEC proliferation, migration and invasion in a dose-dependent manner and can induce an angiogenic response in cultured endothelial cells through $ET_BR$ (Morbidelli *et al.*, 1995; Salani *et al.*, 2000b). ET-1 stimulates invasion and morphological differentiation of HUVEC in matrigel *in vitro*, and may be facilitated *via* ET-1-induced production of MMP-2 by endothelial cells. It has been shown that ET-1 promotes ovarian carcinoma invasion by upregulating and activating the expression of matrix metalloptoeinase (MMP) –2. –9, -3, -7 and 13 through $ET_AR$, and also down regulating tissue inhibitors of matrix metalloproteinases (TIMP) 1 and 2 (Rosano *et al.*, 2001).

ET-1 can also modulate tumor angiogenesis indirectly through the induction of several pro-angiogenic factor *in vitro*, including PDGF and VEGF (Okuda *et al.*, 1998; Pedram *et al.*, 1997). Engagement of the $ET_AR$ by ET-1 induces VEGF production by increasing levels of hypoxia-inducible factor-1 $\alpha$ (Spinella *et al.*, 2002). Moreover, ET-1 and VEGF have stimulatory interactions on each others expression, which may play an important role in concomitant proliferation of endothelial and smooth muscle cells in the vascular wall (Matsuura *et al.*, 1998). During neovascularization, endothelial cells could be initially stimulated by the ET-1/ $ET_BR$ interaction to migrate, and invade surrounding tissue. Thereafter, vessel maturation could be mediated by ET-1/ $ET_AR$ binding in part through the stimulation of VEGF in the existing tumor or vasculature, resulting in angiogenesis (Salani *et al.*, 2000a).

In conclusion, MM and POEMS syndrome represent plasma cell dyscrasias, which interact with the skeleton, but mediate completely disparate osteolytic and osteoblastic bone diseases. This provides us with an opportunity to explore the critical factors required for

physiological and pathological bone remodelling. Although both OAFs and RANKL have been implicated in MM, further studies are required to clarify the precise role of these factors in the complex BM microenvironment of MM. Even though the potential mediator for osteoblastic bone disease, ET-1, has been investigated in cardiac disease and several types of tumours, including prostate cancer with bone metastases, there is no reported studies examining the role of ET-1 in MM-mediated bone disease.

## 1.5 HYPOTHESES

Factors derived from myeloma plasma cells and OBs in response to myeloma cells mediate either sclerotic or osteolytic bone lesions as seen in patients with POEMS or MM, respectively.

## 1.6 PROJECT RATIONALE

We have previously described an *in vitro* model of human OB differentiation (Gronthos *et al.*, 1999), where osteogenic cells derived from human trabecular bone can be separated into four populations charactersistic of different stages of OB differentiation. Separation is based on the expression of the stromal precursor cell marker, STRO-1 and the osteoblastic marker, ALP. This model enables us to explore the role of factors of interest on the phenotype and function of OBs.

As described previously, high serum levels of TNF-α, IL-1β and IL-6 have been noted both in patients with MM and POEMS syndrome. Therefore, the effect of IL-1β, which has the highest serum level compared to TNF-α and IL-6 in patients with POEMS syndrome, on OBs function was explored in this study. In addition, the expression of ET-1 in normal and patient-derived tissue was examined. Finally, previous studies from our laboratory suggest that the potent osteoclastogenesis molecule, RANKL, is most highly expressed by OB-like cells, which display an undifferentiated phenotype. This undifferentiated phenotype is characterised by high levels of expression of the STRO-1 antigen. Therefore, the relationship between RANKL and STRO-1 expression on OBs and BMMNCs was investigated.

## 1.7 SPECIFIC AIMS OF THIS PROJECT

1. Investigate the role of one of major OAFs, IL-1β, in MM and POEMS syndrome with respect to the bone pathology.

2. Define the relationship between RANKL and STRO-1.

3. Examine the role of a potential mediator for osteoblastic bone disease, ET-1, on bone remodelling and tumour growth in patients with MM.

# CHAPTER 2 MATERIALS AND METHODS

## 2.1 CELL CULTURE

All tissue culture was performed in a Class II biohazard cabinet (Gelman Science). Cell cultures were incubated at 37° C (Forma Scientific air incubator), in an atmosphere containing 5% $CO_2$ in air to maintain pH 7.0, and a relative humidity of 97%. Cell densities and viabilities were determined using a haemocytometer and trypan blue dye exclusion. Cells were diluted in 0.4% trypan blue (w/v) in phosphate buffered saline (PBS) and enumerated using a light microscope (Olympus BX40, Japan).

### 2.1.1 Buffers and Media

Tissue culture solutions were made with Milli-Q water which was prepared by purifying the Milli-RO water through two beds of ion exchange resins, a carbon filter and an organic filter using a Milli-QF$^{PLUS}$ system (Millipore Corp.; USA). Prepared solutions and buffers were subsequently sterilised by autoclaving or filter sterilisation as indicated. Filter sterilisation was performed using either a 0.22 μm bottle filter (Nalge Nunc International Corp, USA), Spin-X filter (Costar, MA, USA), or by 0.22 μm Minisart® filters (Sartorius, Germany). Following buffer preparation, all solutions were stored at 4°C, -20°C or -80°C as indicated.

#### 2.1.1.1 2-Mercaptoethanol (2-ME)

A 1M stock solution of 2-ME was prepared by diluting 0.7 ml of 14.27 M β-mercaptoethanol (Sigma, St. Louis, MO, USA, Cat. No. M-3148) into 9.3 ml Hank's Balanced Salt Solution (HBSS, Infectious Diseases Laboratories, IMVS) and was stored at -20°C. The 1 M stock solution was further diluted 1:10 (in HBSS) to prepare a 0.1 M working solution. This was filter sterilised and stored as 500 μl aliquots at -20°C.

#### 2.1.1.2 Double-Strength Iscove's Modified Dulbecco's Medium (2×IMDM)

To prepare 2×IMDM (Cytosystems, Castle Hill, NSW, Australia, Cat. No. 50-016-PA), one sachet of IMDM powder was dissolved in 390 ml of Milli-Q water. To this, sterile stocks of penicillin/streptomycin (CSL Biosciences, Victoria, Australia, Cat. No. 05081901), DEAE-Dextran (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden, Cat. No. 170350-01) and L-asparagine (Sigma, Cat. No. A-0884) were added at concentrations

of 200 i.u./ml, 200 μg/ml, 5.2 mg/ml and 0.4 mg/ml, respectively. Filter sterilised medium was stored as 50 ml aliquots at -20°C or used immediately.

#### 2.1.1.3 10% (w/v) Bovine Serum Albumin (BSA)

To prepare 10% (w/v) BSA, 20 g BSA (CSL Limited, Victoria, Australia, Cat. No. 06711701) was carefully overlayed on the surface of Milli-Q water (88.4 ml), and allowed to sit at 4°C for 24 hrs. Once dissolved, 3 g of AG® 501-X8 (D) Resin (Bio-Rad Laboratories, CA, USA, Cat. No. 142-6425) was used to deionise the BSA. The beads were removed by filtration through Whatman No. 1 paper (Whatman Laboratory Division, England). This process was repeated 3 times to ensure complete removal of residual trace elements and contaminants. An equal volume (100ml) of 2× IMDM (or 2×PBS) was added to the BSA and sterilised by filtration through a 0.22 μm bottle filter, prior to storage at -20°C.

#### 2.1.1.4 Thaw Medium

To prepare 250 ml thaw medium, HBSS was supplemented with 20% (v/v) foetal calf serum (FCS, CSL Limited, Victoria, Australia, Cat. No. 09702301), penicillin (50 i.u./ml)/streptomycin sulphate (50 μg/ml) (JRH Biosciences, USA), 2 mM of glutamine (JRH Biosciences, USA), 1 mM of sodium pyruvate (Sigma), 15 mM of HEPES buffer (JRH Biosciences, USA), 12500 unit Deoxyribonuclease (Dnase I, Sigma, type IV from bovine pancreas) and 0.025 g L-Asparagine (Sigma).

#### 2.1.1.5 Dulbecco' Modified Eagles Medium-10 (DMEM-10)

To prepare 500 ml of DMEM-10, 427.5 ml of Dulbecco' Modified Eagles Medium (JRH Biosciences, USA) was supplemented with penicillin (50 i.u./ml)/streptomycin sulphate (50 μg/ml), 10% (v/v) FCS, 2 mM of glutamine, 1 mM of sodium pyruvate and 15 mM of HEPES buffer. The medium was subsequently filter sterilised and stored at 4°C. The medium was replenished with 2mM L-glutamine at weekly intervals.

#### 2.1.1.6 Roswell Park Memorial Institute Medium (RPMI-1640)-10

To prepare 500 ml of single strength RPMI-10, 427.5 ml of RPMI-1640 (JRH Biosciences, USA) was supplemented with penicillin (50 i.u./ml)/streptomycin sulphate (50 μg /ml), 10% (v/v) FCS, 2 mM of glutamine, 1 mM of sodium pyruvate and 15 mM of

HEPE buffer and 0.45% (w/v) D-glucose (BDH AnalaR®, $C_6H_{12}O_6$, Victoria, Australia). The medium was subsequently filter sterilised and stored at 4°C. The medium was replenished with 2 mM L-glutamine at weekly intervals.

### 2.1.1.7 Alpha Modification of Minimum Essential Medium-10 (α-MEM-10)

To prepare 500 ml of single strength α-MEM-10, 422.5 ml of α-MEM (JRH Biosciences, USA, Cat. No. 51451-500M) was supplemented with penicillin (50 i.u./ml)/streptomycin sulphate (50 μg /ml), 10% (v/v) FCS, 2 mM of glutamine, 1 mM of sodium pyruvate, 15 mM of HEPE buffer and 100 μM L-ascorbate-2-phosphate (WAKO pure chemical industries, LTD, Japan, Cat. No. 013-12061). The medium was subsequently filter sterilised and stored at 4°C. The medium was replenished with 2 mM L-glutamine at weekly intervals. Alternatively, α-MEM-20 refers to α-MEM supplemented with 20% (v/v) FCS and other components described above.

### 2.1.1.8 Serum Deprived Medium (SDM)

The preparation of SDM for the growth of BM CFU-F, was performed essentially as described by Gronthos and Simmons (Gronthos *et al.*, 1995). Single strength α-MEM was supplemented with 10 μg/ml human insulin (Novo Nordisk Pharmaceuticals Ltd, Auckland, New Zealand), 2% (w/v), BSA (Sigma, USA), 20μg/ml iron saturated human transferrin (Sigma, USA), 2 mM L-glutamine, 10 mM HEPES, β-mercaptoethanol ($5\times10^{-5}$M), 50μg/ml human low density lipoprotein (LDL, Sigma, USA), dexamethasone sodium phosphate ($10^{-8}$ M) (Fauldings, Adlaide, Australia), 100 μM L-ascorbate-2-phosphate, and penicillin/streptomycin at 50 i.u./ml and 50 μg/ml, respectively. SDM was supplemented with both PDGF-BB (Pepro Tech Inc., Rocky Hill, NJ) and EGF (Sigma, USA) at 10ng/ml, a concentration previously shown to provide optimal growth to the cultures (Gronthos *et al.*, 1995). The SDM was made fresh as required and was filter sterilised using 0.22 μM low protein binding filter (Millex®-GV, Millipore Corporaiton, USA) prior to its use.

### 2.1.2 Human Myeloma Cell Lines

Myeloma cell lines ARH-77, RPMI8226, and U266 were purchased from American Type Culture Collection. WL2 was kindly provided by Professor Doug Joshua, Royal Prince Alfred Hospital, Sydney. JIMI, LP-1, OPM2, NCI-H929 were kindly provided by A/Professor Andrew Spencer, The Alfred Hospital, Melbourne. ARH-77 is an Epstein-Barr

virus transformed cell line derived from a patient with plasma leukemia. All myeloma cell lines were cultured in RPMI-1640-10. All cell lines were routinely cultured at a density of $2\times10^5$ cells/ml to ensure that a majority of cells were in a logarithmic phase of growth. Cells were subcultured ("split") into fresh media every 2 to 3 days and cultured at 37°C in the presence of 5% $CO_2$.

### 2.1.3 Retroviral Packaging Cell Line PT67

The amphotropic retroviral packaging cell line, PT67, was maintained in DMEM-10. Cultures were grown as monolayers in tissue culture flasks with 75 $cm^2$ surface area, (T-75, Greiner Bio-one, Germany), and passaged every 2 to 3 days. PT67 cultures were harvested by trypsin detachment and following trypsin–inactivation, flasks were reseeded with $5\times10^5$ to $1\times10^6$cells and subsequently incubated at 37°C with 5% $CO_2$.

### 2.1.4 Murine Stromal Fibroblastic Cell Line, Swiss NIH-3T3

The murine fibroblastic cell line Swiss NIH-3T3 was maintained in DMEM-10. Cultures were grown as monolayers in T-75 flasks and subcultured every 3 to 5 days. Fibroblast cultures were harvested by trypsin detachment and following trypsin–inactivation, flasks were reseeded with 0.5×to $1\times10^6$ cells and subsequently incubated at 37°C with 5% $CO_2$.

### 2.1.5 Bone Marrow Mononuclear Cells (BMMNCs)

Fresh bone marrow (BM) aspirates from the iliac crest were obtained from normal adult volunteers (20-35 years old) as part of an institutional ethics committee approved Normal Bone Marrow Donor Program. Bone marrow mononuclear cells and peripheral blood mononuclear cells were routinely obtained on a Lymphoprep gradient (S.G. 1.077 g/ml) (AXIS-SHIELD PoC AS, Oslo, Norway) by centrifugation at 274× *g* for 30 mins. The light density interface cells were harvested and then washed twice in HHF (HBSS supplement with 5% FCS), by centrifugation at 274× *g* for 5 mins at 4°C. The cell pellet was resuspended in fresh HHF and stored on ice before being used for culture and/or immunological studies.

### 2.1.6 BM CFU-F Clonogenic Assay

Twenty four-well cell culture plates were pre-coated with 5 $\mu g/cm^2$ purified fibronectin (Boehringer Mannheim, GMBH, Germany) diluted in PBS for 90 mins at RT. Following this, the fibronectin solution was pipetted off, and the plates rinsed once with PBS. MACS sorted STRO-$1^+$ BMMNCs (please refer to Section 2.4.8) were cultured in SDM supplemented with factors/cytokines as described in the figure legends. Routinely, each condition/treatment was performed in triplicate. Alternatively, MACS sorted STRO-$1^+$ cells were further sorted based on the dual-colour flow cytometric analysis of STRO-1 (FITC) and RANKL (PE) using FACStar$^{PLUS}$ cell sorter (Becton Dickinson, Mountain View, CA, USA). The FACS sorted populations were cultured in α-MEM-20 in 6-well plates without fibronectin coating prior to use. Cultures were incubated at 37° C in the presence of 5% $CO_2$ for 14 days. Cultures were washed three times with PBS at day 14 and subsequently stained with 0.1% (w/v) toluidine blue (Aldrich Chemical Company, Inc. USA) and 1% (v/v) formalin (formaldehyde, ACE Chemical Company, Adelaide, Australia) in PBS for 2 hrs. The cultures were washed with water and air dried. Aggregates of greater than 50 cells were scored as CFU-F using an Olympus SZ-PT dissecting light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) as previously described (Gronthos *et al.*, 2003).

### 2.1.7 Normal Human Bone Cells (NHBC)

Trabecular bone specimens were obtained from rheumatoid arthritis patients during routine knee and hip replacements from the Department of Orthopaedic Surgery and Trauma at the Royal Adelaide Hospital. Bone chips were cultured in T-75 tissue-culture flasks in α-MEM-10. Medium was changed twice weekly for 5-6 weeks, and the cultures were incubated at 37° C in the presence of 5% $CO_2$ until confluent.

### 2.1.8 Normal Osteoblast Donor (NOD)

Osteoblast-like cells were also derived from normal healthy, as part of an Institutional Ethics Committee approved protocol. Briefly, donated bone marrow (as described in Section 2.1.6) was strained using a cell strainer (Becton Dickinson, USA). The small bone chips were washed with HHF and cultured in α-MEM-10. As described above, medium was changed two times per week for 5-6 weeks, and cultures were incubated at 37°C in the presence of 5% $CO_2$ until confluent.

### 2.1.9 Co-culture of OB-like Cells with Myeloma Cells

Human OB-like cells were labelled with CFSE (refer to Section 2.4.6) prior to seeding in T-25 tissue-culture flask at a cell density $1.3\times10^5$ cells in 5 ml α-MEM-10. Approximately $5\times10^5$ myeloma cells RPMI8226 were added to OB-like cells. The mAb IL-1β (MAB 601, R&D Systems) at a concentration 8 μg/ml was added to block the effect of IL-β. Following 3 or 5 days incubation at 37°C in the presence of 5% $CO_2$, cell supernatant was collected for protein detection and OB-like cells were detached using trypsin-EDTA digestion for flow cytometric analysis as described below.

### 2.1.10 Trypsin-EDTA Digestion of Adherent Cells

Adherent cells were routinely detached from tissue culture flasks using Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4 Na, GIBCO Invitrogen Corporation, Canada, Cat. No. 15400-054). Briefly, the medium was removed and the cells rinsed once with 10 ml of sterile PBS. After removal of PBS by aspiration, the 1ml or 2ml of the trypsin solution was added to the cell monolayers for a 25 and 75 $cm^2$ flask, respectively. Alternatively, 2 mM EDTA was used for the detachment of human OB-like cells for RANKL staining. The cultures were incubated at 37°C for 5 mins and the trypsin was inactivated in the presence of FCS by washing the cells once in HHF. The cells were pelleted by centrifugation at 274× *g* for 5 mins in preparation for use in a variety of different assay systems as described elsewhere.

### 2.1.11 Enzymatic Digestion of Established Osteoblast-like Cell Cultures

Single-cell suspensions were obtained from confluent primary OB-like cell cultures by enzymatic digestion. The cells were washed twice in PBS, and digested in a 2 ml solution comprised of collagenase (1 mg/ml, Worthington Biochemical Corporation, NJ, USA) and dispase (1 mg/ml, Boehringer Mannheim, Germany, Cat. No. 165859) per 75 $cm^2$ surface area for 45 mins at 37°C. The detached cells were transferred to a 50 ml polypropylene conical tube (Falcon®, Becton Dickinson Labware, USA). Cells which remained attached were subsequently removed by trypsin detachment as described above (Section 2.1.10). The cells were pooled pelleted by centrifugation at 274× *g* for 5 mins and washed with HHF.

### 2.1.12 Cryopreservation of Cells

Cells were cryopreserved in FCS containing 10% of the cryoprotectant, dimethyl sulphoxide (DMSO, BDH AnalaR® Merck Pty Limited, Victoria, Australia). Immediately prior to freezing, 0.5 ml of pre-cooled sterile freeze mix (20% DMSO in FCS) was added dropwise with mixing to 0.5 ml of FCS containing 5-10 × $10^6$ cells. The cell mixture was kept on ice and immediately transferred to cryoampoules (Greiner Labortechnik) and control-rate cryopreserved in a control rate freezer (KRYO 10 series) at 1°C/minute from 5°C to –12°C, 4°C/minute from –12°C to –20°C, 0°C for 5 minutes, at 1°C/minute from –20°C to –40°C and 3°C/minute from –40°C to –80°C and long term stored in liquid nitrogen storage (-196°C).

### 2.1.13 Thawing Cryopreserved Samples

Following removal from liquid nitrogen storage, cryoampoules were rapidly thawed in a 37°C water bath. The cells were transferred to a 14 ml polypropylene round-bottom tube (Becton Dickinson, NJ, USA) and 3 ml of thaw medium (refer to Section 2.1.13) or appropriate cell culture medium was added. The sample was made up to a final volume of 10 ml and the cells pelleted by centrifugation at 274× *g* for 5 minutes. The sample was subsequently washed twice in medium to remove residual DMSO prior to resuspending cells in appropriate growth as described above.

## 2.2 CELL PROLIFERATION ASSAYS

### 2.2.1 Reagents Used in This Study

Recombinant soluble human Interleukin -1β (IL-1β) and IL-6 was generously provided by Amgen Inc, Thousand Oaks, CA, USA. Recombinant ET-1 and its antagonists BQ123 and BQ788 were purchased from Phoenix Pharmaceuticals, Inc., USA. Recombinant human TNF-α and sIL-6R were purchased from R&D Systems, Inc., USA. Indomethacin was kindly provided by Dr. David Haynes, Deparment of Pathology, The University of Adelaide.

### 2.2.2 Cell Proliferation Assay

Human OB-like cells were seeded in 96-well plates (Nalge Nunc International, Denmark), at a density of 2×$10^3$ cells per well in 100 µl of α-MEM-10. Cells were treated with IL-1β at different concentrations for 1, 3, 5 and 7 days. Alternatively, human OB-like

cells were treated with 10 ng/ml IL-1β and indomethacin at different concentrations as indicated in the figures. The relative number of viable cells in each well was determined using 10 µl per well colorimetric assay reagent WST-1 (Roche Molecular Biochemicals, Cat. No. 1644807). WST-1 is a tetrazolium salt which is cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenase in the sample that leads to an increase in the amount of formazan dye. After a 2 hr incubation at 37°C in the dark, the colour of the medium changed in accord with cell viability and cell number. The absorbance of dye solution was directly measured with an ELISA reader (BIO-RAD Model 3550 microplate reader, CA, USA) using the test wavelength of 450 nm. Different treatment groups were performed in triplicate and the results were compared against controls using the Student's *t*-test.

Alternatively, the cells were treated with recombinant human IL-6 and/or soluble IL-6R, TNF-α, or ET-1 and its antagonists BQ123 and BQ788 at different concentrations. On day1, 3, 5 and 7, the cell number and viability were determined using the WST-1 colourimetric assay as described above.

To measure human OB proliferation in response to enforced expression of ET-1, cells were seeded using the automated cell deposition unit (ACDU) facility of the FACStar$^{PLUS}$ cell sorter. Briefly, human OB-like cells from three different donors were infected with pRUF or pRUF-ET-1 (as described in Section 2.5), and seeded into 96-well plates at a cell density of 4000 cells per well. Cells were incubated at 37°C for 3 and 5 days in α-MEM-10 culture medium and the cell number was determined by WST-1 as described before.

## 2.3 EXAMINATION OF GENE EXPRESSIONIN MYELOMA AND OSTEOBLAST-LIKE CELLS

### 2.3.1 TRIzol™ Isolation of Total RNA

Total RNA was routinely extracted from 0.5-1.0×$10^6$ cells using TRIzol™ (Life technologies, Cat. No. 15596-026) as per manufacturers instructions. Briefly, cell pellets were solubilised by passing the lysate through the pipette a few times. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. To each tube, 0.2 ml of chloroform (APS

Finechem, A division of Asia Pacific Specialty Chemicals Limited CAN, NSW, Australia) was added per 1 ml of TRIzol Reagent. The tubes were shaken vigorously by hand for 15 seconds and incubated at RT for 2 to 3 mins. The samples were centrifuged at 12,000×g for 15 mins in a refrigerated centrifuge to separate the mixture into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The RNA which partitioned with the aqueous phase was carefully removed and transferred to a fresh 1.5 ml eppendorf tube (Scientific Specialties Incorporated Life Technologies, Australasia). The total RNA was precipitated by the addition of 0.5 ml of isopropyl alcohol (BDH Chemicals, Victoria, Australia) per 1 ml of TRIzol Reagent used for the initial homogenization. Following incubation for 10 mins at RT, the RNA was pelleted by centrifugation at 12,000 × *g* for 10 mins at 4°C. The RNA pellet was washed once with 75% (v/v) ethanol, by adding 1 ml of 75% ethanol per 1 ml of TRIzol Reagent used for the initial homogenization. The samples were mixed by vortexing and were centrifuged at 7,500 × *g* for 5 mins at 4°C. The resultant RNA pellet was briefly air-dried at 37°C for 5-10 mins and the resuspended in RNase free diethylpyrocarbonate (DEPC, Sigma Aldrich Ltd)-treated Milli-Q water. Tofacilitate RNA solubility, the samples were incubated for 10 mins at 55 to 60°C. The concentration of RNA was determined by spectrophotometry, and the integrity of the RNA was determined by 1% (w/v) agarose gel electrophoresis. RNA was strored at –80°C or used immediately for cDNA synthesis.

### 2.3.2 Determination of RNA Concentration

The concentration of RNA in solution was determined by measuring the absorption at 260 nm on a Beckman UV spectrophotometer (Beckman Instruments, Mt. Waverley, Victoria, Australia), assuming that an $A_{260}$ of 1.0 represents 40 µg/ml of RNA. Alternatively, the sample was electrophoresed in agarose gels and the intensity of the sample's ethidium bromide-stained bands compared with the intensities of bands containing known concentrations of RNA standards.

### 2.3.3 Reverse Transcription (RT) Polymerase Chain Reaction (PCR) Amplification of DNA

#### 2.3.3.1 Synthesis of Complementary DNA (cDNA)

Total cellular RNA was prepared from cell lines or tissues using TRIzol reagent, as described in Section 2.3.1. The following components were added to a nuclease-free

microcentrifuge tube: 1 µl random hexamers (Geneworks, Cat. No. RP-6) at the concentration of 250 ng/µl, 1µg of total RNA and Rnase-free DEPC-treated Milli-Q water to 12 µl. The mixture was heated at 70°C for 10 mins and quickly chilled on ice. The contents of the tube was collected by brief centrifugation and was then added to a mixture containing 4 µl of 5× first strand buffer, 2 µl of 0.1M DTT, and 2 µl of 5 mM dNTP Mix (5 mM each dATP, dGTP, dCTP and dTTP at neutral pH). The contents of the tube were mixed gently and incubated at room temperature for 10 mins. One µl (200 units) of SUPERSCRIPT™ II (Gibco Life Tech, Cat. No. 18064-014) was added and mixed by pipetting gently up and down. The mixture was incubated at 42°C for 50 mins and the reaction was inactivated by heating at 70°C for 10 mins. The cDNA was then used as a template for amplification in PCR.

### 2.3.3.1 Polymerase Chain Reaction (PCR) Amplification of cDNA

Complementary DNA was amplified by PCR to generate products corresponding to mRNAs of interest. One µl of each cDNA synthesis reaction was utilised as template DNA in each PCR reaction. Routinely, the cDNA mixture was added to a 500 µl microcentrifuge tube, to which 2 µl of 10 × PCR amplification buffer (10 × PCR buffer: 670 mM Tris HCl pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5% Triton-X 100, 2 mg/ml gelatin), 1.2 µl of 25 mM $MgCl_2$, 2 µl of 2 mM dNTP mixture, 100 ng each of the appropriate sense and antisense primers (table 2.1), 0.2 µl (1 Unit) of Amplitaq DNA Polymerase (Applied Biosystems, Roche, Switzerland), and DEPC-treated water were added to a final volume of 20 µl. Amplitaq Gold is a "hot start" DNA polymerase that has an inactivating protein modification that only allows the enzyme to become active at high temperatures. In most instances, amplification was achieved by incubation in a Corbett Research thermal cycler (Corbett Research, N.S.W., Australia). DNA was amplified under the following typical cycling conditions: denaturation at 94°C for 1 min, annealing at the temperatures suitable for different primers for 1 min, extension at 72°C for 1 min for 20-40 cycles, with a final 10 mins extension performed at 72°C. Such that all products were assayed in the exponential phase of the amplification curve. Following amplification, the PCR products (10 µl of each reaction mixture) were separated on 2% (v/v) agarose gel according to molecular weight, stained with ethidium bromide and visualised using a Fluorimager at 532nm wavelength. The product bands were semi-quantitated using ImageQuant software and plotted as a histogram of the ratio of

specific gene expression relative to the expression of GAPDH, a housekeeping gene, which was used as an internal control for mRNA integrity.

### 2.3.4 Primers Used in This Study

All the primers involved in this study are presented in Table 2.1. The cycle numbers were determined by cycle course analysis, and annealing temperatures were determined empirically using AmpliTaq Gold in a gradient thermal cycler.

## 2.4 IMMUNOLOGICAL STUDIES EXAMINING CELL SURFACE ANTIGEN EXPRESSION

### 2.4.1 Buffers and Fixatives

#### 2.4.1.1 Blocking Buffer for Flow Cytometric Analysis

HBSS was supplemented with 0.4% (w/v) bovine serum albumin, 4%(v/v) normal human serum (NHS; Red Cross, Adelaide, South Australia), 5%(v/v) FCS and penicillin (50 i.u./ml)/streptomycin sulphate (50 μg/ml). The buffer was filter sterilised through a 0.22 μm bottle filter and stored at 4°C.

#### 2.4.1.2 Magnetic Activated Cell Sorting Buffer (MACS Buffers)

Single strength $Ca^{2+}$ and $Mn^{2+}$ free PBS was supplemented with 1% (w/v) BSA, 5mM ethylenediaminetetra-acetic acid (EDTA, BDH Chemicals) and 0.01% sodium azide ($NaN_3$, BDH Chemicals). The buffer was filter sterilised and stored at 4°C.

#### 2.4.1.3 Flow Cytometry Fixative (FACS Fix)

Single strength PBS was supplemented with 1% (v/v) formalin, 2% (w/v) D-glucose, and 0.02% (w/v) sodium azide. The buffer was stored at 4°C.

### 2.4.2 Single-Colour Flow Cytometric Analysis

Flow cytometry was performed using an Epics®-XL-MCL analyser (Beckman Coulter). Cells to be analysed were immunostained with primary antibodies that recognise the antigen of interest. Cell populations were analysed on the basis of their forward and side light scattering properties, which is indicative of cell size and intracellular granules, respectively.

Table 2.1: RT-PCR primers and conditions for the specific amplification of human mRNA.

| Target Gene | Sense/ Antisense (5'-3') Primer Sequences | Annealing Temperature (°C) | Cycle number | Product Size | Ref. |
|---|---|---|---|---|---|
| GAPDH | CACTGACACGTTGGCAGTGG/ CATGGAGAAGGCTGGGGCTC | 60 | 18-21 | 417 | 1 |
| IL-1β | AGGAAGATGCTGGTTCCCTCTC/ CAGTTCAGTGATCGTACAGGTGC | 60 | 30 | 151 | 1 |
| IL-1R type I | AAGATGGTGACTCCCTCCTG/ GGCTTGTTCTGTAGATACAG | 62 | 35 | 269 | 2 |
| IL-6 | ATGAACTCCTTCTCCACAAG/ GTGCCTGCAGCTTCGTCAGCA | 62 | 30 | 546 | 2 |
| IL-6R | CCCATGCAGGCACTTACTAC/ GTGGCTCGAGGTATTGTCAG | 62 | 35 | 347 | 2 |
| TNF-α | TCAGATCATCTTCTCGAACC/ CAGATAGATGGGCTCATACC | 62 | 35 | 361 | 2 |
| COX2 | AACACAACAGAGTATGCGATGTG/ CAATGGAAGCCTGTGATACTTTC | 62 | 30 | 434 | 3 |
| M-CSF | CAGTTGTCAAGGACAGCAC/ GCTGGAGGATCCCTCGGACTG | 58 | 35 | 670 | 2 |
| PTHrP | TGGGCTACTCCGTGTCCCTG/ GACATTGGTCACACTTGTGTGG | 58 | 36 | 547 | 2 |
| RANKL (4/5) total | AATAGAATATCAGAAGATGGCACTC/ TAAGGAGGGGTTGGAGACCTCG | 62 | 35 | 665 | 2 |
| RANKL (7/5) total | AACAGGCCTTTCAAGGAGCTG/ TAAGGAGGGGTTGGAGACCTCG | 62 | 35 | 535 | 2 |
| RANKL (8/5)TM | GCAGCGTCGCCCTGTTCTTCTA/ TAAGGAGGGGTTGGAGACCTCG | 62 | 35 | 709 | 4 |
| sRANKL (9/5) | ATCTTCAGAGTTTCGACTTTATCAAC/ TAAGGAGGGGTTGGAGACCTCG | 62 | 35 | 829 | 5 |
| OPG | TGCTGTTCCTACAAAGTTTACTG/ CTTTGAGTGCTTTAGTGCGTG | 62 | 30 | 435 | 2 |
| CBFA-1 | GTGGACGAGGCAAGAGTTTCA/ TGGCAGGTAGGTGTGGTAGTG | 62 | 30 | 632 | 6 |
| BSP | TCAGCATTTTGGGAATGGCC/ GAGGTTGTTGTCTTCGAGGT | 62 | 30 | 666 | 6 |
| OCN | ATGAGAGCCCTCACACTCCTC/ CGTAGAAGCGCCGATAGGC | 62 | 30 | 289 | 6 |
| OPN | AGCCGTGGGAAGGACAGTTATG /GAGTTTCCATGAAGCCA CAAAC | 62 | 30 | 471 | 7 |
| BMP-2 | GGAAGAACTACCAGAAACGAG/ AGATGATCAGCCAGAGGAAAA | 55 | 32 | 656 | 9 |
| ALP (bone type) | TGCTCCCACGCGCTTGTGCCTGGA/ CTGGCACTAAGGAGTTAGTAAG | 58 | 28-30 | 213 | 8 |

| Target Gene | Sense/ Antisense (5'-3') Primer Sequences | Annealing Temperature (°C) | Cycle number | Product Size | Ref. |
|---|---|---|---|---|---|
| ET-1 | GCTCGTCCCTGATGGATAAA/ TTCCTGCTTGGCAAAAATTC | 58 | 30♣ 36♣♣ | 215 | 10 |
| $ET_AR$ | CAGTGAACATCTTAAGCAGCGT/ CGGTCTGTGTTGTGGTTGTTTT | 62 | 31♣ 36♣♣ | 377 | 11 |
| $ET_BR$ | TTGGAGCTGAGATGTGTAAGC/ CAGTGAAGCCATGTTGATACC | 62 | 36 | 625 | 12 |
| $ET_BR\Delta5$ RP | CCAACAGAAAGCTGCTTTAGG (located at the end of exon 4 and at the beginning of exon 6) | 62 | 40♣ 36♣♣ | 455 | 12 |
| $ET_BR$ full length-RP | GACCAGGCAAAAGACGGTTTT (located in exon 5) | 62 | 36 | 481 | 12 |
| ECE-1 | TGCCATCTACAACATGATAG / CTTCTTCACCCAGTTCTG | 58 | 33♣ 35♣♣ | 570 | 13 |
| pRUF | TCAAAGTAGACGGCATCG/ CGACATTGGGTGGAAACA | For sequence | | | |

♣ Osteoblast-like cells ♣♣ Myeloma cell lines

1. Atkins *et al.*, 2000b
2. Atkins *et al.*, 2000a
3. Genebank accession number NM_000963
4. Genebank accession number AF019047
5. Genebank accession number AB037599
6. Gronthos *et al.*, 1999
7. Genebank accession number J04765
8. Genebank accession number NM_000478 (Sato *et al.*, 1994)
9. Genebank accession number NM-001200 (Wordinger *et al.*, 2002)
10. Genebank accession number NM_001955
11. Genebank accession number NM_001957 (Kasperk *et al.*, 1997)
12. Genebank accession number L06623
13. Genebank accession number NM_001397 (Zolk *et al.*, 1999)

Cells were harvested from culture ($2\times10^5$ cells per stain), and pelleted by centrifugation at 274×*g* for 5 mins at 4°C. Prior to immunolabelling, cells were incubated in blocking buffer at a concentration of $5\times10^6$ cells/ml, and were incubated on ice for 30 mins. For each condition, aliquots of $2\times10^5$ cells were incubated with 100 µl of antibody or isotype-matched control (used as culture supernatants or purified mAbs at a final concentration of 10 µg/ml). To examine the specificity of mAb RANKL, 100 µl solutions of mAbs RANKL and its isotype control 1A6.11 at a concentration of 10 µg/ml were incubated with 300 ng/ml SF-21 insect cell-derived RANKL protein (R&D system) for 1 h at 37°C prior to use. After a 45 min incubation at 4°C, the cells were washed twice in chilled HHF to remove unbound antibody, and the pellets resuspended in 50 µl HHF containing 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE) conjugated goat anti-mouse Ig (Southern Biotechnology Associates Inc. Birmingham, AL, USA). Following incubation with secondary antibody for a further 45 mins at 4°C, cells were washed as above and fixed in FACS fix. Typically, for each sample 10,000 events were analysed on the flow cytometer, and stored as list mode data for further analysis using Winmdi software (version 2.8, Joseph Trotter).

### 2.4.3 Two-Colour Flow Cytometric Analysis

Single cell suspensions were incubated in blocking buffer for 30 mins. For each condition, aliquots of $2\times10^5$ cells were incubated with 100 µl of STRO-1 supernatant and 100 µl of B4-78 (anti-ALP mAb) or anti RANKL mAb at the concentration of 10 µg/ml for 1 hr on ice. The isotype matched negative control antibodies, IgM (1A6.12) and $IgG_1$ (1B5) or $IgG_{2b}$ (1A6.11) were used under identical conditions. The cells were then washed in HHF and incubated with a goat anti-mouse IgM-PE (1:50) and a goat anti-mouse IgG–FITC (1:50) for 45 mins on ice. The cells were then washed twice and resuspended in 300 µl of FACS fix for analysis.

### 2.4.4 Three-Colour Flow Cytometric Analysis

To examine the relationship between cycle status and cell surface antigens, three-colour flow cytometric analysis was utilised. Single cell suspension of OB-like cells were dual stained with ALP and STRO-1 using a goat anti-mouse IgG-FITC and IgM-PE conjugated second label as described in Section 2.4.3. After staining, the cells were fixed in

0.5% (wv) paraformaldehyde in PBS ($2\times10^6$ cells/ml) for 30 mins at 4°C. Cells were washed once in TPBA (PBS supplement with 0.1% Trixton 100, 0.1% BSA and 0.1% sodium azide) and resuspended in 500 µl TPBA containing 5µg RNAse (Roche Diagnostics, Castle Hill, NSW, Australia, Cat. No. C109169). The cells were incubated at 37° C for 20 mins and washed once by TPBA. The cells were resuspended in 500 µl PBA (PBS supplement with 0.1% BSA and 0.1% sodium azide) containing 5 µg/ml 7-aminoactinomycin D (7-AAD, Molecular Probes, Netherlands, Cat. No. A-1310) and stored in dark at 4°C until ready to analyse (at least 1 hr after staining).

To examine the characteristics of mesenchymal stem cells derived from normal BMMNCs, three-colour flow cytometric analysis was performed. Single cell suspension of BMMNCs was incubated with 100 µl of STRO-1 supernatant and 100 µl of anti-RANKL mAb at the concentration of 10 µg/ml for 1 hr on ice. The isotype matched negative control antibodies, IgM (1A6.12) and or $IgG_{2b}$ (1A6.11) were used under identical conditions. The cells were washed in HHF and incubated with a goat anti-mouse IgM-biotin (1:50) and a goat anti-mouse IgG–FITC (1:50) for 45 mins on ice. The cells were washed twice in HHF and incubated with Streptavidin-PECy5 (Immunotech, France) and FITC direct conjugated CD3, 14, 19 or glycophorin for 30 mins on ice. The isotype matched negative control antibody, IgG-FITC, was used under identical conditions. Cells were washed twice and resuspended in 300 µl of FACS fix for analysis.

### 2.4.5 Detection of Intracellular Antigens

Single cell suspensions were blocked in blocking buffer for 30 mins on ice and then washed twice in PBS. After fixing in 1% (w/v) paraformaldehyde (BDH Chemical Ltd, England) in PBS for 20 mins at room temperature, cells were washed twice by HHF containing 0.1% saponin (w/v, Sigma Cat. No. S-4521). The cells were resuspended with 50 µl of culture supernatant or purified mAbs at a final concentration of 10 µg/ml (diluted in HHF containing 0.1% saponin). Following a 60 min incubation at 4°C, the cells were washed twice in HHF (containing 0.1% saponin), and resuspended in wash medium containing a 1:50 dilution of goat anti-mouse Ig-FITC or Ig–PE (γ-chain or µ-chain-specific determined by primary antibody Ig class). After a further incubation at 4°C for 45 mins, the cells were washed as described above and fixed in FACS fix for analysis.

### 2.4.6 Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Labelling of OB-like Cells

The cell-permeant fluorescein-based dye CFSE was used to study division-related phenotypic and functional changes during OB-like cell differentiation. CFSE covalently attaches to cytoplasmic components of cells, resulting in uniform bright fluorescence, which upon cell division is equally distributed between daughter cells. This technique allows the resolution of up to eight cycles of cell division by flow cytometry.

The OB-like cells were detached as described previously. The cells were washed once, resuspended in 1 ml of PBS/0.1% BSA and 2 µl of 5 mM CFSE (final 10 µM) was added prior to incubating at 37°C for 10 mins. The staining was quenched by the addition of 5 volumes of ice cold culture medium α-MEM-10 and incubated on ice for 5 mins. The cells were washed three times in the culture medium and *in vitro* cultures were established as described below.

At appropriate time points, the cells were detached by Trypsin-EDTA and stained with STRO-1 or ALP conjugated to PE as described in 2.4.2. Dual-colour flow cytometric analysis was performed to analyse samples.

### 2.4.7 Fluorescence-Activated Cell Sorting (FACS)

Routinely, approximately $1x10^7$ OB-like cells were detached as described in Section 2.1.11 and resuspended in 2 ml (100 µl/ $5\times10^5$ cells) of a saturating concentration of STRO-1 supernatant for 60 minutes on ice. The monoclonal mouse isotype control IgM (1A6.12) was used under identical conditions. The cells were washed in HHF and incubated with a goat anti-mouse IgM–PE (1/50) in a final volume of 1 ml (50 µl/ $5\times10^5$ cells) for 45 mins on ice. The cells were resuspended to a final concentration of $10^6$ cells/ml and passed through a cell strainer to obtain a single cell suspension before analysis using a FACStar$^{PLUS}$ flow cytometer. Positive staining for STRO-1 was defined as the level of fluorescence which was greater than 99% of the corresponding isotype-matched control antibody. STRO-1 negative (STRO-1$^-$) and bright positive (STRO-1$^{bright}$) cells were collected and cultured as described below.

Alternatively, MACS sorted STRO-1$^+$ BMMNCs (see below) were further stained with anti-RANKL mAb using a goat anti-mouse IgG-PE conjugated second label as described in Section 2.4.2. Dual-colour flow cytometric analysis was performed to sort STRO-1$^{bright}$ /RANKL$^{bright}$, STRO-1$^{intermediate}$/RANKL $^{intermediate}$, STRO-1$^-$/RANKL$^+$, STRO-1$^+$/RANKL$^-$, and double negative cells. CFU-F cultures were subsequently cultured in α-MEM-20.

### 2.4.8 Magnetic Activated Cell Sorting (MACS)

This procedure was used to isolate specific populations of cells from BMMNCs, prepared as described in 2.1.6. Briefly, $1\times10^8$ BMMNCs were incubated in blocking buffer for 15 mins on ice. Cells were pelleted by centrifugation, and subsequently resuspended in saturating levels of mouse IgM or IgG mAb supernatant in a 14 ml polypropylene tube and incubated on ice for 60 mins. The cells were then washed twice in HHF or PBS supplemented with 2% (w/v) BSA and resuspended in 1 ml of buffer containing the appropriate second layer isotype specific goat anti-mouse IgM or anti-mouse IgG biotinylated antibody (1/50 dilution) (Southern Biotechnology Associates, Inc. USA), for 45 mins on ice. The cells were washed twice in MACS buffer and resuspended in 900 µl MACS Buffer to which 100µl streptavidin-microbeads (Miltenyi Biotec; Bergisch Gladbach, F.R.G.) was added (10µl of microbeads/$10^7$ cells in 90 µl) and the mixture incubated on ice for 15 mins. Streptavidin-FITC conjugate (1/50) (CALTAG, Burlingame, CA) was added directly to the same suspension in the last 10 mins of incubation. The cells were then washed twice in MACS Buffer and a small aliquot removed for flow cytometric analysis while the remaining cells were separated on a Mini MACS magnetic column (column capacity $2\times10^8$ cells, Miltenyi Biotec) essentially as recommended by the manufacturer. The eluate, containing the negative cell fraction was collected from the column and non-specifically bound cells removed by washing the column 3 times with 0.5 ml MACS buffer. The bead-positive cells bound to the column were isolated by first removing the column from the magnet and then flushing it with 1 ml MACS buffer. To determine the purity and yield of each cell population, a sample of each of the unfractionated bulk, bead-positive and bead-negative cell fractions was analysed by flow cytometry. .

### 2.4.9 Immunofluorescence Staining for Confocal Microscopy.

To examine the localisation of STRO-1 and RANKL on the OB-like cell surface, aliquots of $2\times10^5$ human OB-like cells were incubated in blocking buffer for 30 mins. Cells were incubated with 100 µl of STRO-1 supernatant and 100 µl of anti-RANKL antibody at the concentration of 10 µg/ml for 1 hour on ice. The isotype matched negative control antibodies, $IgG_{2b}$ (1A6.11) and IgM (1A6.12) were used under identical conditions. The cells were then washed in HHF and incubated with a goat anti-mouse IgM–FITC (1:50) and a goat anti-mouse IgG-Texas Red (1:50) (Southern Biotechnology Associates Inc. Birmingham, AL, USA) for 45 mins on ice. Alternatively, MACS sorted STRO-$1^+$ BMMNCs were incubated with 100 µl of mAb RANKL at a concentration of 10 µg/ml for 45 mins on ice. The cells were then washed in HHF and incubated with a goat anti-mouse IgG–FITC (1:50) and a streptavidin-Texas Red conjugate (1:50) (Southern Biotechnology Associates Inc. Birmingham, AL, USA) for 30 mins on ice. The cells were then washed twice and resuspended in 300 µl of FACS fix. The stained cells were subsequently transferred to slides using a Cytospin 2 centrifuge (Shandon) at 500 rpm for 5 mins. The cells were mounted with anti-fade solution (1.8 % w/v of DABCO, 2 mM Tris pH 7.5 in Glycerol) and examined using a Bio-Rad radiance 2100 confocal microscope (Bio-RAD microscience Ltd, UK).

### 2.4.10 Antibodies Used in This Study

All the antibodies used in this study are listed in the table 2.2.

## 2.5 PREPARATION OF A RETROVIRAL CONSTRUCT HARBOURING THE HUMAN ET-1 cDNA

### 2.5.1 Buffers and Reagents

#### 2.5.1.1 Luria-Bertani (LB) Broth

1% Bacto-tryptone (Becton Dickinson Microbiology Systems), 0.5% Bacto™ yeast extract (Becton Dickinson Microbiology Systems), 1% NaCl were made up to 1L with RO water and sterilised by autoclaving.

#### 2.5.1.2 L-agar Plates

1% Bacto-tryptone, 0.5% Bacto™ yeast extract, 1% NaCl, 1.5% Bacto™ agar (Becton Dickinson Microbiology Systems) were made up to 500ml with RO water. Following autoclave sterilisation, the solution was cooled to approximately 60°C prior to the addition

Table 2.2: List of Antibodies Used in This Study

| Antibody Name | Specificity | Isotype | Source |
|---|---|---|---|
| 1A6.11 | Isotype-matched negative control/α-Salmonella | $mIgG_{2b}$ | Dr. L. Ashman[1] |
| 1A6.12 | Isotype-matched negative control/α-Salmonella | mIg M | Dr. L. Ashman |
| 1B5 | Isotype-matched negative control/ α-Giardia | $mIg\ G_1$ | Dr. G. Mayrhofer[2] |
| 1D4.5 | Isotype-matched negative control/α-Salmonella | $mIgG_{2a}$ | Dr. L. Ashman |
| α-MAP | Multiple antigenic peptide(MAP, HBV 21-47 pre $S_1$) | rIg G | Dr. G. Atkins[3] |
| B4-78 | anti-human bone/liver/kidney isoform of ALP | $mIgG_1$ | DSHB[4] |
| CD3-FITC | Main antigen expressed by T cell | $mIgG_1$ | Becton Dickinson[5] |
| CD14-FITC | Main antigen expressed by monocytes | $mIgG_1$ | Immunotech[6] |
| CD19-FITC | Main antigen expressed by B cell | $mIgG_1$ | Immunotech |
| CD38-PE | Main antigen expressed by activated T cell, plasma cells | $mIg\ G_1$ | Immunotech |
| ET-1 | Mouse monoclonal anti-endothelin-1 (ascites fluid, 7mg/ml, 1:1000 dilution for ELISA) | $mIgG_{2a}$ | Sigma[7] |
| ET-1 | Mouse monoclonal anti-endothelin-1 (ascites fluid, 1:250 dilution for immunohistochemistry) | $mIgG_1$ | Affinity BioReagents[8] |
| $ET_BR$ | Rabbit Anti-endothelin receptor type B (stock 0.3mg/ml, 1:200 dilution, 1.5ug/ml) | rIgG | Sigma |
| Glycophorin A | CD235a, main antigen expressed by red cells | $mIgG_1$ | Immunotech |
| $IgG_1$-FITC | Isotype-matched negative control | $mIgG_1$ | Immunotech |
| RANKL | Receptor activator of nuclear factor-κB ligand | mIg G | Santa Cruz[9] |
| STRO-1 | Erythroid, Stromal cells, CFU-F, Endothelial cells | mIgM | DSHB |
| TRANCE | TNF-related activation induced cytokine | $mIgG_{2b}$ | R&D[10] |

Dr. L. Ashman[1]: School of Biomedical Science, The University of Newcastle, NSW, Australia

Dr. G. Mayrhofer[2]: Molecular Lifescience, University of Adelaide, South Australia, Australia

Dr. G. Atkins[3]: Department of Orthopaedics and Trauma, University of Adelaide, South Australia

DSHB[4]: Developmental Studies Hybridoma Bank, University of Iowa, IA, USA

Becton Dickinson[5]: Becton, Dickinson and Company, NJ, USA

Immunotech[6]: Bio Online, Inc. Berkeley, CA, USA

Sigma[7]: Sigma-Aldrich, Inc. Saint Louis, Missouri 63103, USA

Affinity BioReagents[8]: Affinity BioReagents, Inc. 4620 Technology Drive, Suite 600 Golden, CO 80403, USA

Sants Cruz[9]: Santa Cruz Biotechnology, Inc. Santa Cruz, California, 95060, USA

R&D[10]: R&D Systems Inc. Minneapolis, MN, USA

of ampicillin to a final concentration of 100 μg/ml. Approximately 25ml of agar solution was poured into each plate, and allowed to set by incubating in a 37°C incubator overnight.

#### 2.5.1.3 SOC Medium

2% Bacto-tryptone, 5% yeast extract and 10 mM NaCl were dissolved in Milli-Q water, then 2.5 mM KCl (BDH) was added and pH was adjusted using NaOH (BDH). Following sterilisation by autoclaving, sterile 10 mM $MgCl_2$ (BDH) and 20 mM glucose were added to the solution.

### 2.5.2 DNA Synthesis by PCR using Pfu DNA Polymerase

Human OB-like cell cDNA was synthesised as described in Section 2.3. A Proofreading DNA polymerase *Pfu* was used in PCR reaction to obtain high-fidelity DNA synthesis. Briefly, 1 μl of cDNA was added to a 500 μl microcentrifuge tube, to which 2 μl of 10 × Native *Pfu* DNA polymerase reaction buffer, 2 μl of 2 mM dNTP mixture, 100 ng each of the ET-1 sense and antisense primers (Figure 2.1), 0.4 μl (1.2 Unit) of Native *Pfu* DNA Polymerase (Stratagene Cloning Systems, USA & Canada), and DEPC-treated water were added to a final volume of 20 μl. Amplification was achieved by incubation in a Corbett Research thermal cycler as described above, and DNA was amplified under the following typical cycling conditions: denaturation at 95°C for 45 secs, annealing at 58°C for 45 secs, extension at 72°C for 1 min for 30 cycles, with a final 10 min extension performed at 72°C. Following amplification, 2 μl of the PCR product was separated on 2% (v/v) agarose gel and visualised by ethidium bromide staining under UV light at 532 nm. The remaining PCR product was precipitated with sodium acetate (BDH, MERCK Pty, Limited. Victoria, Autralia). Briefly, the PCR product volume was raised to 100 μl by adding 82 μl $H_2O$ and the mixture was removed to a new eppendorf tube. Ten μl of 3M sodium acetate pH 4.6 and 250μl absolute ethanol were added to the mixture and incubated at -70°C for more than 10 mins to precipitate DNA. The precipitated DNA was pelleted by centifugation at 21,000×*g* for 30 mins at 4°C. The DNA pellet was washed in 250 μl of 70% ethanol and air-dried.

### 2.5.3 Restriction Digestion of pRUF*neo* Vector and PCR Product

The retroviral vector pRUF*neo* (figure 2.1) and PCR product were digested with *Bam*HI and *Xho*I restriction enzymes to facilitate the directional cloning of the cDNA into

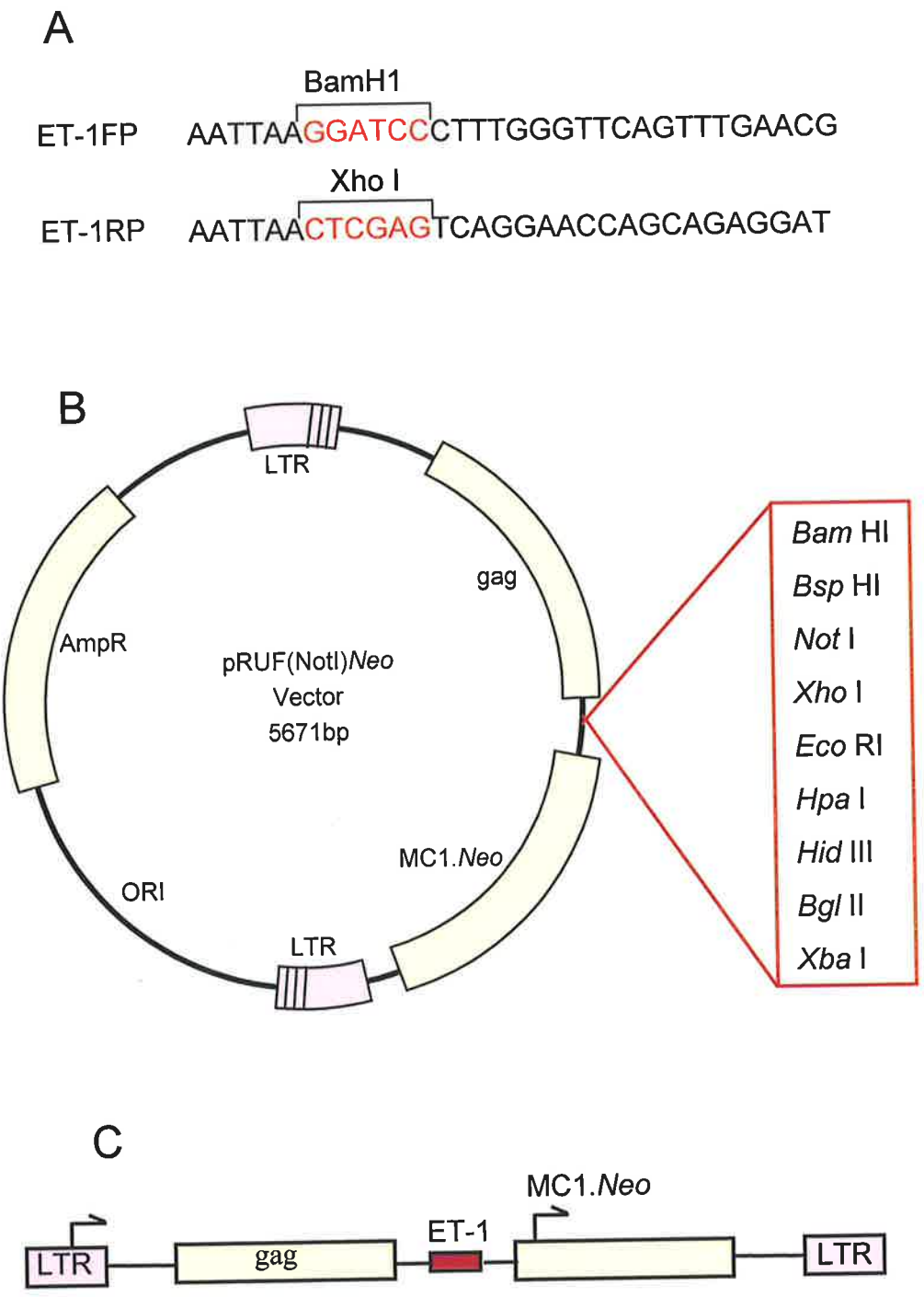



Figure 2.1. Cloning of ET-1 into the retroviral vector pRUF(*neo*). ET-1 primers with restriction enzymes (A), a schematic illustration of retroviral vector pRUF(*neo*) alone (B) and with ET-1 insert (C) are shown.

the unique sites present in the multiple cloning site (MCS) of pRUF*neo*. Digestion was performed as follow: 5 µg of the supercoiled pRUF*neo* vector and precipitated PCR product were resuspended in $H_2O$ to a final volume of 38 µl before the addition of 5 µl 10× *Bam*HI buffer (New England Biolabs, UK) and 10× BSA (10 mg/ml) to provide an optimal (1×) salt concentration for the restriction enzymes. One microliter (20 U) of both *Bam*HI and *Xho*I restriction enzymes (New England Biolabs, UK) was added, and the reaction was allowed to proceed for 2 hrs at 37°C. The entire restriction digest was resolved by 1% agarose gel electrophoresis and DNA was extracted and purified using BRESAclean (Geneworks, Adelaide, Australia).

### 2.5.4 Extraction of DNA from Agarose Gels

Following agarose gel electrophoresis and ethidium bromide staining, separated DNA fragments were visualised by long wave (365nm) ultraviolet light, and excised with a clean scalpel. The volume of agarose was estimated and the assumption made that 1 gram was equal 1ml. Three volumes of BRESA-SALT™ solution was added to the gel and incubated for 5 mins at 55°C to dissolve agarose with occasionally mixing until gel was melted. The BRESA-BIND™ was vortexed well and the silica matrix (a minimum of 5 µl plus 1 µl/µg DNA) was added to the melted gel. The mixture was incubated for 5 mins at RT to bind DNA with regular mixing to keep BRESA-BIND™ in suspension. BRESA-BIND™/DNA complex was pelleted for 5 seconds by centrifugation at 13,000×*g* and the supernatant was removed and set aside. The pellet was washed twice with BRESA-WASH™ by resuspending the pellet in a volume equivalent to the amount of salt used previously. The complex was pelleted again by centrifugation at 13,000×*g* for 5 seconds. All traces of the wash solution were removed by aspirating with a narrow pipette tip. The complex was air dried for 5 mins and DNA was recovered by resuspending the white pellet in water with a volume twice that of the matrix used previously. The pellet was incubated for 5 mins at 45 - 55°C and centrifuged for 1 min. The DNA supernatant was removed immediately after centrifugation and transferred to a new tube. The concentration and purity of DNA fragments were assessed by agarose gel electrophoresis of a proportion of the sample.

### 2.5.5 Ligation of Insert to Vector

The restriction enzyme digested PCR product and vector were semi-quantitated following electrophoresis on 1% agarose gel and stained with ethidium bromide. Typically,

20 ng PCR product (0.8kb) was ligated into 50 ng of vector (5.6kb) with a 3:1 insert:vector molar ratio in a 10 µl reaction volume comprising 1× ligation buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM ATP) and 3 Weiss U of T4 DNA ligase (Promega). After overnight incubation at 12°C, the ligation reaction was ready for transformation.

### 2.5.6 Preparation of Electrocompetent DH10B Cells for Transformations

One litre of superbroth growth medium was innoculated with 10ml of fresh overnight DH10B culture. Cells were incubated at 37°C on a shaking platform until an of $OD_{600}$=0.5-1.0 was achieved. The absorbance was measured using a spectrophotometer at 600nm against superbroth as blank. The flask was chilled on ice for at least 30 mins, and decanted into 4 pre-chilled 250-ml centrifuge tubes. Following centrifugation at 3,000×*g* for 15 mins at 4°C, the supernatant was removed and the pellet was gently resuspend in a small amount of cold water prior to completely filling with cold water. The cultures were centrifuged at 4,000×*g* for 15 mins at 4°C and subsequently resuspended in cold water as above. The cells were pelleted by centrifugation at 5,000×*g* for 15 mins and resuspended in 5 ml cold 10 % (v/v) glycerol in water, and centrifuged at 5,000×*g* for 10 mins at 4°C and resuspended in 1 ml 10% (v/v) glycerol. Cells were dispensed into 1.5 ml eppendorfs in 45 µl aliquots and snap frozen on dry ice and stored at –70°C.

### 2.5.7 Transformation of Competent Cells

One µl of the plasmid DNA and 40 µl of thawed electrocompetent cells (E.coli strain DH10B) were mixed together in a pre-chilled eppendorf tube and incubated on ice for 1 min. The cells were transferred to a pre-chilled electroporation cuvette and electroporated in a Bio-rad Gene Pluser with the following parameters: 1.6kV, 200Ω, 25uFd. One ml of SOC medium was added, and the cells were incubated at 37°C for 1hr with continuous agitation. One hundred µl of transformed bacteria was plated onto LB agar plates supplemented with 100 µg/ml of ampicillin and incubated at 37°C overnight.

### 2.5.8 Isolation and Analysis of Plasmid DNA

Single colonies were isolated from selection plates and cultured overnight in 2 ml of LB broth containing 100 µg/ml ampicillin at 37°C with continuous agitation. Cultures were pelleted by centrifugation at 13,000× *g* for 2 mins at 4°C in a microcentrifuge, and the

supernatants were removed by vacuum aspiration. Plasmid DNA was isolated and purified on QIAGEN™ "Mini-Prep" columns (QIAGEN Inc., Chatsworth, USA) as described by the manufacturers. To identify which recombinant plasmids contained the ET-1 insert, ET-1-specific PCR primers (table 2.1) was used. A 2 ml overnight culture of selected clones were used to inoculate 200 mls of LB medium supplemented with ampicillin, and grown overnight at 37°C with shaking. Bacterial cells were pelleted by centrifugation at 6,000×*g* for 15 mins at 4°C. The plamid DNA was extracted and purified on a QIAGEN-tip 100 column (QIAGEN Inc., Chatsworth, USA) according to the manufacturers recommendations. The eluted plasmid DNA was subsequently precipitated with the addition of 0.7 volumes of isopropanol. The precipitate was pelleted by centrifugation at 11,000×*g* for 30 mins at 4°C, washed in 70% ethanol and vacuum dried. The DNA was resuspended in sterile water, and quantitated by UV spectrophotometry at 260 nm.

### 2.5.9 Automated DNA Sequence Analysis

The ET-1 retroviral construct was directly sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) as recommended by the manufacturer. Routinely, 300 ng of DNA fragments and 100 ng of sequencing primer were added to 8 µl Big Dye Terminator Premix. The reaction was subjected to 25 cycles of PCR using the following cycling parameters: 96 °C/(30 secs), 50 °C/(15 secs), 60 °C/ (4 mins). Upon completion of thermal cycling, the products were precipitated with isopropanol at RT for 15 mins and pelleted by centrifugation at 14,000× *g* for 30 mins at 4 °C. The pellets were washed twice with 70% ethanol, vacuum dried and analysed with an automated DNA sequencer. The resultant polypeptide sequence of ET-1 was deduced from the nucleotide sequence using DNASIS software (Version 2.1, Hitachi Software Engineering Co., Ltd).

### 2.5.10 Transfection of the Retroviral Packaging Cell Line PT67 Using FuGENE 6

The retroviral packaging cell line, PT67, was plated out at $3\times10^5$ cells in 60 mm dishes 1 day prior to transfection. Three µl of FuGENE 6 Reagent (Boehringer Mannheim, Germany) was added directly to 150 µl of serum free DMEM medium and incubated at RT for 5 mins. One to 2 µg of purified DNA in a volume of 1µl was mixed with FuGENE mixture and following 15 mins incubation at RT, was added dropwise to the cells in 3 ml of medium. The cells were incubated at 37°C for 24 hrs, after which they were trypsinized and replated in 75 $cm^2$ flasks in DMEM-10 supplemented with 400 µg/ml G418 (Geneticin

liquid, GIBCO-BRL). The medium was changed every 2-3 days and cells selected for approximately 7 days or until mock transfected cells were dead.

### 2.5.11 Retroviral Infection of OB-Like Cells and Myeloma Cells

Approximately $6\times10^5$ human OB-like cells were plated in 75 $cm^2$ flask 24 hours prior to infection. The PT67-derived culture supernatant (containing the amphotropic retroviral particles) were harvested from either pRUF*neo* or pRUF*neo*-ET-1 transfected PT67 cells when in log phase growth and filtered thorugh a 0.45μm minisart filters (Sartorius, Germany). Aliquots of 10 ml was used to infect subconfluent OB-like cells in 75 $cm^2$ flasks. To facilitate infection, hexadimethrine bromide (Polybrene; Sigma) was added at a final concentration of 4 μg/ml to the virus-containing supernatant. OB-like cells were incubated with fresh viral-supernatant twice over a 48-hr period. Following infection, the infected OB-like cells were selected with G418 at a final concentration 400 μg/ml as described above. The G418 resistant OB-like cells were maintained with regular growth media supplemented with 200 μg/ml G418.

To infect the non-adherent myeloma cell line RPMI-8226, $3\times10^6$ pRUF or pRUF-ET-1 transfected PT67 cells were irradiated with 30Gy of γ radiation prior to culture in a T-75. Following 3 hrs incubation at 37°C in 10 ml DMEM-10, $1.5\times10^6$ RPMI-8226 and polybrene at a final concentration 4 μg/ml were added to the culture. After 2-3 days' co-culture, RPMI-8226 were harvested and selected with G418 at a final concentration 1.8 mg/ml for one week in regular RPMI-1640-10 growth medium. The G418 resistant RPMI-8226 cells were maintained with regular growth media supplemented with 400 μg/ml G418.

## 2.6 *IN VITRO* ASSAY OF BONE FORMING POTENTIAL

The conditions for the induction of human bone marrow stromal cells to develop a mineralised bone matrix *in vitro* have been reported by Gronthos et al (Gronthos *et al.*, 1994). Briefly, primary OB-like cells were seeded into 24-well plates at a cell density of $5\times10^4$/well, in α-MEM supplemented with 10% FCS, β-mercaptoethanol ($5\times10^{-5}$ M), dexamethasone sodium phosphate ($10^{-8}$ M), and $KH_2PO_4$ (1.8 mM) (BDH Chemicals, Australia) at 37°C in the presence of 5% $CO_2$. Human OB-like cells were treated with 10ng/ml of recombinant human IL-1β or ET-1. Alternatively, pRUF and pRUF-ET-1 infected OB-like cells were cultured in osteoinductive medium without any treatment.

Culture medium (1ml/well) was changed at weekly intervals for a period of 6 weeks. The cultures were sacrificed at week 4 and week 6 and calcium levels determined as described below. The cultures were washed three times with $Ca^{2+}$ and $Mg^{2+}$ free PBS and then solublized in 0.6 N HCl (200 µl per well) (Asain Pacific Specialty Chemicals Limited, Australia) overnight. Samples from each well were then reacted with o-cresol-phthalein-complexon, and the colormetric reaction measured at 570 nm. The absolute calcium concentration was determined by a standard curve for calcium according to the manufacturer's recommendations. The method used is based on the cresolphthalein complesone (CPC) method of Moorehead and Briggs (Moorehead *et al.*, 1974). CPC reacts with calcium and magnesium in alkaline solution to form a deeply coloured complex. The intensity of the purple colour formed is proportional to the calcium concentration and can be measured photometrically between 540 and 600 nm with maximum absorbance at 575 nm. Statistical significance ($p \leq 0.05$) in the level of calcium detected between the recombinant factor-treated OB-like cell cultures and the untreated cells or between pRUF and pRUF-ET-1 infected OB-like cells was determined using the paired *t*-test.

## 2.7 *IN VIVO* ASSAYS OF BONE FORMING POTENTIAL

The procedure described below was performed under the approval from the Animal Ethic Committees of the University of Adelaide and the Royal Adelaide Hospital/I.M.V.S., South Australia. The animals used, were six weeks old SCID mice and were housed in sterile microisolator cages in the sterile barrier room located in the animal facility of the Univeristy of Adelaide. All surgical procedures were performed in a non-biohazard laminar flow cabinet using sterile instruments. Prior to the operation, the mice were anaesthetised by a mixed gas flow of nitrous oxide (0.4 L/min) and oxygen (1.0 L/min) and 2-3% halothane and then maintained under anaesthesia with a reduced airflow with 1-2% halothane.

Human OB-like cells derived from two different donors infected with either pRUF*neo* or pRUF*neo*-ET-1 were harvested by trypsin digestion. Approximately $2 \times 10^6$ cells were mixed with 40 mg hydroxyapatite/tricalcium phosphate ceramic particles (HA/TCP, Zimmer Corporation, Warsaw, IN) per one site transplant. Prior to transplantation, 100 µg fibrinogen (Sigma, Cat. No. T8397) and 2 unit thrombin (Sigma, Cat. No. F4385) were added to the cell and particle mixture.

Six-week-old SCID mice were firstly anaesthetised as described above and then shaved on the dorsal side parallel to the shoulders where the site was subsequently swabbed with Betadine. A 1cm long longitudinal incision was made along the skin using small curved Metzenbaum scissors. The skin on the lateral side of the opening was torn away from the underlying tissue using blunt scissors to form two pockets behind each shoulder. Following this, the mixtures of cells and HA/TCP were implanted into subcutaneous pockets. The opening in the skin was then closed by applying 2 to 3 sterile wound clips. The wound was washed liberally with Betadine and the mice were allowed to recover in clean microisolator cages.

Implants were recovered after 8 weeks, fixed in 4% paraformaldelyde for 2 days, then decalcified for a further 10 days in 10% EDTA, prior to embedding in paraffin. For histological analysis, 5 μm sections of the implants were prepared and stained with haematoxylin and eosin (H&E). The human OB-like cells-derived bone was examined observed using 10× objective lens of a light microscope (Olymus BX50, Japan) and the images were recorded using a digital camera (Olympus DP11, Japan). The bone areas were analysed using SCION software (Scion Corporation, USA).

## 2.8 ELISA MEASUREMENT OF SOLUBLE PROTEIN

### 2.8.1 ET-1 Level in BM and PB Plasma and Cell Supernatant

Five millilitres PB and 1 ml BM from same donor were collected using EDTA as an anticoagulant. Plasma was subsequently collected by centrifugation of samples at 274×*g* for 10 mins and stored at -80°C. A total of 49 patients with MM, 6 patients with MGUS, 10 donors without MM or MGUS were included in this study. Among 49 MM patients, 24 patients were male with mean age 64 years (49-80), 25 patients were female with mean age 62.4 years (44-82). Seventeen cases were newly diagnosed MM, 11 cases were relapsed or progressive cases with no response to treatment, and 21 cases were in remission after treatment. The mean age of patients with MGUS was 69.8 years (40-83) with 3 males and 3 females. The mean age of control was 62.3 years (22-85) with 5 male and 5 female subjects. Among 10 control cases, 5 cases were normal bone marrow, three cases were non-Hodgkin's lymphoma and 2 cases had increased megakaryocytes in BM.

The supernatant of myeloma cells was collected by centrifugation of a cell culture at a cell density of $2\times10^5$ cells/ml and stored at -20°C. The 10 ml supernatants derived from human OB-like cells infected with pRUF or pRUF-ET-1 were harvested from $1\times10^6$ cells.

The level of ET-1 in BM and PB plasma and cell line conditioned medium was detected using human ET-1 Immunoassay kit (QuantiGlo®, R&D Systems, USA, Cat. No. QET00). The procedure was performed according to the instruction provided by the manufacturer. Briefly, 100 µl of Assay Diluent QD1-37 was added to each well of the Immunoassay plate. This was followed by the addition of 100 µl of Standard or sample per well. The plate were covered with adhesive strip and incubated for 1.5 hrs at RT on a horizontal orbital microplate shaker (0.3 cm orbit, Crown Scientific) set at 500±50 rpm. The plate was washed four times with 400 µl/well Wash Buffer using a multi-channel pipette. After the last wash, the remaining Wash Buffer was removed by decanting and blotting the plate against clean paper towels. Two hundred µl of ET-1 conjugate was added to each well and the plate was covered with a new adhesive strip and incubated for 3 hrs at RT on the shaker. Following four washes, 200 µl of Substrate Solution was added to each well and the plate was incubated for 30 mins at RT on the benchtop. The relative light unit of each well was determined using single photon count of TopCount Microplate Scintillation Counter (Packard, Canberra, Australia). The absolute ET-1 concentration was determined using a standard curve for ET-1 according to the manufacturer's recommendations.

### 2.8.2 Determination of IL-1β, IL-6, OPG and TNF-α Levels in Culture Supernatants

The amount of IL-1β, IL-6, OPG and TNF-α liberated into the culture medium by the OB-like cells was measured using an enzyme-linked immunosorbent assays (ELISA). The detailed IL-1β ELISA method is described below.

Each well of a 96-well plate (MaxiSorp; Nalge Nunc) was coated overnight with 100µl of 2µg/ml anti-human IL-1β capture mAb (MAB601, R&D Systems, Inc., Minneapolis, MD, USA) diluted in PBS. The plate was washed four times with 100 µl Wash Buffer (PBS containing 0.05 % (v/v) Tween 20), and "blocked" by the addition of 250µl of Blocking Buffer (PBS containing 1% BSA). After a 2 hr incubation at RT, the plate was washed as above. One hundred µl of the IL-1β standard (Cayman chemical company, USA) or a 1/20 dilution of each test sample was added in triplicate wells. The

plates were washed as above, and 100µl of 100 ng/ml biotinylated α-human IL-1β mAb (BAF 201, R&D Systems, Inc., Minneapolis, MN, USA), was diluted in "detection antibody diluent" (Tris Buffered Saline containing 0.1% BSA) and added to each well and incubated for 2 hrs at RT. The plate was washed four times and 100µl of 1:2000 streptavidin-conjugated horse radish peroxidase (Genzyme, Cambridge, Massachusetts, USA) in Blocking Buffer was added to each well and the plate was incubated at RT for 20 mins. Following four washes as above, 100µl of TMB substrate reagent (Sigma Chemical Co., St Louis, Missouri, USA) was added per well and the plate was incubated in the dark for 20 mins at RT. The reaction was stopped with 0.5M sulphuric acid ($H_2SO_4$, Asia Pacific Specialty Chemicals Limited, Australia), and the absorbance measured at 450nm using a BIO-RAD Model 3550 microplate reader.

Alternatively, anti-human OPG mAb (MAB 805), Homodimeric recombinant human OPG-Fc (amino acids 22-401 conjugated to human Fc, glycosylated, 805-OS) and biotinylated α-human OPG mAb (BAF 805) from R&D Systems were used for capture antibody, standard and detection antibody, respectively for OPG ELISA. Anti-human TNF-α mAb (MAB 610), TNF-α standard and biotinylated α-human TNF-α mAb (BAF 210) from R&D Systems were used for capture antibody, standard and detection antibody, respectively for TNF-α ELISA.

## 2.9 WESTERN BLOT ANALYSIS

### 2.9.1 Buffers and Reagents

#### 2.9.1.1 Tris-Saline-EDTA (TSE)

50 mM Tris-HCl, 150 mM sodium Chloride, 1 mM EDTA, 0.1% sodium azide, pH 8.0.

#### 2.9.1.2 1% (v/v) NP40-TSE

1% (v/v) Nonidet P 40 (NP40, Sigma-Aldrich, Castle Hill, NSW. Australia), 50 mM Tris-HCl, 150 mM sodium Chloride, 1 mM EDTA, 0.1% sodium azide, in water, pH 8.0.

#### 2.9.1.3 0.1% (v/v) NP40-TSE

0.1% (v/v) NP40, 50 mM Tris-HCl, 150 mM sodium Chloride, 1 mM EDTA, 0.1% sodium azide, in water, pH 8.0.

#### 2.9.1.4 25× Protease Inhibitor Cocktail

One protease inhibitor cocktail tablet (Roche, Germany) was dissolved in 2 ml Milli-Q water and used as 25 × concentration stock solution. The solution was stored at -20°C.

#### 2.9.1.5 1× Reducing Sample Buffer

1 ml 0.5 M Tris-HCl, pH 6.8, 800 µl glycerol, 1.6 ml 10% sodium dodecyl sulphate (SDS, BDH), 400 µl β-mercaptoethanol, 200 µl 0.05% bromophenol blue and 4 ml Milli-Q water (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β–mercaptoethanol and 0.00125% bromophenol blue).

#### 2.9.1.6 1× Non-reducing Sample Buffer

1 ml 0.5 M Tris-HCl, pH 6.8, 800 µl glycerol, 1.6 ml 10% SDS, 200 µl 0.05% bromophenol blue and 4.4 ml Milli-Q water (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS and 0.00125% bromophenol blue).

#### 2.9.1.7 Electrophoresis "Running" buffer

0.3% Tris-HCl, 1.44% Glycine, 0.1% SDS, pH 8.3 in water.

#### 2.9.1.8 Transfer Buffer

0.3% Tris-HCl, 1.44% Glycine, 20% Methanol, pH 8.3 in water.

#### 2.9.1.9 Separating Buffer

1.5 M Tris-HCl, pH 8.8

#### 2.9.1.10 Stacking Buffer

0.5 M Tris-HCl, pH 6.8

#### 2.9.1.11 PBST

PBS supplemented with 0.05% (v/v) Tween 20.

### 2.9.2 Preparation of Cell Lysates

A confluent T75 flask of human OB-like cells were washed with ice cold PBS twice and lysed in 1 ml of lysis buffer comprised of 1% NP40-TSE supplemented with 1 × protease inhibitor cocktail. The cells were incubated for 30 mins on ice with shaking.

Alternatively, OB-like cells were detached by trypsin digestion, washed 3 time in cold PBS and resuspended at $1\times10^8$ cells per ml and lysed in 1ml of 1% NP40-TSE supplemented with 1 × protease inhibitor cocktail. The cells were incubated on ice for 30 mins with occasional gentle mixing. Lysates were centrifuged to remove detergent-insoluble material and the final protein concentration determined using a BCA protein assay, as recommended by the manufacturer (Pierce, Rockford, IL., U.S.A).

### 2.9.3 Immunoprecipitaiton (IP) with Dynabeads

One ml lysate was pre-cleared by incubation with 25 µl (per IP) uncoated DYNABEADS on rotator at 4°C for 16 hours. The DYNABEADS were washed with 1% NP40 supplemented with 0.5× protease inhibitors three times prior to use. Prior to pre-arming beads, 50 µl (per IP) of Sheep anti-mouse IgG or Rat anti-mouse DYNABEADS were wahsed with 0.1% BSA/PBS three times. Three µg purified mAb or equibalent S/N were added to the beads and incubated on rotator at 4°C for a minimum of 2 hrs (2-24 hrs). The pre-armed beads were washed once with 0.1% BSA/PBS and 1% NP40-TSE supplemented with 0.5 × protease inhibitors. The pre-cleared lysates then were incubated with pre-armed beads on rotator at 4°C for 2 hrs. The immunoprecipitated proteins were washed in 1ml 1% NP 40-TSE three times, in 1 ml 0.1% NP40-TSE twice, and in 1 ml TSE twice. The beads were resuspend in 30 µl 1× Non Reducing Buffer. The protein was liberated from the beads by boiling the samples for 5 mins prior to electrophoresis.

### 2.9.4 Western Blot Analysis

Approximately 20 µg of protein or protein derived from $1\text{-}2\times10^6$ cell equivalents was diluted in an equal volume of 2× reducing buffer. Samples were boiled for 5 mins and proteins separated on 12% SDS-PAG gel prepared according to the following formulation: 6.6 ml distilled water + 8.0 ml 30% acrylamide (Bio-Rad Laboratories, CA, USA) + 5.0 ml 1.5 M Tris pH 8.8 + 0.2 ml 10% SDS. N, N, N' N'-tetramethyl-ethylenediamine (TEMED, ICN Biomedicals Inc. USA) and 10% ammonium persulphate (0.2 ml) (Sigma) were added to the gel solution immediately before pouring. The size of detected proteins was determined by comparison with pre-stained molecular weight standards (Seablue Plus2; San Diego, CA). The gel was electrophoresed at 30 mA until the bromophenol blue buffer front entered the separating gel, at which time the voltage was raised to 40 mA, until the bromophenol blue buffer front migrated to the end of the gel.

Following gel electrophoresis, the proteins were transferred onto Hybond PVDF membranes (Amersham pharmacia biotech, England) overnight at 30 mA using a blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). The membranes were blocked with 5% (w/v) powdered milk in PBST for 1 hour at RT. The blocked membranes were washed in PBS-T, probed with 5 ml of mAb at a concentration 1 μg/ml in PBST for 1 hr. The membranes were subsequently washed six times for 5 mins with PBS-T, and then incubated with an anti-rabbit or anti-mouse Ig alkaline phosphatase (1:10,000, Amersham LIFE SCIENCE) corresponding to Ig class of primary antibody for 1 hr at RT. Following the immunolabelling, the membranes were washed six times as above and the protein bands visualised by adding 1 ml of ECF substrate (diethanolamine, Amersham phamacia biotech) and scanned using a Typhoon™ fluorimager (Molecular Dynamics, CA, USA) at wavelength 488 nm. To ensure equal loading of samples, the membranes were sequentially probed with a goat antibody to β actin (1:500, Santa Cruz), rabbit anti-goat IgG alkaline phosphatase (1:5000, Pierce) and subsequently resolved as described as above.

## 2.10 STATISTICAL ANALYSIS

Results are expressed as the mean±the standard error of the mean (SEM). Student's *t*-test was used to determine the statistical significance of differences between the means of several experiments for data showing normal distribution. Wilcoxon rank sum test was used for related non-parametric data. Mann-Whitney *U* test was used for independent non-parametric data. A probability value $\leq 0.05$ was considered to be statistically significant. Each experiment was repeated at least two times for these analyses.

# CHAPTER 3. THE MULTIPLE MYELOMA-DERIVED PRO-INFLAMMATORY CYTOKINE, IL-1β, INHIBITS BONE FORMATION AND ENHANCES OSTEOCLATOGENESIS BY INCREASING THE NUMBER OF RANKL-EXPRESSING STRO-1 POSITIVE OSTEOPROGENITOR CELLS

## 3.1 INTRODUCTION

It is generally accepted that the focal or diffuse osteolytic lesions seen in MM patients are due to enhanced and uncontrolled osteoclastic bone resorption adjacent to areas of plasma cell infiltrates. This assumption is supported by histomorphometric analyses, which have identified characteristic abnormalities in the bone remodelling process in patients with MM. It has shown that MM patients display an increased eroded bone surface due to an elevated number and activity of OCs (Bataille *et al.*, 1991; Roux *et al.*, 1994). Malignant PCs are thought to be responsible for this increase, as indices of bone resorption are highest when BM infiltration by malignant plasma cells is greatest (Martin *et al.*, 1994). The increased bone resorption is widely considered to be due to the local stimulation of OCs by OAFs released by myeloma cells and/or the BM microenvironment in response to tumour cells, including IL-1β, IL-6, TNF-α, TNF-β, PTHrP (Bataille *et al.*, 1992; Caligaris-Cappio *et al.*, 1992; Filella *et al.*, 1996; Lichtenstein *et al.*, 1989) and RANKL (Farrugia *et al.*, 2003).

Under normal homeostatic bone remodelling conditions, the bone resorbed by OCs is replaced by a quantitatively similar amount of bone formation in what is known as "coupled response". It is thought that loss of bone in MM is compounded by the inadequate regeneration of new bone to replace the resorbed bone due to the inhibition of OB function. This is supported by studies which show that there is no evidence of new bone formation within the skeletal lesions or in their vicinity (Silvestris *et al.*, 2003). Surprisingly however, histomorphometric studies show that the numbers of OBs and bone formation rate are increased in early stage MM or when the degree of BM PC involvement is modest (5–50% of the mononuclear cell compartment) (Bataille *et al.*, 1991; Taube *et al.*, 1992). However, in the later stages of MM or when the tumour burden is greater than 50%, the bone formation rate is similar to that observed in normal subjects or patients with MGUS (Bataille *et al.*, 1991; Taube *et al.*, 1992). These changes in bone formation rate are associated with an increase in the OB number and osteoid surface area or osteoid volume (Bataille *et al.*, 1991). However, this increased bone formation rate does not correlate with an increased

mineral apposition rate, which remains unchanged (Bataille *et al.*, 1991). Thus, the abnormalities of bone remodelling in patients with MM can be summarised as follows: an increase in bone resorption, followed by an increase in the number of OBs, and a failure of these cells to replace completely the bone resorbed. The mechanisms of how increased OBs fail to completely replace the resorbed bone remain unknown. Although the function of OAFs on OCs is well defined (Jimi *et al.*, 1998; Tamura *et al.*, 1993; Thomson *et al.*, 1986), the mechanisms for the observed changes in the OB compartment and the activity of OBs in MM remain to be fully elucidated. This increased OB number may be due to increased OB recruitment or increased OB proliferation on the bone surface induced by cytokines present in the bone microenvironment. It is well known that OBs play a pivotal role during the biological response of bone to agents including hormones, polypeptide growth factors and cytokines that stimulate or inhibit bone resorption and/or bone formation. In addition, OBs are essential for both osteoclastogenesis (Udagawa *et al.*, 1990) and bone formation (Mundy, 1995). As detailed in the general introduction, pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, have been shown to be elevated in the serum of patients with MM (Filella *et al.*, 1996; Jourdan *et al.*, 1991; Lichtenstein *et al.*, 1989; Ohtake *et al.*, 1990; Yamamoto *et al.*, 1989). Although their direct role in osteoclastogenesis is well established (Cozzolino *et al.*, 1989; Karadag *et al.*, 2000b; Kurihara *et al.*, 1990; Pfeilschifter *et al.*, 1989; Tamura *et al.*, 1993; Yamamoto *et al.*, 1989), the way in which these factors influence bone cell function remains to be fully elucidated.

In an attempt to identify the underlying mechanisms for observed changes in the OB compartment in MM, we screened a large panel of cytokines/factors, secreted in abundance in MM bone microenvironment, to identify those which preferentially affect the proliferation and differentiation of OB-like cells. We have previously described an *in vitro* model of human OB differentiation based on the expression of the stromal precursor cell marker, STRO-1, and the osteoblastic marker, ALP. In this model, osteogenic cells derived from human trabecular bone can be separated into four populations characteristic of different stages of OB differentiation. The proceeding chapter details studies examining the hypothesis that MM PCs-derived pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, serve to increase the number of immature OB cells, as defined by STRO-1 and AP expression, in the early stages of MM.

## 3.2 RESULTS

### 3.2.1 IL-1β is a Highly Mitogenic Cytokine for Human OB-like Cells

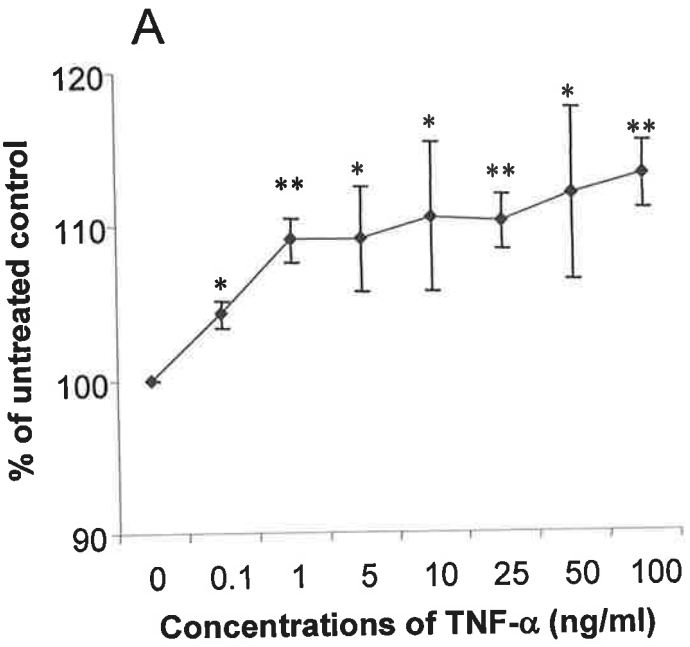
Preliminary experiments examining the mitogenic effects of the various cytokines were reliant on the use of the colorimetric reagent, WST-1. WST-1 has been widely used as an indirect measure of cell proliferation and viable cell number in a variety of systems (Bedard *et al.*, 1999; Dong *et al.*, 1999; Harkacz *et al.*, 1997; Zygmunt *et al.*, 1998), and was used throughout work outlined in this thesis. Prior to its use, a series of studies examining its suitability and reliability were performed as summarised in Appendix 1. As shown in Appendix 1, the absorbance at 450nm wavelength (A450) of WST-1 exhibited a linear correlation with the number of viable cells. The non adherent cell ARH-77 was in the linear range of A450 at cell density between $1\times10^4$ and $8\times10^4$(Appendix 1A). In contrast, adherent human OB-like cells were in the linear range of A450 at cell density between $2\times10^3$ and $1.6\times10^4$(Appendix1B). When used in these defined ranges, WST-1 was considered a reliable method to measure OB and myeloma cell number.

Initial experiments were designed to monitor the effect of TNF-α, IL-6 and IL-1β on the growth of normal human OB-like cells. As shown in Figure 3.1, culture of OB-like cells in the presence of TNF-α, resulted in a dose-dependent increase in cell number. At both d5 (Figure 3.1A) and d7 (Figure 3.1B), TNF-α promoted a significant dose-dependent increase in cell number at a concentration as low as 0.1 ng/ml ($P$=0.005, Student $t$-test). TNF-α increased cell number to 131.1±3.5% of untreated control ($P$=0.0004, Student $t$-test) at a concentration of 100 ng/ml at d7 (Figure 3.1B).

In contrast, IL-6 only had mitogenic effects on human OB-like cells at high concentrations (Figure 3.2). Although IL-6 and sIL-6R did not increase the cell number at lower concentrations, they both significantly increased cell number at a concentration of 100 ng/ml at both d5 (Figure 3.2A) and d7 (Figure 3.2B). Notably, a synergistic effect of IL-6 and sIL-6R on the cell proliferation of human OB-like cells was observed (124.4±2.5% of control cultures, $P$=0.0003, Student $t$-test) at a concentration of 100 ng/ml at d7 (Figure 3.2B).

Interestingly, amongst the three cytokines tested, IL-1β had the most mitogenic effect on human OB-like cells. As shown in Figure 3.3, IL-1β induced a significant increase in

Figure 3.1. TNF-α dose dependently increases the proliferation of human OB-like cells. Human OB-like cells were seeded into 96-well plates at a cell density of 2,000 cells/well. The cells were cultured in the presence or absence of TNF-α at the indicated concentrations and the cell number and viability quantitated at d1, d3, d5 and d7 using WST-1, as described in the methods. At both d5 (A) and d7 (B), TNF-α induced a significant dose-dependent increase (* $P<0.05$, ** $P<0.005$, Student *t*-test) in cell number. It significantly promoted cell growth at a concentration as low as 0.1 ng/ml ($P=0.005$, Student *t*-test). TNF-α increased cell number to 131.1±3.5% of negative control ($P=0.0004$, Student *t*-test) at a concentration of 100 ng/ml at d7. Values represent means ± SEM of triplicate cultures of each concentration. The results displayed are a representative example of 3 independent experiments.


A
120
110
100
90
% of untreated control
0
0.1
1
5
10
25
50
100
Concentrations of TNF-α (ng/ml)

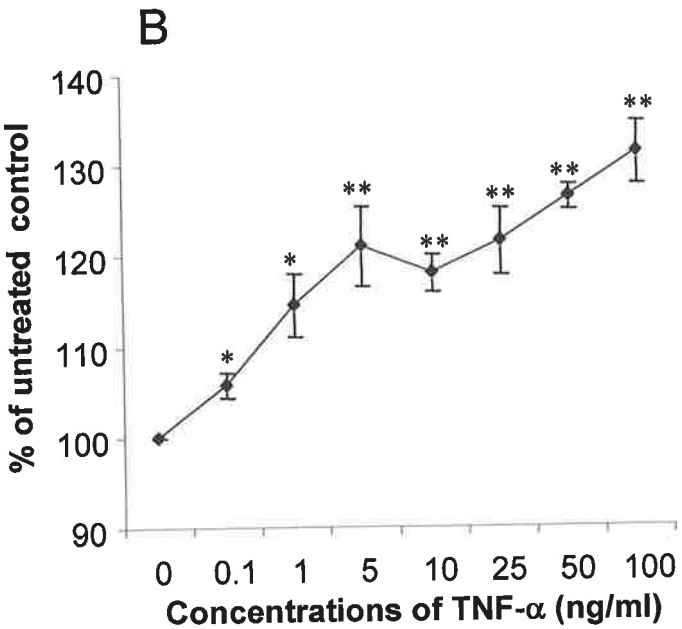
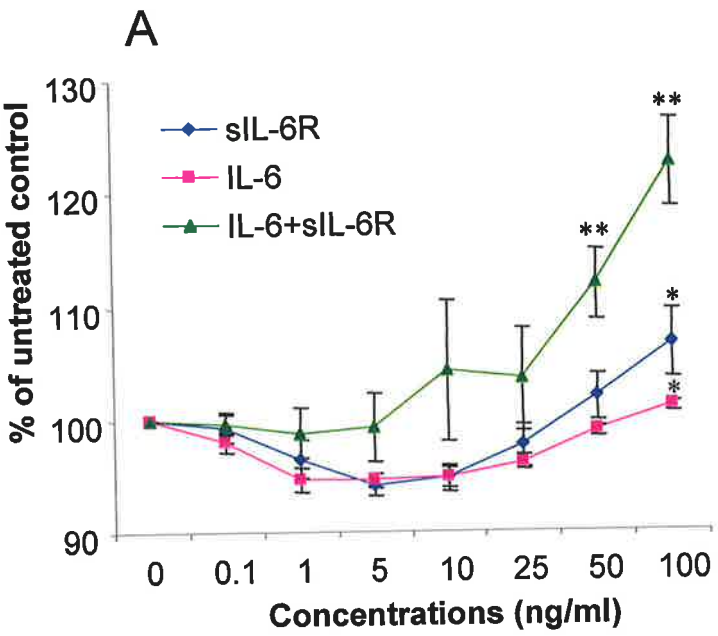
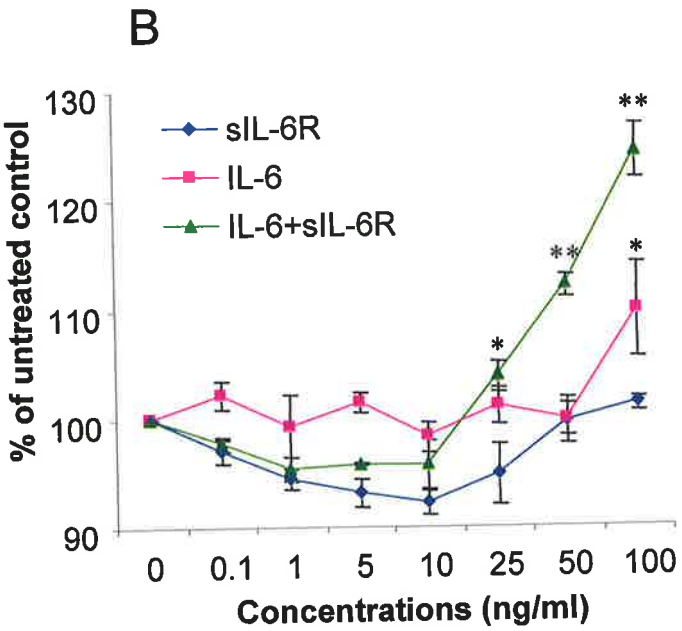

B
140
130
120
110
100
90
% of untreated control
0
0.1
1
5
10
25
50
100
Concentrations of TNF-α (ng/ml)

Figure 3.2. IL-6 is mitogenic for human OB-like cells at high concentrations. Human OB-like cells were seeded in 96-well plates at a cell density of 2,000 cells/ well. The cells were cultured in the presence or absence of IL-6 and/or soluble IL-6 receptor (sIL-6R) at different concentrations, and the cell number and viability quantitated at d1, d3, d5 and d7 using WST-1, as described in the methods. Although IL-6 and sIL-6R did not increase the cell number at lower concentration, both factors significantly (* $P<0.05$, ** $P<0.005$, Student *t*-test) increased cell number at a concentration of 100 ng/ml at both d5 (A) and d7 (B). The synergistic effect of IL-6 and sIL-6R on the cell proliferation of human OB-like cells was noted. The combination of IL-6 and sIL-6R increased the cell number of human OB-like cells to 124.4±2.5% of control cultures ($P=0.0003$, Student *t*-test) at a concentration of 100 ng/ml at d7. Values represent means ± SEM of triplicate cultures of each concentration. The results displayed are a representative example of 3 independent experiments.


A
130
120
110
100
90
% of untreated control
sIL-6R
IL-6
IL-6+sIL-6R
**
**
*
*
0
0.1
1
5
10
25
50
100
Concentrations (ng/ml)


B
130
120
110
100
90
% of untreated control
sIL-6R
IL-6
IL-6+sIL-6R
**
**
*
*
0
0.1
1
5
10
25
50
100
Concentrations (ng/ml)

cell number at a concentration of 0.01 ng/ml as early as at d1 (Figure 3.3A, $P$=0.01, Student $t$-test). At d7, IL-1β at concentration 0.01ng/ml, significantly increased cell number to 136.6±1.2% of untreated control cultures (Figure 3.3D, $P$=0.000003, Student $t$-test). A plateau effect was observed at concentrations greater than 0.1 ng/ml. The mitogenic effect of IL-1β was observed in cultures established in both α-MEM-10 medium (Figure 3.3A-D) and serum-deprived medium (Figure 3.3E&F). Because of the dramatic mitogenic effect of IL-1β on OB-like cells and its highest serum level among TNF-α, IL-6 and IL-1β in patients with POEMS syndrome (161±73pg/ml) (Gherardi *et al.*, 1996), IL-1β was further invested in this study as a representative of pro-inflammatory cytokines.

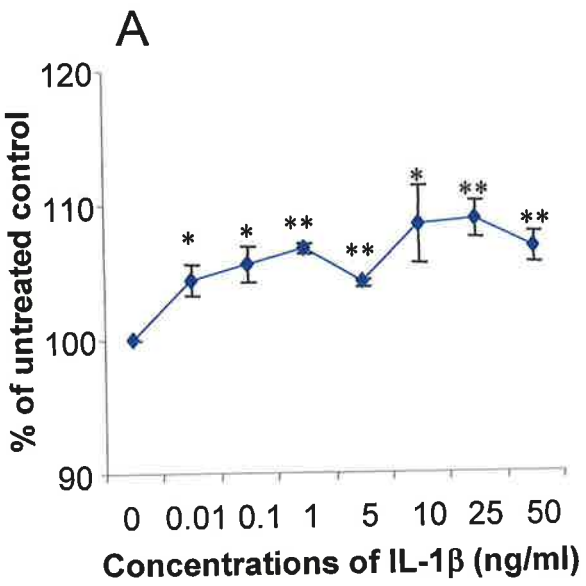
### 3.2.2 IL-1β May Regulate Expression of IL-1β, TNF-α and IL-6 genes *via* $PGE_2$ Synthesis Pathway

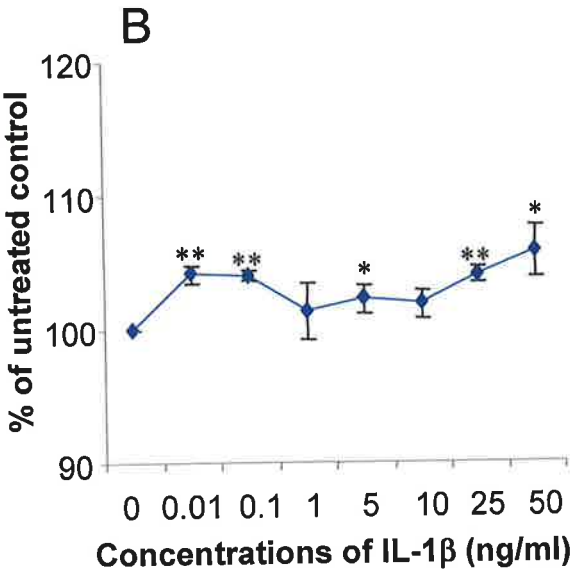
To examine whether IL-1β promoted cell growth by regulating the expression of certain genes, semi-quantitative RT-PCR was used as described in the methods. As seen in Figure 3.4, IL-1β significantly upregulated TNF-α (Figure 3.4A), IL-1β (Figure 3.4B), IL-6 (Figure 3.4C) and COX-2 (Figure 3.4D) gene expression in human OB-like cells (* $P$<0.05, ** $P$<0.005, Student $t$-test). Interestingly, the IL-1β-induced upregulation of genes, including IL-1β (Figure 3.5 B), TNF-α (Figure 3.5C), and COX-2 (Figure 3.5E) was maintained until d21.

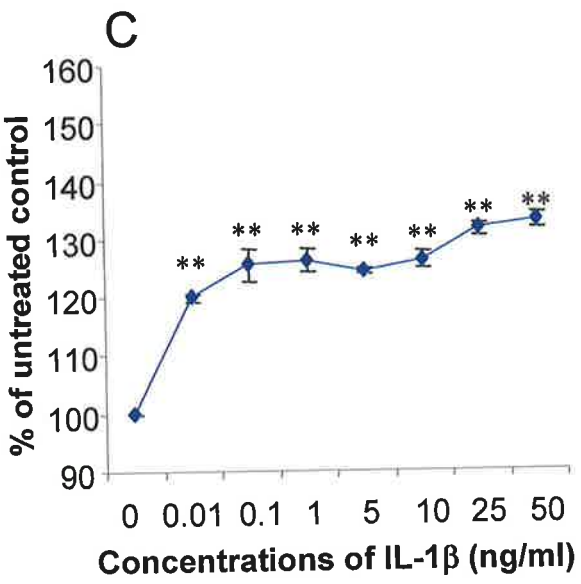
Although TNF-α and IL-1β protein could not be detected by ELISA following IL-1β stimulation (data not shown), IL-1β appeared to stimulate the synthesis of IL-6 protein in OB-like cells. As seen in Figure 3.6, a significant increase of IL-6 level in OB-like cells was observed as early as 6h following exposure to 10ng/ml IL-1β. The IL-6 levels continued to increase from 0.65±0.08, 0.97±0.1, 1.17±0.08 and 2.48±0.08ng/ml to 2.13±0.07, 3.73±0.01, 4.45±0.02 and 4.36±0.08ng/ml at 6h, 24h, 72h and 120h, respectively ($P$=0.0008, 0.0013, 0.0008, 0.003, respectively, Students $t$-test).

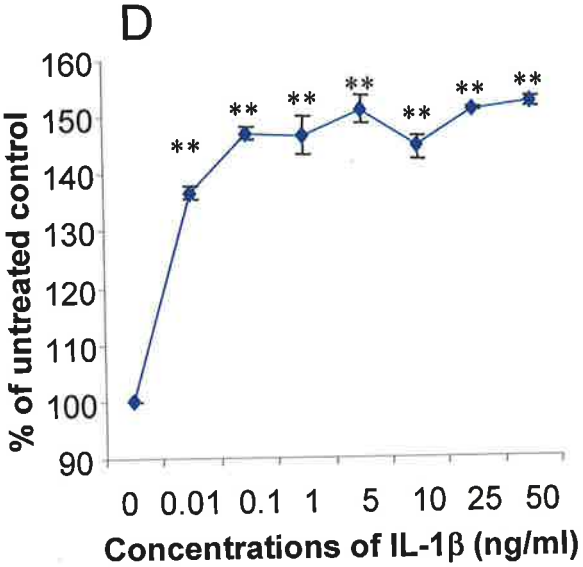
Although COX2 gene expression was significantly upregulated by IL-1β, indomethacin, a potent COX1 and COX2 inhibitor, did not block IL-1β induced mitogenesis of OB-like cells (Figure 3.7). Consistent with the findings shown in Figure 3.3, IL-1β induced a significant increase in cell number at a concentration of 10 ng/ml at d5 (Figure 3.7A, $P$=0.0006, Student $t$-test) and d7 (Figure 3.7B, $P$=0.00007, Student $t$-test). However,

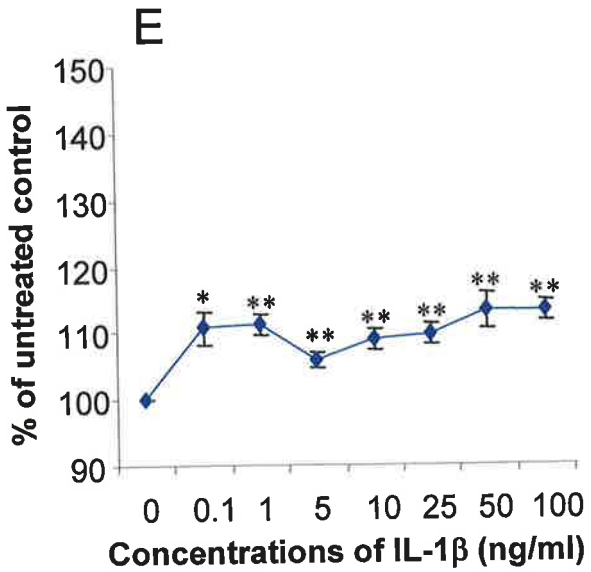
Figure 3.3. IL-1β is the most mitogenic cytokine among the three cytokines tested on human OB-like cells. Human OB-like cells were seeded in 96-well plates at a cell density of 2,000 cells/ well and cultured either in α-MEM-10 or SDM. Cultures were supplemented with IL-1β at the indicated concentrations and the cell number and viability quantitated at d1, d3, d5 and d7 using WST-1, as described in the methods. IL-1β induced a significant increase (* $P<0.05$, ** $P<0.005$, Student *t*-test) in cell number at a concentration of 0.01 ng/ml as early as at d1 (A, $P=0.01$, Student *t*-test). At d7, IL-1β at concentration 0.01ng/ml significantly increased cell number to 136.6±1.2% of untreated control cultures (D, $P=0.000003$, Student *t*-test). A plateau effect was achieved at concentrations greater than 0.1 ng/ml. The mitogenic effect of IL-1β was observed in cultures established in α-MEM-10 (A-D) and SDM (E&F). When cells were cultured in SDM, the mitogenic effect of IL-1β was also observed at d3 (E) and d5 (F). Values represent means ± SEM of triplicate cultures of each concentration. The results displayed are a representative example of 3 independent experiments.


A
120
110
100
90
% of untreated control
0 0.01 0.1 1 5 10 25 50
Concentrations of IL-1β (ng/ml)


B
120
110
100
90
% of untreated control
0 0.01 0.1 1 5 10 25 50
Concentrations of IL-1β (ng/ml)


C
160
150
140
130
120
110
100
90
% of untreated control
0 0.01 0.1 1 5 10 25 50
Concentrations of IL-1β (ng/ml)


D
160
150
140
130
120
110
100
90
% of untreated control
0 0.01 0.1 1 5 10 25 50
Concentrations of IL-1β (ng/ml)


E
150
140
130
120
110
100
90
% of untreated control
*
**
**
**
**
**
**
0 0.1 1 5 10 25 50 100
Concentrations of IL-1β (ng/ml)

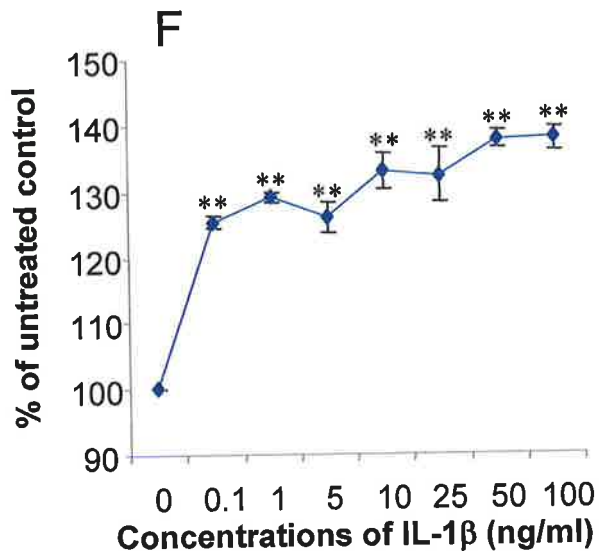
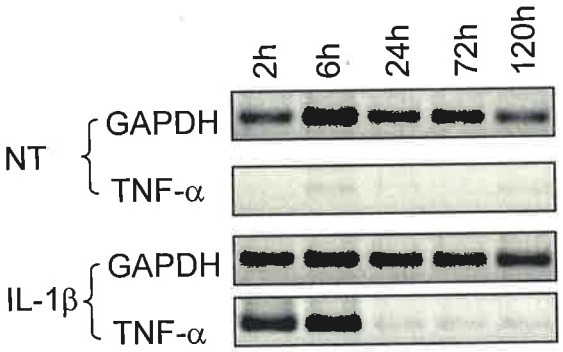
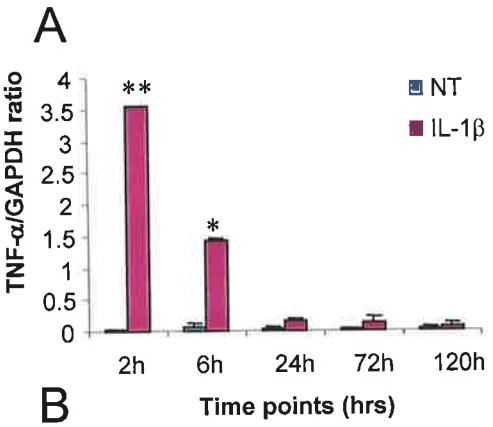
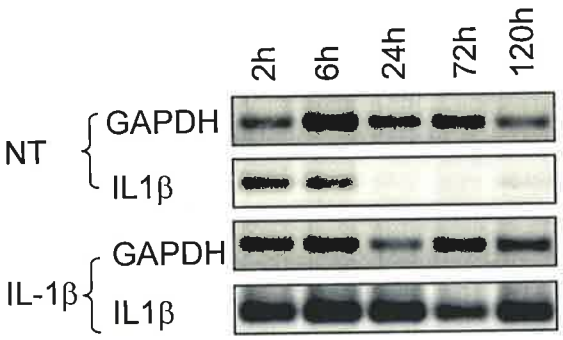
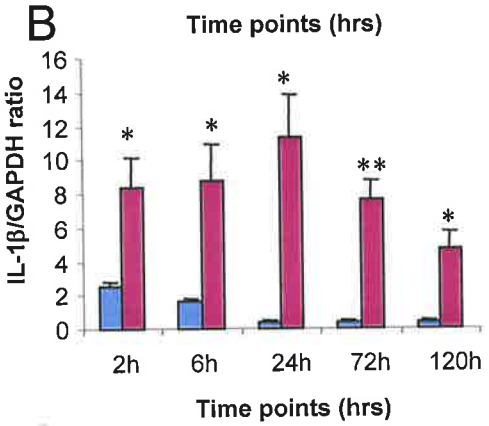

F
150
140
130
120
110
100
90
% of untreated control
**
**
**
**
**
**
**
0 0.1 1 5 10 25 50 100
Concentrations of IL-1β (ng/ml)

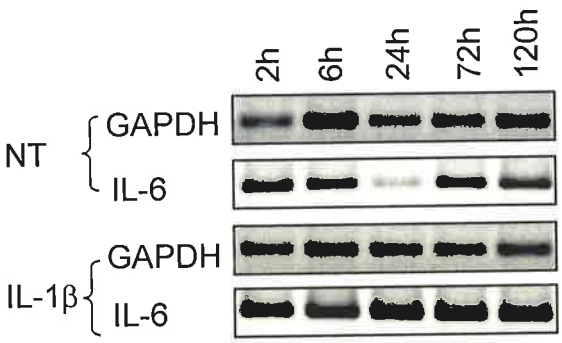
Figure 3.4. IL-1β regulates human OB-like cell gene expression. Human OB-like cells were seeded in a 6-well plate at a cell density of $6\times10^4$/well in 3ml of α-MOD. Following their attachment, cells were washed once with PBS and cultured in SDM alone or supplemented with IL-1β at a concentration of 10 ng/ml. Cells were harvested at the indicated time points over a 5 day period. Total RNA was isolated and semi-quantitative RT-PCR was performed as described in the methods. The house-keeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression assessment. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager at 532nm wavelength. The product bands were semi-quantitated using ImageQuant software and plotted as a histogram of the ratio of specific gene expression relative to GAPDH. IL-1β significantly upregulated TNF-α (A), IL-1β (B), IL-6 (C) and COX2 (D) gene expression in human OB-like cells (* $P<0.05$, ** $P<0.005$, Student *t*-test). The experiments were performed three times and a similar trend was observed in OB-like cells derived from three different donors.

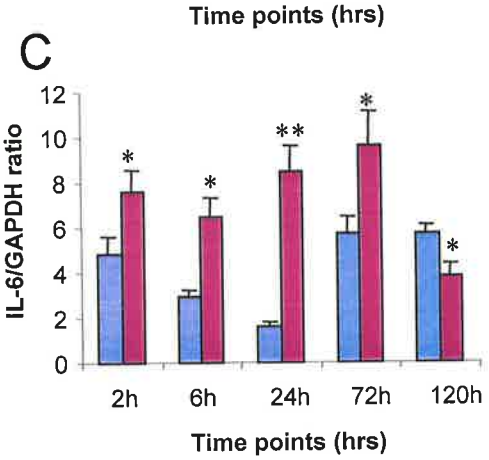

2h
6h
24h
72h
120h
NT
GAPDH
TNF-α
IL-1β
GAPDH
TNF-α

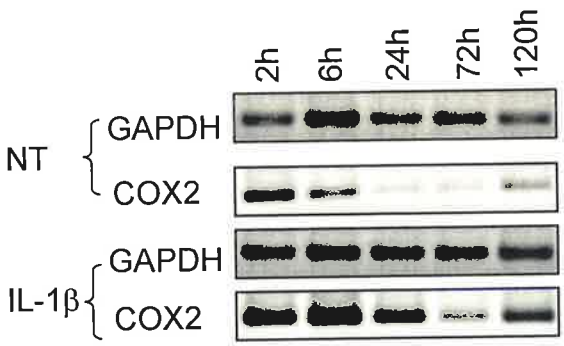

A
TNF-α/GAPDH ratio
NT
IL-1β
**
*
2h
6h
24h
72h
120h
Time points (hrs)


2h
6h
24h
72h
120h
NT
GAPDH
IL1β
IL-1β
GAPDH
IL1β


B
IL-1β/GAPDH ratio
*
**
2h
6h
24h
72h
120h
Time points (hrs)


2h
6h
24h
72h
120h
NT
GAPDH
IL-6
IL-1β
GAPDH
IL-6


C
IL-6/GAPDH ratio
*
**
2h
6h
24h
72h
120h
Time points (hrs)


2h
6h
24h
72h
120h
NT
GAPDH
COX2
IL-1β
GAPDH
COX2

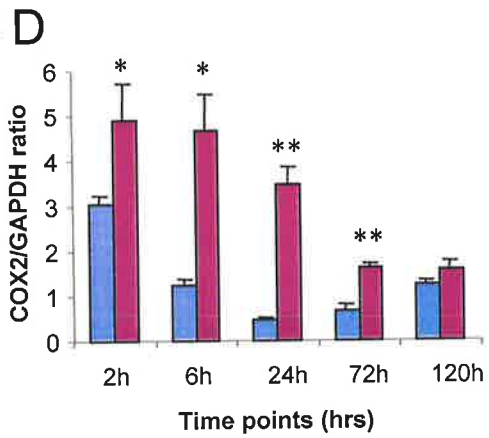

D
COX2/GAPDH ratio
*
**
2h
6h
24h
72h
120h
Time points (hrs)

Figure 3.5. Long term exposure of OB-like cells to IL-1β regulates OB-like cell gene expression. Human OB-like cells were treated with IL-1β at a concentration of 10 ng/ml and harvested at d7, d14 and d21. Total RNA was isolated and semi-quantitative RT-PCR was performed as described in the methods. The house-keeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression assessment. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager. The product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to GAPDH. IL-1β significantly upregulated IL-1β (A), TNF-α (B), COX2 (D) gene expression in human OB-like cells (* $P<0.05$, ** $P<0.005$, Student *t*-test). The experiments were performed three times and a similar trend was observed in two different donors.

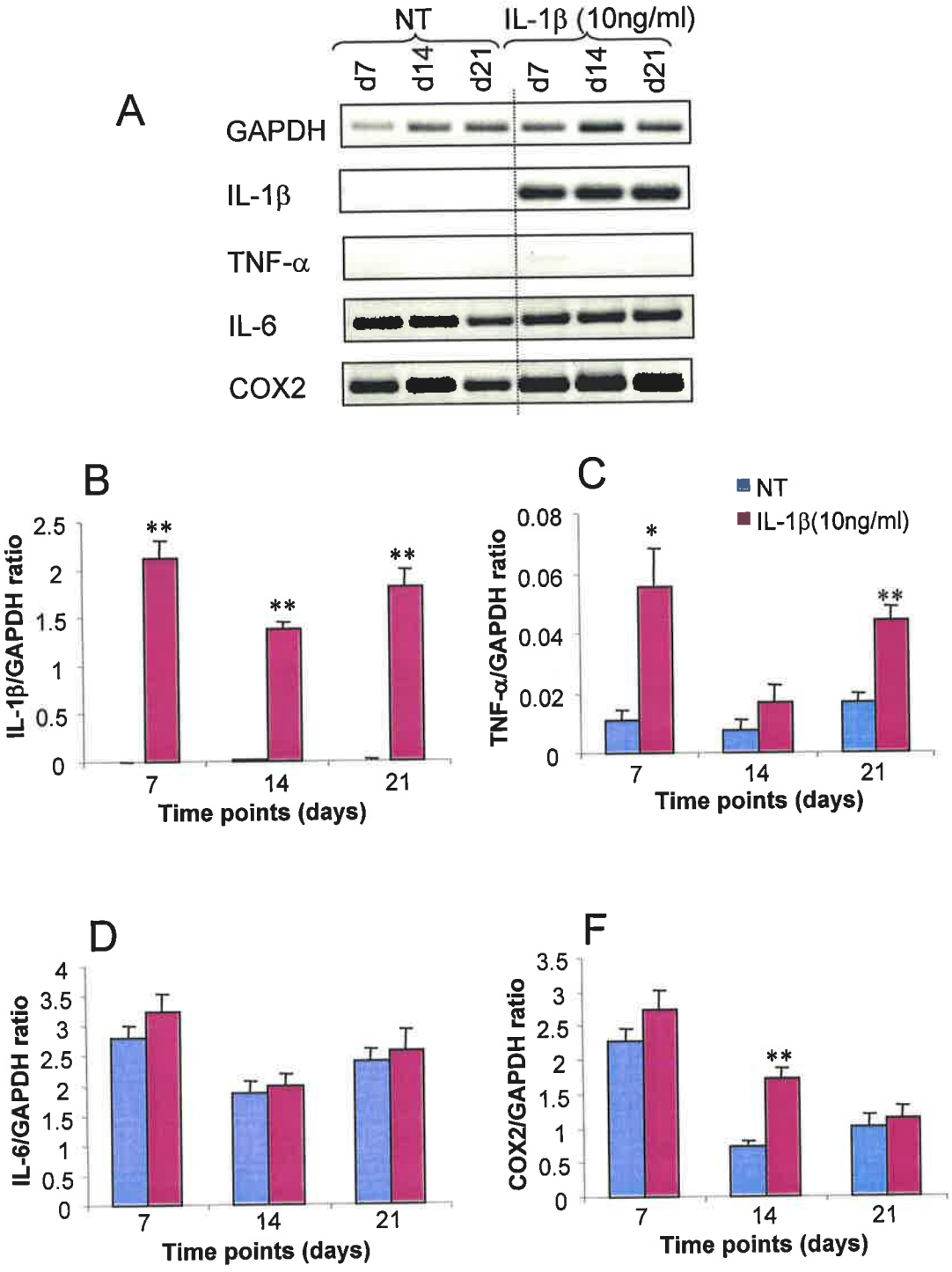

A
NT
IL-1β (10ng/ml)
d7
d14
d21
d7
d14
d21
GAPDH
IL-1β
TNF-α
IL-6
COX2
B
IL-1β/GAPDH ratio
2.5
2
1.5
1
0.5
0
**
**
**
7
14
21
Time points (days)
C
NT
IL-1β(10ng/ml)
TNF-α/GAPDH ratio
0.08
0.06
0.04
0.02
0
*
**
7
14
21
Time points (days)
D
IL-6/GAPDH ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
7
14
21
Time points (days)
F
COX2/GAPDH ratio
3.5
3
2.5
2
1.5
1
0.5
0
**
7
14
21
Time points (days)

Figure 3.6. IL-1β increases IL-6 protein expression in human OB-like cells. IL-6 protein levels in conditioned medium harvested from human OB-like cells at the indicated time points was measured by ELISA. A significant increase in IL-6 protein level at four time points was observed when cells were treated with IL-1β at a concentration of 10ng/ml. The IL-6 levels were increased from 0.65±0.08, 0.97±0.1, 1.17±0.08 and 2.48±0.08ng/ml to 2.13±0.07, 3.73±0.01, 4.45±0.02 and 4.36±0.08ng/ml at 6h, 24h, 72h and 120h, respectively ($P$=0.0008, 0.0013, 0.0008, 0.003, respectively, Students $t$-test). Two independent experiments were performed and similar results were observed from both donors.

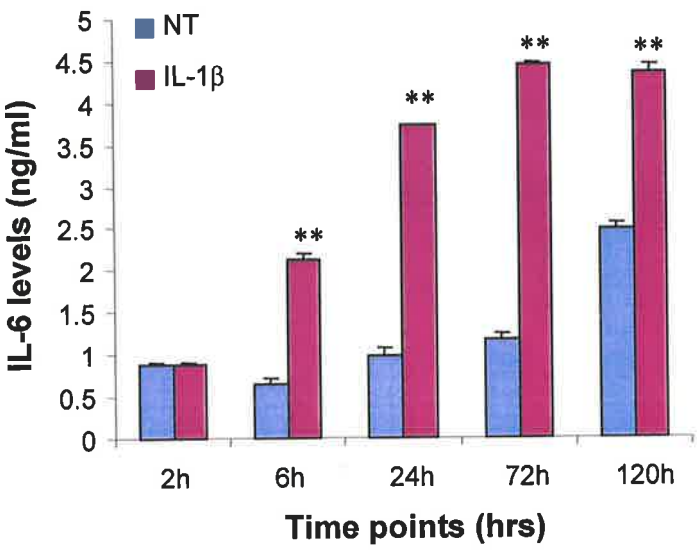
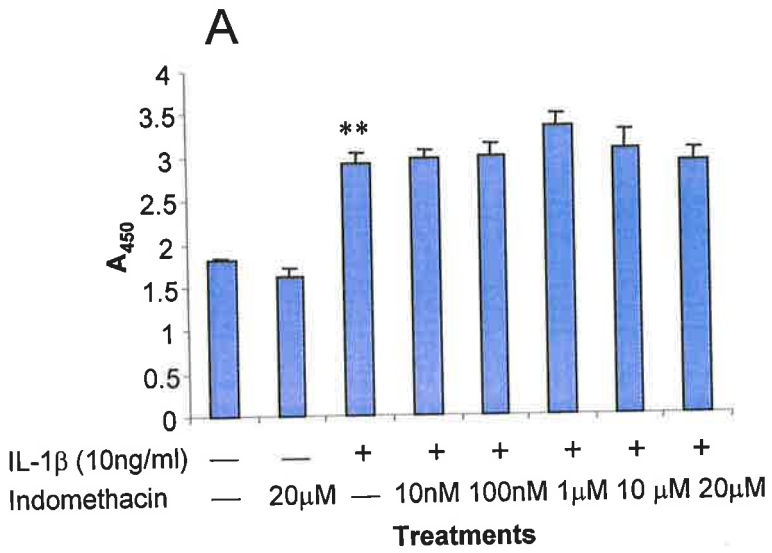
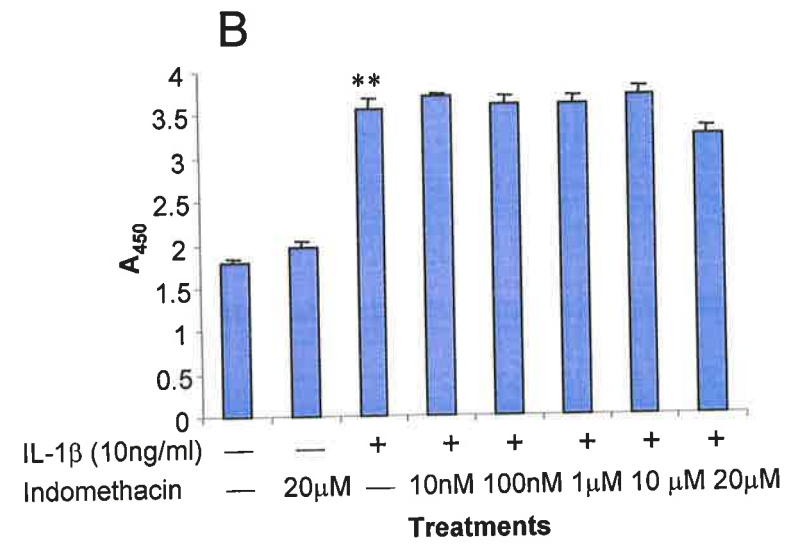

NT
IL-1β
IL-6 levels (ng/ml)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
**
**
2h
6h
24h
72h
120h
Time points (hrs)

Figure 3.7. Indomethacin does not block IL-1β-induced mitogenesis. Human OB-like cells were seeded into 96-well plates at a cell density of 2,000 cells/well and cultured in the presence of IL-1β and/or indomethacin at the indicated concentrations. The cell number and viability were quantitated at d3, d5 and d7 using WST-1, as described in the methods. IL-1β induced a significant increase (** $P<0.05$, Student *t*-test) in cell number at a concentration of 10 ng/ml at d5 (A, $P=0.0006$, Student *t*-test) and d7 (B, $P=0.00007$, Student *t*-test). Indomethacin treatment did not affect OB-like cell growth and did not block the IL-1β induced mitogenic effect. Values represent means ± SEM of triplicate cultures of each concentration. The results displayed are representative examples of 3 independent experiments.


A
4
3.5
3
2.5
2
1.5
1
0.5
0
$A_{450}$
**
IL-1β (10ng/ml) — — + + + + + +
Indomethacin — 20μM — 10nM 100nM 1μM 10 μM 20μM
Treatments


B
4
3.5
3
2.5
2
1.5
1
0.5
0
$A_{450}$
**
IL-1β (10ng/ml) — — + + + + + +
Indomethacin — 20μM — 10nM 100nM 1μM 10 μM 20μM
Treatments

culture of OB-like cells with indomethacin did not inhibit the IL-1β-induced mitogenesis, suggesting that cell proliferation mediated by IL-1β was independent of the COX2 pathway.

### 3.2.3 Myeloma-derived IL-1β Mediates its Effects by Increasing the Proliferation of OB-like Cells by Augmenting the Percentage of Cells Expressing High Levels of the STRO-1 Antigen

As described in Section 1.2.3.2, normal human bone cells can be separated on the basis of their expression of the stromal precursor cell marker, STRO-1, and the osteoblastic marker, ALP. According to this model, cells with the surface phenotype STRO-$1^+$/$ALP^-$, STRO-$1^+$/$ALP^+$, STRO-$1^-$/$ALP^+$ and STRO-$1^-$/$ALP^-$ represent osteoprogenitors, pre-OB, maturing OBs and bone lining cells/osteocytes, respectively. Using mAb antibodies STRO-1 and B4-78 (directed against the bone/liver/kidney isoenzyme of ALP) and dual-colour flow cytometric analysis, the effect of IL-1β on the phenotype of OB-like cells was examined. As seen in Figure 3.8, culture of OB-like cells in 10 ng/ml IL-1β resulted in an increase in the expression of both STRO-1 and ALP cell surface markers. IL-1β increased the percentage of cells expressing STRO-1 antigen, and decreased the percentage of cells found in the STRO-$1^-$/$ALP^-$ compartment. At d3 (Figure 3.8 C & D), there was a substantial increase in the number of STRO-$1^+$ cells (from 28.8% to 53%), with a concomitant decrease in the number of STRO-$1^-$ cells (from 36.4% to 26.7%). At d5 (Figure 3.8 E & F), the percentage of cells expressing STRO-1 almost trebled, with up to 77.5% expressing STRO-1. These observations were consistent in all donor cell cultures tested (n=12).

Although it is well known that the level of IL-1β in patients with MM is elevated in comparison with control individuals (Filella *et al.*, 1996; Lichtenstein *et al.*, 1989; Yamamoto *et al.*, 1989), the effect of co-culture of myeloma cells with OB-like cells on OB phenotype, has been poorly studied. Thus, OB-like cells were co-cultured with the well-characterised myeloma cell line RPMI8226, and changes in the expression of STRO-1 monitored over time. As seen in Figure 3.9, following 5 days co-culture with RPMI8226, OB-like cells displayed an increase in STRO-1 expression with a change in mean fluorescence intensity (MFI) from 193 (Figure 3.9A) to 334 (Figure 3.9B). The role of MM cell line-derived IL-1β was assessed by adding a function-blocking mAb to IL-1β. As displayed in Figure 3.9C, blockade of MM-derived IL-1β resulted in a significant

Figure 3.8. IL-1β modulates the expression of the STRO-1 and ALP antigens on the surface of human OB-like cells. A model of bone cell development has been previously described where cultures of normal human bone cells can be separated on the basis of their expression of the stromal precursor cell marker, STRO-1, and the osteoblastic marker, ALP (refer to section 1.3.3.2). According to this model, cells with the surface phenotype STRO-$1^+$/$ALP^-$, STRO-$1^+$/$ALP^+$, STRO-$1^-$/$ALP^+$ and STRO-$1^-$/$ALP^-$ represent osteoprogenitors, pre-OB, maturing OBs and bone lining cells/osteocytes, respectively. Dual-colour immunofluorescence and flow cytometric analysis was performed using OB-like cells stained with the mAbs STRO-1 (anti-stromal precursors marker) and B4-78 (anti-ALP Ab). Immunoreactivity was revealed by incubation with anti IgM-PE (y-axis) and IgG-FITC (x-axis), as described in the methods. The dot plot histogram represents 10,000 events collected as listmode data. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, 1B5 (FITC) and 1A6.12 (PE), respectively. The results showed that IL-1β altered the proportion of cells expressing STRO-1 and ALP cell surface markers. IL-1β increased the percentage of cells expressing STRO-1 antigen, and decreased the percentage of cells expressing the STRO-$1^-$/$ALP^-$ phenotype. At d3, there was a substantive increase in the number of STRO-$1^+$ cells (from 28.8% in C to 53% in D), with a concomitant decrease in the number of STRO-$1^-$ cells (from 36.4% in C to 26.7% in D). At d5, the increase in the number of cells expressing STRO-1 was greatest (77.5%, F). These observations were consistent in all donor cell cultures tested. A representative example of the results obtained from three experiments is shown.

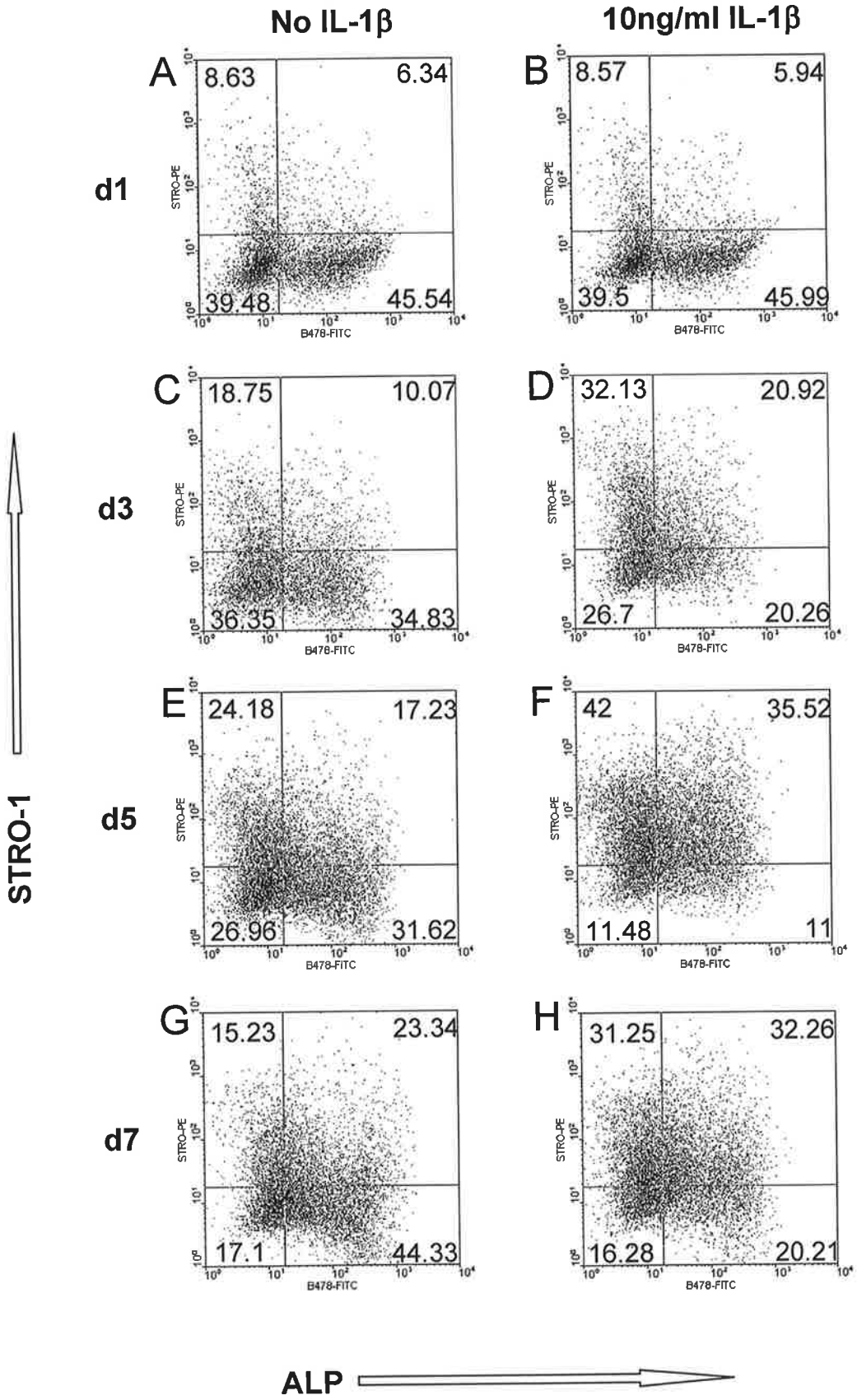
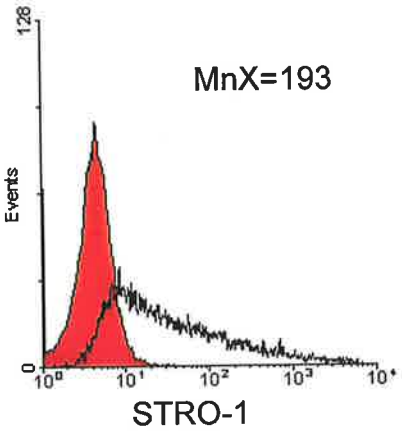

No IL-1β
10ng/ml IL-1β
A
8.63
6.34
39.48
45.54
B
8.57
5.94
39.5
45.99
C
18.75
10.07
36.35
34.83
D
32.13
20.92
26.7
20.26
E
24.18
17.23
26.96
31.62
F
42
35.52
11.48
11
G
15.23
23.34
17.1
44.33
H
31.25
32.26
16.28
20.21
d1
d3
d5
d7
STRO-1
ALP
STRO-PE
B478-FITC

Figure 3.9. Myeloma cells-derived IL-1β increases the percentage of OB-like cells that expressed high levels of the STRO-1 antigen. Myeloma cell line RPMI8226 was co-cultured with OB-like cells for 3 and 5 days as described in methods. Cells were subsequently stained with STRO-1-PE. Fluorescence histograms depict the expression of surface STRO-1 protein. The data is expressed as the relative cell count (y-axis) versus the intensity of STRO-1 expression (log scale). Ten thousand events were collected as listmode data. Isotype matched mAb, 1A6.12, was used as a negative control. An increase of membrane STRO-1 expression was observed when cells were co-cultured with RPMI8226 for 5 days. Co-culture resulted in an increase in mean fluorescence of STRO-1 expression from 193 (A) to 334 (B). Addition of a function-blocking anti-IL-1β mAb abolished the effect and the mean fluorescence decreased to 218 (C). The experiments were performed three times using OB-like cells derived from three different donors.

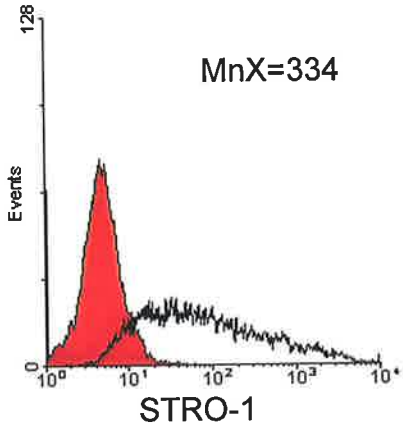
A

OB



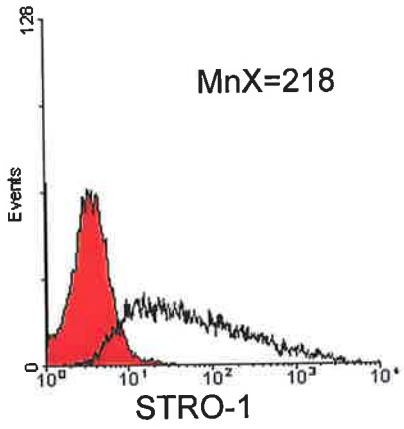

B

OB + RPMI8226




C

OB + RPMI8226
+ mAb IL-1β



diminution of STRO-1 expression to a level similar to OB cultures grown in the absence of RPMI8226 (MFI of 218).

Consistent with our previous findings (Figure 3.6), which showed that exposure of OB-like cells to IL-1β resulted in a substantial increase in OB-like cell expression of IL-6, co-culture of RPMI8226 with OB-like cells also resulted in an increase in the level of IL-6 protein detectable in the conditioned medium. As seen in Figure 3.10, a significant increase ($P$=0.008, Student $t$-test) of IL-6 protein was observed when RPMI8226 were co-cultured with OB-like cells with an increase from undetectable levels and 4.62±0.12ng/ml in RPMI8226 and OB-like cell monocultures, respectively, to 5.74±0.03ng/ml in co-culture supernatant at d3. Similarly, the level of detectable IL-6 was found to increase from 5.82±0.03ng/ml in OB culture to 6.56±0.15ng/ml in co-culture at d5 ($P$=0.018, Students $t$-test). Although the function-blocking anti-IL-1β mAb resulted in a slight decrease in IL-6 measurable in the culture supernatant, this decrease was not significant, suggesting that the IL-6 response observed may be multi-factorial and not dependent entirely on MM-derived IL-1β.

### 3.2.4 STRO-1$^+$ Cells Have a Greater Proliferative Potential than STRO-1$^-$ and Represent a Greater Source of IL-6 Protein.

To further investigate the apparent association between cell proliferation and STRO-1 expression by human OB-like cells, carboxyfluorescein diacetate succinimiyl ester (CFSE) was used (Lyons, 1999; Lyons, 2000). OB-like cells were labelled with CFSE and cultured for 3 days, and subsequently stained with STRO-1-PE as described in the methods. As seen in Figure 3.11, OB-like cells were partitioned into STRO-1$^{bright}$ (R1) and STRO-1$^{negative/dim}$ population (R2) (Figure 3.11A), and their mitogenic capacity analysed with reference to the level of CFSE fluorescence. Interestingly, the STRO-1$^{bright}$ cells (Figure 3.11C) were found to have a significantly greater capacity to proliferate when compared with the STRO-1$^{negative/dim}$ population (Figure 3.11B). When the STRO-1$^{bright}$ cells were examined in isolation (Figure 3.11C, gate R1), a total of 70% of cells were found to have undergone at least one cell division, whilst 22% of cells had undergone 3 cell divisions. In contrast, when the STRO-1$^{negative/dim}$ population was examined (Figure 3.11B, gate R2), 37% of cells had undergone at least one cell division while only 14% had undergone 3 divisions. Consistent with the data presented in Figures 3.3 and 3.8, culture of OB-like cells with 10

Figure 3.10. The level of IL-6 protein is increased in supernatants of OB-like cell and myeloma cell co-culture. The level of IL-6 protein in the conditioned medium harvested from human OB-like cells or co-culture at d3 and d5 was measured by ELISA. A significant increase of IL-6 protein was found when RPMI8226 was co-cultured with OB-like cells. The IL-6 level was undetectable in supernatant of RPMI8226 cell culture. However, the IL-6 level increased to 5.74±0.03ng/ml in co-culture supernatants at d3, which was significantly higher than that found in OB-like cell culture (4.62±0.12ng/ml, $P$=0.008, Student $t$-test). Similarly, the IL-6 level was increased from 5.82±0.03ng/ml in OB culture to 6.56±0.15ng/ml in co-cultures at d5 ($P$=0.018, Students $t$-test). The mAb IL-1β slightly decreased IL-6 levels in co-culture, but was non-significant. Two independent experiments were performed and the similar results were observed.

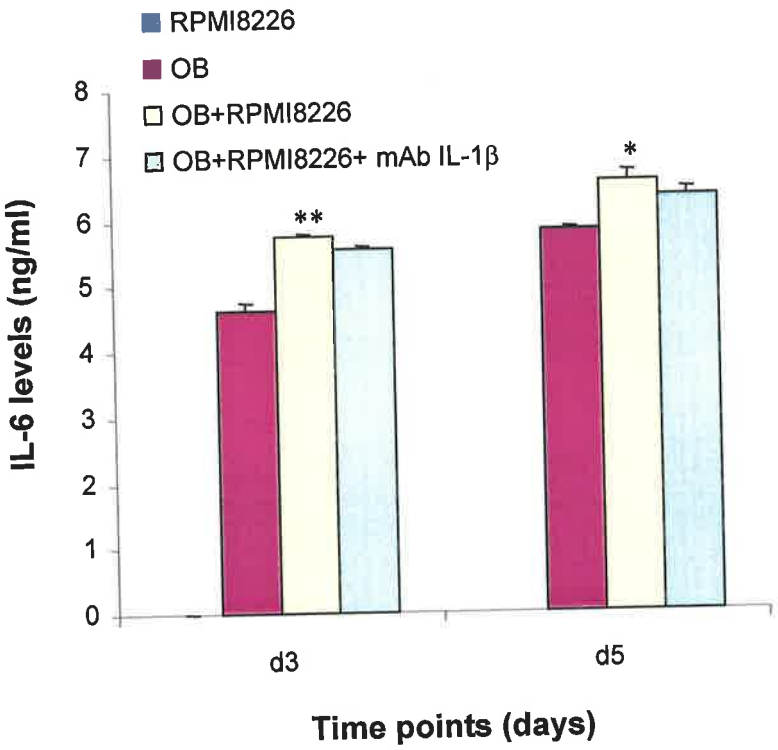
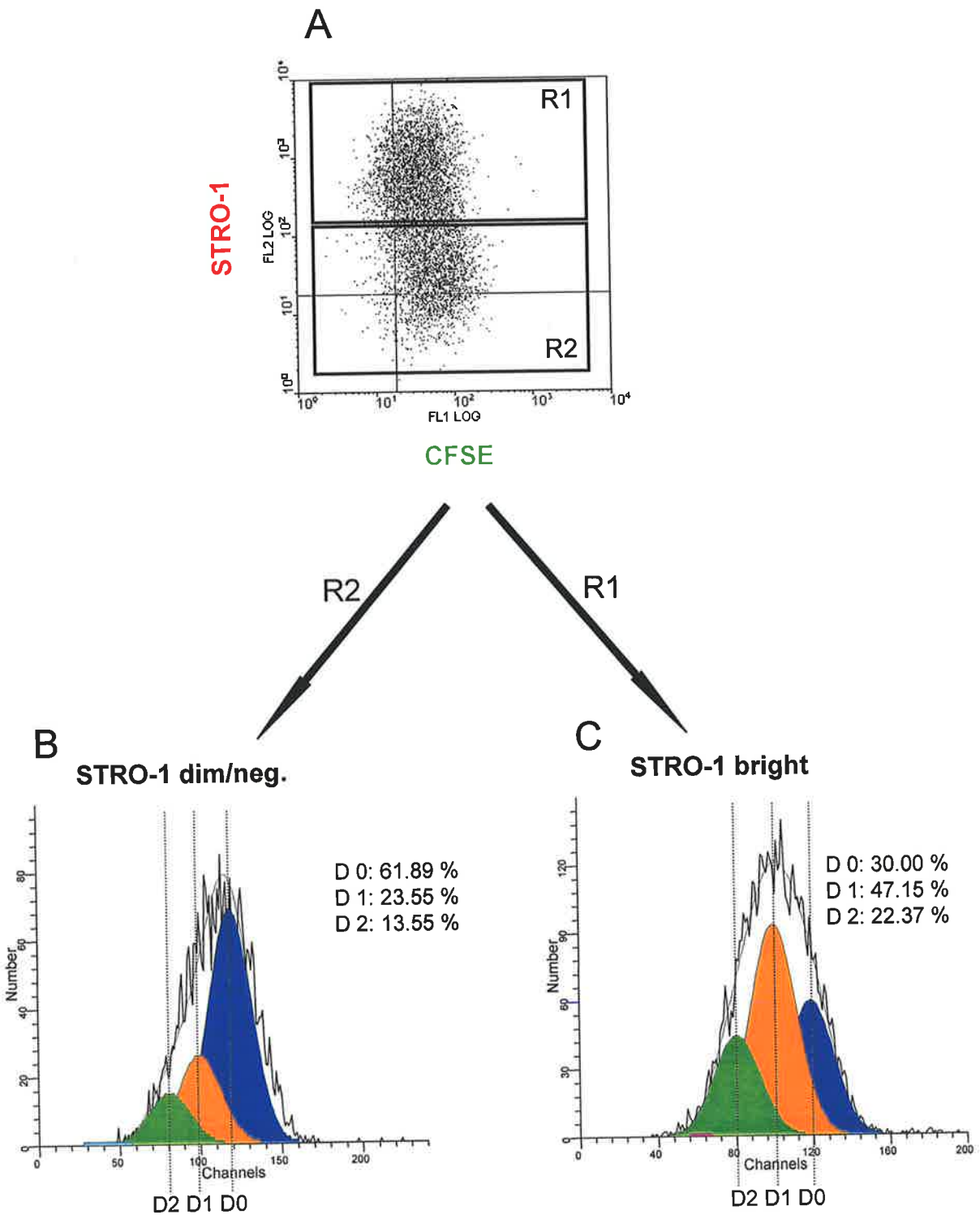

RPMI8226
OB
OB+RPMI8226
OB+RPMI8226+ mAb IL-1β
IL-6 levels (ng/ml)
0
1
2
3
4
5
6
7
8
**
*
d3
d5
Time points (days)

Figure 3.11. The STRO1$^{bright}$ fraction of OB-like cells possess a greater proliferative capacity than the STRO-1$^{negative/dim}$ fraction. OB-like cells were labelled with CFSE as described in the methods. Unlabelled OB-like cells were used to establish a negative control (auto-fluorescence). Colcemid® (100 ng/ml) was used to inhibit cell division and provided an input labelling index. After 3 days culture, cells were subsequently stained with STRO-1-PE as described in the methods and the cell proliferation was analysed using the ModFit LT for win 32 (Version 2.0). The STRO-1$^{bright}$ cells (R1) were found to have a significantly greater capacity to proliferate when compared with the STRO-1$^{negative/dim}$ population (R2).


A
R1
R2
STRO-1
FL2 LOG
FL1 LOG
CFSE
R2
R1
B
STRO-1 dim/neg.
D 0: 61.89 %
D 1: 23.55 %
D 2: 13.55 %
Number
Channels
D2 D1 D0
C
STRO-1 bright
D 0: 30.00 %
D 1: 47.15 %
D 2: 22.37 %
Number
Channels
D2 D1 D0

ng/ml IL-1β was associated with a significant increase in cell proliferation and an increase in the number of cells expressing the STRO-1 antigen (Figure 3.12). In the representative experiment presented in Figure 3.12, 17%, 43% and 16% untreated cells had undergone 1, 2 and 3 divisions, respectively at day 5 (Figure 3.12C), whilst in the presence of 10 ng/ml IL-1β, 8%, 21%, 31% and 25% of cells had undergone 1, 2, 3 and 4 divisions, respectively (Figure 3.12D). These studies strongly suggest that the expression of STRO-1 cell surface molecule is strongly associated with actively dividing OB-like cells

Although the levels of TNF-α and IL-1β protein were undetectable by ELISA, it was noted that STRO-1$^+$ cells expressed higher levels of IL-6 protein, when compared with STRO-1$^-$ cells. As seen in Figure 3.13, a significantly higher level of IL-6 protein was observed in STRO-1$^+$ cells (0.44±0.01ng/ml) compared with STRO-1$^-$ cells (0.29±0.02ng/ml) at 6h ($P$=0.04, Student $t$-test).

### 3.2.5 IL-1β Maintains the Immature Phenotype of OB-like Cells by Increasing STRO-1 Antigen Expression.

Previous studies (Gronthos *et al.*, 1999) suggest that bone-related gene expression was correlated with the phenotype of OB-like cells and the effect of IL-1β on the bone-related gene expression was therefore further explored. As seen in Figure 3.14, IL-1β significantly down regulated ALP (Figure 3.14A & B) and OCN (Figure 3.14C & D) gene expression in human OB-like cells (* $P$<0.05, ** $P$<0.005, Student $t$-test). These effects were maintained until day 21. As shown in Figure 3.15, IL-1β significantly down regulated ALP (Figure 3.15B), OCN (Figure 3.15C) and BSP (Figure 3.15D) gene expression in human OB-like cells until day 21. Furthermore, as seen in Figure 3.16, the decreased cell surface protein expression of the osteoblastic marker, ALP, in human OB-like cells was confirmed using immunofluorescence and flow cytometric analysis. A decrease in mean fluorescence of membrane ALP expression was observed when cells were exposed to 10 ng/ml IL-1β treatment at d7 (Figure 3.16A versus E), 14 (Figure 3.16B versus F), 21 (Figure 3.16C versus G) and 28 (Figure 3.16D versus H). In addition, exposure of OB-like cells to IL-1β, resulted in a significant elevation in ET-1 (Figure 3.15E) gene expression. As further discussed in Chapter 5, ET-1 may be critical for regulating OB bone formation.

Figure 3.12. IL-1β increases the proliferative potential of OB-like cells. Osteoblast-like cells were labelled with CFSE as described in the methods. Cells were subsequently cultured in the presence of 10 ng/ml IL-1β for 5 days, stained with STRO-1 mAb and analysed as described above. Of note, IL-1β enhanced cell division by increasing the number of bright STRO-$1^+$ osteoprogenitor cells. The results displayed are a representative example of 3 independent experiments.

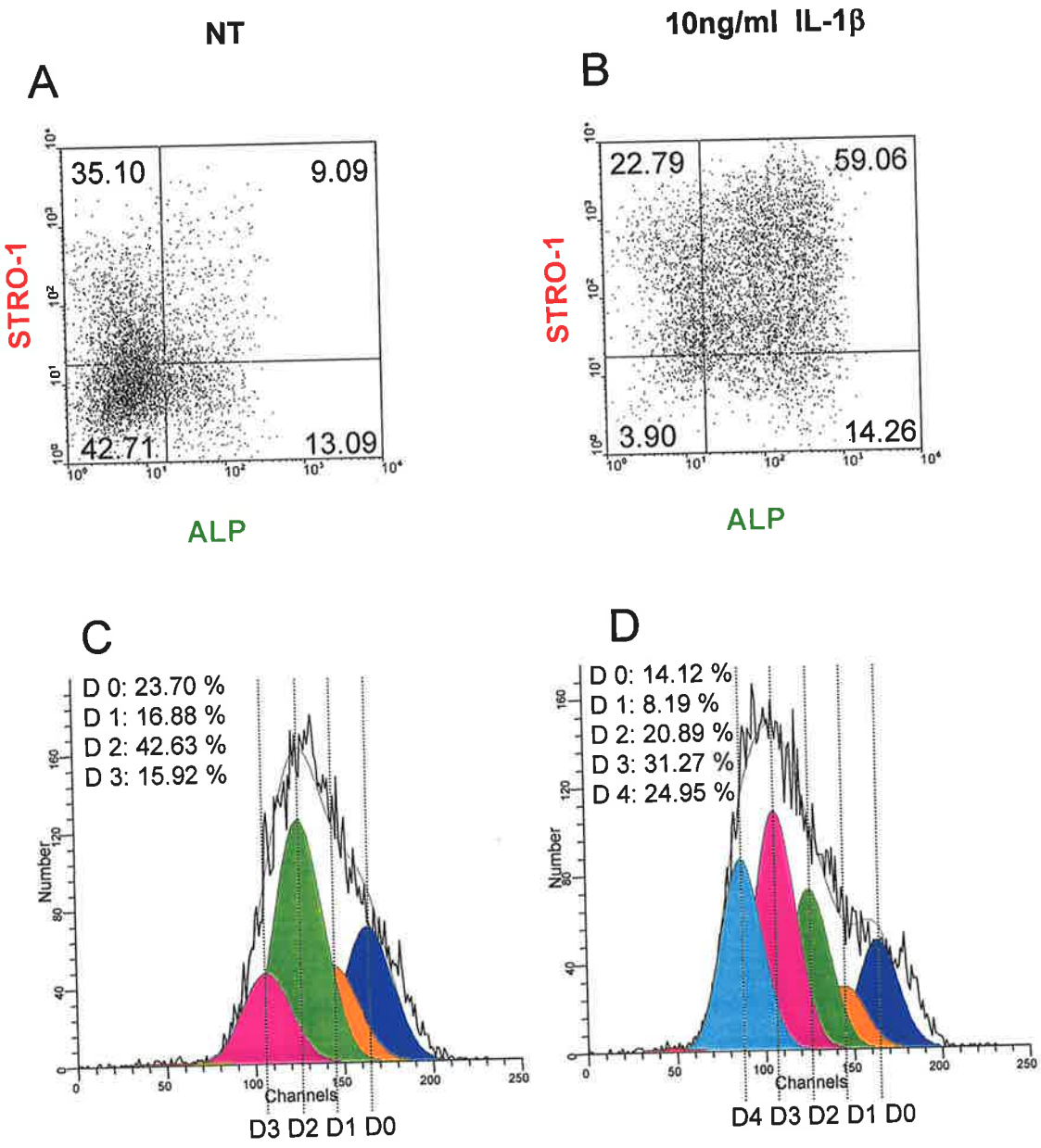

NT
10ng/ml IL-1β
A
B
35.10
9.09
42.71
13.09
22.79
59.06
3.90
14.26
STRO-1
ALP
C
D
D 0: 23.70 %
D 1: 16.88 %
D 2: 42.63 %
D 3: 15.92 %
D 0: 14.12 %
D 1: 8.19 %
D 2: 20.89 %
D 3: 31.27 %
D 4: 24.95 %
Number
Channels
D3 D2 D1 D0
D4 D3 D2 D1 D0

Figure 3.13. STRO-$1^+$ OB-like cells express higher levels of IL-6 protein compared with STRO-$1^-$ cells. Human OB-like cells were sorted using a FACStar$^{PLUS}$ cell sorter into STRO-$1^-$ and STRO-$1^+$ populations as described in the methods. Cells were cultured in a 6-well plate and the conditioned medium harvested at the indicated time points. The IL-6 level in the conditioned medium was measured by ELISA. A significant higher IL-6 level was observed in STRO-$1^+$ cells (0.44±0.01ng/ml) compared to STRO-$1^-$ cells (0.29±0.02ng/ml) at 6h ($P$=0.04, Student $t$-test). Two independent experiments were performed and the similar results were observed.

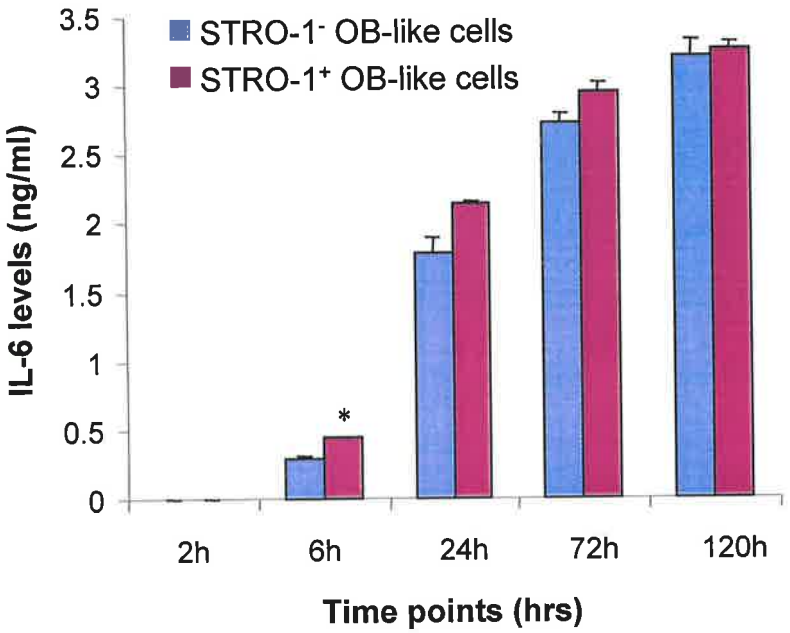

STRO-1⁻ OB-like cells
STRO-1⁺ OB-like cells
IL-6 levels (ng/ml)
3.5
3
2.5
2
1.5
1
0.5
0
*
2h
6h
24h
72h
120h
Time points (hrs)

Figure 3.14. IL-1β treatment mediates a down regulation of bone-related genes. Human OB-like cells were cultured in the presence of 10 ng/ml IL-1β and harvested at the indicated time points over a period of 5 days as described above. Total RNA was isolated and semi-quantitative RT-PCR was performed as described in the methods. The house-keeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression assessment. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager. The product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to the expression of GAPDH. The data showed that IL-1β significantly down regulated ALP (A and B) and OCN (C and D) gene expression in human OB-like cells (* $P<0.05$, ** $P<0.005$, Student *t*-test). The experiments were performed three times and a similar trend was observed in three different donors.

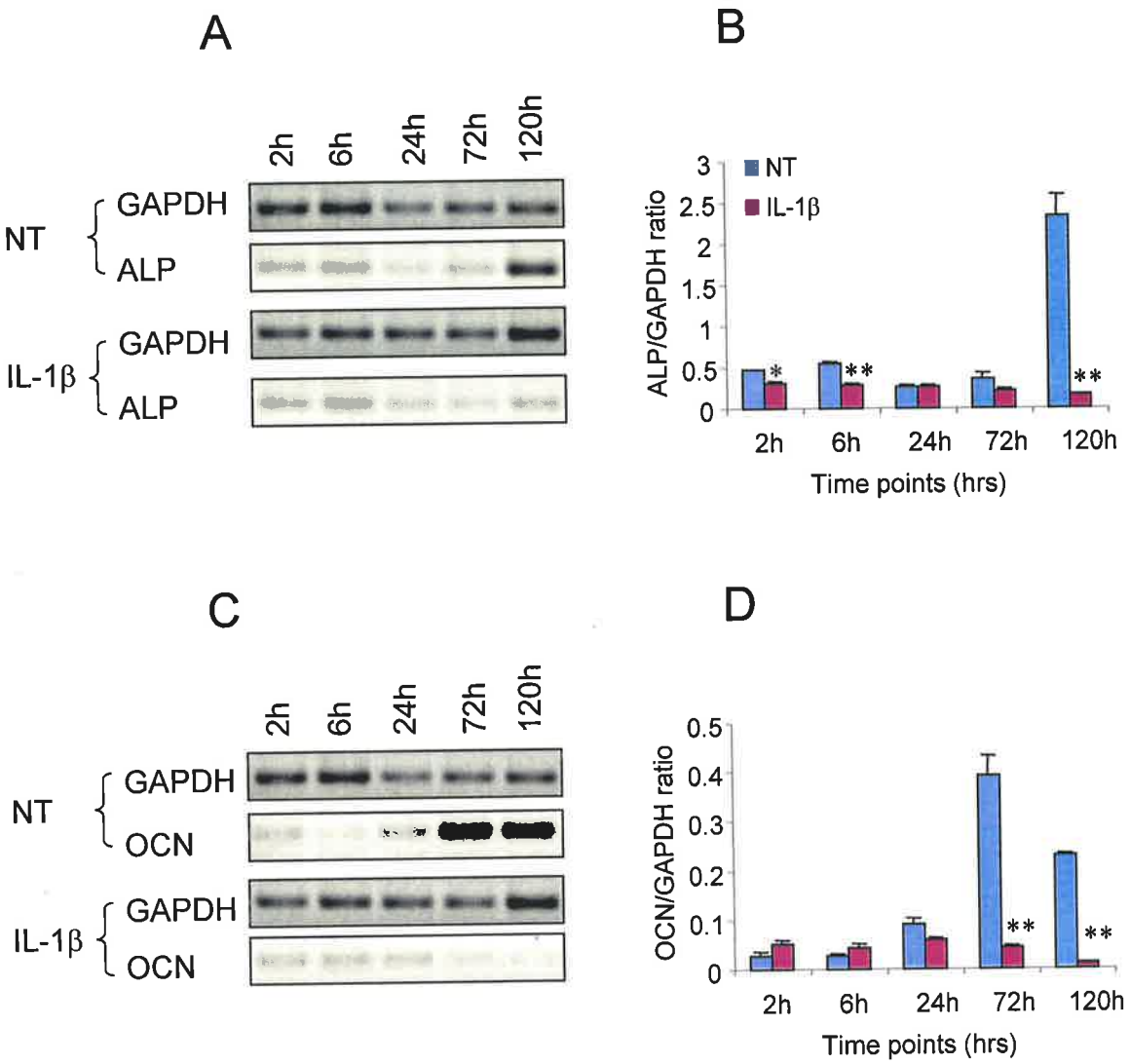

A
2h
6h
24h
72h
120h
NT
GAPDH
ALP
IL-1β
GAPDH
ALP
B
NT
IL-1β
ALP/GAPDH ratio
3
2.5
2
1.5
1
0.5
0
*
**
**
2h
6h
24h
72h
120h
Time points (hrs)
C
2h
6h
24h
72h
120h
NT
GAPDH
OCN
IL-1β
GAPDH
OCN
D
OCN/GAPDH ratio
0.5
0.4
0.3
0.2
0.1
0
**
**
2h
6h
24h
72h
120h
Time points (hrs)

Figure 3.15. Long term exposure of OB-like cells to IL-1β regulates bone-related gene expression. Human OB-like cells were treated with IL-1β at a concentration of 10 ng/ml and harvested at d7, d14 and d21. Total RNA was isolated and semi-quantitative RT-PCR was performed as described in the methods. The house-keeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression assessment. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager. The product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to the expression of GAPDH. IL-1β significantly down regulated ALP (B), OCN (C) and BSP (D) gene expression in human OB-like cells (* $P<0.05$, ** $P<0.005$, Student *t*-test). However, IL-1β significantly upregulated ET-1 (E) gene expression in human OB-like cells (* $P<0.05$, ** $P<0.005$, Student *t*-test). The experiments were performed three times and a similar trend was observed in two different donors.

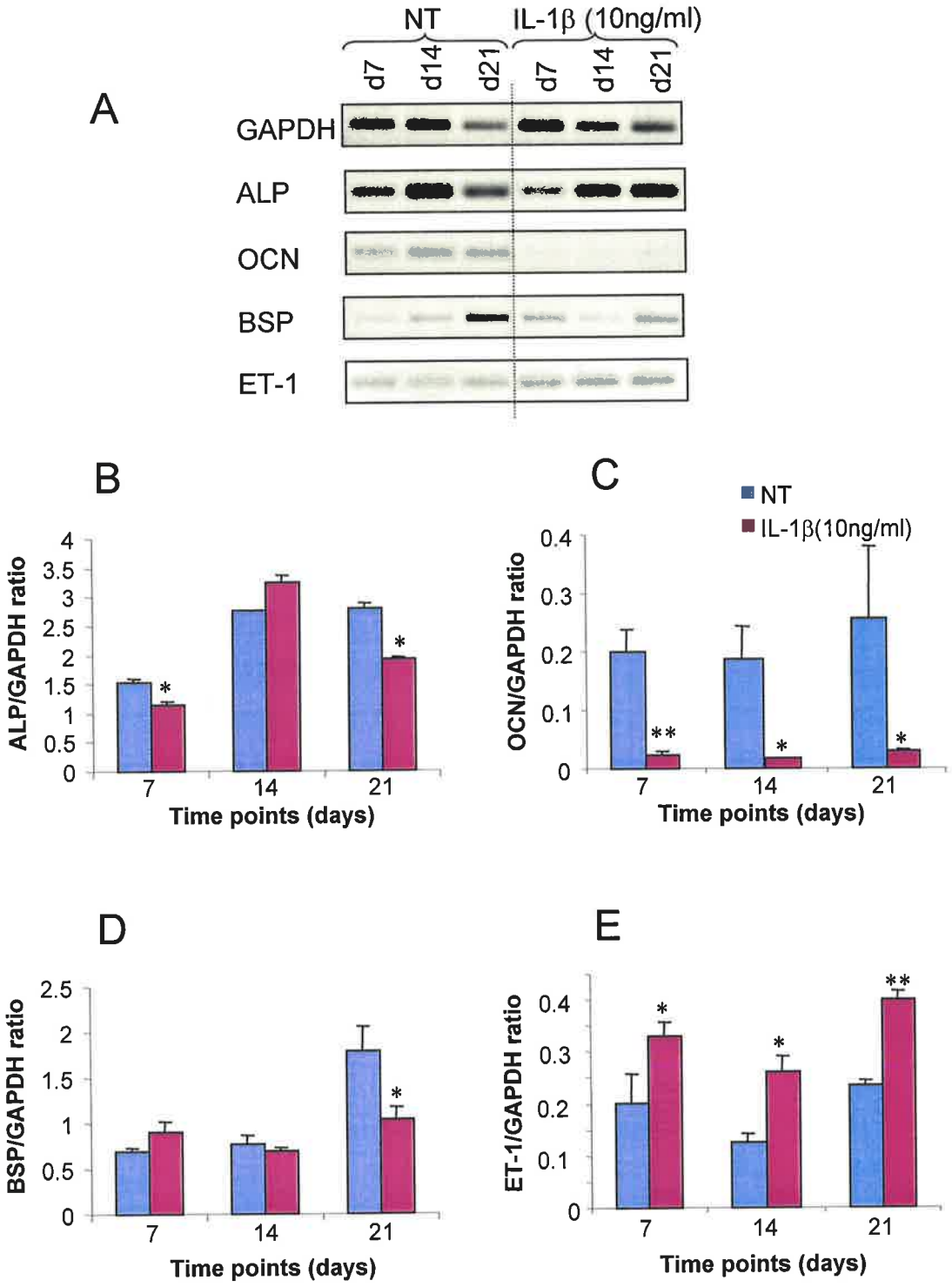
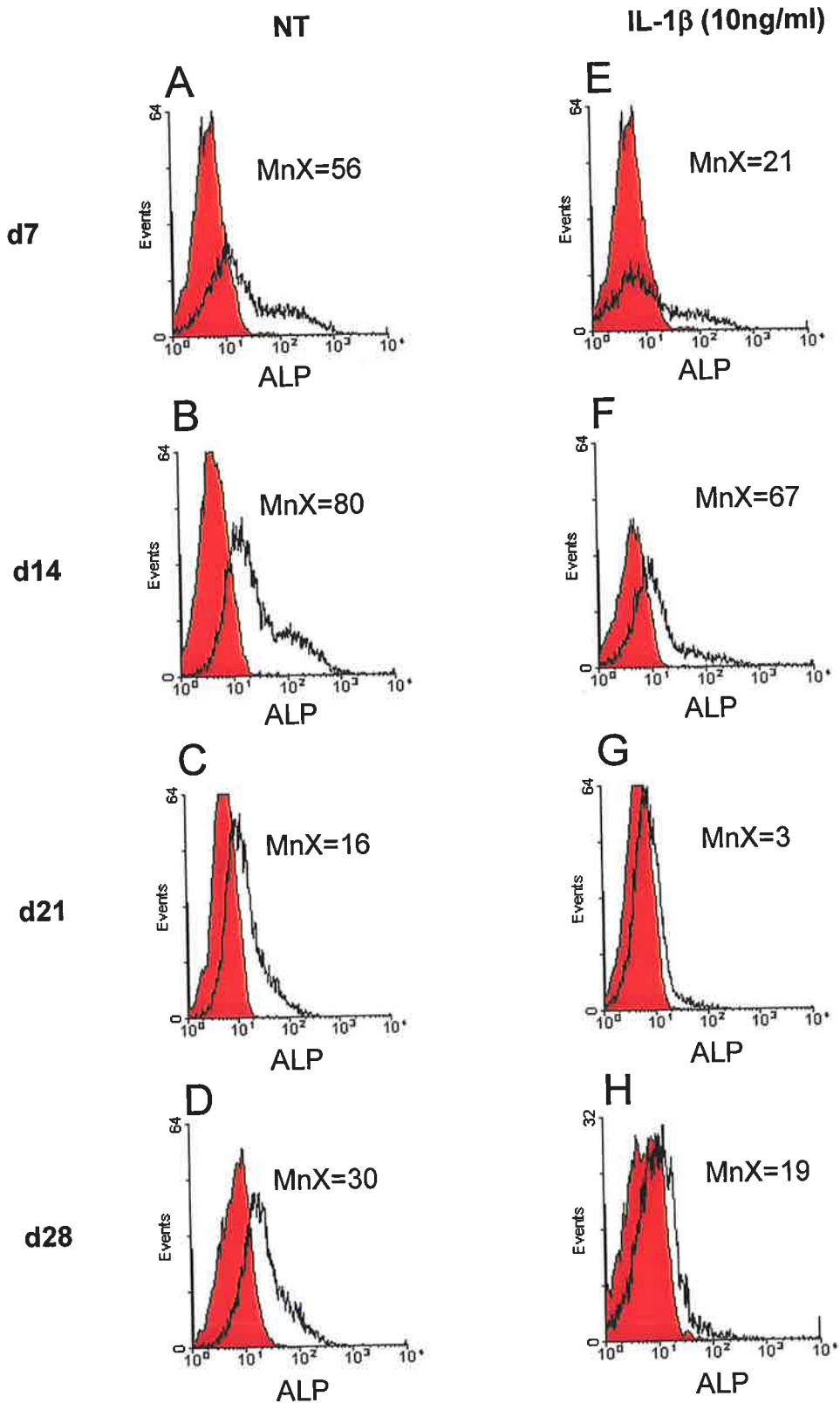

A
NT
IL-1β (10ng/ml)
d7
d14
d21
d7
d14
d21
GAPDH
ALP
OCN
BSP
ET-1
B
ALP/GAPDH ratio
Time points (days)
C
NT
IL-1β(10ng/ml)
OCN/GAPDH ratio
Time points (days)
D
BSP/GAPDH ratio
Time points (days)
E
ET-1/GAPDH ratio
Time points (days)

Figure 3.16. IL-1β decreases cell surface protein expression of the osteoblastic marker, ALP, in human OB-like cells. Cells were cultured in 75 $cm^2$ flask at a cell density of $4\times10^5$ for the period of 28 days in the presence/absence of 10 ng/ml IL-1β. The media was changed twice per week for the duration of culture. Cells were detached at weekly intervals and $4\times10^5$ cells were re-cultured whilst the remaining cells were stained with ALP-FITC. Fluorescence histograms depict the expression of surface ALP protein. The data is expressed as the relative cell count (y-axis) versus the intensity of ALP expression (log scale). Ten thousand events were collected as listmode data. Isotype matched mAb, 1B5, was used as a negative control. A decrease in mean fluorescence of membrane ALP expression was observed when cells were exposed to 10 ng/ml IL-1β treatment at d7 (A versus E), 14 (B versus F), 21 (C versus G) and 28 (D versus H). The experiments were performed three times using OB-like cells derived from three different donors.


NT
IL-1β (10ng/ml)
d7
d14
d21
d28
A
B
C
D
E
F
G
H
MnX=56
MnX=80
MnX=16
MnX=30
MnX=21
MnX=67
MnX=3
MnX=19
Events
ALP

### 3.2.6 The Phenotype of OB-Like Cells Derived from Patients with MM are Similar to Normal OBs Which Have Been Subjected to Long Term IL-1β Exposure

Numerous studies have been published, which report that primary OBs from individuals with skeletal disease maintain an altered phenotype in culture when compared with control OBs (Chenoufi *et al.*, 2001; Massicotte *et al.*, 2002; Yudoh *et al.*, 2000). In accord with these findings, we observed that OB-like cells derived from patients with MM and MGUS exhibited differential STRO-1 and ALP expression when compared with normal OB-like cells (Figure 3.17A-C). Notably, OB-like cells derived from MGUS and MM expressed less surface ALP.

Interestingly, we found that long term exposure of normal OB-like cells to IL-1β resulted in a decrease in STRO-1 and ALP expression. As shown in Figure 3.17, in the absence of IL-1β, the expression of STRO-1 and ALP altered as a function of "time in culture" (Figure 3.17 D to F). When cultured with IL-1β, OB-like cells displayed an initial increase in STRO-1 (from 64.42% to 76.51%) and ALP (from 58.13% to 66.95%) expression at d7 (Figure 3.17D versus G), which was followed by a reduction of both STRO-1 and ALP expression in OB-like cells at the later time points examined. At d14 (Figure 3.17H) and d21 (Figure 3.17I), IL-1β treated OB-like cells were more homogenous and expressed lower levels of both STRO-1 and ALP compared with untreated control (Figure 3.17E & F). As shown in Figure 3.18, following long term culture in IL-1β, OB-like cells assumed a more homogeneous morphology with a highly organised orientation and fibroblastic appearance.

Considering the differences in OB-like cell phenotype between cells derived from MM, MGUS patients and normal donors, we next examined if these cells were still capable of responding to the mitogenic effects of IL-1β. Osteoblasts derived from 4 NHB, 4 MGUS and 4 MM were cultured in the presence of 10 ng/ml IL-1β and the cell number and viability measured using WST-1. As seen in Figure 3.19, IL-1β induced a significant increase (* $P<0.05$, ** $P<0.005$, Student *t*-test) in cell number in OB-like cells derived from all donors tested at d5.

Figure 3.17. The phenotype of OB-like cells derived from MM is found to be phenotypically similar to normal OBs exposed to IL-1β long term. Cells derived from normal donors, patients with MGUS or MM were cultured in 75 $cm^2$ flask for three days and dual-colour immunofluorescence and flow cytometric analysis performed using mAbs STRO-1 and B4-78. Alternatively, normal OB-like cells were cultured in 25 $cm^2$ flasks at a cell density of $1.3\times10^5$ and the media changed two times per week with/without 10 ng/ml IL-1β. Cells were detached by trypsin every week and dual-colour immunofluorescence and flow cytometric analysis was performed using mAbs STRO-1 and B4-78. Immunoreactivity was revealed by incubation with an anti IgM-PE (y-axis) and IgG-FITC (x-axis), as described in the methods. The dot plot histogram represents 10,000 events collected as listmode data. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, 1B5 (FITC) and 1A6.12 (PE), respectively. The phenotype of OB-like cells derived from MGUS (B) and MM (C) displayed differential STRO-1 and ALP expression compared to cells derived from normal donor (A). Notably, OB-like cells derived from MGUS and MM expressed less surface ALP, which was consistent with the previous data (figure 3.16) showing that long term exposure of OB to IL-1β resulted in a reduction of ALP expression. Interestingly, long term exposure of IL-1β decreased both STRO-1 and ALP expression on human OB-like cells. The expression pattern of STRO-1 and ALP in human OB-like cells differed at different time points d7 (D), d14 (E) and d21 (F). Although IL-1β increased STRO-1 (from 64.42% to 76.51%) and ALP (from 58.13% to 66.95%) expression at d7 (D versus G), it reduced both STRO-1 and ALP expression in OB-like cells following long term exposure. At d14 (H) and d21 (I), IL-1β treated OB-like cells were more homogenous and had lower expression of both STRO-1 and ALP antigen compared with untreated controls (E & F). A representative example of the results obtained from three independent experiments is shown.

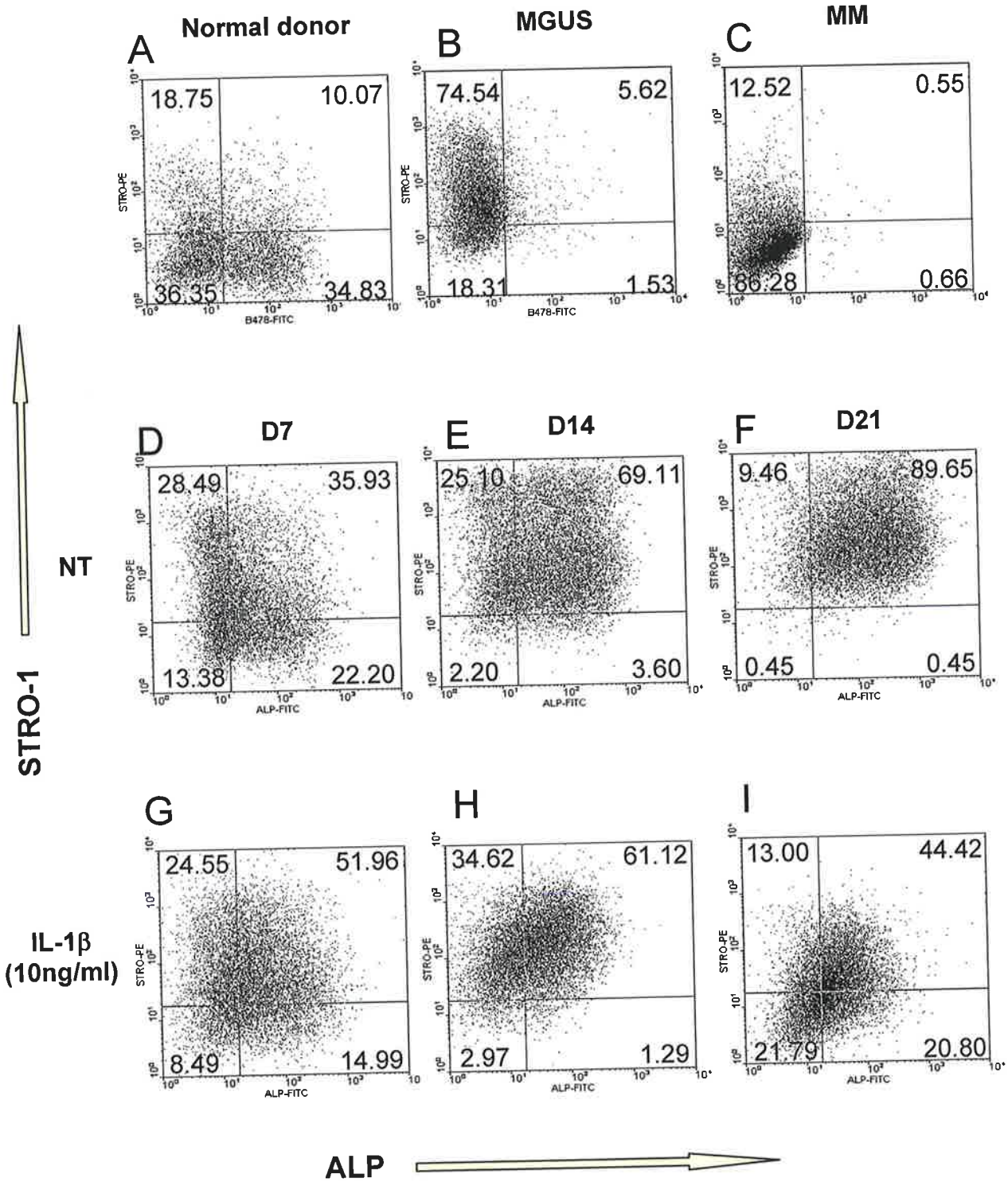
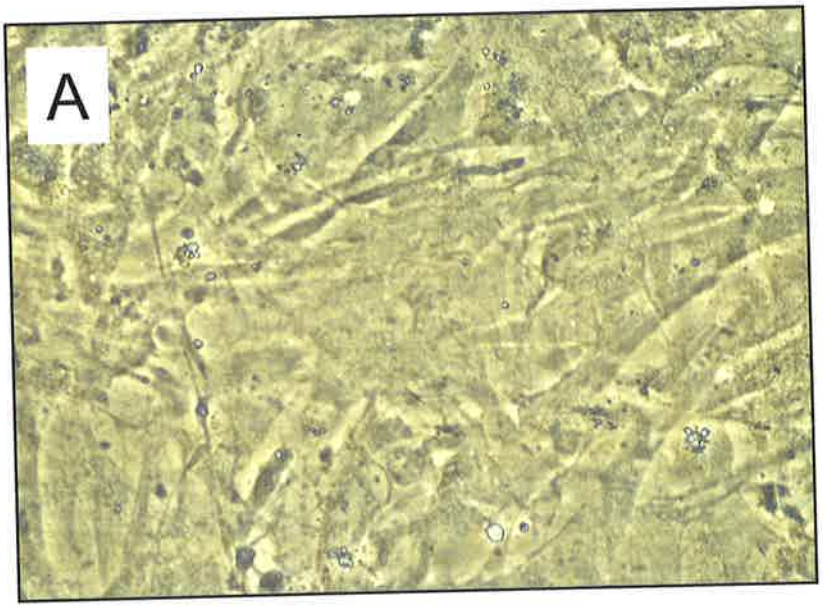

Normal donor
MGUS
MM
A
B
C
18.75
10.07
36.35
34.83
74.54
5.62
18.31
1.53
12.52
0.55
86.28
0.66
STRO-PE
B478-FITC
D7
D14
D21
D
E
F
28.49
35.93
13.38
22.20
25.10
69.11
2.20
3.60
9.46
89.65
0.45
0.45
ALP-FITC
NT
STRO-1
G
H
I
24.55
51.96
8.49
14.99
34.62
61.12
2.97
1.29
13.00
44.42
21.79
20.80
IL-1β
(10ng/ml)
ALP

Figure 3.18. Long term exposure of IL-1β alters OB-like cell morphology. Human OB-like cells were cultured in 25 $cm^2$ flasks at a cell density of $1.3\times10^5$ and the media were changed twice a week with/without 10 ng/ml IL-1β. The morphological changes of OB-like cells treated with IL-1β were examined by phase-contrast light microscopy at d21. OB-like cells exposed to IL-1β displayed a more homogeneous morphology with a highly organised orientation and fibroblastic appearance (B) compared with untreated control (A). A representative example of three experiments is shown. Original magnification, ×20


A

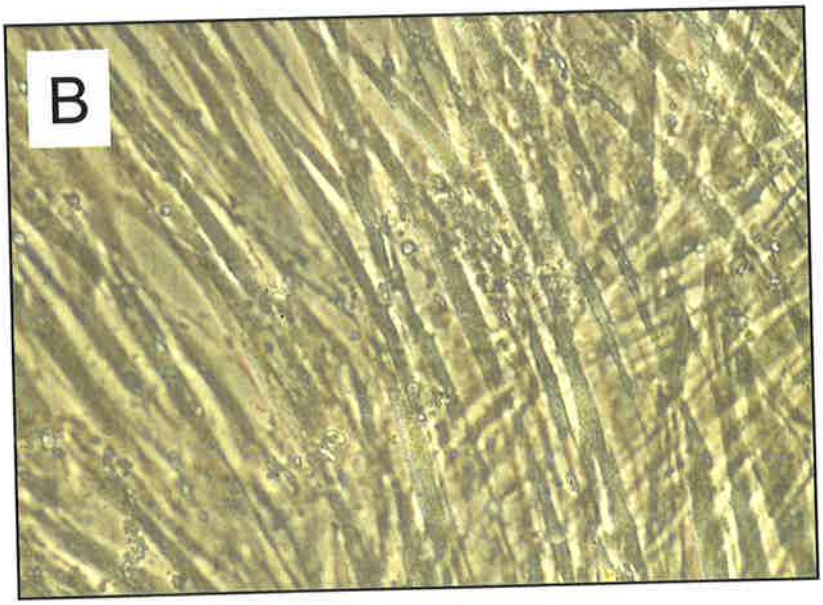
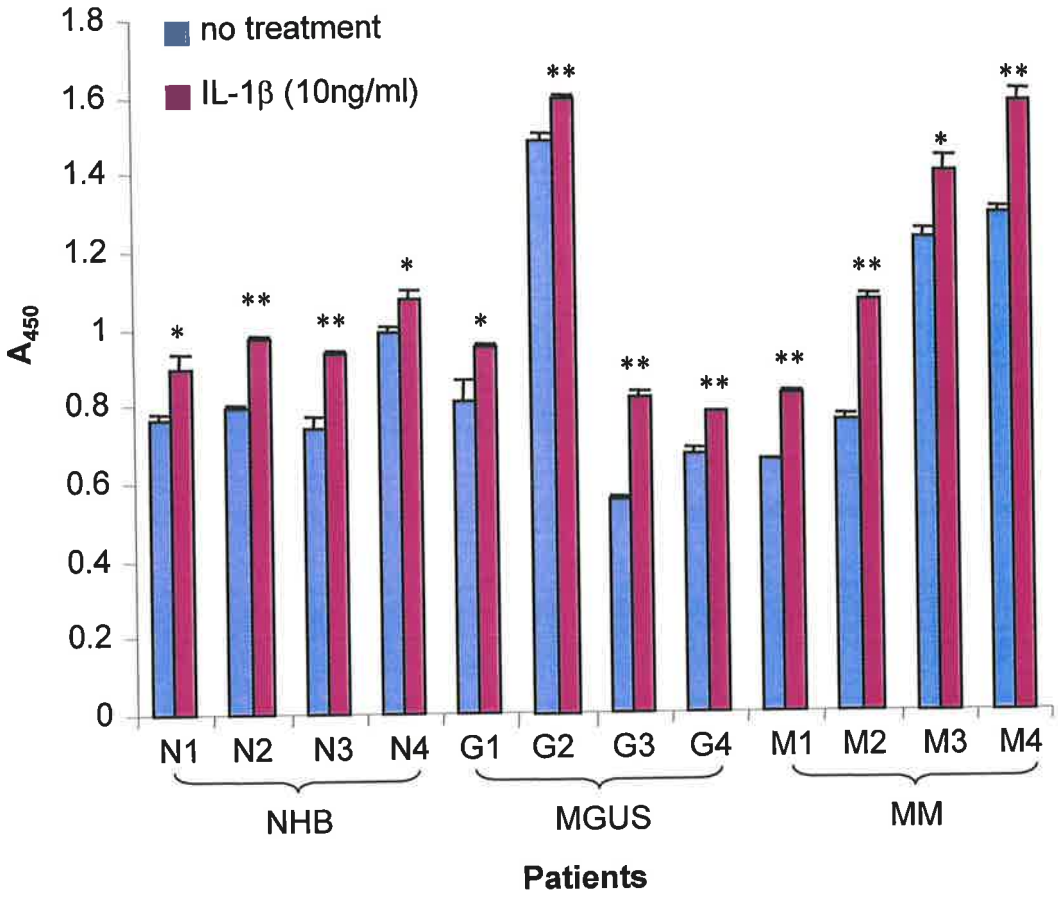

B

Figure 3.19. Human OB-like cells derived from MGUS (G) and MM (M) exhibit a similar proliferative response to IL-1β compared with OB-like cells derived from normal donors (N). Human OB-like cells were seeded into 96-well plates at a cell density of 2,000 cells/well. The cells were cultured in the presence or absence of 10ng/ml IL-1β and the cell number and viability quantitated at d5 using WST-1, as described in the methods. IL-1β induced a significant increase (* $P<0.05$, ** $P<0.005$, Student *t*-test) in cell number in all donors at d5. Values represent means ± SEM of triplicate cultures at each concentration. The results displayed are a representative example of 3 independent experiments. N: NHB (normal human bone cells); G: MGUS; M: MM.


no treatment
IL-1β (10ng/ml)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A450
N1 N2 N3 N4 G1 G2 G3 G4 M1 M2 M3 M4
NHB
MGUS
MM
Patients

### 3.2.7 IL-1β Does not Impair the Bone Mineral Forming Capacity of OB-Like Cells under Osteoinductive Condition

We next examined whether exposure to IL-1β demonstrably altered the function of OB-like cells. Although one effect of IL-1β was to maintain the immature phenotype of OB-like cells (as evidenced by down regulated bone-related genes and upregulated STRO-1 antigen expression), IL-1β did not appear to affect the bone mineral forming potential of OB-like cells when cultured in osteoinductive conditions. As shown in Figure 3.20, the mineral deposition was increased in cells treated with IL-1β (Figure 3.20B) compared with untreated cells (Figure 3.20A). The calcium level in IL-1β treated cells was significantly higher than that in untreated cells at both week 4 (**$P$=0.00009, Student $t$-test) and week 6 (**$P$=0.00004, Student $t$-test) (Figure 3.20C).

### 3.2.8 IL-1β-upregulated STRO-1$^+$ OB-like Cells Possess an Enhanced Capacity to Support Osteoclastogenesis.

Besides bone formation, OBs are essential for component of the osteoclastogenic process. We therefore examined the changes in OB phenotype in relation to changes in expression of the potent osteoclastogenic protein RANKL following IL-1β stimulation. Interestingly, the cell surface STRO-1 protein expression on human OB-like cells was closely associated with RANKL expression. As seen in Figure 3.21, the dual-colour histogram, showed that STRO-1 and RANKL expression was co-expressed, with cells expressing high levels of STRO-1, also expressing high RANKL expression.

Numerous studies have shown that osteoclastogenesis is determined principally by the local ratio of RANKL to OPG. In accord with this, we next examined the level of OPG in the conditioned medium following IL-1β treatment of OB-like cells. As seen in Figure 3.22, IL-1β significantly increased the level of OPG protein from 4.57±0.27, 15.58±1.09 and 39.01±2.97ng/ml to 7.74±0.34ng/ml, 31.53±1.02 and 68.2±3.71ng/ml at 24h ($P$=0.008, Student $t$-test), 72h ($P$=0.0004, Student $t$-test) and 120h ($P$=0.024, Student $t$-test), respectively (Figure 3.22A). Moreover, a significantly higher OPG level was observed in STRO-1$^+$ cells (15.48±0.89ng/ml) compared with STRO-1$^-$ cells (10.1±0.39ng/ml) at 72h ($P$=0.007, Student $t$-test) (Figure 3.22B). However, although co-culture of OB-like cells with RPMI8226 resulted in an increased in the number of STRO-1$^+$ OB-like cells, it was associated with a significant decrease in the OPG level from 43.38±3.59ng/ml to

Figure 3.20. IL-1β does not impair the bone forming capacity of OB-like cells. Osteoblast-like cells were seeded into 24-well plates at a cell density of $5\times10^4$/well in triplicate, and cultured in osteoinductive conditions, as described in the methods. The cells were treated with IL-1β at a concentration 10 ng/ml and cultures were "fed" weekly with fresh medium containing IL-1β. The release of free calcium from the matrix was achieved by treating the adherent cell layers under acidic condition as described in the methods. Samples were reacted with o-cresol-phthalein-complexon and the colorimetric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium. The results showed that mineral deposition was increased in cells treated with IL-1β (B) compared to untreated cells (A). The calcium level in IL-1β treated cells was significantly higher than that in untreated cells at both week 4 (**$P$=0.00009, Student $t$-test) and week 6 (**$P$=0.00004, Student $t$-test). The experiments were performed three times using OB-like cells derived from three different donors.

A

B

C

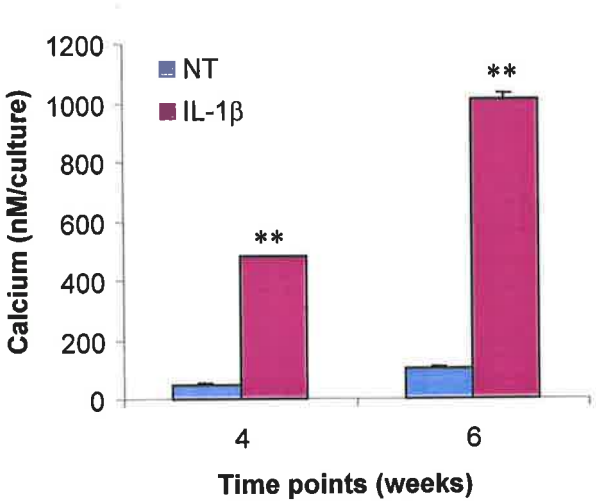
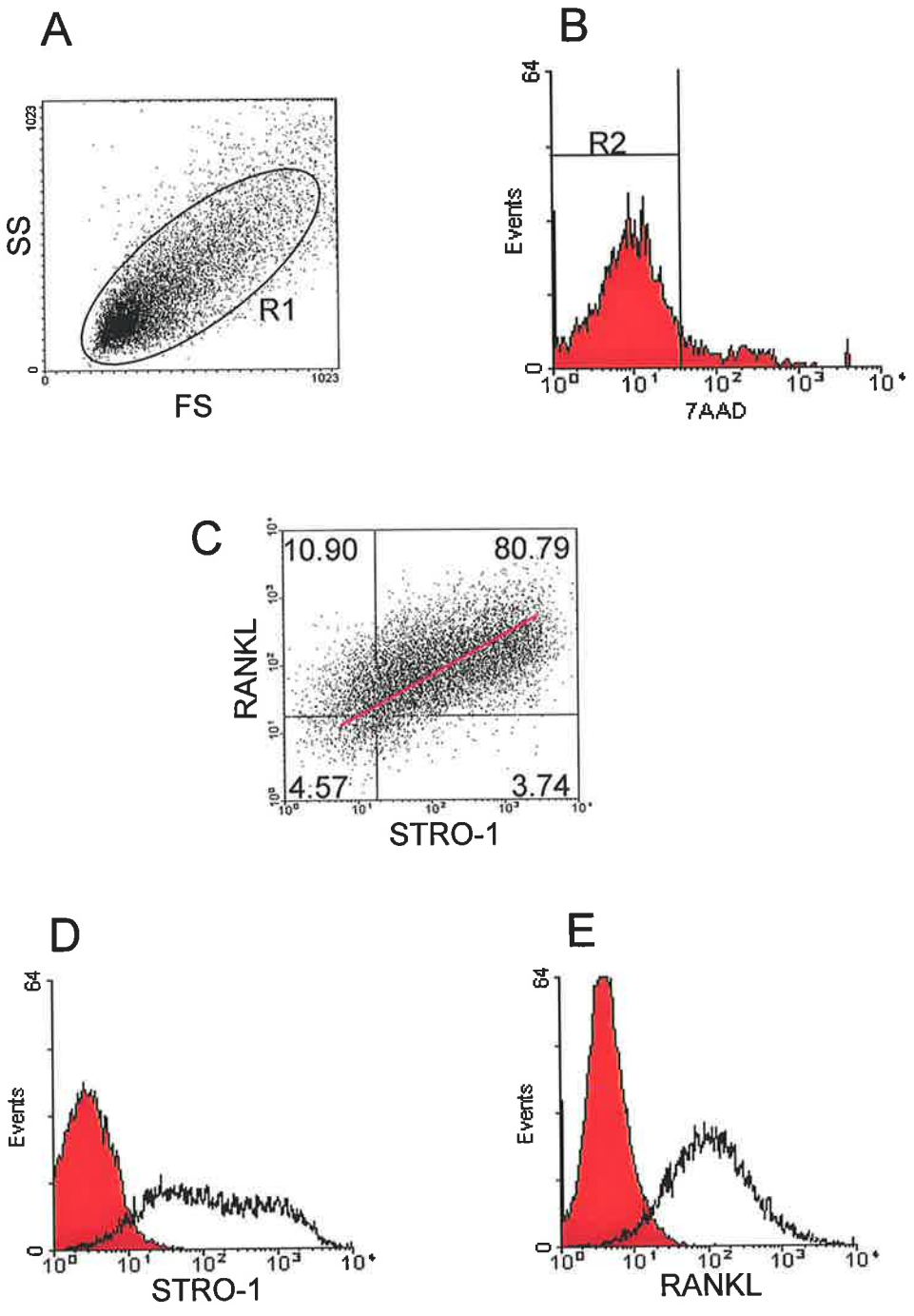

1200
1000
800
600
400
200
0
Calcium (nM/culture)
NT
IL-1β
**
**
4
6
Time points (weeks)

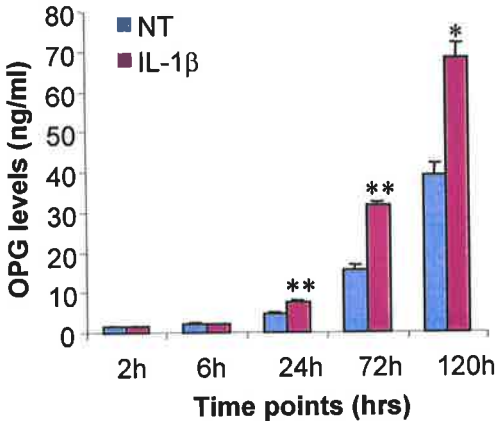
Figure 3.21. Cell surface STRO-1 protein expression on human OB-like cells is positively associated with RANKL expression. Subconfluent human OB-like cells cultured in 75 $cm^2$ flask were detached using EDTA as described in the methods. Dual-colour immunofluorescence and flow cytometric analysis was performed using OB-like cells stained with the mAbs STRO-1 and RANKL (MAB626, R&D). Immunoreactivity was revealed by incubation with anti IgM-FITC (x-axis) and IgG-PE (y-axis), as described in the methods. The histogram represents 10,000 events collected as listmode data. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, 1A6.12 (FITC) and 1A6.11 (PE), respectively. 7-AAD was used to exclude dead cells. Viable OB-like cells were gated on combination of R1 (A) and R2 (B) gates. The dot plot histogram showed that STRO-1 and RANKL expression was tightly associated (C). Fluorescence histograms depict the expression of STRO-1 (D) and RANKL (E) protein on the cell surface of OB-like cells.


A
SS
FS
R1
1023
0
1023
B
64
0
Events
R2
7AAD
C
RANKL
STRO-1
10.90
80.79
4.57
3.74
D
Events
STRO-1
E
Events
RANKL

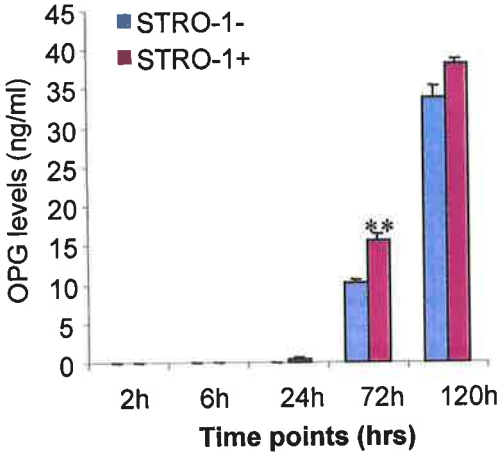
34.54±1.79ng/ml at d5 ($P$=0.046, Student $t$-test) (Figure 3.22C), suggesting that myeloma cells may play a role in reducing OPG level in the co-culture.

Figure 3.22. Although IL-1β increases OPG level, co-culture of RPMI8226 with OB-like cells decreases OPG level. The OPG protein levels in medium harvested from IL-1β treated cultures, sorted STRO-1$^-$ and STRO-1$^+$ cultures and co-cultures were measured by ELISA as described in the methods. Notably, IL-1β significantly increased OPG levels in OB-like cells (A). The IL-1β treatment increased OPG levels from 4.57±0.27, 15.58±1.09 and 39.01±2.97ng/ml to 7.74±0.34, 31.53±1.02 and 68.2±3.71ng/ml at 24h ($P$=0.008, Student $t$-test), 72h ($P$=0.0004, Student $t$-test) and 120h ($P$=0.024, Student $t$-test), respectively. A significantly higher level of OPG was observed in STRO-1$^+$ cells (15.48±0.89ng/ml) compared with STRO-1$^-$ cells (10.1±0.39ng/ml) at 72h ($P$=0.007, Student $t$-test) (B). Surprisingly, co-culture RPMI8226 with OB-like cells significantly decreased OPG level from 43.38±3.59ng/ml to 34.54±1.79ng/ml at d5 ($P$=0.046, Student $t$-test) (C). Two independent experiments were performed and similar results were observed in each case.

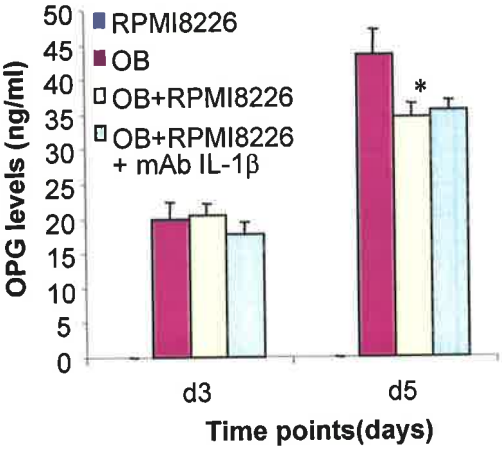
A


NT
IL-1β
OPG levels (ng/ml)
80
70
60
50
40
30
20
10
0
**
**
*
2h
6h
24h
72h
120h
Time points (hrs)

B


STRO-1-
STRO-1+
OPG levels (ng/ml)
45
40
35
30
25
20
15
10
5
0
**
2h
6h
24h
72h
120h
Time points (hrs)

C


RPMI8226
OB
OB+RPMI8226
OB+RPMI8226
+ mAb IL-1β
OPG levels (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
*
d3
d5
Time points(days)

## 3.3 DISCUSSION

Bone destruction is a characteristic feature in more than 80% of patients diagnosed with MM. Whilst numerous studies have focused on the excessive osteoclastic bone resorption in patients with advanced MM, few have investigated the function of OBs in MM. The bone microenvironment is a heterogeneous tissue comprised of numerous cell types, including stromal/OB cells, endothelial cells, OCs and haemopoietic cells which respond to a multitude of signals that they encounter in the extracellular environment. It is therefore reasonable to assume that the mechanisms responsible for the osteolytic bone lesions are the result of a complex series of interactions between the different types of cells and factors produced within the BM microenvironment. Because OBs are essential for both bone formation and for their support of OC formation, we have focused on the effects of a number of cytokines, which are elevated in the most cases of MM, on these cells.

No common mediator of MM-induced osteoclastogenesis has been identified. In fact, when cytokine levels are examined in patients with MM, the levels detected in serum are often heterogeneous. Despite this, it is commonly reported that the levels of IL-1β and TNF-α in patients with MM are higher than that of normal controls (Lichtenstein *et al.*, 1989). Whilst it remains controversial as to whether myeloma cells secrete these cytokines directly, most reports suggest that myeloma cells produce several of these OAFs directly (Costes *et al.*, 1998; Cozzolino *et al.*, 1989; Donovan *et al.*, 2002; Donovan *et al.*, 1998; Lacy *et al.*, 1999; Yamamoto *et al.*, 1989). IL-1β has been detected using both *in situ* hybridization and immunochemistry techniques in patients with MM (Donovan *et al.*, 2002; Donovan *et al.*, 1998; Lacy *et al.*, 1999). In addition, increased IL-1β have been detected in the supernatant of myeloma cell cultures, which have been isolated from MM patients with flow cytometric cell sorting methods (Costes *et al.*, 1998; Cozzolino *et al.*, 1989; Yamamoto *et al.*, 1989). However, some studies have presented inconclusive results (Borset *et al.*, 1993; Sati *et al.*, 1999). Using immunomagnetic separation techniques, purified human myeloma cells were shown not to be the source of IL-1 or of IL-6 (Borset *et al.*, 1993). According to these data, production of IL-1 and IL-6 in myeloma tumour was derived from non-myeloma cells such as endothelium, OBs, monocytes, macrophages, B cells and activated T cells. BM stromal cells have been shown to produce high levels of IL-6 and measurable amount of IL-1β (Caligaris-Cappio *et al.*, 1991). In addition, the adhesion of T cells to human primary OBs has been reported to result in the synthesis of IL-6 by OBs

(Rifas *et al.*, 1999). Furthermore, it has known that macrophages are capable of producing a plethora of cytokines, including TNF-α, IL-1 and IL-6. (Borset *et al.*, 1993; Sati *et al.*, 1999). No matter which type cells are responsible for the increased OAFs in patients with MM, these cytokines appear to contribute significantly to the formation of osteolytic lesion in MM.

Although it is exceedingly difficult to predict *in vivo* effects and roles of individual cytokines based on *in vitro* experimental models, it is still represents a valid and practical approach to investigate the underlying mechanisms responsible for the bone resorption induced by these pro-inflammatory cytokines. With this in mind, we first examined the effect of pro-inflammatory cytokines, IL-1β, TNF-α and IL-6, for their mitogenic effects on human OB-like cells. Consistent with the findings of others (Evans *et al.*, 1990; Evans *et al.*, 1989; Gowen *et al.*, 1985; Ikeda *et al.*, 1988), IL-1β was found to produce the greatest mitogenic effect on OB-like cells among the three cytokines tested. TNF-α was also found to significantly increases the cell growth of OB-like cells in a dose dependent manner (Frost *et al.*, 1997; Harbour *et al.*, 2000; Kim *et al.*, 2002a; Morioka *et al.*, 2000). Although other studies have shown that IL-6 does not affect the proliferation rate of human OB-like cells (Frost *et al.*, 1997; Modrowski *et al.*, 1995), we found that IL-6 displayed mitogenic effects on OB-like cells at high concentrations exceeding 100ng/ml. It is well accepted that different cytokines may exert similar and overlapping functions on certain cell types. For example, IL-6 has been shown to elicit similar biological effects when compared with IL-11 (Oleksowicz *et al.*, 1994) due to their common use of the gp130 signal transducing polypeptide (Klein *et al.*, 1995). Moreover, many cytokines, including IL-1β, TNF-α and IL-6 operate synergistically to enhance their individual actions on OB-like cells (Costes *et al.*, 1998; Linkhart *et al.*, 1991; Lu *et al.*, 1995; Thomas *et al.*, 1998).

In addition to examining gene expression, we also measured the levels of IL-1β, TNF-α and IL-6 protein secreted by OB-like cells in response to recombinant cytokine stimulation. Consistent with the findings of others (Gowen *et al.*, 1990; Linkhart *et al.*, 1991), our studies showed that IL-1β treatment significantly upregulated gene expression of TNF-α and IL-1β in OB-like cells. In keeping with the published observations (Chaudhary *et al.*, 1992; Gori *et al.*, 2000; Linkhart *et al.*, 1991), our studies showed that TNF-α and IL-1β were undetectable in the culture supernatant of OB-like cells. Linkhart *et al* (1991)

demonstrated that while recombinant IL-1β resulted in an increase in IL-1β mRNA, it was unable to increase cellular or secreted levels of IL-1β protein in human OBs (Linkhart *et al.*, 1991). In addition, Chaudhary *et al* (1992) were unable to detect TNF-α protein following IL-1β stimulation of human OB-like cells (Chaudhary *et al.*, 1992). Contrary to these reports, Keeting and colleagues (1991) reported the detection of 1.3±0.3pg/ml IL-1β in conditioned medium harvested from normal human OB-like cells (Keeting *et al.*, 1991). In keeping with this, studies by Calligaris-Cappio and co-workers (1991) repeatedly detected 7-10 pg/ml of IL-1β in the supernatant of a 7 week BM stromal cell culture (Caligaris-Cappio *et al.*, 1991). Therefore, it is reasonable to conclude that whilst OB-like cells are likely to secrete measurable levels of TNF-α and IL-1β, these levels are low and sensitive methods of detection are required to measure any subtle changes caused by exogenous treatment. In addition, the dissociation phenomena of transcription from translation has been reported in cytokines IL-1β and TNF-α, and this phenomena may also be the possible reason for undetectable IL-1β and TNF-α protein in our study (Schindler *et al.*, 1990a; Schindler *et al.*, 1990b).

Our data also suggest that IL-1β increases both gene expression and protein synthesis of IL-6. Furthermore, COX2 gene expression is also upregulated following IL-1β stimulation, suggesting that these down-stream events may occur *via* a $PGE_2$ synthesis pathway. Cyclooxygenase (COX) is a family of enzymes that catalyse the conversion of arachidonic acid to prostaglandins (for review see (Hinz *et al.*, 2002)). Two COX isoforms have been identified: COX1 and COX2. COX1 is constitutively expressed as a "housekeeping" enzyme and is ubiquitous in its distribution. COX2 is inducible and can be upregulated by various pro-inflammatory agents, including lipopolysaccharide (LPS), cytokines, and growth factors. The induction of COX2 has been shown to promote cell growth, inhibit apoptosis, and enhance cell motility and adhesion in a number of cells and animal models. It has reported that IL-1β dose-dependently increases IL-6 synthesis (Carter *et al.*, 1990; Chaudhary *et al.*, 1992; Linkhart *et al.*, 1991) through the production of $PGE_2$ in human OB cells (Li *et al.*, 1992; Takaoka *et al.*, 1999). In bone, IL-1 stimulates prostaglandin production by a rapid and transient activation of prostaglandin G/H synthase 2 gene expression (Harrison *et al.*, 2000). This cytokine-induced $PGE_2$ synthesis and COX2 activation are regulated by a nitric oxide-dependent and -independent mechanism (Hughes *et al.*, 1999; Hukkanen *et al.*, 1995).

Because IL-1β induces expression of itself and IL-6 synthesis, one may conclude that this, at least in part, is the reason why IL-1β has the greatest mitogenic effect amongst the three cytokines. Although indomethacin, a common inhibitor of COX1 and COX2, has been shown to inhibit rhIL-1β-induced $PGE_2$ (Takiguchi *et al.*, 1999) and IL-6 production (Lu *et al.*, 1995), our studies suggest that it is unable to abolish the mitogenic effects of recombinant IL-1β and TNF-α (Frost *et al.*, 1997; Modrowski *et al.*, 1995). These findings and those of others (Frost *et al.*, 1997; Modrowski *et al.*, 1995) suggest that the mitogenic effect of IL-1β and TNF-α on OBs is not due to the activation of the $PGE_2$ synthesis pathway, but *via* other mechanisms. Studies have shown that IL-1β and TNF-α are both potent activator of nuclear transcription factors, AP-1 (Granet *et al.*, 2004), NF-κB (Duh *et al.*, 1989; Granet *et al.*, 2004; Osborn *et al.*, 1989) and early growth response-1 (Egr-1) gene (Chaudhary *et al.*, 1996; Granet *et al.*, 2004). IL-1β can preferentially activate JNK pathway in normal human OBs and rat UMR-106 cells to regulate the cell growth and differentiation (Chaudhary *et al.*, 1998).

In addition to the cytokine itself, its receptors and naturally occuring inhibitors also play an important role in the regulation of the cytokines effects *in vivo*. In the study described here, although IL-6 displayed a limited mitogenic response at low concentration, it was found to act synergistically with sIL-6R on human OB-like cells. Thus, the regulation of IL-1β on OB-like cells may not only include cytokine gene expression, corresponding protein synthesis and secretion, but may also extend to the expression of cell surface receptors, soluble receptors and receptor antagonists.

Since IL-1β was shown to have the most profound effect on OB-like cell proliferation, we next examined the effect of IL-1β on the OB phenotype. Using an *in vitro* model of OB differentiation, our studies suggest that IL-1β dramatically increases the number of OB-like cells which express the STRO-1 antigen. A similar change in OB phenotype was observed when OB-like cells were co-cultured with myeloma cells. In addition, the increased STRO-1 expression can be partially blocked by anti-IL-1β monoclonal antibody, suggesting myeloma cells produce small amount of IL-1β or induce OB-like cell to produce IL-1β. Similar to the effects of IL-1β on OB-like cells, co-culture of OB-like cells with RPMI8226 was found to result in an increase in IL-6 detectable in conditioned medium harvested from

these co-cultures. It is likely that myeloma cells increase the production of IL-6 in these co-cultures by stimulating OB-like cell production of IL-6 (Uchiyama *et al.*, 1993). Therefore, it is highly likely that pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6, produced by myeloma cells or OBs in response to myeloma cells, alter the phenotype of OB-like cells *in vivo* to control the bone formation and bone resorption in patients with MM.

Previous studies from our laboratory have shown that STRO-$1^+$ cells possess a greater proliferative capacity compared with STRO-$1^-$ cells (Pan *et al.*, 2004b). Studies presented in this chapter, extend these findings and now show that the IL-1β-induced increase in the rate of cell proliferation was correlated with an increase in STRO-1 expression, suggesting that STRO-1 expression in human OB-like cells represents a surrogate index of cell proliferative potential. Interestingly, BM mesenchymal stem cells express high levels of the STRO-1 antigen but do not divide *in vivo*, suggesting that this phenomenon may only be applicable in human OB-like cells (Gronthos *et al.*, 2003). It has shown that OBs isolated from postmenopausal osteoporosis patients with high bone formation exhibited a 2-fold higher proliferative rate *in vitro* compared with OBs from patients with normal or low bone formation or OB cells from age-matched controls (Marie *et al.*, 1993). In addition, BM stromal cells derived from MM have been noted to possess a "striking" proliferative ability that is absent in MGUS and normal donors of the same age group (Gregoretti *et al.*, 1994). Our data suggest that the different proliferative capacity between stromal derived from MM and normal donors may due to their different stage of differentiation.

Our data shows that the IL-1β-induced increase in the number of STRO-$1^+$ OB-like cells is associated with an increase in IL-6 synthesis in OB-like cells, suggesting that the IL-6 production by OB-like cells may be related to the differentiation stage of OB-like cells. These observations were confirmed by examining the expression of IL-6 protein by sorted populations of STRO-$1^-$ and STRO-$1^+$ cells. Our data provide direct evidence that myeloma cells and IL-1β increase the proportion of high IL-6 expressing STRO-$1^+$ cells, a stromal layer that preferentially supports the florid growth of the MM clone (Wunder *et al.*, 1995), to accelerate the growth of MM cells (Karadag *et al.*, 2000a; Kawano *et al.*, 1989a).

Increased OB proliferation has been shown to result in an inhibition of OB differentiation, collagen synthesis and mineralisation (Gilbert *et al.*, 2000; Ikeda *et al.*, 1988; Kuroki *et al.*, 1994; Martinez *et al.*, 1996; Santibanez *et al.*, 1996; Stashenko *et al.*, 1987).

Comparative studies show that a major difference between MM and normal donor BM stromal cells involves lower deposition and simpler organisation of the extracellular matrix proteins (fibronectin, laminin, collagen type IV) deposited by MM OB-like cells (Gregoretti *et al.*, 1994). In addition, myeloma cells have been shown to decrease the production of OCN by osteosarcoma cells in co-culture experiments (Barille *et al.*, 1995). Consistent with these findings, we show that culture of OBs in the presence of IL-1β, suppresses ALP activity (Ikeda *et al.*, 1988) and inhibits OCN synthesis (Evans *et al.*, 1990; Taichman *et al.*, 1992). ALP activity and OCN expression are indexes of osteoblastic differentiation. ALP is widely considered to be an early differentiation marker which is "switched on" during bone formation, whilst OCN is thought to represent a later stage differentiation marker (Weinreb *et al.*, 1990). Therefore, it would appear that IL-1β inhibits the differentiation of OB-like cells and maintains them in an immature STRO-$1^+$ OB-like phenotype.

To further examine the long-term effects of OB-like cell exposure to pro-inflammatory cytokines, we assessed the phenotype of OBs derived from MM and MGUS patients. To our surprise, we found that MM patient-derived BM stromal cells displayed a distinct lack of STRO-$1^+$/ALP$^-$osteoprogenitors and STRO-$1^+$/ALP$^+$ pre-osteoblastic cells. Osteoblasts derived from MGUS also displayed a lack of ALP expression, but expressed more STRO-1 antigen when compared with OB-like cells derived from MM. Considering OB cells derived from MM patients, would in all likelihood have been exposed to pro-inflammatory cytokines for long periods, we next exposed the normal OB-like cells to IL-1β for periods up to 21 days. Interestingly, whilst OB-like cells exhibited an early increase in STRO-1 expression, over time this level was found to decrease to levels below that seen at day 21 in untreated cultures. Notably, based on STRO-1 and ALP expression, normal OB-like cells cultured in the presence of IL-1β for long periods began to resemble cultures of OB-like cells derived from patients with MGUS and MM. In addition, it was also evident that the morphology of the OB-like cells assumed a more homogeneous morphology with a highly organised orientation and fibroblastic appearance. The reduced STRO-$1^+$ cells in MM may indicate that long term exposure of OB-like cells to IL-1β may finally deplete the number of osteoprogenitor cells within the BM microenvironment.

As described by Gregoretti *et al* (Gregoretti *et al.*, 1994), when OB-like cells are cultured *in vitro*, some OBs derived from MM and MGUS have higher proliferative rate

than normal OBs. The proliferative rate of these cells, may in part, be reflective of the disease stage at the time of cell isolation. Although their proliferate rate is different, OB-like cells derived either from MGUS, MM patients or normal donors appear to have a similar response to IL-1β induced mitogenesis *in vitro*. Therefore, *in vivo*, it is possible that OB-like cells will continue to proliferate at high levels under the mitogenic influence of IL-1β (and other pro-inflammatory cytokines) when sufficient space for their proliferation was provided by activated OCs. Our findings indicate that MM stromal cells are in a state of activation (Caligaris-Cappio *et al.*, 1991) and these immature cells express high STRO-1 and low ALP antigen on the cell surface.

Although we found that IL-1β inhibits the differentiation of OBs, it does not impair the bone mineral formation capacity under osteoinductive condition. Previous data suggests that IL-1β can inhibit the expression of OCN, which may play a role in bone mineralisation. OCN is not present in areas of crystal formation, but in the fully mineralised matrix. It is thought that its role may be to control the size and speed of crystal formation (Roach, 1994) and depletion of OCN from newly formed bone results in no impairment of mineralisation (Price *et al.*, 1982). In addition, OCN could act as an inhibitor rather than a promoter of the mineralisation process (Ducy *et al.*, 1996; Luo *et al.*, 1997).

*In vitro* assays of mineral formation potential are reliant on the inclusion of the glucocorticosteroid, DEX, which is known to induce OB differentiation (Cheng *et al.*, 1994; Rickard *et al.*, 1994). Our data suggests that IL-1β maintains a more "immature OB-like phenotype", which when cultured in the presence of DEX, can be induced to differentiate and form mineral. As IL-1β exposure results in an increase in OB-like cell number, the actual calcium levels per culture in IL-1β treated cells are significantly higher than that in untreated cells. In addition, we have found that IL-1β activates COX2 and the $PGE_2$ synthesis pathway, both important factors in bone formation. In inflammation associated with injury, COX2 is induced in the early phase of the bone reparative process and triggers the differentiation of mesenchymal stem cells into OBs to commence the bone repair process. In keeping with this, the COX2 knock out mouse, displays reduced bone formation (Zhang *et al.*, 2002). Moreover, systemic or local injection of PGE2 can stimulate the bone formation process in young rats (Weinreb *et al.*, 1997). Based on these findings, we suggest that the increased mineralisation observed in IL-1β-treated cultures is largely due to the

combined effects of both the osteoinductive influence of DEX and the activation of the $PGE_2$ synthesis pathway. Therefore, other factors may be responsible for the impaired bone formation.

The crucial events in adult bone formation are the recruitment, proliferation, differentiation and apoptosis of OBs. Besides affecting cell proliferation and differentiation of OB-like cells, IL-1β may also affect on OB apoptosis and recruitment. At the bone injury site, addition of IL-1β was found to significantly increase the number of OBs and decrease apoptotic cells (Olmedo *et al.*, 1999). Like TNF-α [Gilbert, 2000 #717], IL-1β inhibits entry of precursor cells into the differentiation pathway rather than cause decreased survival of mature OBs once formed. Although IL-1β and TNF-α alone could not induce the apoptosis of human OBs, studies suggest that they can increase Fas-mediated apoptosis (Tsuboi *et al.*, 1999). Osteoblasts derived from MM patients have been found to over-express Fas and TRAIL death receptors (DR4 and DR5) and they are highly susceptible to apoptogenic stimuli produced by myeloma cells and promptly undergo apoptosis *in vitro* after interaction with malignant plasma cells (Silvestris *et al.*, 2003). As a result, IL-1β increases the rate of cell proliferation in the normal OB-like cells without inducing apoptosis in the short time. In contrast, cells may be induced to express Fas or death receptors following long term exposure to pro-inflammatory cytokines and undergo apoptosis in the presence of apoptogenic stimuli produced by myeloma cells. This mechanism could accelerate the exit of OBs or their precursors from the functional pool.

Two processes of bone remodelling, bone resorption and formation, are closely coordinated. The osteoclastogenesis is dependent on haemopoietic precursors being exposed to the appropriate OBs or stromal cells in an environment that provides appropriate simulatory factors (Lam *et al.*, 2000; Suda *et al.*, 1999). Numerous recent studies highlight the factor that OC activation is closely controlled by the local ratio of RANKL to OPG (Hofbauer *et al.*, 2000; Hofbauer *et al.*, 2001). Therefore, the IL-1β-induced phenotypic change of OB-like cells will greatly affect osteoclastogenesis. It has been reported that TNF-α, IL-1β (Kimble *et al.*, 1997; Kimble *et al.*, 1995; Kitazawa *et al.*, 1994) and IL-6 (Jilka *et al.*, 1992) play a central role in the bone loss caused by oestrogen deficiency. IL-1β and TNF-α can increase the stromal cell production of IL-7, which can stimulate OC formation by up-regulating the T-cell production of soluble osteoclastogenic cytokines

(Weitzmann *et al.*, 2000). More direct evidence provided by Hofbauer *et al* (Hofbauer *et al.*, 1999) suggests that IL-1β and TNF-α may stimulate osteoclastogenesis by inducing the expression of RANKL in human OB-like cells. Interestingly, we found that STRO-1 expression on the surface of OB-like cells strongly co-associated with TM-RANKL expression. Therefore, the increased STRO-1 expression induced by IL-1β results in an increased RANKL expression by OB-like cells. This developmental regulation of RANKL/OPG ratio in human OBs has been reported by Gori *et al* (2000), who showed that the ratio of RANKL to OPG decreases when immortalised stromal cells start to differentiate (Gori *et al.*, 2000). Although our results have shown that STRO-$1^+$ cells synthesise more OPG protein than STRO-$1^-$ cells, co-culture of the myeloma cell line RPMI8226 with human OB-like cells resulted in a decrease in the measurable OPG protein found in the culture medium. This may due to the internalisation and degradation of OPG by myeloma cells (Standal *et al.*, 2002). Therefore, our studies suggest that osteoclastogenesis is related to the stage of OB differentiation and myeloma cell-derived IL-1β acts to increase osteoclastogenesis by maintaining the number of STRO-1 expressing immature OBs. In normal bone remodelling, after activating the OCs to resorb bone, undifferentiated stromal cells differentiate toward the mature OB phenotype and lose their ability to support osteoclastogenesis. Following termination of the resorption phase by apoptosis of OCs, the fully mature OBs would respond to chemotactic signals by migrating to the empty resorption surface to initiate bone formation (Gori *et al.*, 2000). In MM, the elevated pro-inflammatory cytokines inhibit the differentiation of OBs, which would serve to interrupt the coordinated development of OCs and OBs and result in the excessive osteoclastic bone resorption.

Besides undifferentiated OBs, increased vascularity and vessel tortuosity characteristic of MM (Vacca *et al.*, 2001; Vacca *et al.*, 1994) and of synovial membrane in rheumatoid arthritis (Choy *et al.*, 2001) are noted. This increased angiogenesis may facilitate enhanced recruitment and entry of OC precursors into the bone surface. IL-1β enhances endothelial activation and thereby stimulates mobilisation of peripheral blood mononuclear cells (PBMNCs) from luminal to abluminal spaces across the endothelium (Tokukoda *et al.*, 2001). Osteoclast precursors can adhere to activated endothelial cells that have been stimulated by proinflammatory cytokines including IL-1β and TNF-α (Collin-Osdoby *et al.*, 2001). Exposure to IL-1β and TNF-α could induce the expression of fibronectin and

vitronectin receptors on endothelial cells, facilitating OC precursors binding and tissue migration (McGowan *et al.*, 2001). Thus, IL-1β may contribute to the increased angiogenesis in MM patients (Vacca *et al.*, 2001; Vacca *et al.*, 1994; Voronov *et al.*, 2003) and indirectly promote osteoclastogenesis.

In conclusion, the network of BM cells and cytokines in BM microenvironment may act in concert to facilitate the formation of osteolytic bone lesion in MM. Pro-inflammatory cytokines, such as IL-1β, which is produced by myeloma cells or OBs in response to MM cells, would serve to increase the number of highly proliferative STRO-1 expressing OB-like cells. IL-1β may lengthen the period in which OB cells express this immature, OC-supportive phenotype, and disrupt or delay the sequence of OB differentiation. Furthermore, the high STRO-1 expressing OBs express high levels of TM-RANKL, thus creating a BM microenvironment which is more supportive of osteoclastogenesis by virtue of the increased ratio of RANKL to OPG. Additionally, the undifferentiated OB-like cells are more sensitive to the apoptogenic stimuli produced by myeloma cells, thereby reducing OB-mediated bone formation. The STRO-1 expressing OBs also synthesis high levels of IL-6 protein, which provides a favourable BM microenvironment for myeloma cell growth. The amplified growth of myeloma cells would result in a heightened secretion of pro-inflammatory factors by myeloma cells and/or OBs, thereby, promoting the "vicious cycle" of disease.

# CHAPTER 4. THE POTENT OSTEOCLASTOGENIC FACTOR, RANKL, IS A MARKER OF IMMATURE OSTEOPROGENITOR CELLS AND BONE MARROW STROMAL STEM CELLS.

## 4.1 INTRODUCTION

To maintain the mechanical strength and mineral homeostasis, the mammalian skeleton is continuously remodelled throughout life (Dempster, 1999). This process requires the coordinated action of two cell types, namely the bone resorbing OCs and the bone-forming OBs. The OB cell lineage belongs to the stromal fibroblastic system of the BM, which includes other stromal tissue, such as cartilage, smooth muscle, and fat (Beresford, 1989; Owen *et al.*, 1988). Although there is heterogeneity within the OB lineage with regard to their proliferative capacity and functional state, these cells have a common stromal cell precursor, known as bone marrow stromal stem cells (BMSSCs) (Gronthos *et al.*, 2003) or mesenchymal stem cells (MSCs), capable of multilineage differentiation (Liu *et al.*, 1997). Recent studies have demonstrated that BMSSCs or MSCs (these terms will be used interchangeably throughout this chapter) are able to differentiate to chondrocytes, adipocytes and OBs in culture (Gronthos *et al.*, 2003; Pittenger *et al.*, 1999).

Although the mechanisms whereby the most primitive pluripotent stem cells develop into the undifferentiated multipotential MSCs remains unknown, it is well accepted that immature osteoprogenitors are derived from BMSSCs through the concerted action of local and systemic growth factors and cell-cell and cell-extracellular matrix interactions. Whilst the identity of all the factors involved in this process remain to be determined, many cytokines, growth factors and hormones have been identified (Mundy, 1995). A key obstacle in the area of developmental bone biology has been the inability to clearly identify early progenitor cells of the osteogenic and related lineages prior to their expression of lineage-related markers. The osteogenic potential of the marrow stroma has been attributed to the presence of a small population of highly adherent, clonogenic cells, termed CFU-F (Owen *et al.*, 1988). Using the characterisation of haemopoietic stem cells as a paradigm, several groups have developed antibodies to cell surface proteins using putative marrow stromal cell populations as the immunogen. Of these, the mAb HOP-26, which identifies the tetraspanin, CD63, reacts strongly with cells within the BM which are capable of forming stromal colonies. In addition, its expression appears to be down regulated following the induction of ALP activity or when cell confluence is reached, suggesting that

HOP-26 identifies a primitive BMSSCs (Joyner *et al.*, 1997; Zannettino *et al.*, 2003). The mAbs, SH2, SH3, and SH4, identify 3 different cell surface antigens expressed by marrow-derived mesenchymal cells, and whose expression is lost on OBs or osteocytes (Haynesworth *et al.*, 1992; Horwitz *et al.*, 1999). To date, mAb STRO-1 represents the most well studied mAb, which exhibits specificity for BMSSCs. Whilst the identity of the cell surface molecule to which STRO-1 binds remains to be determined, studies show that mAb STRO-1 recognises a trypsin-resistant, cell surface antigen present on the osteoprogenitor stem cell subpopulation of BM cells (Simmons *et al.*, 1991). BM mononuclear cells sorted on the basis of STRO-1 expression are capable of establishing an adherent stromal layer *in vitro*, consisting of a number of phenotypically distinct stromal cell types, including fibroblasts, smooth muscle cells, adipocytes, and OB-like cells (Simmons *et al.*, 1991). In addition, it has been demonstrated that the STRO-$1^+$ population of human BM cells is capable of osteogenic differentiation and that osteoprogenitors are almost exclusively present within the STRO-$1^+$ population (Gronthos *et al.*, 1994). Furthermore, STRO-$1^{bright}$ BMMNCs represent MSCs that have extensive proliferation potential and retain the capacity to differentiation into bone, cartilage and adipose tissue *in vitro* (Gronthos *et al.*, 2003).

As detailed in Chapter 3 of this thesis, our studies suggest that the expression of STRO-1 is positively associated with the surface expression of tumour necrosis factor (TNF)-ligand family member, RANKL, on OB-like cells. Receptor activator of nuclear factor-κB ligand plays a pivotal role in OC development and survival by binding to the TNF receptor family member, RANK, expressed on the OC precursors (Anderson *et al.*, 1997; Lacey *et al.*, 1998). A close association between STRO-1 and RANKL has been observed in OB-like cells, suggesting that RANKL may also be expressed by BMSSCs. This raises the possibility that antibodies to RANKL may be used to further enrich the populations of osteoprogenitor cells for experimental studies of osteogenic differentiation. Therefore, in this study we examined the expression of RANKL on MSCs and attempted to reveal the relationship between STRO-1 and RANKL protein in BMSSCs and OB-like cells.

## 4.2 RESULTS

### 4.2.1 Human OB-like Cells Co-express STRO-1 and TM-RANKL.

Our previous studies (please see Figure 3.21) have shown that the expression of the STRO-1 protein at the cell surface of human OB-like cells was positively associated with the expression of TM-RANKL. As seen in Figure 4.1, this initial observation was confirmed by examining the expression of STRO-1 and RANKL on OB-like cells derived from three different donors using dual-colour immunofluorescence and flow cytometric analysis. Confocal microscopy was next employed to further examine the apparent relationship between STRO-1 and RANKL on human OB-like cells. The STRO-1 antigen was identified with a FITC-labelled secondary reagent, whilst the RANKL antigen was stained with Texas Red as described in the Methods. Figure 4.2 shows an example of a single OB-like cell stained with either STRO-1 (green) or RANKL (red) protein or both (yellow/orange merged colour). As can be seen from this figure, these two antigens are only partially co-localised on these cells.

### 4.2.2 Human BMMNCs Co-express STRO-1 and TM-RANKL on the Cell Surface

Previous studies show that approximately 10% of BMMNCs express the STRO-1 antigen (Simmons *et al.*, 1991). We next compared the expression of RANKL and STRO-1 on multiple (n=10) normal donor-derived BMMNC preparations. Similar to human OB-like cells, normal BMMNCs which exhibited high levels of STRO-1 expression were found to co-express high levels of TM-RANKL protein (Figure 4.3). BMMNCs were gated based on perpendicular light scatter and forward scatter parameters (Figure 4.3A) and in a representative example shown in Figure 4.3, 14.42% of the BMMNC population expressed the RANKL antigen in the absence of any STRO-1, 3.86% cells expressed STRO-1 alone and 19.73% cells co-expressed both RANKL and STRO-1 protein (Figure 4.3B). Notably, high STRO-1 expressing cells, expressed high level of transmembrane RANKL protein on the cell surface (figure 4.3B). Whilst displaying normal donor-donor variation, all samples (n=10) tested exhibited a similar degree of co-expression of STRO-1 and RANKL (Figure 4.3E-H).

Confocal microscopy was once again employed to examine the localisation of STRO-1 and RANKL in the BMMNCs as described in the Methods. To enrich the positive cells, the STRO-$1^+$ BMMNCs were first isolated by MACS and stained for both STRO-1 (Texas Red) and RANKL (FITC). As seen in Figure 4.4, the pattern of STRO-1 and RANKL

Figure 4.1. Cell surface STRO-1 protein expression on human OB-like cells is positively correlated with the TM-RANKL expression. Dual-colour immunofluorescence and flow cytometric analysis was performed as described in figure 3.21. STRO-1 and RANKL expression on human OB-like cells was found to be highly associated. This association between STRO-1 and RANKL was observed in human OB-like cells derived from three different donors (A-C).

A

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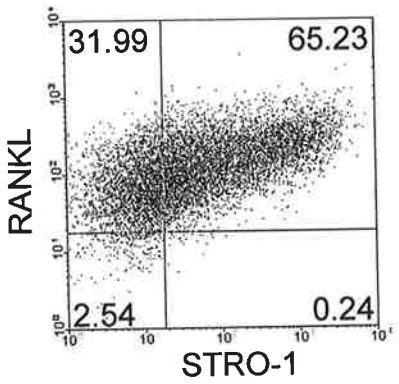


B

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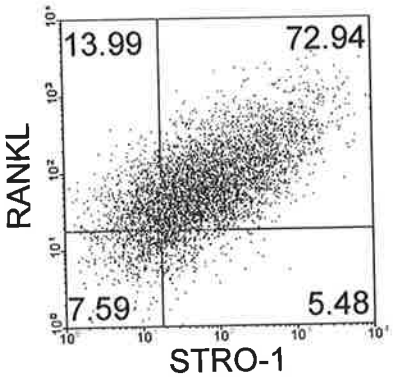


C

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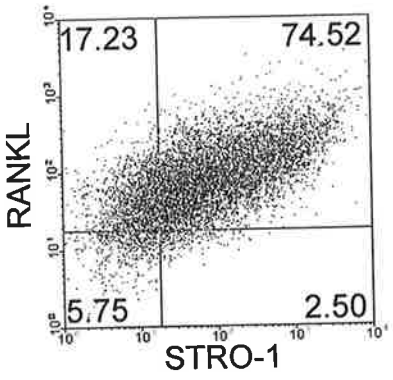
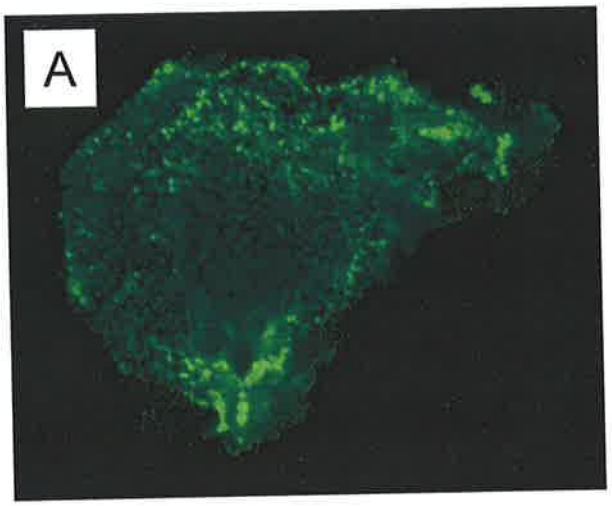
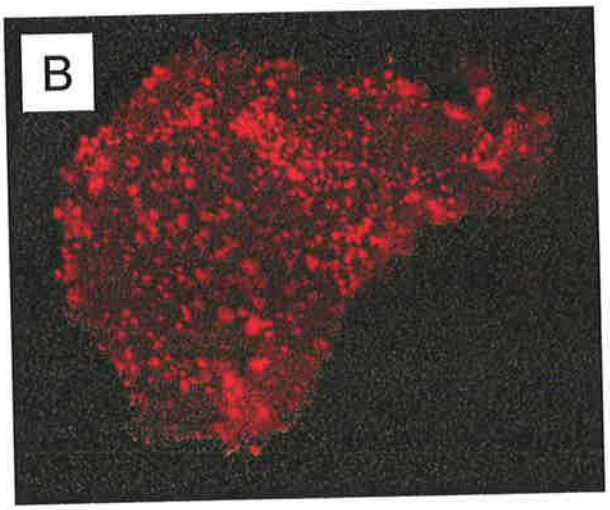


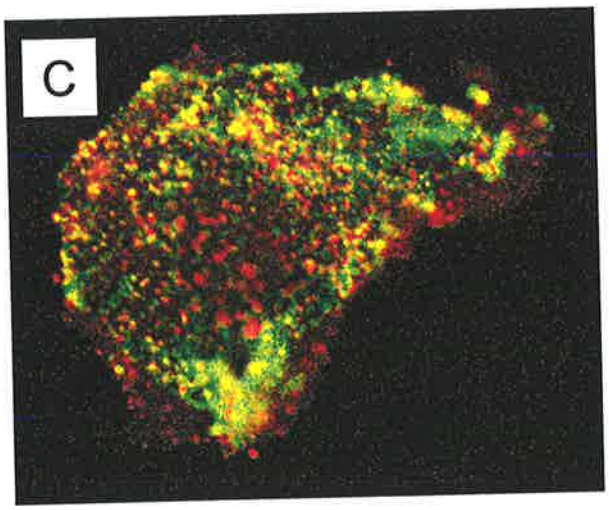
Figure 4.2. STRO-1 and RANKL proteins on human OB-like cells are partially co-localised. Human OB-like cells were detached using 2 mM EDTA and the slides prepared as described in the methods. STRO-1 antigen was stained with FITC and RANKL was stained with Texas Red. A single cell stained with STRO-1 (A) and RANKL (B) is shown. When the expression of these two antigens was merged, a small amount of co-localisation (yellow staining) was observed. The result displayed is a representative example of 3 independent experiments. Original magnification, ×40




STRO-1



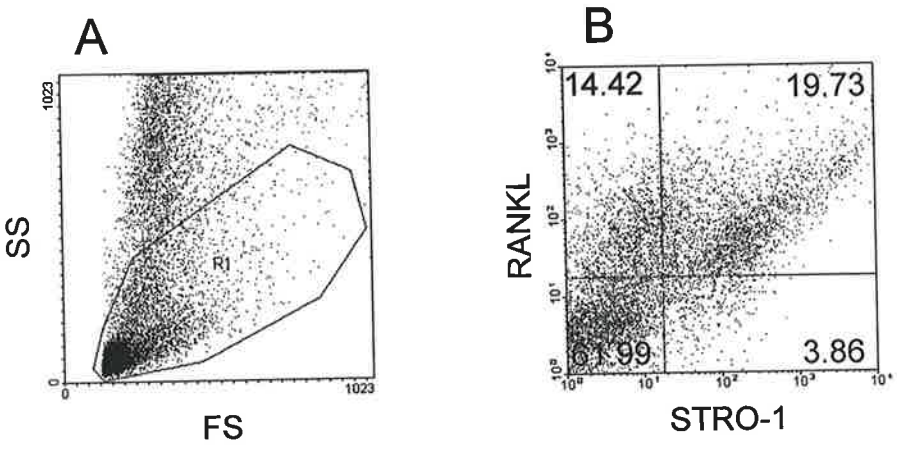

RANKL




STRO-1 &
RANKL

Figure 4.3. Normal BMMNCs co-express high levels of TM-RANKL and STRO-1 proteins. BMMNCs obtained from normal donors were isolated using Lymphoprep as described in methods. Dual-colour immunofluorescence and flow cytometric analysis was performed using BMMNCs stained with the mAbs STRO-1 and RANKL. Immunoreactivity was revealed by incubation with an anti IgM-FITC (x-axis) and IgG-PE (y-axis), as described in the methods. The dot plot histogram represents 10,000 events collected as listmode data. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, 1A6.12 (FITC) and 1A6.11 (PE), respectively. Cells for analysis were gated based on the perpendicular light scatter and forward scatter parameters (A). The dot plot histogram showed that 14.42% cells expressed RANKL antigen, 3.86% cells expressed STRO-1 and 19.73% cells co-expressed RANKL and STRO-1 protein. Notably, high STRO-1 expressing cells expressed high levels of transmembrane RANKL protein on the cell surface (B). Fluorescence histograms depict the expression of STRO-1 (C) and RANKL (D) protein at the cell surface. The data is expressed as the relative cell count (y-axis) versus the intensity of STRO-1 or RANKL expression (log scale). A similar and reproducible linear co-expression pattern between STRO-1 and RANKL was observed on human BMMNCs derived from ten different donors (n=10). Representative examples of 4 donors are shown (E-H).

Donor #1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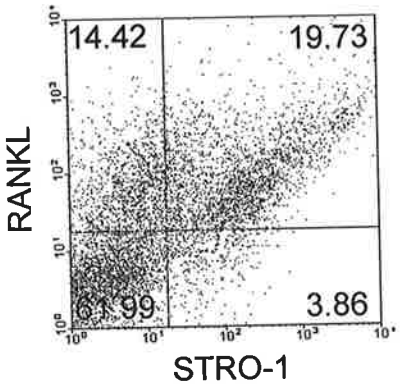
SS
FS
R1
1023
0
1023
B
RANKL
STRO-1
14.42
19.73
61.99
3.86
C
Events
MnX=124
STRO-1
32
0
D
Events
MnX=75
RANKL
64
0

E

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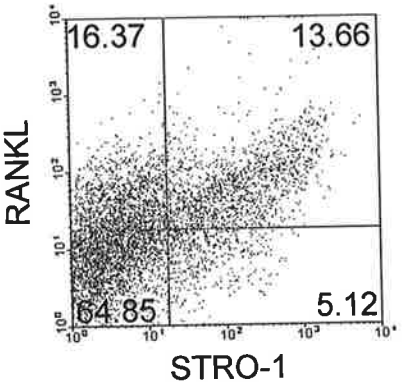


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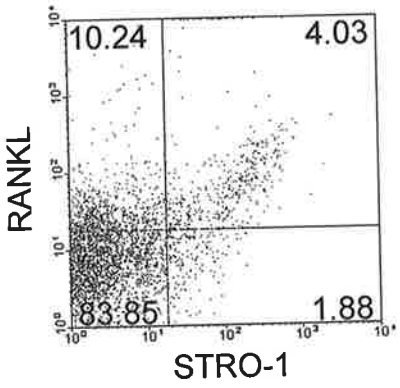


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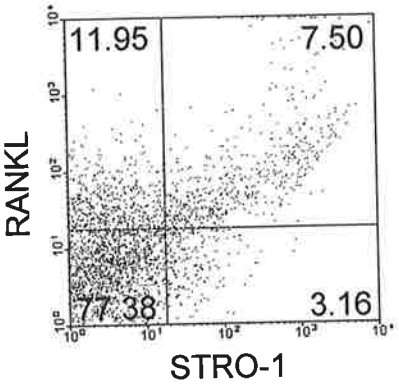



Donor #4

staining on BMMNCs was almost identical to that seen in human OB-like cells. Figure 4.4 shows an example of a single BMMNC stained with either STRO-1 (Figure 4.4A) or RANKL (Figure 4.4B). When the expression of these two antigens was merged (Figure 4.4C), only a small proportion of these molecules were found to be co-localised.

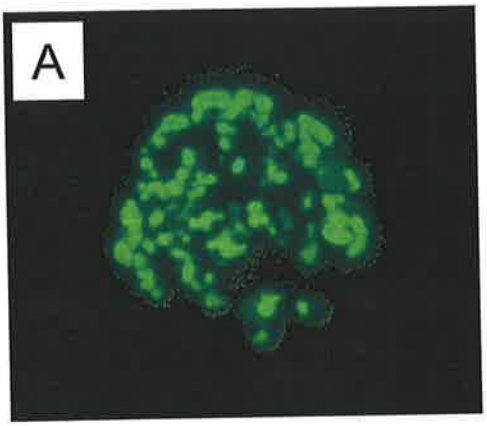
### 4.2.3 Distribution of STRO-1 and RANKL on BMMNCs

Previous studies (Simmons *et al.*, 1991) suggest that in addition to BMMSCs, STRO-1 identifies other cellular subsets within the bone marrow including $CD19^+$ B cells and Glycophorin $A^+$ nucleated erythroid progenitors. To determine which haemopoietic lineages express the RANKL antigen, BMMNCs were stained with RANKL, STRO-1 and a panel of antibodies directed against lineage-restricted. As seen in Figure 4.5, RANKL and STRO-1 exhibited a similar pattern of expression on the myeloid and lymphoid cell populations. Consistent with Figure 4.3, high STRO-1 expressing BMMNCs were found to express high levels of TM-RANKL protein on the cell surface (Figure 4.5B). The expression pattern of RANKL and STRO-1 on lymphoid (CD3 for T cells, CD19 for B cells) and myeloid populations (CD14 for monocyte, glycophorin A for erythroid cells) was also similar (Figure 4.5C-J). The co-expression of RANKL and STRO-1 within the lymphoid and myeloid populations is summarised in Table 4.1. Approximately 11.76±3.06% BMMNCs expressed both RANKL and STRO-1 antigen. All haemopoietic lineages in BM were reactive with RANKL and STRO-1. Approximately 8.96±2.73%, 8.40±4.03%, 5.92±1.41% and 11.12±2.96% cells co-expressed RANKL with CD3, CD14, CD19 and gycophorin A, respectively. Although the expression pattern of STRO-1 on lymphoid and myeloid populations were similar to that of RANKL (Figure 4.5C-J), the mean percentage of BMMNCs derived from 5 donors which were positive for STRO-1 and a lineage-defining marker was lower than that found for RANKL. Approximately 2.62±1.12%, 1.16±0.28% and 1.52±0.60% cells were found to co-express STRO-1 with CD3, CD14, and CD19, respectively. Notably, similar numbers of nucleated erythroid cells expressing Glycophorin A were found to express the STRO-1 (10.90±3.14%) and RANKL (11.12±2.96%) antigens.

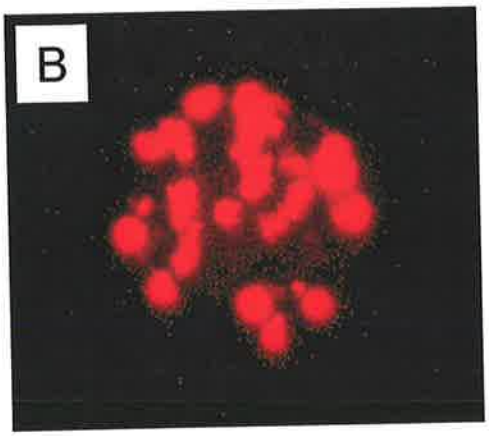
### 4.2.4 Colony Forming Units-Fibroblastic (CFU-F) are Restricted to a Population of BMMNCs Which Express RANKL and STRO-1 at High Levels

STRO-1 has been utilised to prospectively isolate clonogenic CFU-F from aspirates of human BM (Gronthos *et al.*, 1994; Gronthos *et al.*, 2003; Simmons *et al.*, 1991). Since

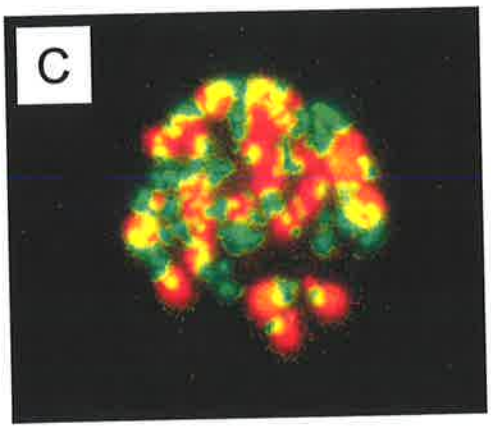
Figure 4.4. STRO-1 and RANKL proteins are partially co-localised on human BMMNCs. The STRO-$1^+$ BMMNCs were first isolated by MACS and then stained with STRO-1 (Texas Red) and RANKL (FITC). The slides for confocal microscope were prepared as described in the methods. A single cell stained with RANKL (A) and STRO-1 (B) is shown. When the expression of these two antigens was merged, co-localisation of STRO-1 (red) and RANKL (green) was noted in some instances (yellow staining). Original magnification, ×60




RANKL




STRO-1




RANKL &
STRO-1

Figure 4.5. RANKL and STRO-1 exhibit similar expression on the myeloid and lymphoid cell populations. BMMNCs obtained from normal bone marrow donors were isolated using Lymphoprep as described in methods and three-colour immunofluorescence and flow cytometric analysis performed. The dot plot histogram represents 10,000 events collected as listmode data. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, $IgG_1$ (FITC), 1A6.11 (PE) and 1A6.12 (PECy5), respectively. Cells for analysis were gated (R1) based on the perpendicular light scatter and forward scatter parameters (A). Consistent with figure 4.3, high STRO-1 expressing BMMNCs expressed high levels of TM-RANKL protein on the cell surface (B). The expression pattern of RANKL and STRO-1 on lymphoid (CD3 for T cells, CD19 for B cells) and myeloid populations (CD14 for monocyte, glycophorin A for erythroid cells) was similar (figure C-J). The result displayed is a representative example of 5 independent experiments.

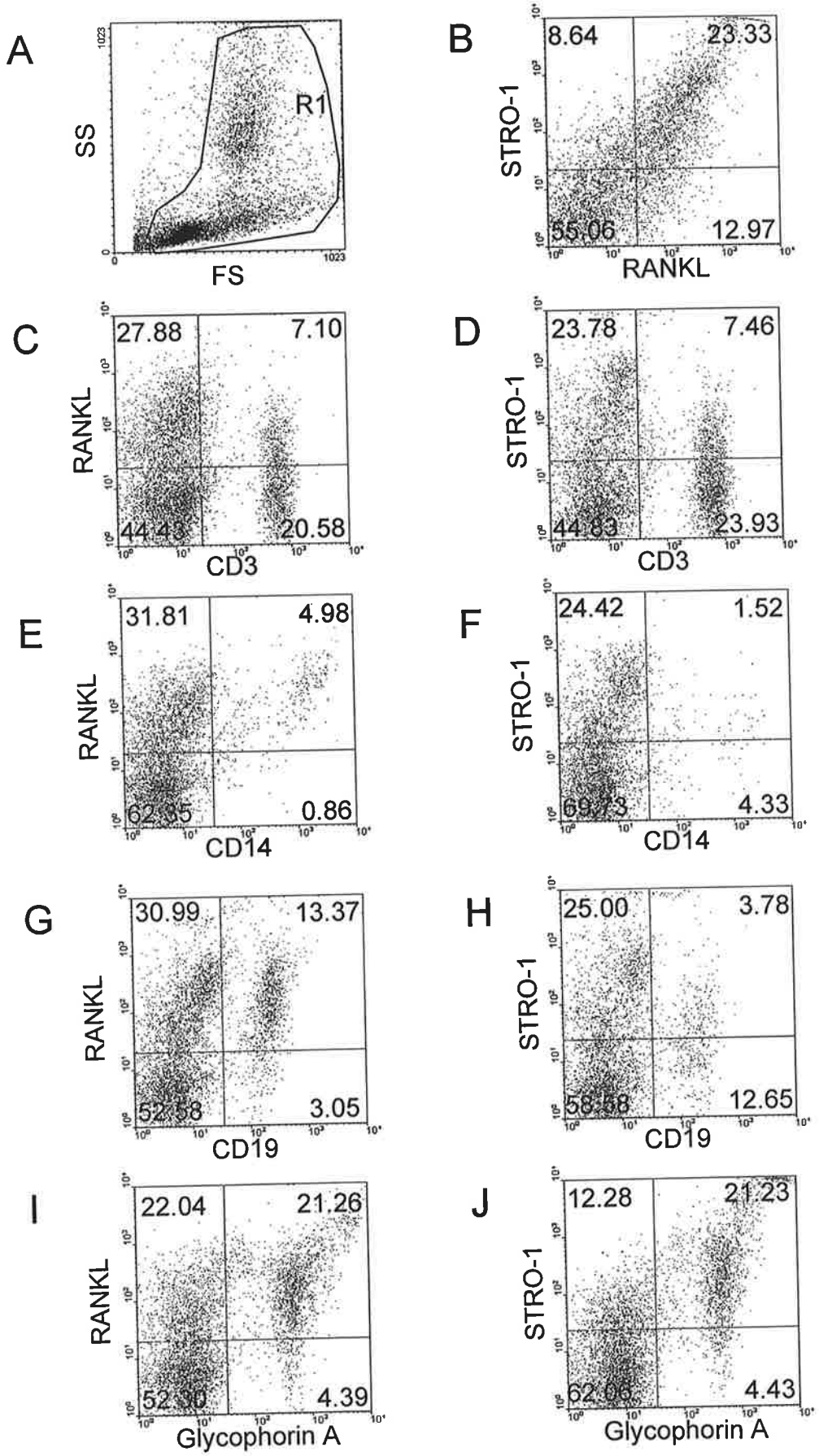
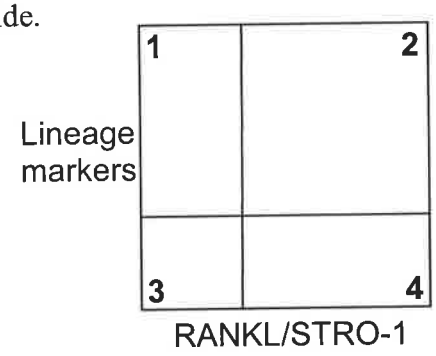

A
SS
FS
R1
1023
0
1023
B
STRO-1
RANKL
8.64
23.33
55.06
12.97
C
RANKL
CD3
27.88
7.10
44.43
20.58
D
STRO-1
CD3
23.78
7.46
44.83
23.93
E
RANKL
CD14
31.81
4.98
62.35
0.86
F
STRO-1
CD14
24.42
1.52
69.73
4.33
G
RANKL
CD19
30.99
13.37
52.58
3.05
H
STRO-1
CD19
25.00
3.78
58.58
12.65
I
RANKL
Glycophorin A
22.04
21.26
52.30
4.39
J
STRO-1
Glycophorin A
12.28
21.23
62.06
4.43

Table 4.1. Cell surface protein expression by BMMNCs derived from normal donors (age 20-35 years old). Summary of the data obtained from three-colour immunofluorescence and flow cytometric analysis demonstrating the co-expression of RANKL and STRO-1 with lymphoid and myeloid populations. Information generated from listmode data, and represent $1\times 10^4$ collected events. The mean positive percentage of BMMNCs derived from 5 donors ± SEM is shown.

| Antibody | **Expression of antigen by total BMMNCs** (Expressed by percentage positive) | **Expression of lineage antigen by RANKL+ BMMNCs** | **Expression of lineage antigen by STRO-1+ BMMNCs** |
|---|---|---|---|
| Quadrant number | Region 2+4 | Region 2 | Region 2 |
| CD3 | **42.6±5.1** | **8.96±2.73** | **2.62±1.12** |
| CD14 | **12.5±5.9** | **8.40±4.03** | **1.16±0.28** |
| CD19 | **8.0±2.4** | **5.92±1.41** | **1.52±0.60** |
| Glycophorin A | **15.8±4.9** | **11.12±2.96** | **10.90±3.14** |
| RANKL | **10.7±0.7** | – | – |
| STRO-1 | **10.4±2.4** | **11.76±3.06** | – |

The regions are indicated in the right side.



STRO-1 expression is, in most instances, associated with RANKL expression, we next examined if a combination of both STRO-1 and RANKL could effectively isolate all MSCs from BM. The STRO-1$^+$ BMMNCs were first isolated by MACS and subsequently co-labelled with RANKL and sorted as described in the Methods (Section 2.4.7 & 2.4.8). As seen in Figure 4.6, five populations were sorted based on the level of STRO-1 and RANKL expression. The sorted cells were then cultured in α-MEM supplemented with 20% FCS for 14 days, fixed, and enumerated. As seen in Figure 4.6, essentially all of the colony forming potential (CFU-F) was localised to the population of BMMNCs which expressed high levels of both STRO-1 and RANKL (R5) (Figure 4.6B). STRO-1 and RANKL bright cells produced significantly more ($P$=0.001, Student's $t$-test) CFU-F (1841.1±65.4 CFU-F per $10^5$ cells at d14) than cells expressing intermediate levels of RANKL and STRO-1 (R4) (2±1 CFU-F per $10^5$ cells at d14) (Figure 4.6C). No colonies were found in double negative population (R1) or cells expressing either STRO-1 (R3) or RANKL (R2) antigen alone (Figure 4.6C).

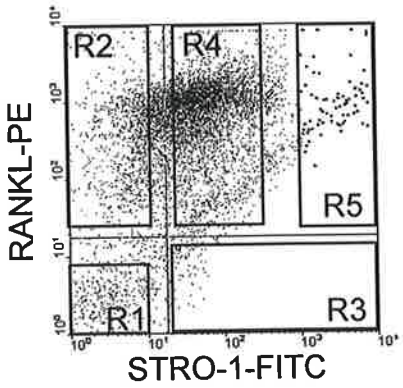
### 4.2.5 RANKL and STRO-1 are Highly Associated Molecules

Although STRO-1 and RANKL are highly associated molecules, they also exhibit disparate binding characteristics. In experiments using dual-colour immunofluorescence and flow cytometric analysis, it was noted that the incubation of mAb STRO-1 with RANKL appeared to influence the binding capacity of mAb RANKL to its cell surface antigen. Notably, BMMNCs expressed low levels of cell surface RANKL when cells were incubated with anti-RANKL mAb and STRO-1 isotype-matched control 1A6.12 (Figure 4.7A). However, BMMNCs were found to express significantly higher levels of RANKL on the cell surface when cells were incubated with both anti-RANKL and STRO-1 mAbs (Figure 4.7C), with the mean fluorescence of RANKL expression significantly increasing from 20.7±4.2 to 62.0±8.8 (n=10, $P$=0.0005, Student's $t$-test) (Figure 4.7E). However, the incubation of STRO-1 with RANKL mAb did not affect the level of STRO-1 expression ($P$=0.46, Student's $t$-test, Figure 4.7B, D and E).

Because STRO-1 mAb increased the capacity of mAb RANKL to bind to TM-RANKL on the cell surface, we further examined the specificity of RANKL mAb with regard to the binding potential with RANKL. The expression of membrane-associated RANKL by RPMI8226 was measured using indirect immunofluorescence and flow

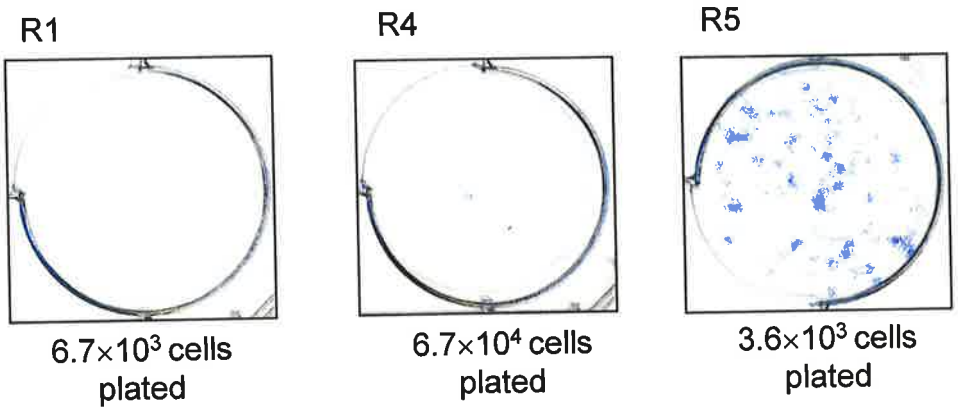
Figure 4.6. Colony forming units-fibroblastic (CFU-F) are restricted to a population of BMMNCs which express RANKL and STRO-1 at high levels. The STRO-1$^{+}$ BMMNCs were first isolated by MACS and then sorted based on the dual-colour flow cytometric analysis of STRO-1 (FITC) and RANKL (PE) expression using a FACStar$^{PLUS}$ cell sorter (A). Five populations were sorted based on the gates shown in A and cells were then cultured in α-MEM supplemented with 20% FCS. The cultures were fixed and stained toluidine blue at d14. An example of the BM CFU-F formed from cells expressing bright STRO-1 and RANKL (R5) antigen (B) is shown. STRO-1 and RANKL bright cells produced 1841.1±65.4 CFU-F per $10^5$ cells at d14 ($P$=0.001, Student's $t$-test) (C). Whilst the cells expressing intermediate RANKL and STRO-1 (R4) produced 2±1 CFU-F per $10^5$ cells at d14 (C). No colonies were found in double negative population (R1) or cells expressing either STRO-1 (R3) or RANKL (R2) (C). The data represent the mean number of day 14 CFU-F per $10^5$ cells plated ± SEM (n= 3 different BM samples).

A




R1 4.55%
R2 12.56%
R3 0.83%
R4 58.95%
R5 0.91%

B




C

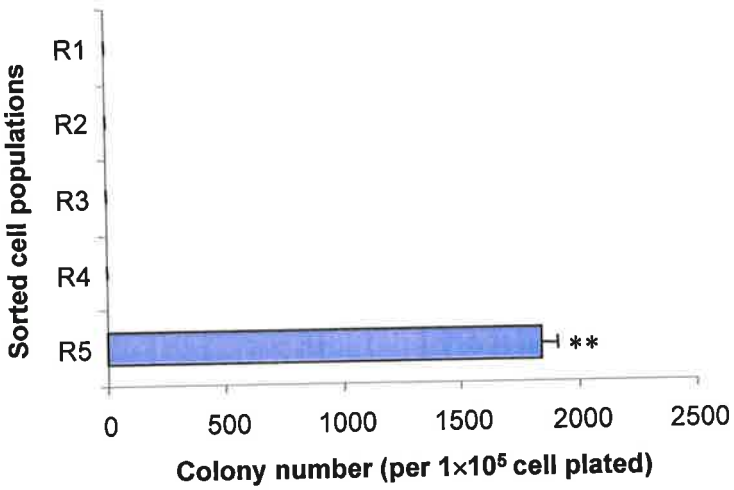
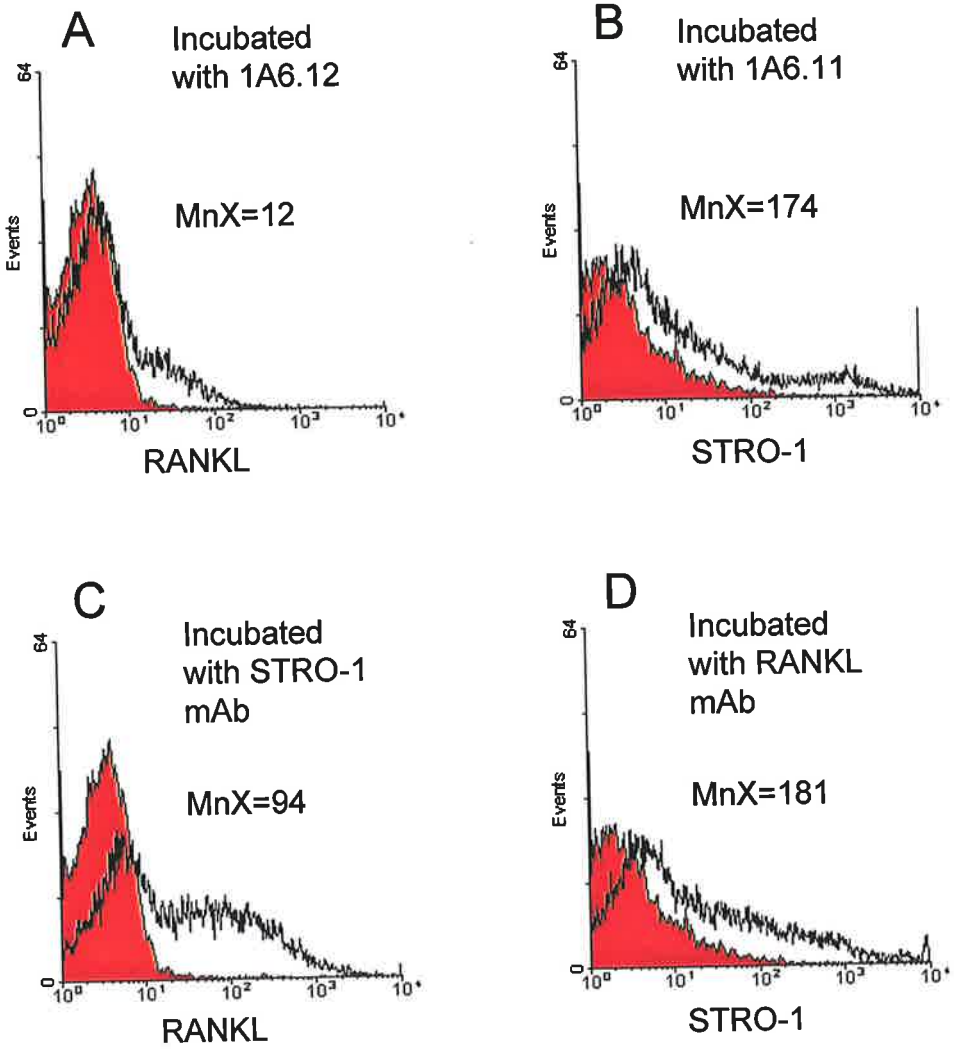
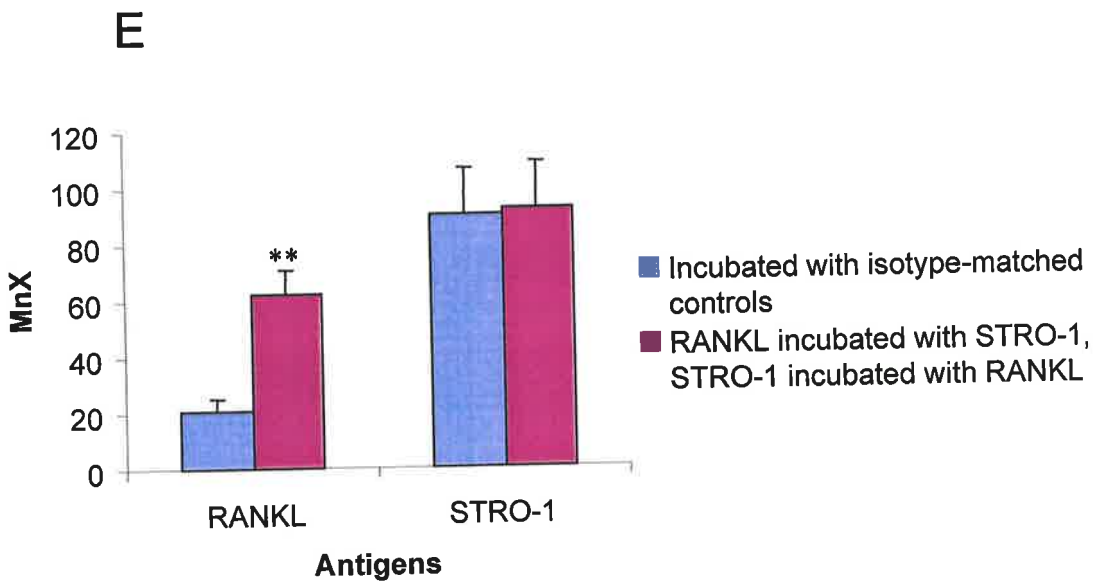


Figure 4.7. Monoclonal antibody, STRO-1, influences the binding characteristics of the mAb RANKL to its antigen. Dual-colour immunofluorescence and flow cytometric analysis was performed as described in figure 4.3. Fluorescence histograms depict the expression of STRO-1 and RANKL protein at the cell surface. The data is expressed as the relative cell count (y-axis) versus the intensity of STRO-1 or RANKL expression (log scale). BMMNCs expressed low levels of RANKL on the cell surface when cells were incubated with mAb RANKL and the isotype-matched control 1A6.12 (A). BMMNCs expressed significantly higher levels of RANKL on the cell surface when cells were incubated with both mAbs RANKL and STRO-1 (C). The mean fluorescence of RANKL expression was significantly increased from 20.7±4.2 to 62.0±8.8 ($P$=0.0005, Student's $t$-test) (E). However, the incubation of mAb STRO-1 with RANKL did not affect the level of STRO-1 binding ($P$=0.46, Student's $t$-test, B, D and E). A reproducibly similar phenomena was observed on human BMMNCs derived from ten different donors (n=10). A representative example is presented.


A
Incubated with 1A6.12
MnX=12
Events
RANKL
B
Incubated with 1A6.11
MnX=174
Events
STRO-1
C
Incubated with STRO-1 mAb
MnX=94
Events
RANKL
D
Incubated with RANKL mAb
MnX=181
Events
STRO-1

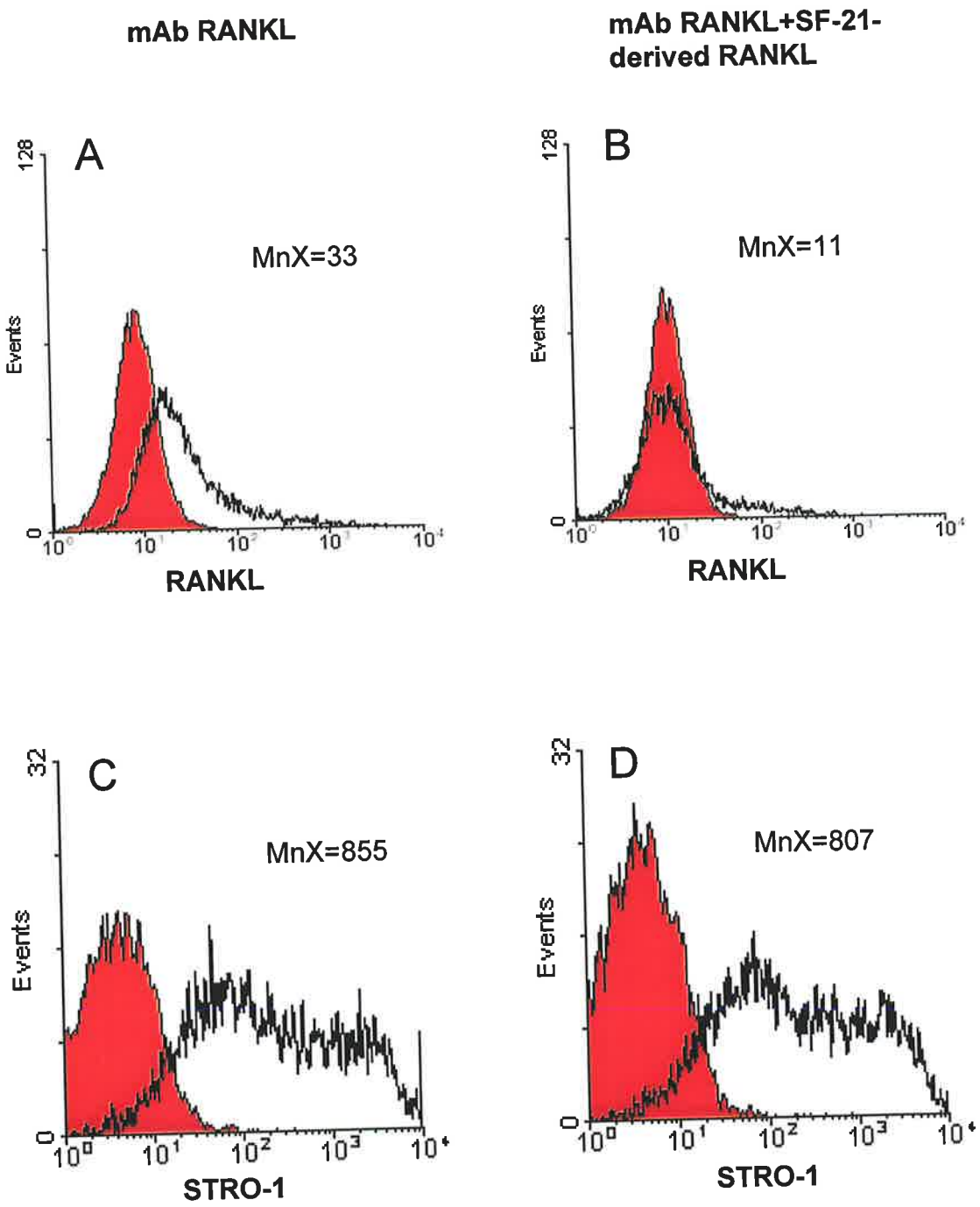

E
120
100
80
60
40
20
0
MnX
**
RANKL
STRO-1
Antigens
Incubated with isotype-matched controls
RANKL incubated with STRO-1, STRO-1 incubated with RANKL

cytometry. Approximately $2\times10^5$ cells were stained with a 100 µl solution of anti-RANKL mAb (MAB626) at a concentration of 10 µg/ml and specific binding was revealed by incubation with saturating concentration of goat-anti-mouse IgG-FITC. As seen in Figure 4.8, myeloma cell line RPMI8226 expressed RANKL protein (blank histogram) on the cell surface with a mean fluorescence 33 after subtraction of the mean fluorescence of isotype-matched control, 1A6.11 (red histogram). To examine the specificity of anti-RANKL mAb, 100 µl solutions of anti-RANKL mAb and negative control 1A6.11 at a concentration of 10 µg/ml were incubated with 300 ng/ml SF-21 insect cell-derived RANKL protein for 1 h at 37°C before staining the RPMI8226. The reactivity of anti-RANKL mAb was almost completely inhibited by prior incubation with SF-21-derived recombinant RANKL. The mean fluorescence of TM-RANKL expression on RPMI8226 was decreased from 33 (Figure 4.8A) to 11 (Figure 4.8B). Thus, the mAb RANKL was found to be specific for RANKL protein on the cell surface.

Since RANKL and STRO-1 were highly associated, we further examined whether SF-21-derived RANKL protein could block the STRO-1 binding. The expression of STRO-1 by human OB-like cells was measured using indirect immunofluorescence and flow cytometry. Approximately $2\times10^5$ OB-like cells were stained with a 100 µl supernatant of mAb STRO-1 and specific binding was revealed by incubation with saturating concentration of goat-anti-mouse IgG-FITC. As seen in Figure 4.8C, human OB-like cells expressed STRO-1 protein (blank histogram) on the cell surface with the mean fluorescence 855. When 100 µl supernatant of mAb STRO-1 and negative control 1A6.12 were incubated with 300 ng/ml SF-21-derived RANKL protein for 1 h at 37°C before staining the OB-like cells, the reactivity of mAb STRO-1 was marginally inhibited by prior incubation with SF-21-derived RANKL protein compared with untreated cells. The mean fluorescence of STRO-1 expression was decreased from 855 (Figure 4.8C) to 807 (Figure 4.8D).

Figure 4.8. The mAb RANKL is specific for RANKL protein. The expression of membrane-associated RANKL by RPMI8226 was measured using indirect immunofluorescence and flow cytometry. $2\times10^5$ cells were stained with a 100 µl solution of mAb RANKL at a concentration of 10 µg/ml. Specific binding was revealed by incubation with saturating concentrations of goat-anti-mouse IgG-FITC. The expression of RANKL protein (blank histogram) on RPMI 8226 is shown (A). The isotype-matched control, 1A6.11, was used under identical conditions (red histogram). To examine the specificity of mAb RANKL, 100 µl solutions of mAb RANKL and negative control 1A6.11 at a concentration of 10 µg/ml were incubated with 300 ng/ml SF-21 insect cell-derived RANKL protein for 1 h at 37°C before staining the RPMI8226. The reactivity of mAb RANKL is almost completely inhibited by prior incubation with SF-21-derived recombinant RANKL. The mean fluorescence of transmembrane RANKL expression on RPMI8226 was decreased from 33 (A) to 11 (B).

SF-21-derived RANKL protein was also used to examine whether it could block STRO-1 binding. The expression of STRO-1 by human OB-like cells was measured using indirect immunofluorescence and flow cytometry. Approximately $2\times10^5$ OB-like cells were stained with a 100 µl supernatant of mAb STRO-1 and specific binding was revealed by incubation with saturating concentration of goat-anti-mouse IgG-FITC. Human OB-like cells expressed STRO-1 protein (blank histogram) on the cell surface with the mean fluorescence 855. When 100 µl supernatant of mAb STRO-1 and negative control 1A6.12 were incubated with 300 ng/ml SF-21-derived RANKL protein for 1 h at 37°C before staining the OB-like cells, the reactivity of mAb STRO-1 was marginally inhibited by prior incubation with SF-21-derived RANKL protein compared with untreated cells. The mean fluorescence of STRO-1 expression was decreased from 855 (C) to 807 (D).


mAb RANKL
mAb RANKL+SF-21-derived RANKL
A
MnX=33
Events
RANKL
B
MnX=11
Events
RANKL
C
MnX=855
Events
STRO-1
D
MnX=807
Events
STRO-1

## 4.3 DISCUSSION

Accumulating evidence supports the notion of the existence of multi-potent stem cell population from which the stromal tissue of the BM is derived. These stem cells possess the capacity to differentiate into the many mesenchymal cell lineages of the BM, including fat, bone, cartilage, tendon and ligament (Gronthos *et al.*, 2003; Pittenger *et al.*, 1999). For more than a decade, the mAb STRO-1, has been used to isolate stromal precursors in human BM (Simmons *et al.*, 1991). STRO-1 binds to an as yet uncharacterised cell surface antigen expressed by a subpopulation of human BMMNCs, which harbour all the osteogenic precursors (Gronthos *et al.*, 1994; Simmons *et al.*, 1991). Although STRO-1 binds heterogeneously to populations of BM mononuclear cells, the majority of BM MSCs are contained within the STRO-$1^{bright}$ fraction (Gronthos *et al.*, 2003). This fraction has been further enriched using a combination of two-colour flow cytometric cell sorting and an antibody to vascular adhesion molecule-1 (VCAM-1), a cell adhesion molecule constitutively expressed by marrow stromal tissue (Gronthos *et al.*, 2003; Simmons *et al.*, 1994). In addition to VCAM, MSCs have been enriched using a number of additional markers including mAbs to CD146 and the pericyte-associated antigen 3G5(Seo *et al.*, 2004; Shi *et al.*, 2003).

Studies presented in this chapter reveal MSCs, which express high levels of STRO-1, also co-express RANKL at high levels. This finding suggests that antibodies to RANKL may therefore facilitate enrichment of BM-derived MSC. Using STRO-1 in combination with mAb to RANKL, we are able to isolate a minor (approximately 1% of MACS sorted STRO-$1^{+}$ cells) of BM cells which contain 99.9% CFU-F.

As previously discussed, *in vivo* and *in vitro* studies show that RANKL is an essential OC differentiation factor. RANKL-RANK signalling pathway is the ultimate common mediator of humoral signals that regulate osteoclastogenesis (Anderson *et al.*, 1997; Lacey *et al.*, 1998). RANKL provides an essential signal to OC progenitors through the membrane-anchored receptor RANK, initiating osteoclastogenic signal transduction (Anderson *et al.*, 1997; Hofbauer *et al.*, 2000; Hofbauer *et al.*, 2001; Hsu *et al.*, 1999), to enable their differentiation into OCs (Yasuda *et al.*, 1999; Yasuda *et al.*, 1998b). This signalling pathway can be blocked by a naturally occurring decoy receptor for RANKL, OPG, by disrupting the interaction between RANKL and RANK (Yasuda *et al.*, 1998a; Yasuda *et al.*, 1999). RANKL has been found to be expressed on the surface of BM

OB/stromal cells, chondrocytes, mesenchymal cells of the periosteum, OCs, some endothelial cells and activated T cells (Anderson *et al.*, 1997; Kartsogiannis *et al.*, 1999; Lacey *et al.*, 1998; Yasuda *et al.*, 1998b). We therefore, for the first time, show that in addition to these cell types, BM MSCs also express the RANKL protein at high levels on the cell surface.

The implications of this finding remain to be determined. However, considering the pivotal role of RANKL in osteoclastogenesis, these studies imply that in addition to maintenance of the stromal tissue of the BM, MSCs may play a critical role in OC development. It is well accepted that the bone remodelling process includes four distinct phases: activation, resorption, reversal, and formation (Dempster, 1999). Although it is thought that during the reversal phase, a coupling signal is emanated from the resorption cavity to recruit OB progenitors into the resorption area (Dempster, 1999), it is possible that the bone remodelling may commence with recruitment of both OC precursors and MSCs. The migration of MSCs to a site targeted for remodelling might initially provide a stromal layer conducive to OC development and survival, by virtue of their ability to express high levels of RANKL.

The exact location of BM MSCs in the marrow spaces remains a subject of significant conjecture. Histological evidence suggests that multipotential progenitors may reside in the soft fibrous tissue of the marrow stroma, where they are in intimate association with the marrow vasculature (Bianco *et al.*, 2001). Endothelial cells and subendothelial pericytes which constitute the vessels have been found to be physiologically similar to osteoprogenitors, and both endothelial cells and pericytes display osteoblastic features (Bianco *et al.*, 2001). OC precursors reside in BM and can circulate in the peripheral blood. When migrating to sites of bone resorption from BM or peripheral blood, OC precursors encounter vascular endothelial cells (VECs). During and following their transmigration across the VECs of blood vessels, molecules displayed on the vascular surface may activate OC precursors. If marrow pericytes and marrow stromal precursors are similar in nature or indeed, one in the same, RANKL expression by MSCs may represent a "calling card", facilitating the recruitment and activation of OC precursors. In addition, RANKL can stimulate capillary-like tube formation in primary cultured human umbilical vascular endothelial cells (HUVECs) through the Src-PLC-$Ca^{2+}$ signalling cascade in endothelial cells (Kim *et al.*, 2002b), suggesting high RANKL expressing MSCs have an important role

in the neoangiogenic process-an important factor for the recruitment of OCs (Collin-Osdoby *et al.*, 2002; Formigli *et al.*, 1995).

In addition to its utility in isolating MSCs, STRO-1 has also been employed to define the differentiation stage of human OB-like cells in combination with ALP (Gronthos *et al.*, 1999). Numerous studies now show that OB-like cell differentiation is associated with a loss of STRO-1 antigen expression (Gronthos *et al.*, 1999, Stewart, 1999 #64; Pan *et al.*, 2004b; Simmons *et al.*, 1991). Emerging reports suggest that the expression of RANKL is linked to the differentiation state of OB-like cells (Gori *et al.*, 2000). In support of this notion, our recent study suggests that RANKL mRNA expression is associated positively with the number of STRO-$1^+$ human OB-like cells within a mixed population of OB-like cells in response to pro-osteoclastogenic stimuli (Atkins *et al.*, 2003). The observation that the expression of the STRO-1 and RANKL protein on the human OB-like cells is highly associated provides direct evidence that RANKL expression is, indeed related to the differentiation stage of human OB-like cells. Moreover, it implies that undifferentiated OB-like cells would be more supportive of osteoclastogenesis when compared with their differentiated counterparts, by virtue of their expression of high levels of TM-RANKL.

The positive association between STRO-1 and RANKL is not only evident in human OB-like cells, but also in human BMMNCs, further supporting the idea that these two molecules are highly associated. Given the positive association between STRO-1 and RANKL, it is not surprising to see that RANKL and STRO-1 exhibit similar patterns of expression on both myeloid and lymphoid cell populations. Consistent with the findings of others (Simmons *et al.*, 1991), approximately 10% of STRO-1 and RANKL positive cells express glycophorin A, a marker of erythroid cells. Furthermore, another striking feature of the inter-relatedness comes from the observation that mAb STRO-1 was found to influence the capacity of mAb RANKL to bind to cell surface RANKL. Pre-incubation of cells with mAb STRO-1 resulted in mAb RANKL exhibiting the ability to bind to more than three times the number of RANKL molecules on these cells. To rule out the possibility of non-specific binding of the RANKL, SF-21 insect cell-derived recombinant RANKL was employed and was found to completely block the mAb RANKL binding to cell surface RANKL expressed by the myeloma cell line RPMI8226. These studies raise the intriguing possibility that mAb RANKL and STRO-1 may recognise different epitopes on the same molecule.

Although the localisation of STRO-1 and RANKL on human OB-like cells was similar to that of BMMNCs, differences in the expression pattern between RANKL and STRO-1 were noted. In cells which expressed both STRO-1 and RANKL antigen, the frequency of RANKL staining was found to be higher than that seen with STRO-1. In addition, on BMMNCs, the staining of RANKL was found to be more punctate than that seen with STRO-1. Although a large number of BMMNCs and OB-like cells expressed both STRO-1 and RANKL, cells exclusively expressing either of these markers were also present in these preparations. Furthermore, in contrast to the specific reduction seen on the TM-RANKL expression by SF-21-drived RANKL protein, limited inhibition of STRO-1 binding was observed in the presence of this recombinant protein. If we suppose that mAb RANKL and STRO-1 recognise different epitopes on RANKL, the apparent differences in binding character may be attributed to the subtle differences in epitope specificity of STRO-1. Whilst beyond the scope of this thesis, future studies examining this hypothesis, could examine whether STRO-1 does identify an epitope of RANKL which is dependent upon specific glycosylation, comformation or multimerisation of the RANKL molecule. This could be achieved using a range of strategies including RNAi to knock down RANKL, or the generation of a RANKL transfectant in a cell line capable of appropriate glycosylation.

In conclusion, our studies show that BM MSCs expressed high levels of the pro-osteoclastogenic molecule RANKL. Moreover, RANKL expression is positively correlated with STRO-1 expression on the cell surface of both OB-like cells and BMMNCs. Whilst inconclusive, our studies lead us to conclude that mAb STRO-1 may identify a cryptic epitope on the RANKL cell surface protein. Further studies are required to reveal the true relationship between these two molecules.

# CHAPTER 5. ELEVATED SERUM LEVELS OF THE VASOPEPTIDE, ET-1, MAY ACCOUNT FOR THE SUPPRESSION OF BONE FORMATION IN MYELOMA PATIENTS

## 5.1 INTRODUCTION

Endothelin-1 (ET-1) is a 21 amino acid peptide generated by sequential cleavage of the preproendothelin-1 by a furin convertase and an endothelin converting enzyme-1 (ECE-1) (Yanagisawa *et al.*, 1988). ET-1 produces its biological effect *via* binding to G-protein coupled receptors, $ET_AR$ and $ET_BR$ (Arai *et al.*, 1990; Lin *et al.*, 1991; Sakurai *et al.*, 1990). In addition, a non-signaling isoform of $ET_BR$, termed $ET_BR\Delta5$, has been described (Tanoue *et al.*, 2002) in which the fifth exon of the primary $ET_BR$ transcript has been deleted by alternate splicing (Tanoue *et al.*, 2002). ET-1 and its receptors have been found in many tissues and cell types, including epithelial, mesangial, leukocyte, macrophages, smooth muscle cells, cardiomyocytes, neuronal and glial cells (Arai *et al.*, 1990; Elshourbagy *et al.*, 1993; Luscher *et al.*, 2000; Nunez *et al.*, 1990; Ogawa *et al.*, 1991). ET-1 has been demonstrated to act as an autocrine/paracrine growth factor in a variety of cell types *in vitro,* including vascular smooth muscle cells, mouse embryonic Swiss 3T3 fibroblasts, glomerular mesangial cells and airway smooth muscle cells (Grant *et al.*, 2003; Komuro *et al.*, 1988; Noveral *et al.*, 1992; Simonson *et al.*, 1989; Sugitani *et al.*, 2001; Takuwa *et al.*, 1989a). ET-1 is not only involved in a wide spectrum of normal physiological situations (Calogero *et al.*, 1998; Grant *et al.*, 2003; Kanyicska *et al.*, 1998), but has also been implicated in numerous pathological conditions including carcinogenesis (Bogoyevitch *et al.*, 1994; Dupuis *et al.*, 1998; Grant *et al.*, 2003; Shi-Wen *et al.*, 2001; Zolk *et al.*, 1999). ET-1 is produced by several human cancer cell types derived from tissues of the digestive, reproductive and respiratory systems (Ahmed *et al.*, 2000; Asham *et al.*, 2001; Bagnato *et al.*, 1999; de Matteis *et al.*, 2001; Grant *et al.*, 1997; Kojima *et al.*, 1995; Pekonen *et al.*, 1992; Salani *et al.*, 2000a; Shichiri *et al.*, 1991; Simpson *et al.*, 2000; Yamashita *et al.*, 1991). Moreover, the level of plasma ET-1 and $ET_AR$ expression has been shown to be elevated in malignant tissue derived from patients with colorectal liver metastasis (Shankar *et al.*, 1998), ovarian (Bagnato *et al.*, 1999) and prostate tumours (Nelson *et al.*, 1996). *In vitro* experiments have shown that exogenous and endogenous ET-1 can directly stimulate the proliferation of human cancer cell lines (Kikuchi *et al.*, 1996; Kusuhara *et al.*, 1990; Shichiri *et al.*, 1991).

In addition to its role in cancer progression, ET-1 and $ET_AR$ appears to play a role in the formation of bone and cartilage as demonstrated in ET-1 (Kurihara *et al.*, 1994) and $ET_AR$ (Clouthier *et al.*, 1998; Clouthier *et al.*, 2000) knockout mice. ET-1 expression has been localised to young osteocytes, OBs, OCs and vascular endothelial cells in the vascular-rich bone metaphyseal region in rat (Sasaki *et al.*, 1993). As well as its mitogenic role in other cell types, ET-1 has been shown to stimulate human osteoblastic cell proliferation in a time- and dose-dependent manner (Kasperk *et al.*, 1997; Takuwa *et al.*, 1990; Tatrai *et al.*, 1992). Despite these findings, the effects of ET-1 on OB function remain controversial. Some studies show that ET-1 reduces cellular ALP activity in osteoblastic cells (Hagiwara *et al.*, 1996; Takuwa *et al.*, 1990; Takuwa *et al.*, 1989b) and inhibits the expression of OCN mRNA (Hiruma *et al.*, 1998b) and the mineralisation of MC3T3-E1 cells (Hiruma *et al.*, 1998a). However, other studies suggest that ET-1 increases ALP, type-I collagen (Kasperk *et al.*, 1997; Tatrai *et al.*, 1992) and enhances OCN and OPN mRNA expression in OB-like cell lines (Nambi *et al.*, 1995). Despite these controversial *in vitro* findings, *in vivo* studies have shown that ET-1 may be the major factor responsible for initiating sclerotic bone lesions in prostate and breast cancer with bone metastases (Guise *et al.*, 2003; Nelson *et al.*, 1999).

As discussed, MM is a B cell malignancy, characterised by the presence of abnormal PCs, M-protein and lytic bone lesions. In contrast, the plasma dyscrasia, POEMS syndrome, is characterised by the development of osteoblastic bone lesions. To date, no report has been published on the expression and function of ET-1 and its receptors in patients with MM or POEMS syndrome. Using microarray analysis, recent studies from our laboratory (Zannettino AC & Gronthos S, *unpublished data*) suggest that BMMNCs derived from patients with MM express higher levels of ET-1 when compared with BMMNCs from controls subjects. Although ET-1 has been implicated in the prostate carcinoma and breast cancer with osteoblastic metastases, the role of ET-1 in the osteolytic bone lesion induced by MM and osteoblastic lesion induced by POEMS syndrome remain unknown. Therefore, the aim of this study was to investigate whether ET-1, its receptors and receptor splice variant were dysregulated in the patients with MM. In addition, as the function of ET-1 on OBs *in vitro* and *in vivo* remains controversial, studies in this chapter were designed to examine the direct effect of ET-1 on cultured bone-derived OB-like cells following enforced expression with a retrovirus expressing ET-1.

## 5.2 RESULTS

### 5.2.1 The Expression of ET-1 and $ET_AR$ is Elevated in Myeloma Cells

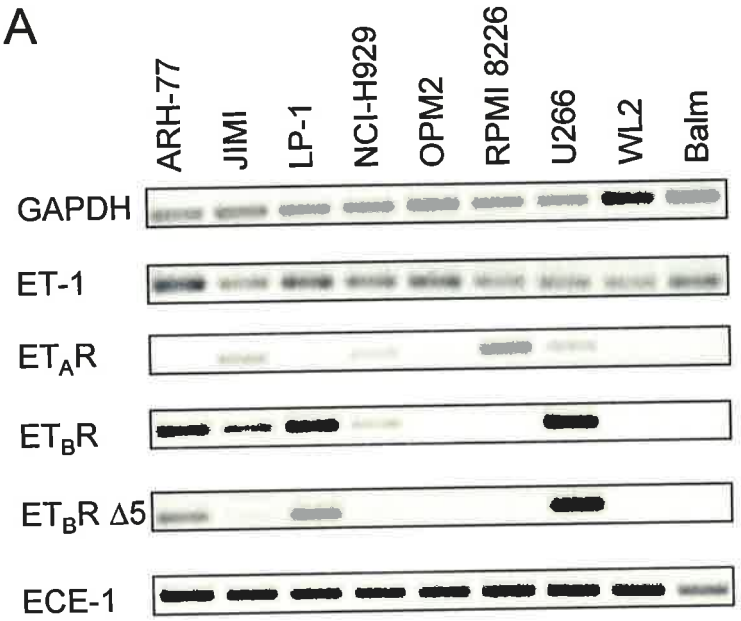
The expression of ET-1, its receptors $ET_AR$, $ET_BR$ and the sliced variant $ET_BR\Delta5$ were examined in a variety of myeloma and B cell lines using semi-quantitative RT-PCR. As seen in Figure 5.1, all eight myeloma cell lines and the B cell line, Balm, constitutively expressed ET-1 and ECE-1 genes. Myeloma cell lines JIMI, NCI-H929 and U266 expressed both $ET_AR$ and $ET_BR$ mRNAs. In contrast, the myeloma cell line RPMI8226 only expressed $ET_AR$, whilst ARH-77 and LP-1 only expressed $ET_BR$. Interestingly, when cell lines expressed high levels of $ET_BR$ gene, they also appeared to express the $ET_BR$ splice variant, $ET_BR\Delta5$ (Figure 5.1A). Although the existence of $ET_BR\Delta5$ has been reported (Tanoue *et al.*, 2002), the expression and function of this splice variant remains unknown. We next examined the expression profile and the relative level of $ET_BR\Delta5$ expression in a range of human tissue samples. As seen in Appendix 2, all the human tissues tested, expressed both $ET_BR$ and its splice variant $ET_BR\Delta5$ (Appendix 2A). When the product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to the expression of GAPDH, it showed that spleen expressed highest level of $ET_BR$, with high levels in the lung, prostate, placenta, uterus and foetal liver (Appendix 2B). Consistent with Figure 5.1, the level of $ET_BR$ delta 5 expression was positively associated with $ET_BR$ expression.

To confirm that ET-1 mRNA expression in myeloma cell lines gave rise to functional ET-1 protein expression, we next quantitated ET-1 protein using a commercially available human ET-1 Immunoassay. Although ET-1 protein expression was, in all instances, detectable in the supernatant harvested from myeloma cell lines, it was found to be low (Figure 5.1B). Among the cell lines tested, ARH-77 and RPMI8226 were found to express the highest levels of ET-1, with 0.156 pg and 0.338 pg per $2\times10^5$ cells, respectively. In contrast, JIMI and U266 were found to express the lowest levels of ET-1 with 0.021 and 0.014pg per $2\times10^5$ cells.

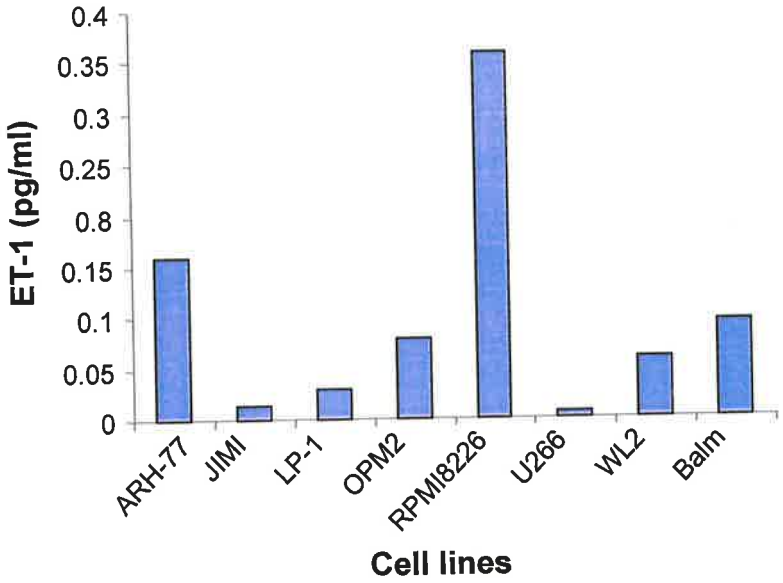
To confirm the preliminary microarray analysis which suggested that patients with MM expressed higher levels of ET-1 when compared with a normal donor, we next surveyed BM expression of ET-1 and its associated genes in a cohort of MM patients and normal donors using semi-quantitative PCR. As seen in Figure 5.2, all 20 samples of

Figure 5.1. Multiple myeloma cell lines express ET-1. Multiple myeloma cell lines were cultured in RPMI-10 and subsequently harvested for RNA isolation. Semi-quantitative RT-PCR was performed as described in the methods. The housekeeping gene, GAPDH was used as an internal control for mRNA integrity. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager. (A) All eight myeloma cell lines and one B cell line, Balm, expressed ET-1 and ECE-1 genes. Cell lines JIMI, NCI-H929 and U266 expressed both $ET_AR$ and $ET_BR$. Cell line RPMI8226 only expressed $ET_AR$. However, cell lines ARH-77 and LP-1 only expressed $ET_BR$. Interestingly, when cell lines expressed strong $ET_BR$ gene, they also expressed $ET_BR$ splice variant, $ET_BR\Delta5$. (B) The ET-1 protein level in the supernatant harvested from myeloma cell lines was assessed using human ET-1 Immunoassay kit.

A


ARH-77
JIMI
LP-1
NCI-H929
OPM2
RPMI 8226
U266
WL2
Balm
GAPDH
ET-1
ETAR
ETBR
ETBR Δ5
ECE-1

B


ET-1 (pg/ml)
0.4
0.35
0.3
0.25
0.8
0.15
0.1
0.05
0
ARH-77
JIMI
LP-1
OPM2
RPMI8226
U266
WL2
Balm
Cell lines

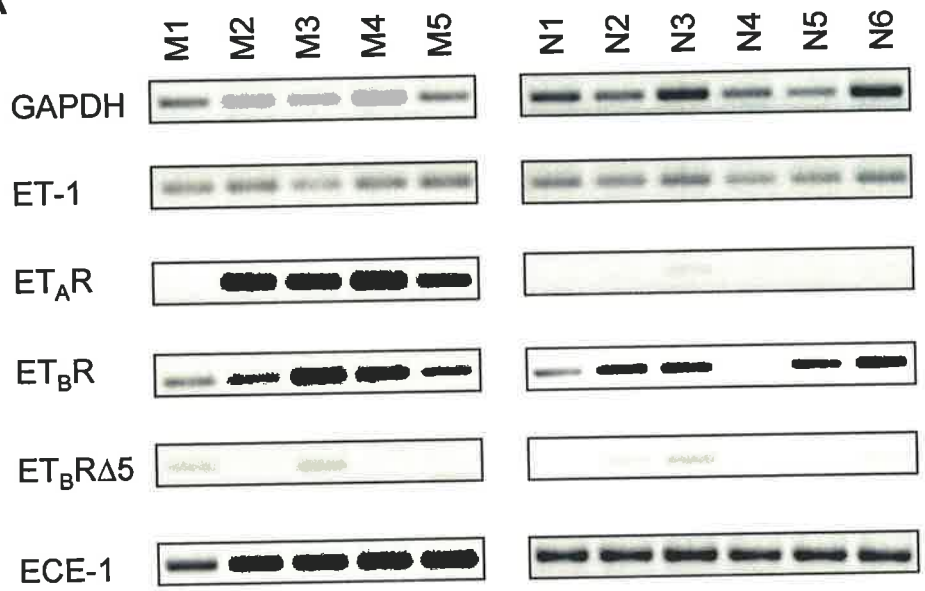
BMMNCs derived from MM patients (an example of 5 MM patients is shown in Figure 5.2) and 6 samples of BMMNCs derived from normal donors expressed ET-1, $ET_BR$ and ECE-1 genes. Consistent with expression pattern of myeloma cell lines, when BMMNCs expressed strong $ET_BR$ gene, they also expressed the $ET_BR$ splice variant, $ET_BR\Delta5$ (Figure 5.2A). Surprisingly, and in contrast to preliminary findings, we found no significant difference in the mRNA expression levels of ET-1, $ET_BR$, $ET_BR\Delta5$ and ECE-1 between BMMNCs derived from MM and normal donors (Figure 5.2C-F). However, the $ET_AR$ gene expression in BMMNCs derived from MM (n=20) was found to be significantly higher when compared with normal donors (n=6) ($P<0.025$, Mann-Whitney *U* test) (Figure 5.2B). In addition, highly purified, flow cytometrically sorted populations of $CD38^{bright}$ myeloma PCs were found to express the ET-1 gene (data not shown).

### 5.2.2 Plasma Levels of ET-1 are Increased in MM Patients

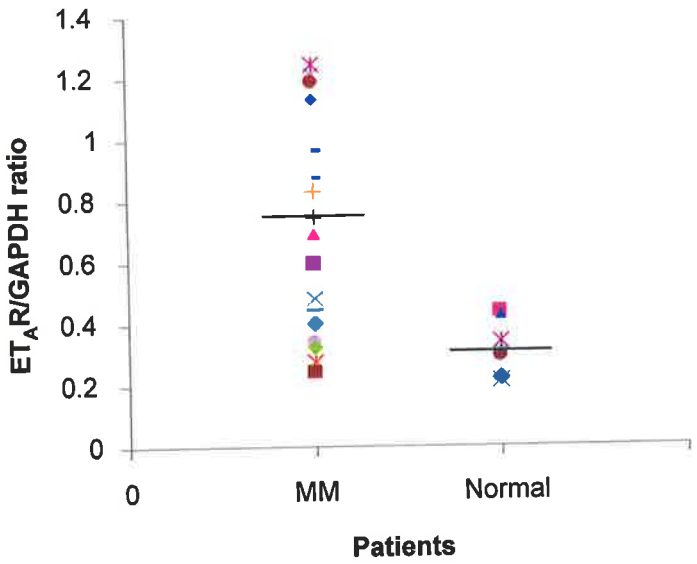
To further examine the expression of ET-1 in patients with MM, a commercially available human ET-1 Immunoassay was employed, as described above. BM and peripheral blood plasma was harvested from patients diagnosed with MM, pre-myloma, MGUS, or normal age-matched control subjects. As seen in Figure 5.3, and in contrast to the gene expression studies described above, BM plasma ET-1 levels were found to be elevated in the patients with MM. The average ET-1 levels in BM were 0.185±0.062 pg/ml (range 0.018-0.845pg/ml, n=49) in a cohort of 49 patients with MM; 0.112±0.036 pg/ml in a cohort of 6 patients with MGUS (range 0.023-0.236 pg/ml, n=6); and 0.109±0.015 pg/ml (range 0.003-0.157pg/ml, n=10) in a cohort of 10 normal, age-matched subjects (Figure 5.3A). Although the difference between MM and MGUS, MM and control groups was approaching significance ($P$=0.0885 and 0.0618, respectively, Mann-Whitney *U* test), significance was not achieved (Figure 5.3A). In addition, the levels of plasma ET-1 in MM patients had no apparent correlation with BM PC number and the presence/absence of osteolytic bone lesion (data not shown). Moreover, there was no apparent gender bias in the level of BM ET-1 between male and female patients ($P$=0.97, Mann-Whitney *U* test) (Figure 5.3B). Unexpectedly, the ET-1 level in the peripheral blood (0.489±0.069pg/ml) was significantly higher than that in BM (0.202±0.077pg/ml) in 10 paired MM patients ($P<0.005$, Wilcoxon rank sum test, n=10) (Figure 5.3C).

Figure 5.2. BMMNCs derived from MM express higher levels of $ET_AR$ compared to BMMNCs derived from normal donors. BMMNCs were isolated using Lymphoprep, RNA prepared using TRIzol and semi-quantitative RT-PCR performed as described in the methods. The housekeeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression to be assessed. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager. (A) The data revealed that all the BMMNCs expressed ET-1, $ET_BR$ and ECE-1 genes. Examples of 5 MM patients and 6 normal donors are shown. (B) BMMNCs derived from MM (n=20) displayed higher $ET_AR$ gene expression compared with normal donors (n=6) ($P<0.025$, Mann-Whitney $U$ test). $ET_AR$ bands were semi-quantitated and their ratio to the expression of GAPDH was plotted as a scatter dot. However, the expression of ET-1, $ET_BR$, $ET_BR\Delta5$ and ECE-1 displayed no significant difference between the BMMNCs derived from MM and normal control (C-F).

A


M1
M2
M3
M4
M5
N1
N2
N3
N4
N5
N6
GAPDH
ET-1
ETAR
ETBR
ETBRΔ5
ECE-1

B


1.4
1.2
1
0.8
0.6
0.4
0.2
0
ETAR/GAPDH ratio
0
MM
Normal
Patients

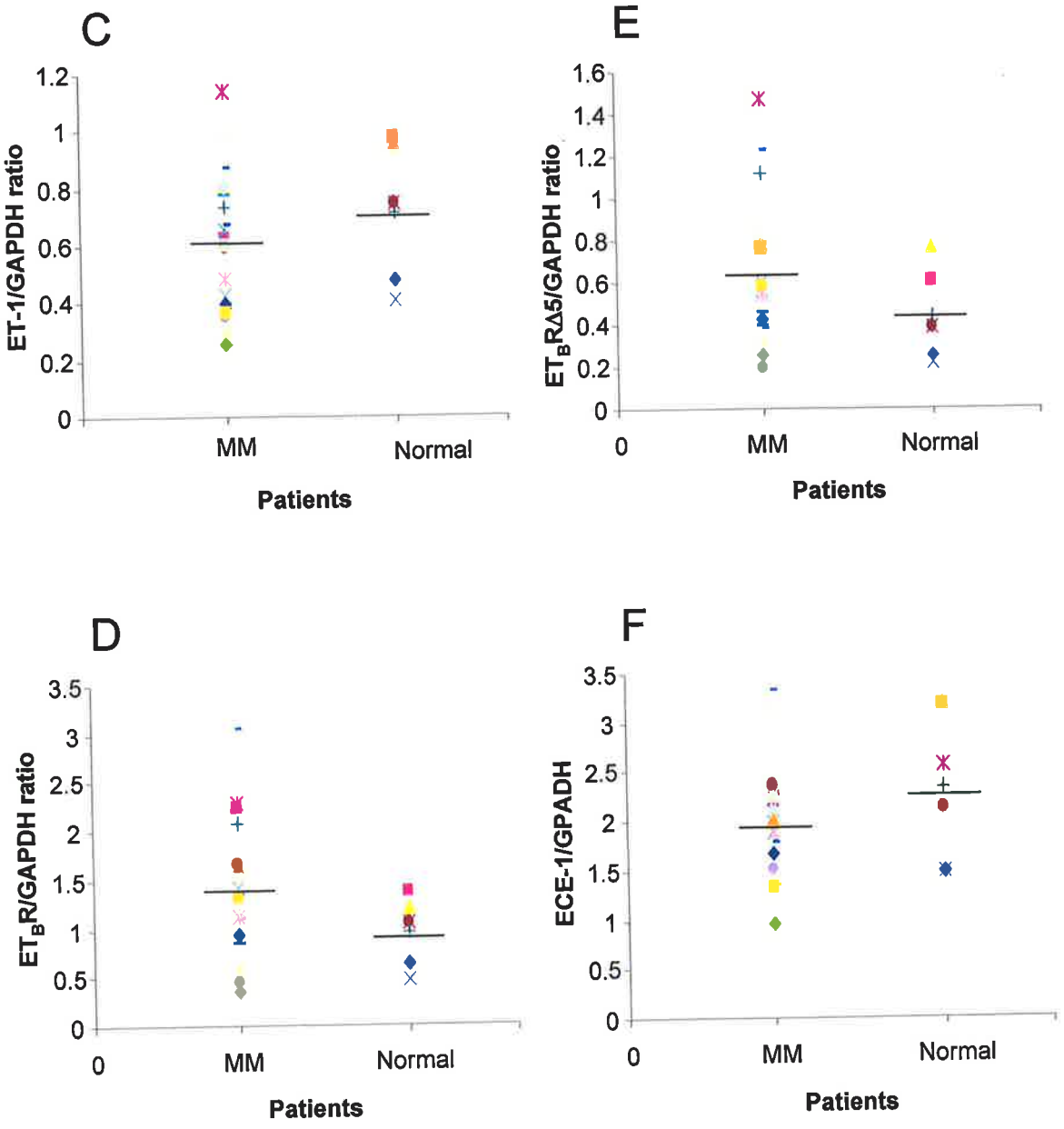
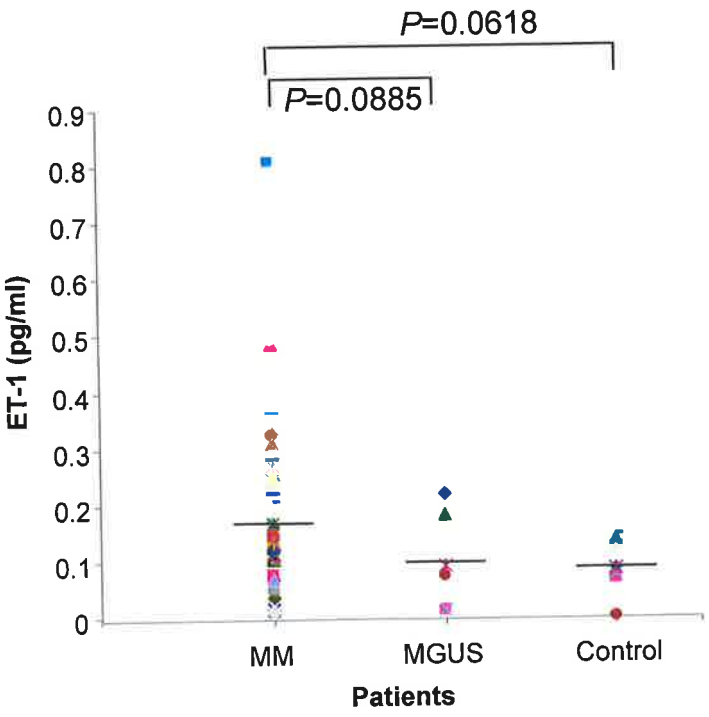
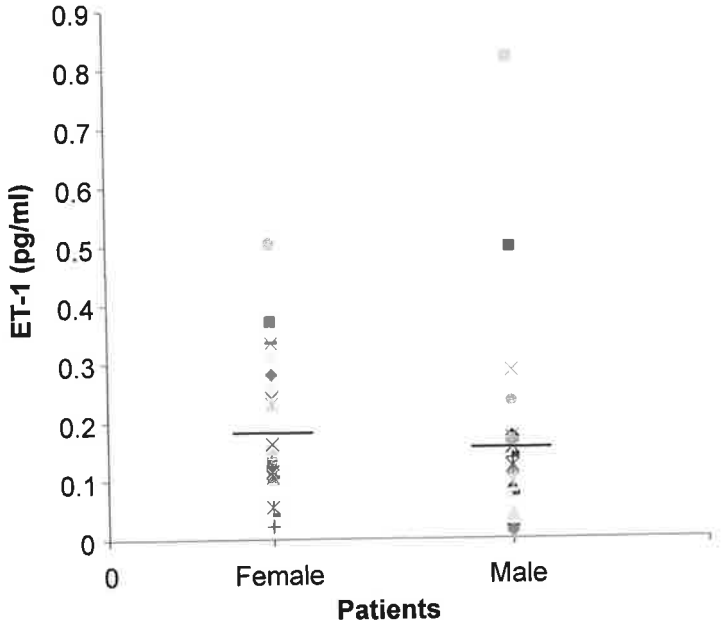

C
ET-1/GAPDH ratio
1.2
1
0.8
0.6
0.4
0.2
0
MM
Normal
Patients
E
ET$_B$RΔ5/GAPDH ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
MM
Normal
Patients
D
ET$_B$R/GAPDH ratio
3.5
3
2.5
2
1.5
1
0.5
0
0
MM
Normal
Patients
F
ECE-1/GPADH
3.5
3
2.5
2
1.5
1
0.5
0
0
MM
Normal
Patients

Figure 5.3. Plasma ET-1 levels were increased in the patients with MM. The ET-1 levels were 0.185±0.062 pg/ml (range 0.018-0.845pg/ml, n=49) in cohort of patients with MM, 0.112±0.036 pg/ml in MGUS (range 0.023-0.236 pg/ml, n=6), and 0.109±0.015 pg/ml in control (0.003-0.157pg/ml, n=10) (A). However, the difference between MM and MGUS, MM and control was non-significant ($P$=0.0885 and 0.0618, respectively, Mann-Whitney $U$ test) (A). Moreover, there was no significant difference in ET-1 levels between male and female patients ($P$=0.97, Mann-Whitney $U$ test) (B). Unexpectedly, the ET-1 level in the peripheral blood (0.489±0.069pg/ml) was significantly higher than that in BM (0.202±0.077pg/ml) in a paired 10 MM patients (P<0.005, Wilcoxon rank sum test) (C).

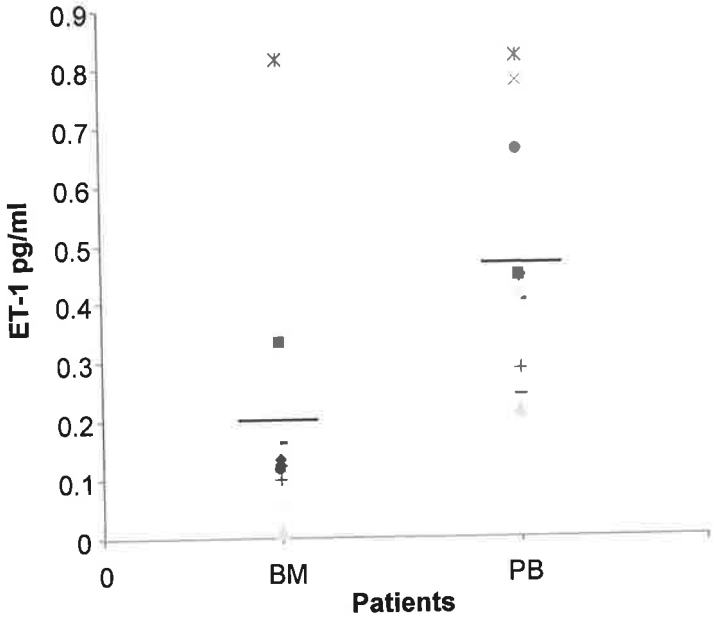
A


P=0.0618
P=0.0885
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ET-1 (pg/ml)
MM
MGUS
Control
Patients

B


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ET-1 (pg/ml)
0
Female
Male
Patients

C


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ET-1 pg/ml
0
BM
PB
Patients

### 5.2.3 OB-like Cells Derived from MM Patients Express Higher Levels of Endogenous ET-1 and Lower Levels of the $ET_BR$ Splice Variant, $ET_BR\Delta5$, Compared with Control Cultures

Marrow stromal/OB cells are anatomically located close to endosteal and trabecular surfaces (Hughes *et al.*, 1996) where myeloma cells normally reside. Previous data from our laboratory and those of others (Caligaris-Cappio *et al.*, 1991; Pearse *et al.*, 2001) show that stromal/OB cells derived from patients with MM are phenotypically, genotypically and functionally different to control cultures, suggesting that myeloma cells in some way affect their local microenvironment. In addition, while the MM patients, on average, presented with higher levels of plasma ET-1, there was no correlation with myeloma PC number, suggesting that the myeloma PCs may induce the surrounding microenvironmental cells to produce more ET-1. In light of this, we next examined the expression of the ET-1, $ET_AR$, $ET_BR$, $ET_BR\Delta5$ and ECE-1 in OB-like cells derived from patients with MM. As MGUS often precedes MM, but is free of the associated bone complications seen in MM, OB-like cells from patients with MGUS were used as controls in this study. As shown in Figure 5.4, human OB-like cells expressed ET-1, $ET_AR$, $ET_BR$ and ECE-1 (Figure 5.4A). Among these genes, OB-like cells derived from MM patients expressed significantly higher ET-1 than cells derived from MGUS patients ($P$=0.05, Mann-Whitney $U$ test) (Figure 5.4B). Although the gene expression of $ET_AR$, $ET_BR$ and ECE-1 in the patients with MM was higher than that in the patients with MGUS, there was no significant difference ($P$>0.05, Mann-Whitney $U$ test). Interestingly, we found that OB-like cells derived from patients with MM expressed significantly lower $ET_BR\Delta5$ mRNA compared with cells derived from patients with MGUS ($P$=0.01, Mann-Whitney $U$ test, Figure 5.5). Unlike what is observed in normal tissue, the expression of $ET_BR\Delta5$ was uncoupled from the expression of $ET_BR$.

### 5.2.4 Recombinant Human ET-1 (rhET-1) is not Mitogenic for Myeloma Cells or Human OB-like Cells

Having established that ET-1 and its receptors were expressed by both myeloma cells and human OB-like cells, we next examined the effect of rhET-1 on these two cell types. Unlike previous published findings (Kikuchi *et al.*, 1996; Kusuhara *et al.*, 1990; Shichiri *et al.*, 1991), rhET-1 had no effect on the cell proliferation of myeloma cells (Figure 5.6A). This was confirmed using the $ET_AR$ antagonist BQ123 (Figure 5.6B) and $ET_BR$ antagonist BQ788 (Figure 5.6C), which were also unable to block the effect of endogenous ET-1. Similarly, rhET-1 and its receptor antagonists BQ123 and BQ788 had no effect on cell

Figure 5.4. OB-like cells derived from patients with MM express higher ET-1 mRNA compared with cells derived from patients with MGUS. Human OB-like cells were derived from normal donors and patients as described in the methods. Cells from passage 2 were harvested for RNA isolation and semi-quantitative RT-PCR was performed as described in the methods. The house-keeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression to be assessed. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager at 532nm. The product bands were semi-quantitated and plotted as a dot plot of the ratio of specific gene expression relative to the expression of GAPDH. OB-like cells were found to express ET-1, $ET_AR$, $ET_BR$ and ECE-1 (A). Interestingly, OB-like cells derived from MM patients expressed significantly higher ET-1 than cells derived from MGUS patients ($P$=0.05, Mann-Whitney $U$ test) (B). Although the gene expression of $ET_AR$, $ET_BR$ and ECE-1 in patients with MM was higher than that in patients with MGUS, there was no significant difference ($P$>0.05, Mann-Whitney $U$ test). M: MM; G: MGUS.

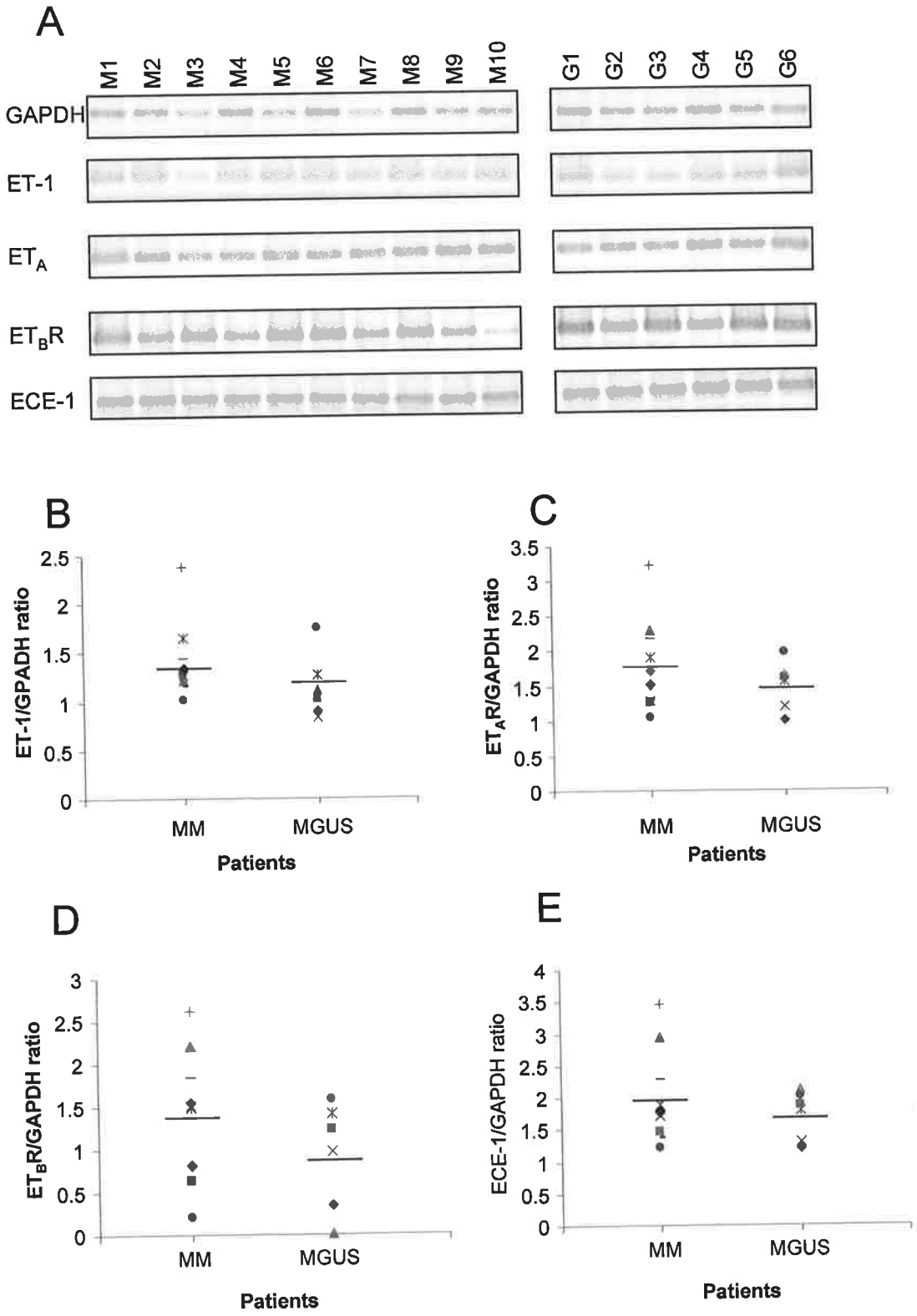
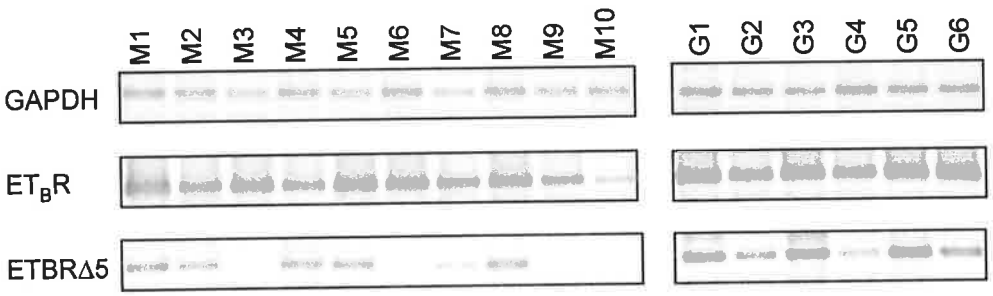

A
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
G1
G2
G3
G4
G5
G6
GAPDH
ET-1
ET$_A$
ET$_B$R
ECE-1
B
ET-1/GPADH ratio
2.5
2
1.5
1
0.5
0
MM
MGUS
Patients
C
ET$_A$R/GAPDH ratio
3.5
3
2.5
2
1.5
1
0.5
0
MM
MGUS
Patients
D
ET$_B$R/GAPDH ratio
3
2.5
2
1.5
1
0.5
0
MM
MGUS
Patients
E
ECE-1/GAPDH ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
MM
MGUS
Patients

Figure 5.5. OB-like cells derived from patients with MM express lower $ET_BR\Delta5$ mRNA compared with cells derived from patients with MGUS. Experiments were performed as described in figure 5.3. OB-like cells derived from MM patients were found to express significantly lower $ET_BR\Delta5$ than cells derived from MGUS patients ($P$=0.01, Mann-Whitney $U$ test) (A&B). M: MM; G: MGUS.

A


M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
G1
G2
G3
G4
G5
G6
GAPDH
ETBR
ETBRΔ5

B

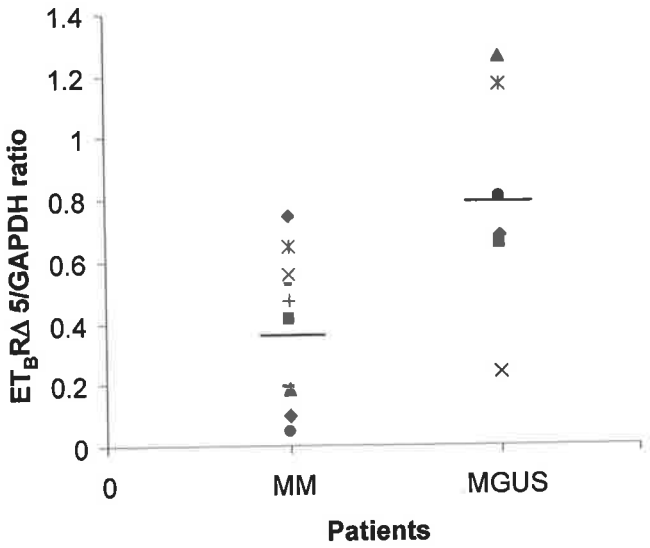
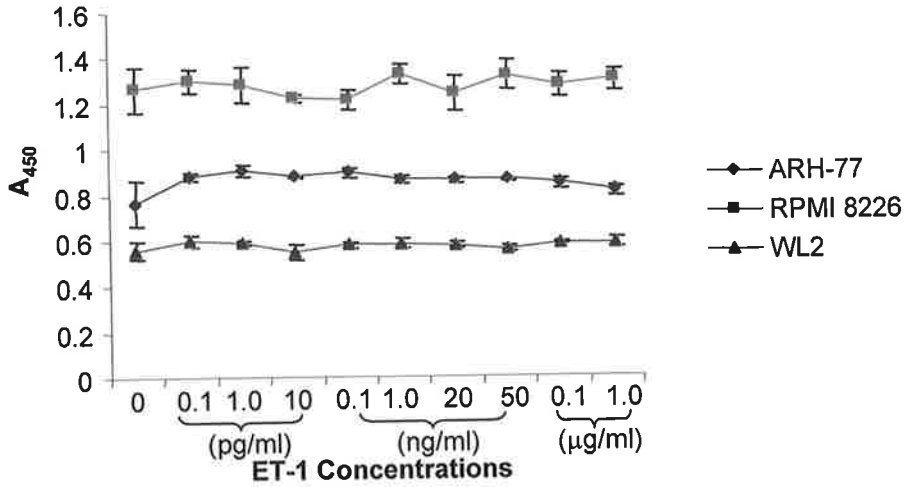
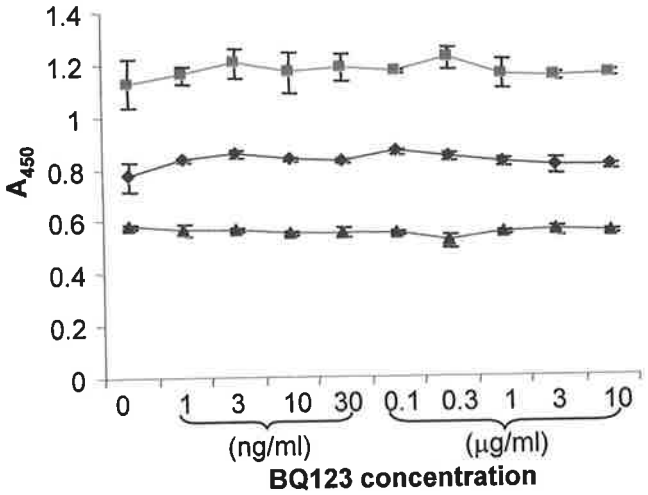

1.4
1.2
1
0.8
0.6
0.4
0.2
0
ETBRΔ 5/GAPDH ratio
0
MM
MGUS
Patients

Figure 5.6. Recombinant hET-1 has no effect on human myeloma proliferation. Human myeloma cell lines ARH-77, RPMI8226 and WL2 were seeded into 96-well plates at a cell density of 10,000 cells/ well. The cells were cultured in the presence or absence of rhET-1 and its receptor antagonists BQ123 and BQ788 at the indicated concentrations. The cell number and viability was assessed at d3 using WST-1, as described in the methods. Recombinant hET-1 had no effect on the proliferation of myeloma cell lines (A). $ET_AR$ antagonist BQ123 (B) and $ET_BR$ antagonist BQ788 (C) could not block the effect of endogenous ET-1. Values represent means ± SEM of triplicate cultures of each concentration. The results displayed are representative of 3 independent experiments.

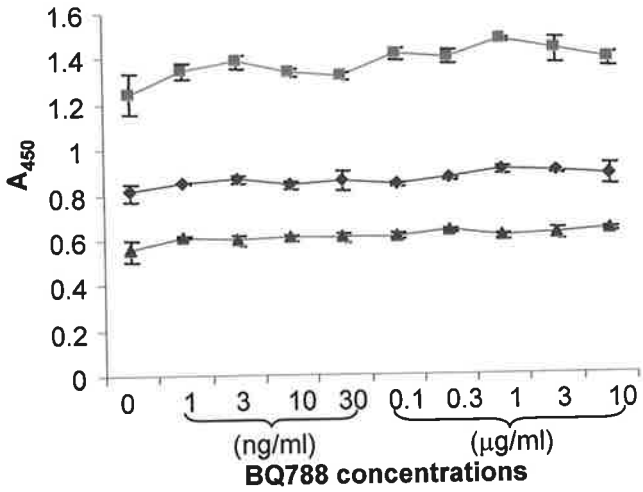
A


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
$A_{450}$
0 0.1 1.0 10 0.1 1.0 20 50 0.1 1.0
(pg/ml) (ng/ml) (µg/ml)
ET-1 Concentrations
ARH-77
RPMI 8226
WL2

B


1.4
1.2
1
0.8
0.6
0.4
0.2
0
$A_{450}$
0 1 3 10 30 0.1 0.3 1 3 10
(ng/ml) (µg/ml)
BQ123 concentration

C


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
$A_{450}$
0 1 3 10 30 0.1 0.3 1 3 10
(ng/ml) (µg/ml)
BQ788 concentrations

proliferation of human OB-like cells at d3 (Figure 5.7A), d5 (Figure 5.7B) and d7 (Figure 5.7C) when cells were cultured in α-MEM-10 medium. Moreover, to eliminate the mitogenic effects of foetal bovine serum, the assay was performed in SDM. Recombinant hET-1 had no effect on OB-like cell proliferation when cultured in SDM at d3 (Figure 5.7D) and d5 (Figure 5.7F).

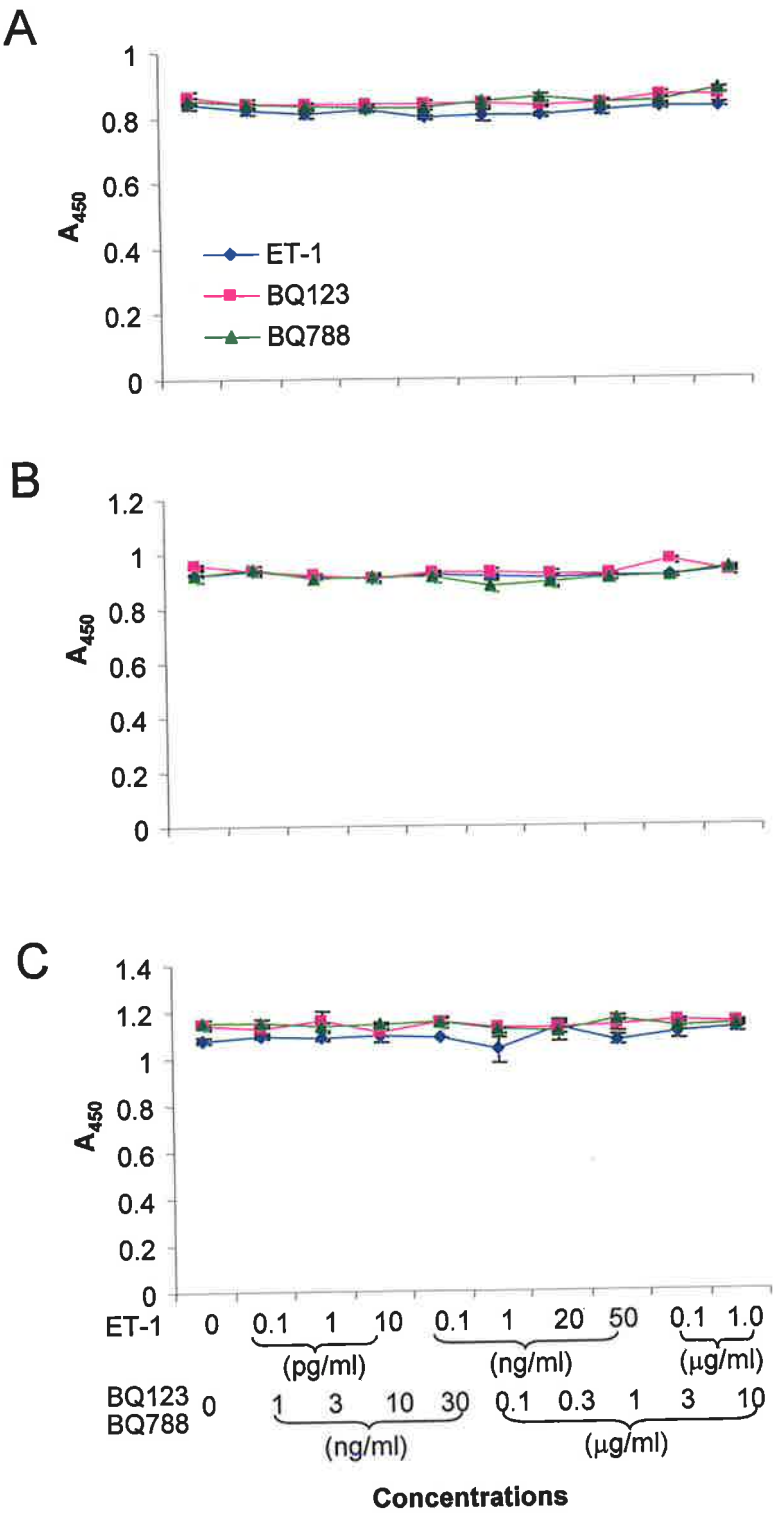
### 5.2.5 Recombinant hET-1 Alters the Differentiation State of *ex vivo* Cultured OB-like Cells

As described above, the effects of ET-1 on the OB differentiation remain inconclusive (Hagiwara *et al.*, 1996; Hiruma *et al.*, 1998a; Hiruma *et al.*, 1998b; Kasperk *et al.*, 1997; Nambi *et al.*, 1995; Takuwa *et al.*, 1990; Takuwa *et al.*, 1989b; Tatrai *et al.*, 1992). Using dual-colour immunofluorescence and flow cytometric analysis, the effects of rhET-1 on OB differentiation were monitored using the model of human OB differentiation described elsewhere in this thesis (refer to Section 1.3.3.2). As shown in Figure 5.8, rhET-1 at a concentration of 25ng/ml, was found to increase the level of expression of the osteoprogenitor marker, STRO-1, in cultures of normal human OB-like cells (Figure 5.8F versus E). In contrast, 25ng/ml rhET-1 was found to decrease the cell surface expression of the mature OB marker, ALP (Figure 5.8D versus C). Although the extent of change in the expression of both STRO-1 and ALP was variable in normal OB-cultures derived from 3 different donors, a similar trend was observed.

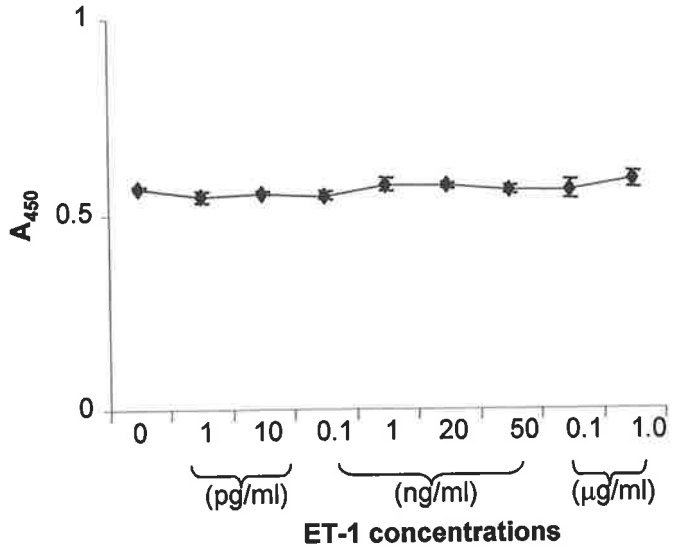
### 5.2.6 Enforced Over-expression of Human ET-1 in Human OB-like Cells: Preparation of the Retroviral Expression Construct

Due to the short plasma half-life of ET-1 (de Nucci *et al.*, 1988), we decided to mimic constant high level exposure of OB-like cells to ET-1, by enforced over-expression. The human ET-1 gene was amplified by PCR and cloned into the retroviral vector pRUF (*neo*) as described in the Methods (Section 2.5) and the retrovirus used to transduce primary human OB-like cells. The nucleotide sequence of cloned ET-1 was deduced by automated sequence analysis and the resultant sequence compared with sequences submitted to the GenBank database *via* standard "nucleotide-nucleotide BLAST". Using pRUF sequence forward primer (Appendix 3A) and reverse primer (Appendix 3B) (refer to table 2.1), a one base pair substitution was identified at position 797 (T → G) (Appendix 3). This observation was consistent in two clones tested in which ET-1 cDNA was synthetised using mRNA isolated from human normal OB-like cells or myeloma cell line ARH-77. The

Figure 5.7. Recombinant hET-1 has no effect on human OB-like cell proliferation. Human OB-like cells were seeded into 96-well plates at a cell density of 2,000 cells/well. The cells were cultured in the presence or absence of rhET-1 or its receptor antagonists BQ123 and BQ788 at the indicated concentrations and the cell number and viability assessed at d3, d5 and d7 using WST-1, as described in the methods. Recombinant hET-1 and its receptor antagonists BQ123 and BQ788 had no effect on cell proliferation of human OB-like cells at d3 (A), d5 (B) and d7 (C) when cells were cultured in α-MEM-10 medium. Moreover, rhET-1 had no effect on OB-like cell proliferation when cultured in SDM at d3 (D) and d5 (F). Values represent means ± SEM of triplicate cultures of each concentration. The results displayed are a representative example of 3 independent experiments.


A
1
0.8
0.6
0.4
0.2
0
$A_{450}$
ET-1
BQ123
BQ788
B
1.2
1
0.8
0.6
0.4
0.2
0
$A_{450}$
C
1.4
1.2
1
0.8
0.6
0.4
0.2
0
$A_{450}$
ET-1 0 0.1 1 10 0.1 1 20 50 0.1 1.0
(pg/ml)
(ng/ml)
(μg/ml)
BQ123 0 1 3 10 30 0.1 0.3 1 3 10
BQ788
(ng/ml)
(μg/ml)
Concentrations

D


1
0.5
0
$A_{450}$
0
1
10
0.1
1
20
50
0.1
1.0
(pg/ml)
(ng/ml)
(μg/ml)
ET-1 concentrations

F

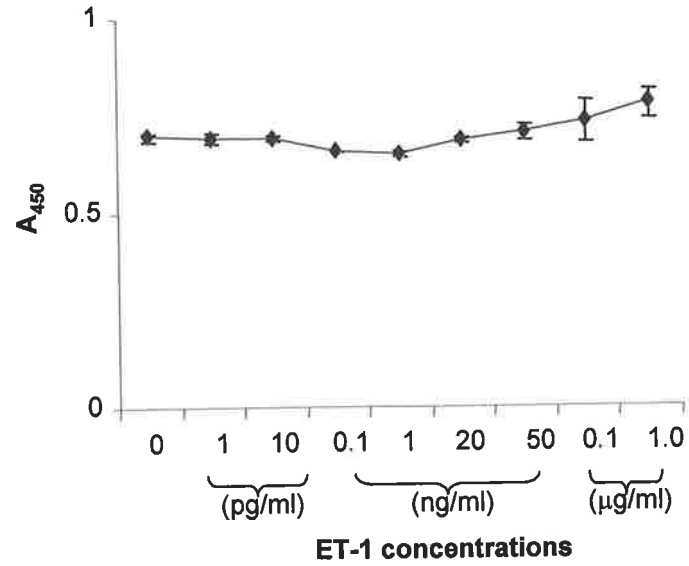
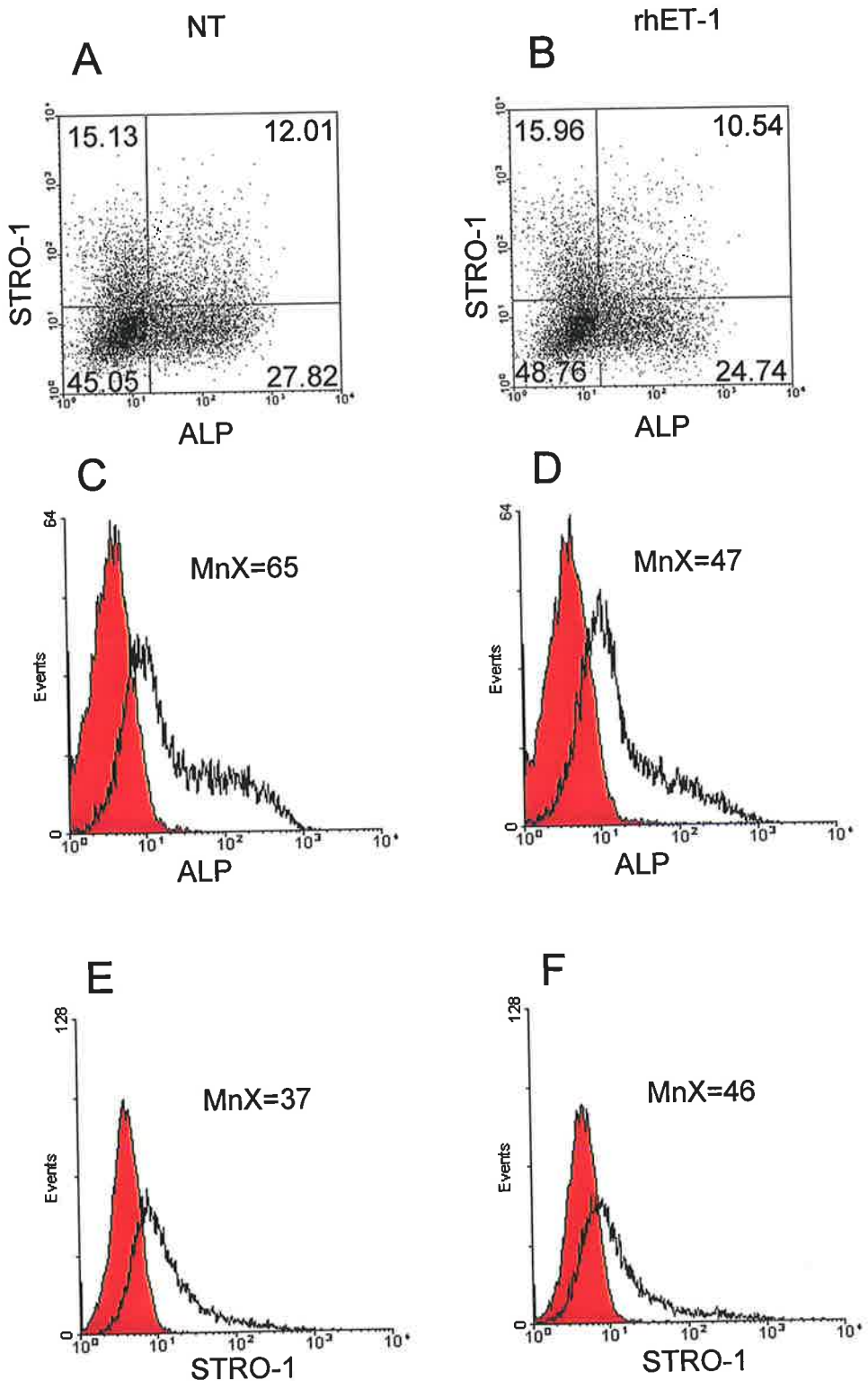

1
0.5
0
$A_{450}$
0
1
10
0.1
1
20
50
0.1
1.0
(pg/ml)
(ng/ml)
(μg/ml)
ET-1 concentrations

Figure 5.8. Recombinant hET-1 reduces cell surface ALP protein expression in human OB-like cells. Osteoblast-like cells were treated with rhET-1 at a concentration of 25ng/ml and dual-colour immunofluorescence and flow cytometric analysis was performed using mAbs STRO-1 and B4-78 at d3. Immunoreactivity was revealed by incubation with an anti-IgM-PE (y-axis) and IgG-FITC (x-axis), as described in the methods. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, 1B5 (FITC) and 1A6.12 (PE), respectively. Fluorescence histograms depict the relative cell count (y-axis) versus the intensity of ALP or STRO-1 expression (log scale) with isotype matched mAb, 1B5 or 1A6.12, shown in red histogram. Recombinant hET-1 was found to increase STRO-1 expression (F versus E). In contrast, ET-1 decreased cell surface ALP expression (D versus C). These observations were consistent in all three donors tested. A representative example from one donor is shown. A,C,E: untreated; B,D,F: treated with 25ng/ml ET-1.


NT
rhET-1
A
B
15.13
12.01
45.05
27.82
15.96
10.54
48.76
24.74
STRO-1
ALP
C
D
MnX=65
MnX=47
Events
ALP
E
F
MnX=37
MnX=46
Events
STRO-1

resultant polypeptide sequence of ET-1 was deduced from the nucleotide using DNASIS software, as described in the Methods (Section 2.5.9). The single bp substitution at position 797 (T → G) resulted in an amino acid substitution at position 198 (Asn → Lys) of the deduced amino acid sequence (indicated in blue, Appendix 4). As the amino acid substitution was contained only in the preproendothelin-1 (Yanagisawa *et al.*, 1988) and not within the mature, active 21 amino acid ET-1 peptide, it had no effect on the production of active ET-1.

Successful expression of ET-1 by transduced OB-like cells was confirmed by measuring the culture supernatant from both "empty vector" and ET-1-transduced OB-like cells using the commercially available ET-1 Immunoassay as described above. As seen in Figure 5.9, the human ET-1 infected OB-like cells produced significantly more ET-1 protein compared with OB-like cells infected with empty vector with pRUF-ET-1 and pRUF infected cells producing 171.19±32.44pg and 3.55±3.14pg ET-1 protein per $1\times10^5$ cells, respectively ($P$=0.018, Student $t$-test).

### 5.2.7 Human ET-1 Reduces the Bone Forming Capacity of Human OB-like Cells by Inhibiting Cellular Differentiation

Following successful infection of OB-like cells with human ET-1, we examined the effect of constant exposure of ET-1 on the OB-like cell proliferation. Infected human OB-like cells were seeded in 96-well plates at a density of 2,000 cells per well and the cell number was assessed at d3 and d5 using WST-1 as described in the Methods (Section 2.2.2). As seen in Figure 5.10, cells infected with ET-1 displayed a similar proliferative capacity compared with cells infected with empty vector pRUF(*neo*) at both d3 (Figure 5.10A) and d5 (Figure 5.10B). Similarly, independent experiments examining the mitogenic effects of enforced ET-1 expression in the myeloma cell line RPMI8226, revealed no difference in proliferative index of these cells when compared with their empty vector infected counterparts (data not shown).

We next examined whether constant exposure of ET-1 had any significant effects on cellular differentiation as measured by their expression of STRO-1 and ALP as described above. Consistent with the effect of exogenous rhET-1, pRUF-ET-1 infected human OB-like cells expressed lower levels of cell surface ALP protein (Figure 5.11D) compared with cells infected with empty vector (Figure 5.11C). The mean fluorescence of cell surface ALP

Figure 5.9. Human ET-1 (pRUF-ET-1) infected human OB-like cells produce more ET-1 protein compared with OB-like cells infected with empty vector. Supernatant collected from $1\times10^6$ OB-like cells infected with pRUF or pRUF-ET-1 was analysed using the human ET-1 Immunoassay kit. The level of ET-1 in supernatants collected from pRUF infected OB-like cells was found to be $3.55\pm3.14$pg/$1\times10^5$ cells. In contrast, pRUF-ET-1 infected OB-like cells secreted significantly more ET-1 protein ($171.19\pm32.44$pg/$1\times10^5$ cells, $P$=0.018, Student $t$-test). Similar results were obtained from OB-like cells derived from two different donors.

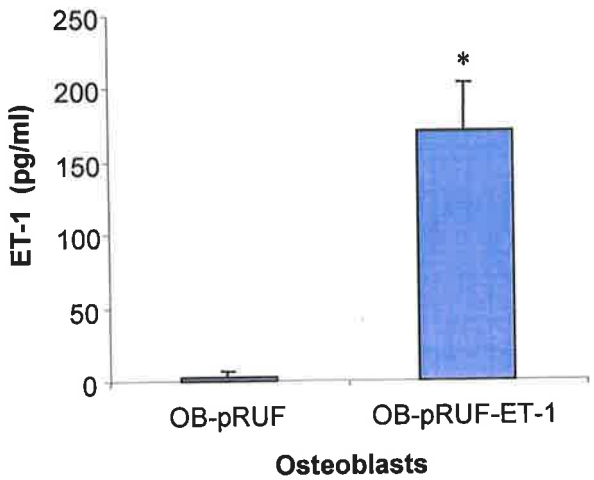
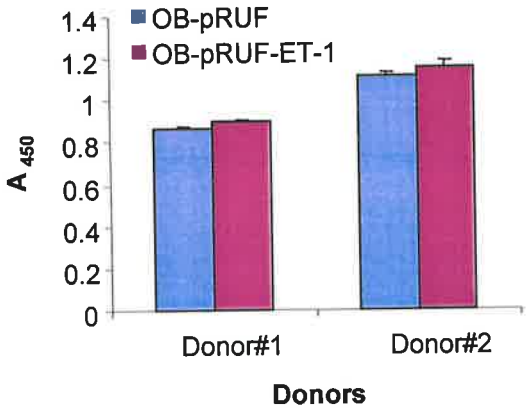
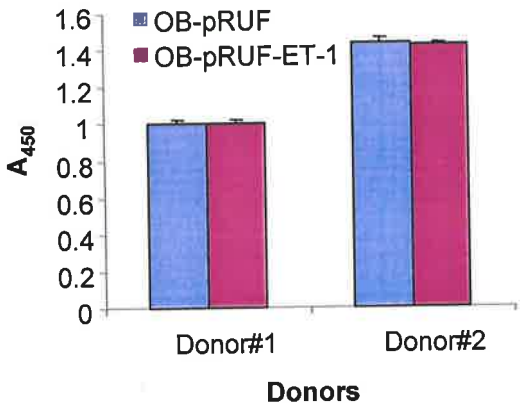

250
200
150
100
50
0
ET-1 (pg/ml)
*
OB-pRUF
OB-pRUF-ET-1
Osteoblasts

Figure 5.10. Human OB-like cells infected with pRUF-ET-1 possess a similar proliferative capacity to cells infected with empty vector. Infected human OB-like cells were seeded into 96-well plates at a cell density of 2,000 cells/well using ACDU facility of the FACStar cell sorter. The cells were cultured and the number and viability quantitated at d3 (A) and d5 (B) using WST-1, as described in the methods. Cells infected with ET-1 displayed a similar proliferative capacity compared with cells infected with empty vector pRUF(*neo*). Values represent means ± SEM of triplicate cultures of each concentration. The results displayed are a representative example of 3 independent experiments.

A


OB-pRUF
OB-pRUF-ET-1
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A 450
Donor#1
Donor#2
Donors

B


OB-pRUF
OB-pRUF-ET-1
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A450
Donor#1
Donor#2
Donors

protein expression decreased from 122 in OB-like cells infected with empty vector to 51 in cells infected with the ET-1 virus. Moreover, pRUF-ET-1 infected human OB-like cells exhibited higher levels of STRO-1 expression on the cell surface (Figure 5.11F) when compared with cells infected with the empty vector (Figure 5.11E).

Based on the well-described model of OB-like cell differentiation (Gronthos *et al.*, 1999; Stewart *et al.*, 1999), these studies suggested that ET-1 may affect the functional capacity of these cells by limiting their differentiative capacity. In order to test this hypothesis, we examined whether the bone mineral formation capacity of ET-1 infected cells differed from cells infected with the empty vector *in vitro* assay of mineral formation. Consistent with the observed change in OB-like phenotype following infection with the ET-1 retrovirus, the mineralisation potential of pRUF-ET-1 infected OB-like cells was considerably decreased (Figure 5.12B), compared with the pRUF infected OB-like cells (Figure 5.12A). The degree of mineralisation of the extracellular (as indirectly measured by acid-liberated calcium) was significantly lower in pRUF-ET-1 infected cells compared with the pRUF infected cells at both week 4 ($P$=0.0007, Student $t$-test) and week 6 ($P$=0.0003, Student $t$-test) (Figure 5.12C). The calcium levels decreased from 780.5±15.6 nM/culture in empty vector infected OB-like cells to 43.0±12.0 nM/culture in ET-1 infected cells at week 4. Similarly, the calcium levels decreased from 1167.5±20.7 nM/culture in empty vector infected cells to 161.3±28.7 nM/culture in ET-1 infected cells at week 6 (Figure 5.12).

The ability of a cell to produce a mineralised matrix *in vitro,* may not be predictive of their capacity to produce an organised bone tissue *in vivo*. Thus, the ET-1 and empty vector infected OB-like cells were assayed for their capacity to develop a human bone tissue following ectopic transplantation in SCID mice, using HA/TCP particles as a osteo-conductive/-inductive carrier vehicle (Gronthos *et al.*, 2000; Gronthos *et al.*, 2003). Histological examination of harvested transplants at week 8, showed that OB-like cells infected with empty vector had the capacity to form new bone reminiscent of mature lamellar bone containing osteocytes (arrow) developing at the HA/TCP particle surfaces together with surrounding fibrous and haemopoietic tissue (Figure 5.13A). In contrast, bone was rarely found in cells infected with pRUF-ET-1 (Figure 5.13B). The total bone area formed in each of the transplants was determined using SCION software and the ratio of bone area to total tissue area was determined (Figure 5.13C). Human OB-like cells infected with empty vector significantly (Figure 5.13C, $P$=0.0002, 0.004 for donor 1 and 2,

Figure 5.11. Human OB-like cells infected with pRUF-ET-1 express lower ALP and higher STRO-1 protein compared with cells infected with empty vector. Dual-colour immunofluorescence and flow cytometric analysis was performed using infected OB-like cells stained with the mAbs STRO-1 and B4-78. Immunoreactivity was revealed by incubation with an anti IgM-PE (y-axis) and IgG-FITC (x-axis), as described in the methods. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, 1B5 (FITC) and 1A6.12 (PE), respectively. Fluorescence histograms depict the relative cell count (y-axis) versus the intensity of ALP or STRO-1 expression (log scale). Isotype matched mAb, 1B5 and 1A6.12, were shown in red histogram. pRUF-ET-1 infected human OB-like cells were found to express dramatically lower levels of cell surface ALP protein (D) compared with cells infected with empty vector (C). Moreover, pRUF-ET-1 infected human OB-like cells expressed higher STRO-1 protein (F) on the cell surface compared with cells infected with empty vector (E). These observations were consistent in all three donors tested. Representative distributions from three experiments are shown. A,C,E: pRUF infected OB-like cells; B,D,F: pRUF-ET-1 infected OB-like cells.

OB-pRUF

OB-pRUF-ET-1

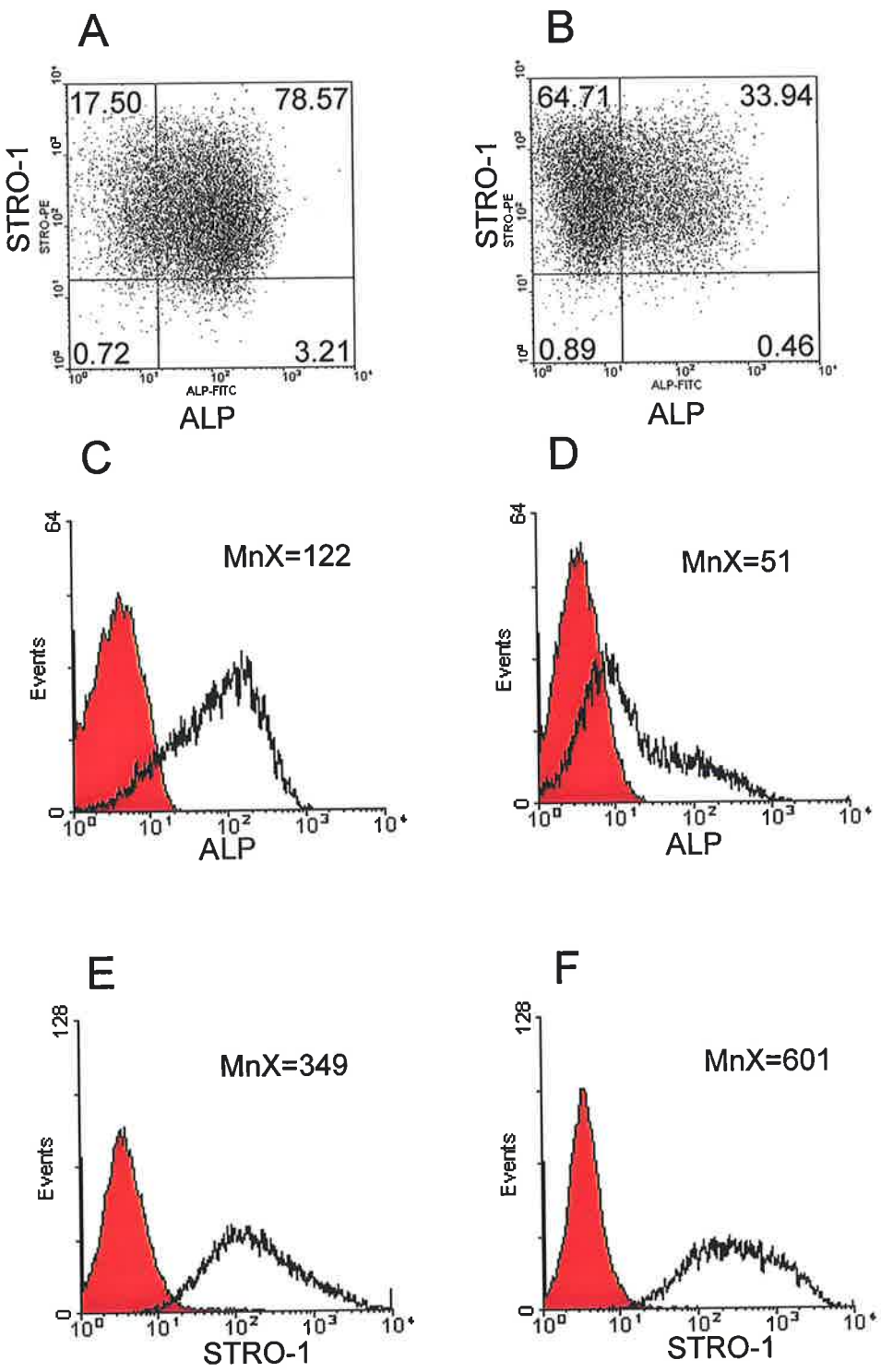
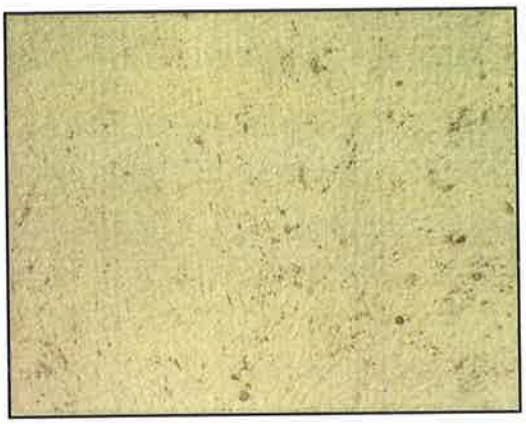


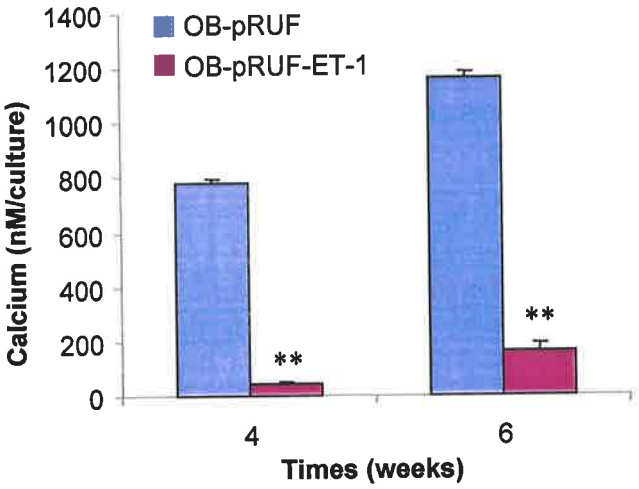
Figure 5.12. The mineralisation potential of pRUF-ET-1 infected OB-like cells is decreased compared to cells infected with empty vector. Infected OB-like cells were seeded into 24-well plate at a cell density of $5\times10^4$/well in triplicate and cultured in osteoinductive conditions, as described in the methods. The cells were "fed" weekly with fresh medium. The release of free calcium from the matrix was achieved by treating the adherent cell layers under acidic condition as described in the methods. Samples then were reacted with o-cresol-phthalein-complexon and the colorimetric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium. pRUF infected OB-like cells (A, original magnification 10×) were found to produce more mineral than cells infected with pRUF-ET-1 (B, original magnification 10×) at week 6. The calcium level was significantly lower in pRUF-ET-1 infected cells than that in pRUF infected cells (**$P$=0.0007, 0.0003 at week 4 and week 6, respectively, Student $t$-test). The calcium levels in pRUF infected cells were 780.5±15.6 nM/culture at week 4 and 1167.5±20.7 nM/culture at week 6. In contrast, the calcium levels in pRUF-ET-1 infected cells were 43.0±12.0 nM/culture at week 4 and 161.3±28.7 nM/culture at week 6. The experiments were performed twice using different OB-like cells derived from three donors.

A

B



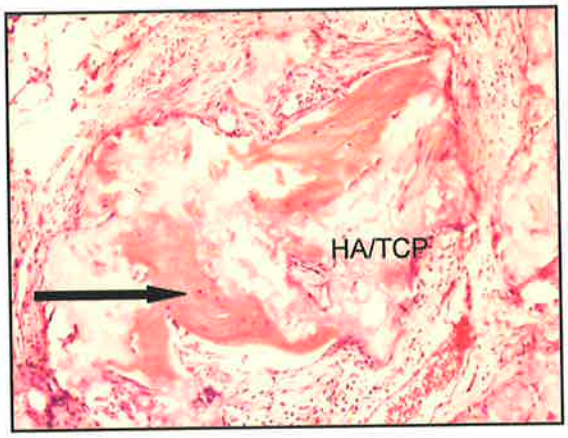
C


1400
1200
1000
800
600
400
200
0
OB-pRUF
OB-pRUF-ET-1
Calcium (nM/culture)
**
**
4
6
Times (weeks)

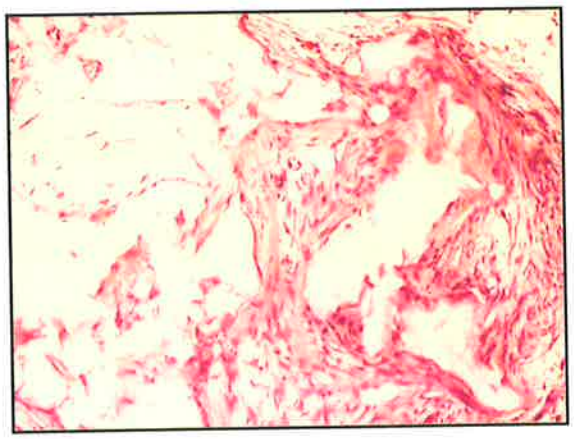
respectively, Student *t*-test) form more bone *in vivo* (5.23±0.29%, 7.08±0.37% of total area for donor 1 and 2, respectively) when compared with cells infected with pRUF-ET-1 (0%, 0.37±0.22% of total area for donor 1 and 2, respectively). We therefore conclude that exposure of OB to high levels of ET-1 significantly inhibits their bone forming potential *in vivo*.

Figure 5.13. *In vivo* bone forming potential of human OB-like cells infected with pRUF-ET-1 was decreased compared with cells infected with empty vector. The infected OB-like cells were expanded *in vitro* and implanted subcutaneously into SCID mice using HA/TCP carrier. Implants were harvested 8 weeks after the transplant, fixed and paraffin embedded as described in the methods. Five μM sections were cut and counter stained with haematoxylin and eosin. New bone formation containing osteocytes (arrow) was observed for OB-like cells infected with empty vector developing at the HA/TCP particle surfaces together with surrounding fibrous and haematopoietic tissue (original magnification, 10×) (A). Bone was rarely found in cells infected with pRUF-ET-1 (B). The total bone area formed was determined using SCION software and the ratio of bone area to total tissue area was determined. Human OB-like cells infected with empty vector formed significantly (C, $P$=0.0002, 0.004 for donor 1 and 2, respectively, Student $t$-test) more bone *in vivo* (5.23±0.29%, 7.08±0.37% of total area for donor 1 and 2, respectively) when compared with cells infected with pRUF-ET-1 (0%, 0.37±0.22% of total area for donor 1 and 2, respectively).

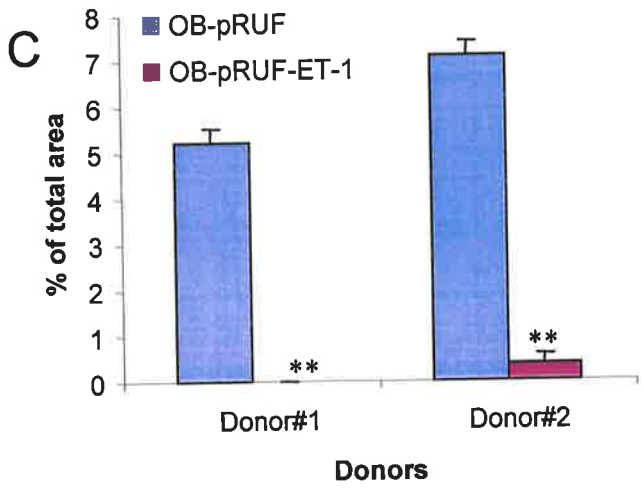
A


HA/TCP

B



C


OB-pRUF
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## 5.3 DISCUSSION

Although previous studies have shown that ET-1 is expressed by many cell types, including epithelial, mesangial, leukocyte, macrophages, smooth muscle cells, cardiomyocytes, neuronal and glial cells (reviewed in (Luscher *et al.*, 2000)), the studies described here, represent the first description of ET-1 in myeloma PCs. Our data suggest that myeloma cell lines not only express ET-1 gene, but also synthesise ET-1 protein. In addition, BMMNCs derived from the patients with MM also express the ET-1 gene. Because of the diversity of cell types in BM which may express ET-1, $CD38^{bright}$ PCs (Joshua *et al.*, 2002), were isolated using preparative cell sorting and ET-1 gene expression confirmed by RT-PCR. Consistent with published findings (Sasaki *et al.*, 1993), our studies also show that OB-like cells express ET-1 at appreciable levels. Although increased ET-1 gene expression in BMMNCs derived from MM patients was not consistently observed, OB-like cells derived from patients with MM displayed significantly higher ET-1 gene expression compared with cells isolated from subjects with the pre-myeloma disease, MGUS. These studies suggest that OB-like cells may, in response to myeloma PCs secrete more ET-1. Interestingly, the level of ET-1 secreted by OBs (Figure 5.9, $3.55\pm3.14$pg/$1\times10^5$ cells) was found to be significantly higher than that secreted by myeloma cell lines (Figure 5.1B, RPMI8226 0.338pg/$2\times10^5$ cells).

Consistent with these findings, assessment of plasma levels of ET-1 in patients with MM exhibited higher ET-1 plasma levels when compared with plasma from patients with MGUS and age-matched controls. Whilst the differences in plasma ET-1 concentration between patients with MM and normal subjects or patients with MGUS, was not statistically significant ($P$=0.0618 and 0.0885, respectively), we feel that this was a reflection of the fact that we had comparatively few MGUS and "normal" samples compared with MM samples. Future studies examining this question will require the accrual of a larger study sample.

Notably, whilst the ET-1 mRNA was strongly expressed by myeloma cell lines and BMMNCs derived from MM patients, the level of ET-1 protein found in myeloma cell line conditioned media and patients' plasma was extremely low. It must be remembered that levels of ET-1 measurable in the plasma are determined not only by the synthesis of, but also the clearance and breakdown rate of ET-1. The elevated levels of ET-1 found in MM patients may in part due to (1) enhanced synthesis of preproendothelin-1; (2) enhanced conversion of ET-1 precursor peptide to active ET-1; (3) decreased clearance of ET-1. The

intracellular half-life of the preproendothelin-1 mRNA has been estimated to be approximately 15 minutes (Inoue *et al.*, 1989b). The transcription of ET-1 mRNA and the synthesis and secretion of ET-1 can be induced by a variety of stimuli including hypoxia, ischemia or shear stress within minutes (Noll *et al.*, 1996), suggesting the ET-1 synthesis is precisely controlled by subtle environmental changes. ET-1 has been detected on the rough endoplasmic reticulum, Golgi vesicles, small vesicles beneath the cell membrane and the lysosomes, but not in secretory granules in both endothelial cells and OBs (Nakamura *et al.*, 1990; Sasaki *et al.*, 1993). This suggests that ET-1 production may be regulated predominantly at the level of mRNA transcription, rather than *via* the release of pre-formed protein from storage vesicles following activation. ET-1 is cleared mostly by pulmonary circulation (de Nucci *et al.*, 1988) which may contribute to the short half-life of ET-1 protein in the plasma. The low plasma concentrations of ET-1 strongly supports the notion that ET-1 may act as an autocrine/paracrine signalling peptide rather than as circulating hormones.

Most studies which have examined the level of plasma ET-1 have restricted their examination to the peripheral blood of healthy subjects (Haapaniemi *et al.*, 2000; Haufschild *et al.*, 2004; Maeda *et al.*, 2003; Properzi *et al.*, 2001; Roland *et al.*, 2001). The lowest average level of PB plasma ET-1 reported was 0.54±0.3 pg/ml (Roland *et al.*, 2001) and this level has been reported to increase with age (Maeda *et al.*, 2003). In view of the abundance of endothelial cells in bone marrow and the proximity of these cells to other ET-1 producing cells such as OBs and BMMNCs, it is conceivable that ET-1 level in local BM environment would be higher than that found in the peripheral circulation. Unexpectedly, our findings suggest that the plasma level of ET-1 in BM is lower than that reported in PB. Furthermore, in a paired cohort study of 10 MM patients in which the level of ET-1 in BM was compared with that found in PB, confirmed that the PB plasma levels of ET-1 are, in all instances, higher than that found in BM plasma. In should be remembered that ET-1 was first isolated from porcine aortic endothelial cells and plays an important role in maintaining blood pressure (Yanagisawa *et al.*, 1988). In addition, blood vessel smooth muscle cells and leukocytes within the blood vessel represent a considerable source of ET-1 in PB (Luscher *et al.*, 2000). This may, in part, explain why the level of ET-1 in PB is higher than that in BM. Interestingly and in keeping with the findings of others regarding the level of ET-1 in PB (Hirai *et al.*, 2004; Telemaque-Potts *et al.*, 2002), there was no apparent gender bias in the level of BM ET-1 between male and female patients.

ET-1 exerts its physiological effect *via* its receptors, $ET_AR$ and/or $ET_BR$. In addition to the level of ET-1 protein, the effect of ET-1 on the targeted cells also depends on the receptor expression by the target cells. Interestingly, BMMNCs derived from MM patients were found to express higher levels of $ET_AR$ compared with BMMNCs from normal control subjects, suggesting that ET-1 may mediate its mitogenic effect on myeloma cells through its increased expression of $ET_AR$. The increased $ET_AR$ expression in malignant tissue has been reported using immunohistochemistry and/or autoradiography in several types of cancers including colorectal (Asham *et al.*, 2001; Simpson *et al.*, 2000), ovarian (Bagnato *et al.*, 1999) and prostate tumours (Nelson *et al.*, 1996). Furthermore, the increased secretion of ET-1 co-ordinated with increased expression of ECE-1a are intrinsically involved in the hyperpigmentation mechanism of a benign epidermal tumour seborrhoeic keratosis (Manaka *et al.*, 2001). In addition, the ectoenzyme ECE-1c is the most commonly expressed isoform in lung cancer, suggesting that preproendothelin produced by tumour cells could be activated by the over-expression of ECE-1c, causing autocrine and/or paracrine growth stimulation of surrounding cells *via* $ET_AR$ (Ahmed *et al.*, 2000). Nevertheless, our studies show that the ECE-1 expression in myeloma cells and human OB-like cells are not regulated.

Although the $ET_AR$ and $ET_BR$ are not regulated in OB-like cells, the $ET_BR$ splice variant, $ET_BR\Delta5$, is down regulated. $ET_BR$ and $ET_BR\Delta5$ represent alternatively spliced variants of a single gene. Receptor subtypes can arise through divergent genes, such as $ET_AR$ and $ET_BR$, whilst additional variants within a subtype can arise by alternative RNA splicing. The loss of the entire fifth exon of $ET_BR\Delta5$ results in a premature termination of translation and loss of the cytoplasmic signalling domain (Tanoue *et al.*, 2002). Although the splice variant displayed the same binding properties as the wild type, this receptor is incapable of stimulating a functional response due to the loss of the cytoplasmic signalling domain (Cramer *et al.*, 2001). Studies show that the intracellular COOH-terminal domain of the 7 transmembrane receptors is critical for G protein coupling and functional responses. Hence it is tempting to speculate that this receptor may function as a clearance receptor for the ET-1 peptide. If so, the decreased $ET_BR\Delta5$ in MM may decrease the local sequestration and clearance of activated ET-1 in the BM microenvironment, thereby increasing the biological effect of ET-1. However, it is still possible that the $ET_BR\Delta5$ could mediate another, yet unidentified, response.

The production of ET-1 has been shown in several other human cancer cells, including breast (Kojima *et al.*, 1995; Yamashita *et al.*, 1991), colon (Asham *et al.*, 2001; Simpson *et al.*, 2000), cervix (Shichiri *et al.*, 1991), larynx (Shichiri *et al.*, 1991), endometrium (Pekonen *et al.*, 1992), prostate (de Matteis *et al.*, 2001; Grant *et al.*, 1997), ovary (Salani *et al.*, 2000a) and lung (Ahmed *et al.*, 2000; Bagnato *et al.*, 1999). In addition, the presence of ET-1 correlates with tumour vascularity and malignancy in well-vascularised brain tumours (Stiles *et al.*, 1997) and colorectal cancer (Egidy *et al.*, 2000). Moreover, $ET_BR$ is almost undetectable in normal colon mucosa, but are abundant in all vascularized areas of the cancer stroma (Egidy *et al.*, 2000) and $ET_AR$ expression is localised in carcinoma cells as well as in vascular smooth muscle cells (VSMCs) (Salani *et al.*, 2000a). These observations suggest that ET-1 is a positive regulator of the angiogenic process. There is accumulating evidence to suggest that MM patients with progressive disease display increased angiogenesis compared with patients with non-active MM and MGUS (Vacca *et al.*, 1994). In addition, the increased angiogenesis is negatively correlated with survival rate of MM (Pruneri *et al.*, 2002). Therefore, it may be possible that the elevated expression of ET-1 in MM patients may play an important role in MM angiogenesis through increased $ET_AR$ and decreased $ET_BR\Delta5$. ET-1 can modulate tumour angiogenesis indirectly through the induction of several pro-angiogenic factor *in vitro*, including PDGF and VEGF (Okuda *et al.*, 1998; Pedram *et al.*, 1997). Engagement of the $ET_AR$ by ET-1 induces VEGF production by increasing levels of hypoxia-inducible factor-1 $\alpha$ (Spinella *et al.*, 2002). Moreover, ET-1 and VEGF have reciprocal stimulatory effects, which may play an important role in concomitant proliferation of endothelial and smooth muscle cells in the vascular wall (Matsuura *et al.*, 1998). In addition, myeloma cells and/or surrounding OBs produce a plethora of growth factors and inflammatory cytokines, such as IL-1$\beta$ and TNF-$\alpha$, that can upregulate the expression of ET-1 as detailed in Chapter 3 (Saleh *et al.*, 1997, Le Brun, 1999 #765). Taken together, the regulated expression of ET-1 and its receptors in MM may have a significant role in the pathogenesis of MM.

In addition to the elevated expression of ET-1 and $ET_AR$ expression in many types of malignant tissues, *in vitro* experiments has shown that exogenous and endogenous ET-1 directly stimulates proliferation of cancer cell lines *via* $ET_AR$ or $ET_BR$ (Kikuchi *et al.*, 1996; Kusuhara *et al.*, 1990; Shichiri *et al.*, 1991). We therefore examined the role of exogenous rhET-1 on the growth of myeloma cell lines. Three different cell lines were chosen based on the different gene expression of two receptors: ARH-77 expresses ET-1 and $ET_BR$;

RPMI8226 expresses ET-1 and $ET_AR$; and WL2 expresses ET-1 without expression of two receptors (Figure 5.1). Unexpectedly, none of these three cell lines responded mitogenically to the addition of rhET-1. Similarly, exogenous rhET-1 also had no effect on human OB-like cells either in serum-deprived medium or FCS supplemented medium. Furthermore, $ET_AR$ antagonist BQ123 and $ET_BR$ antagonist BQ788 had no effect on the cell growth of myeloma cell lines or human OB-like cells, which further confirms that endogenous ET-1 has no mitogenic effect on these two types of cells.

BQ-123, a cyclic pentapeptide (-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-), hampers ET-1 induced hydrolysis of phosphoinositide and DNA synthesis in rat VSMCs by inhibiting ET-1 binding to cell surface $ET_AR$ (Eguchi *et al.*, 1992). The IC50 value for $ET_AR$ on porcine aortic smooth muscle cells and rat VSMCs are 7.3 nM (Ihara *et al.*, 1992) and 20 nM (Eguchi *et al.*, 1992), respectively. However, 15% of specific binding of [$^{125}$I] ET-1 to rat VSMC could not be displaced by BQ123 even at 1 μM (Eguchi *et al.*, 1992), suggesting that cultured rat VSMC may process small amount of non-isopeptide-selective $ET_BR$ through which ET-1 may also stimulate DNA synthesis. BQ788 [N-cis-2,6-dimethylpiperidinocarbonly-L-γ-methylleucyl-D-1-methoxy-carbonlyl-trypto-phanyl-D-norleucine] is a potent, specific, and selective $ET_BR$ antagonist with no agonist activity (Ishikawa *et al.*, 1994). BQ788 potently inhibits ET-1 binding to $ET_BR$ both in porcine cerebellar membranes and on human Girardi heart cells, with IC50 value of 0.9 nM and 1.2 nM, respectively (Ishikawa *et al.*, 1994). In this study, we used BQ123 and BQ788 at concentrations as high as 10μg/ml (equal to 16.4 μM for BQ123 and 15.1 μM for BQ788) which should have blocked any potential mitogenic effect of ET-1 on myeloma cells and human OB-like cells.

Some studies have reported that the mitogenic activity produced by ET-1 on smooth muscle cells is only apparent in the presence of serum (Sugitani *et al.*, 2001), platelet-derived growth factor (Weissberg *et al.*, 1990) or insulin (Komuro *et al.*, 1988). Although rat aortic smooth muscle cells express functional ET-1 cell surface receptors but do not proliferate when cultured in serum-free culture medium supplemented with ET-1 (Alberts *et al.*, 1994). However, our study suggest that exogenous rhET-1 has no mitogenic effect on human OB-like cells either in the presence or absence of serum. It has been reported that the constitutive ET-1 exposure, following transgene expression of ET-1, can promote smooth muscle cell proliferation (Alberts *et al.*, 1994). This observation suggests that

extracellular ET-1 may be rapidly degraded by smooth muscle cell-derived proteases and long term exposure to biologically active ET-1 may be necessary for mitogenesis. Therefore, the physiological activity of ET-1 may not be mimicked by the addition of exogenous injection of ET-1 into circulating blood *in vivo*, because the exogenously supplied ET-1 may rapidly inactivated by proteases expressed by many types of cells which capable of degrading extracellular ET-1. In light of this, we decided to clone the human ET-1 gene and examine whether constitutive over-expression of ET-1 may produce mitogenic effects on human myeloma cell lines and OB-like cells.

The level of ET-1 protein produced by the ET-1 infected OB-like cells was detected using human ET-1 Immunoassay, and was found to expressed more than 48 times of that in OB-like cells infected with empty vector. We examined the cell proliferation rate using ACDU FACS sort mode, which guaranteed an accurate input cell number. However, no significant difference of cell growth was observed between ET-1 infected human OB-like cells and empty vector infected cells. The ET-1 induced mitogenic action *in vitro* has been observed in mouse embryonic Swiss 3T3 fibroblasts (Takuwa *et al.*, 1989a), rat calvariae (Takuwa *et al.*, 1990; Tatrai *et al.*, 1992) and human OB-like cells (Kasperk *et al.*, 1997). However some reports suggest that exogenous ET-1 has no mitogenic effect on certain cell types including prostatic epithelial and stromal cells (Grant *et al.*, 1997). Given the variability in the experimental design, cell types and culture condition used, it is almost impossible to determine the underlying reasons for these apparent discrepancies.

Although displaying no measurable mitogenic effect on human OB-like cells, our studies show that ET-1 has an effect on cellular differentiation. Exogenous rhET-1, at a concentration of 25 μg/ml, was found to decrease the expression of the OB differentiation marker, ALP (Weinreb *et al.*, 1990). Similarly, OB-like cells infected with ET-1 were found to express less cell surface ALP protein and higher STRO-1 antigen compared with the control cells. The inhibitory effects of ET-1 on human osteoblastic cell differentiation (decreased ALP production) provides a basis for a regulatory role of ET-1 in human bone formation because the number of differentiated OBs is the most important determinant for the bone-forming capacity of a bone compartment. In accord with the phenotypic change which accompanied ET-1 over-expression, OB-like cells exhibited a reduced capacity to form bone mineral *in vitro*. Other studies also have shown that ET-1 reduces cellular ALP activity in a dose-dependent manner in osteoblastic cells (Hagiwara *et al.*, 1996; Takuwa *et*

*al.*, 1990; Takuwa *et al.*, 1989b). In addition, ET-1 inhibits the expression of mRNA for OCN, which is also considered a seminal marker of OB maturation. (Hiruma *et al.*, 1998b). Consistent with our data, ET-1 has been found to inhibit the mineralisation of MC3T3-E1 cells through the $ET_AR$ (Hagiwara *et al.*, 1996; Hiruma *et al.*, 1998a; Hiruma *et al.*, 1998b). However, other studies suggest that ET-1 increases markers of the OB differentiation such as ALP, type-I collagen in a dose-dependent manner (Kasperk *et al.*, 1997; Tatrai *et al.*, 1992). In addition, ET-1 has also been found to enhance mRNA expression of OCN and OPN in the OB-like cells lines *via* the $ET_AR$ (Nambi *et al.*, 1995).

To further investigate the biological effects of chronic exposure to ET-1, OB-like cells transduced with an ET-1 retrovirus were transplanted into the SCID mice and assessed for their capacity to produce an organised bone tissue *in vivo*. Consistent with the reduced bone mineral formation capacity *in vitro*, human OB-like cells infected with human ET-1 displayed a reduced capacity to form an organised bone tissue *in vivo*, suggesting that ET-1 has the ability to inhibit OB-like cells to form an organised and mineralised bone. In contrast to our findings, studies by others (Guise *et al.*, 2003; Nelson *et al.*, 1999; Yin *et al.*, 2003) suggest that ET-1 over-expressing tumour cell lines play a prominent role in the osteoblastic metastasis seen in individuals with prostate and breast cancer. The amnion-derived WISH human tumour cell line, made to over-express ET-1, was found to induce abundant local new bone formation when inoculated into the mouse tibia (Nelson *et al.*, 1999). In addition, the breast cancer cell line ZR-75-1, which expresses an abundance of ET-1, caused osteoblastic metastases when inoculated into the left cardiac ventricle of nude mice (Guise *et al.*, 2003; Yin *et al.*, 2003). These effects were specifically blocked by $ET_AR$ antagonist, A127722 (Nelson *et al.*, 1999) and ABT-627 (Guise *et al.*, 2003; Yin *et al.*, 2003). These findings lead us to speculate that osteoblastic metastasis which arise following inoculation of animals with ET-1 over-expressing cancer cells, occurs *via* indirect mechanism(s). Firstly, the cancer cell itself, may in response to ET-1 over-expression, produce bone cell active mitogen(s) which stimulate bone formation (Yin *et al.*, 2003). In addition, ET-1 expression by these cancer cells, may induce other cell types within the inoculation site, such as endothelial cells, to produce bone cell active mitogen(s) (Guenther *et al.*, 1986).

BM endothelial cells play an important role in tumour-associated angiogenesis in bone. Interestingly, ET-1 can act directly on endothelial cells initially *via* the $ET_BR$ to

modulates different stages of neovascularization, including proliferation, migration, invasion, protease production and morphogenesis (Salani *et al.*, 2000a). In addition, it also mediates vessel maturation by binding to $ET_AR$ resulting in neovascularization *in vivo* (Morbidelli *et al.*, 1995; Salani *et al.*, 2000b). As detailed in the Introduction (please refer to Section 1.4.2), the pattern of vascularization determines the way in which bone develops in the process of intramembranous or endochondral ossification (Pechak *et al.*, 1986; Pritchard *et al.*, 1956). In addition, it has been documented that endothelial cells can promote calvarial cell osteogenesis (Villanueva *et al.*, 1990). Therefore, neovascularization may play an important role in ET-1 producing cancer cell line-induced osteoblastic metastases. However, how angiogenesis affects bone formation or how epithelial cells and mesenchymal cells react to induce osteogenesis is largely unknown. The development of embryonic craniofacial complex has been closely investigated to explore the determinants of chondrogenesis and osteogenesis (Hall, 1982; Hall, 1991; Hall *et al.*, 2000). The regulatory factors derived mainly from a discrete region of the ectoderm, which induces cranial neural crest-derived ectomesenchymal cells to became osteogenic and chondrogenic structures (Hall, 1991). The notion that ET-1 plays an important role in osteogenesis came from the studies of ET-1 and its receptor $ET_AR$ knockout mice (Clouthier *et al.*, 1998; Clouthier *et al.*, 2000; Kurihara *et al.*, 1994) in which craniofacial abnormalities were noted. The impaired facial bone formation observed in both ET-1 (Kurihara *et al.*, 1994) and $ET_AR$ (Clouthier *et al.*, 1998; Clouthier *et al.*, 2000) knockout mice suggests that ET-1 may participate in the epithelial-mesenchymal interactions to promote pharyngeal arch development whose origin is mainly neural crest-derived ectomesenchymal cells. Epithelial-mesenchymal interactions are pivotal events in organ development in the pharyngeal region as well as in many other regions. Defects at any stage of mandibular development can lead to craniofacial malformations. Absence of the neural crest, abnormal migration of neural crest-derived cells, abnormal extracellular environments, altered inductive tissue interactions, and deficiencies in epigenetic interactions between components of the mandible can all lead to craniofacial malformations (Hall, 1982). *In situ* hybridisation studies have shown that ET-1 is found in the human foetal endothelial cells of the oral cavity and both $ET_AR$ and $ET_BR$ are detected in the human osteogenic cells along craniofacial bones (Barni *et al.*, 1995). Whilst our data suggest that ET-1 had no effect on the recruitment (data not shown) and proliferation of OB-like cells, it is possible that ET-1 may serve as an inductive signal to induce mesenchymal cell condensation and craniofacial development (Hall *et al.*, 2000).

In conclusion, we report for the first time that MM patients express significantly higher levels of ET-1 protein in BM. The ET-1 level in BM is significantly lower than that in PB. Our data suggest that myeloma cells may promote OB-like cells to produce excess ET-1 in BM microenvironment. In addition, these OB-like cells produce less $ET_BR\Delta5$, thereby decreasing the clearance of active ET-1. The increased ET-1 may produce important role in MM disease progression. Most importantly, the increased ET-1 directly inhibits the bone formation of OB-like cells in the patients with MM. The apparent divergent roles of ET-1 in prostate cancer with osteoblastic bone metastases (Nelson *et al.*, 1996; Nelson *et al.*, 1995; Nelson *et al.*, 1999) and MM with osteolytic bone lesions, suggests that the role of ET-1 in bone biology is complicated. Elucidation of the role of ET-1 may come from future studies examining the plasma cell dyscrasia, POEMS syndrome which displays osteoblastic bone lesions.

# CHAPTER 6. GENERAL DISCUSSION AND FUTURE CONSIDERATIONS

The mechanisms of MM-mediated osteolytic bone disease are attributed to the increased osteoclastic bone resorption caused by the over-expression of OC activating factors by myeloma cells or BM microenvironment in response to the myeloma cells. In addition to its direct role in promoting OC formation, studies detailed here show that the pro-inflammatory cytokine, IL-1β, indirectly promotes osteoclastic bone resorption by modifying the OB phenotype and its profile of OC-supporting cytokines. Furthermore, our studies suggest that the vasopeptide ET-1, previously implicated in other maliganancies, is also involved in the suppression of new bone formation in MM. Finally, we show that multi-potential MSCs express high level of the OC-supporting factor, RANKL, and present data supporting the notion that STRO-1 and RANKL proteins are highly related.

In the BM microenvironment of patients with MM, the interactions between myeloma cells, OBs, OCs, endothelial cells and other haemopoietic elements is complex and not well defined. This situation is further complicated by the large number of growth factors, cytokines, endocrine factors and adhesive interactions which are active between these various cell types (Ducy *et al.*, 2000). The factors responsible for communication between cells of the OB lineage and those OCs are the subject of much interest and speculation. Many studies show (Gowen *et al.*, 1990; Karadag *et al.*, 2000a; Keeting *et al.*, 1991; Michigami *et al.*, 2000; Pearse *et al.*, 2001; Roodman, 2002) that OBs are an abundant source of numerous local acting factors, which in association with endocrine factors, act to tightly regulate bone remodelling.

Studies presented in this thesis show that in the early phase of MM, pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6, which are produced by myeloma cells or OBs in response to MM cells, serve to increase the number of OB precursor cells by increasing cell proliferation. In addition, whilst IL-1β upregulates the expression of its own gene and that of TNF-α and IL-6 in OB-like cells *via* activation of the $PGE_2$ synthesis pathway, this pathway does not appear to be responsible for the IL-1β induced mitogenic effect on the OB-like cells.

As previously discussed, OBs are crucial for normal OC development *in vivo*. Osteoblast-derived RANKL is an essential deterministic factor in this process and is required for normal OC development and activity (Azuma *et al.*, 2000; Lacey *et al.*, 1998). Our studies showed that exposure of OB-like cells to IL-1β resulted in a concomitant increase in the rate of cell proliferation and STRO-1 expression by OB-like cells. In addition, STRO-1 expression was found to be positively associated with TM-RANKL expression by human OB-like cells suggesting that IL-1β acts, in part, to increase the number of undifferentiated STRO-$1^+$ osteoprogenitor cells which appear more capable of supporting OC formation.

Under normal physiological conditions, as osteoclastogenesis proceeds, the OB-like cells are thought to differentiate towards the mature OB phenotype and lose their ability to support osteoclastogenesis (Gori *et al.*, 2000). In MM, the increased levels of pro-inflammatory cytokines, such as IL-1β, may serve to extend the period in which OB-like cells are maintained in an "immature" OC-supportive phenotype. This would result in the disruption of the coordinated sequence of OC and OB differentiation during the bone remodelling cycle and the increased RANKL expression would result in increased osteoclastogenesis. These findings are consistent with studies which show histomorphometrically that a critical early event in the progression of MM is an increase in the number of osteoprogenitor cells and associated osteoclastogenesis (Bataille *et al.*, 1991). It is now well accepted that OBs are not the only source of RANKL protein in patients with MM. Our previous studies show that freshly isolated myeloma cells from patients with MM secrete RANKL protein which is able to induce OC formation in a co-culture system in the absence of OBs (Farrugia *et al.*, 2003). Interestingly, the phenotypic changes observed in the OB-like cells following the IL-1β treatment (or in response to myeloma cells) were not only associated with an increase in RANKL expression but also in the production of IL-6. STRO-$1^+$ OBs also synthesise higher levels of IL-6 protein compared with their STRO-$1^-$ counterparts, which would provide a more favourable BM microenvironment for myeloma cell growth (Kawano *et al.*, 1988; Klein *et al.*, 1989; Klein *et al.*, 1995; Nilsson *et al.*, 1990; Ohtake *et al.*, 1990). Therefore, IL-1β stimulation would result in an increase of OB-like cells which secrete more of the MM mitogen, IL-6. The amplified growth of myeloma cells would subsequently result in the secretion of more RANKL and pro-inflammatory factors by myeloma cells and/or OBs. In addition, the presence of myeloma cells in the BM

microenvironment would result in a decrease in the level of the soluble RANKL decoy receptor, OPG, *via* receptor mediated internalisation by MM cells. Thus, the net ratio of RANKL to OPG in the BM microenvironment of MM patients would be elevated resulting in more osteoclastogenesis (Hofbauer *et al.*, 2000; Hofbauer *et al.*, 2001). Our *in vitro* bone mineral formation data suggests that IL-1β, does not affect bone mineral forming potential of OB-like cells when placed in osteoinductive culture conditions, suggesting that IL-1β may inhibit bone formation *in vivo* by other mechanisms. One possibility is that the IL-1β-induced undifferentiated OB-like cells may be sensitive to apoptogenic stimuli produced by myeloma cells that may accelerate the exit of OBs or their precursors from their functional pool.

In addition to IL-1β, we have identified an additional myeloma cell-derived factor which appears to have a direct effect on bone formation. Our studies show, for the first time, that MM patients express higher plasma levels of ET-1 protein in BM compared to MGUS and normal control. This increased plasma level of ET-1 may be due to the increased secretion of ET-1 by OBs in response to myeloma cells. Additionally, the increased plasma level of ET-1 may also be due to the decreased expression of $ET_BR\Delta5$ by OBs, thereby decreasing the clearance of active ET-1. The increased ET-1 may subsequently produce effects on myeloma BMMNCs *via* over-expression of $ET_AR$. Furthermore, our *in vitro* and *in vivo* studies suggest that ET-1 directly inhibits bone formation by inhibiting the differentiation of OB-like cells.

Interestingly, ET-1 and IL-1β may produce their effect on cells *via* the same pathway. IL-1β can dose-dependently increase IL-6 synthesis (Carter *et al.*, 1990; Chaudhary *et al.*, 1992; Linkhart *et al.*, 1991) through the production of $PGE_2$ in human OB cells (Li *et al.*, 1992; Takaoka *et al.*, 1999). Likewise, ET-1 stimulates arachidonic acid release from phosphatidylinositol phosphate through $ET_AR$, putatively *via* phospholipase $A_2$ and phospholipase D activation (Kawanabe *et al.*, 2003; Kozawa *et al.*, 1997; Wu-Wong *et al.*, 1996), and promotes $PGE_2$ synthesis which increases cell proliferation (Baylink *et al.*, 1996). In addition, ET-1 can elicit a dose-dependent stimulatory effect on IL-6 expression in primary human osteoblastic cells (Hierl *et al.*, 1998). Interestingly, our data also suggests that IL-1β can significantly upregulate the expression of ET-1 mRNA.

A number of studies suggest that IL-1β (Margetts *et al.*, 2002; Voronov *et al.*, 2003), RANKL (Kim *et al.*, 2002b), and ET-1 (Morbidelli *et al.*, 1995; Salani *et al.*, 2000b) play important roles in angiogenesis. The increased angiogenesis may facilitate enhanced recruitment and entry of OC precursors into the bone surface. IL-1β- and TNF-α-stimulated endothelial cell activation would serve to increase the recruitment of OC precursors (Formigli *et al.*, 1995; McGowan *et al.*, 2001; Tokukoda *et al.*, 2001), which may be regulated by elevated RANKL expression on the endothelial cell surface (Collin-Osdoby *et al.*, 2001). Exposure to IL-1β and TNF-α could induce the expression of fibronectin and vitronectin receptors on endothelial cells, facilitating OC precursors binding and tissue migration (McGowan *et al.*, 2001). In addition, our data suggest that BM MSCs express high level of RANKL protein. Although the function of high RANKL expressing MSCs remains unknown, these cells may also serve to facilitate OC recruitment and development since marrow stromal precursors possess an intimate relationship with the marrow vascularity (Bianco *et al.*, 2001). Therefore, the increased circulating OC precursors in patients with aggressive MM develop into functionally active OC-like cells once they come in contact with the OC-inductive environment of the BM (Gregoretti *et al.*, 1995).

Considering our findings, it would appear that the strategies to neutralise the effect of cytokines, such as IL-1β, would go some way to control osteolytic bone disease. However, given the complexity of cytokine interactions and the multiplicity of cytokine targets, this may present a number of problems. An alternative approach maybe to use compounds which induce the differentiation of OB like cells such as bisphosphonate compounds (Giuliani *et al.*, 1998; Pan *et al.*, 2004b; Reinholz *et al.*, 2000), which would act to reduce the RANKL to OPG ratio within the BM microenvironment.

Although this study has provided some insight into mechanisms which underlie the MM-mediated osteolytic bone disease, further studies are required. Firstly, our data suggests that ET-1 inhibits bone formation both *in vitro* and *in vivo* by inhibiting differentiation of OB-like cells. In contrast, whilst causing a decrease in ALP expression by OB-like cells, IL-1β does not appear to impair the bone mineral formation capacity *in vitro*. This difference may be attributed to the differential mitogenic effects of both of these factors. Further investigations are required to clarify the reason for this disparate observation.

Secondly, OBs derived from MM patients have been found to over-express Fas and TRAIL death receptors (DR4 and DR5), making them highly susceptible to apoptogenic stimuli produced by myeloma cells, leading to MM-mediated programmed cell death of OB-like cells (Silvestris *et al.*, 2003). Our studies suggest that the increased number of osteoprogenitor cells in the early stage of progression of MM (Bataille *et al.*, 1991) is mediated, in part, by the increased proliferation of immature OB-like cells. Future studies directed at examining whether undifferentiated STRO-$1^+$ OB-like cells express more Fas or death receptors would be informative.

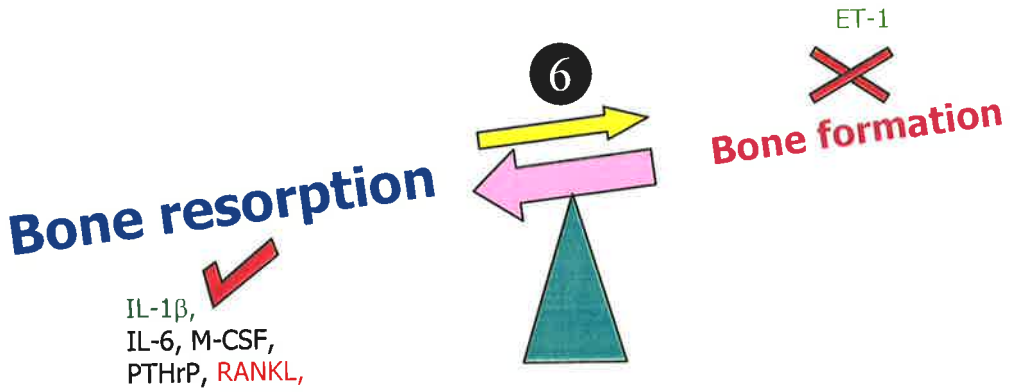
Thirdly, although we have examined the gene expression of ET-1 and its receptors in myeloma plasma cells and OB-like cells, our studies have not examined the protein expression. Future studies examining the expression of ET-1 and its receptor expression at the protein level on BM trephine sections obtained from MM patients using *in situ* immunofluorescence would provide valuable data and further evidence to support our results.

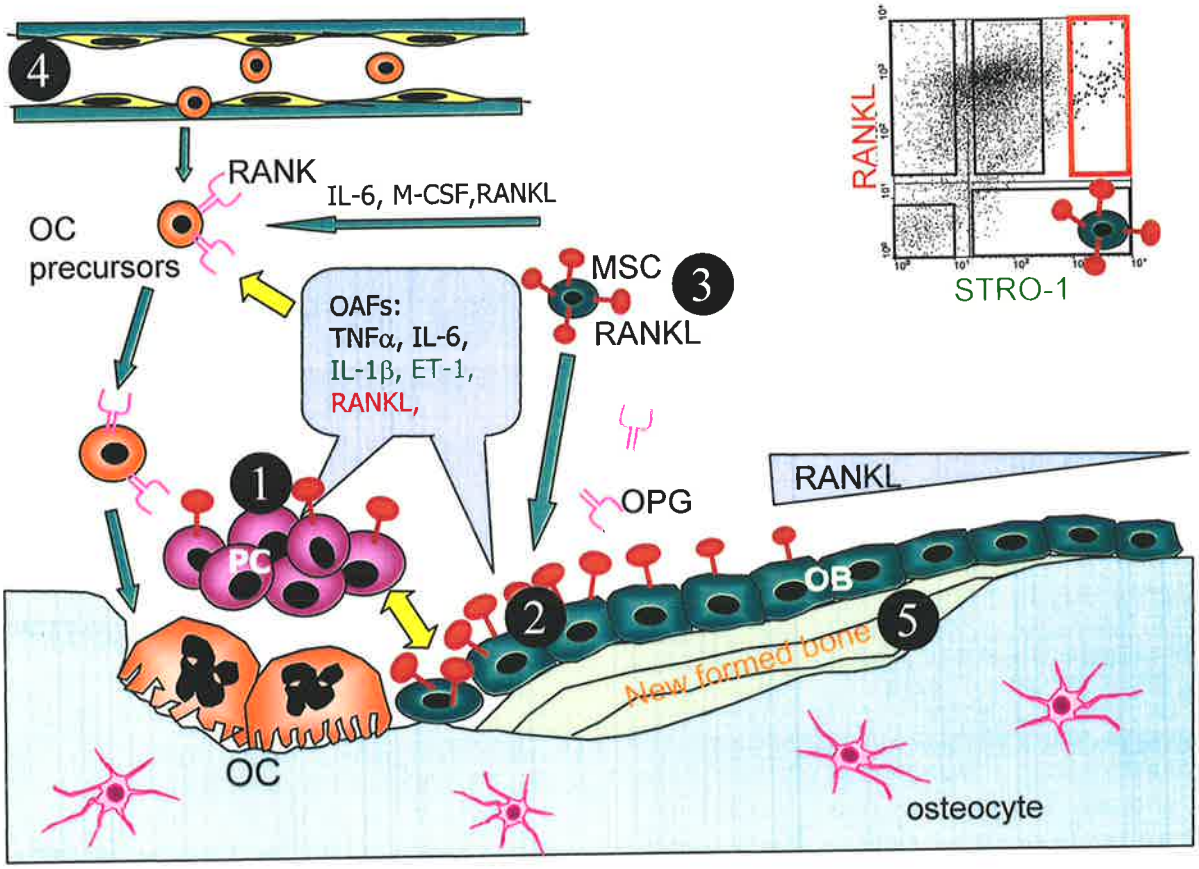
Finally, our data suggests that the direct function of ET-1 on the human OB-like cells is to inhibit bone formation. However, the osteoblastic metastasis have been reported in both prostate and breast cancer cell lines, which seems to express excess ET-1 (Guise *et al.*, 2003; Nelson *et al.*, 1999; Yin *et al.*, 2003). ET-1 can regulate bone metabolism by inhibiting osteoclastic bone resorption (Alam *et al.*, 1992; Chiao *et al.*, 2000), which may contribute to osteoblastic metastasis seen in cancer cell lines. Therefore, further studies are required to examine whether cancer cell line-derived ET-1 has indirect effects on OB-like cells by inducing other cells to produce factors which contribute to the osteoblastic bone lesions. Furthermore, it would be of value to identify the differences in expression of ET-1 in patients with POEMS syndrome and MM and what the role of IL-1β is in POEMS syndrome.

In conclusion, this study further supports the notion that bone destruction in MM is a likely result of the complex interactions between myeloma cells, OBs, OCs and endothelial cells. Myeloma cells secrete RANKL, ET-1 and OC activating factors, which results in the inhibition of OB differentiation. The immature OBs- and stromal precursors-produced high level of RANKL, which in co-operation with IL-1β and ET-1, promotes angiogenesis,

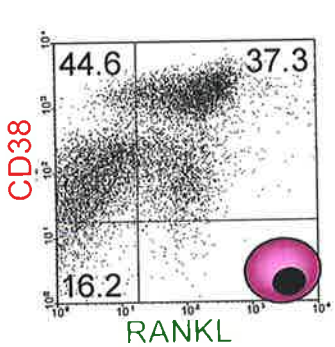
increases the recruitment of OC precursors and enhances osteoclastic bone resorption. Finally, ET-1 contributes to the loss of bone by directly inhibiting bone formation, thereby leading to a net bone loss in patients with MM. These concepts are summarised in Figure 6.1.

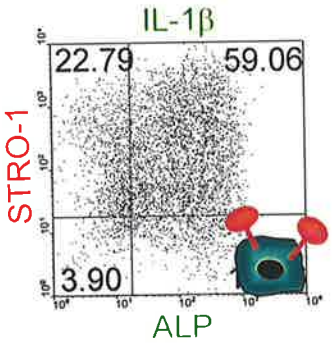
Figure 6.1. The schematic diagram highlighting the relationship between myeloma cells, OBs, OCs and endothelial cells in patients with MM. Myeloma cells secrete RANKL, ET-1 and OC activating factors, which result in the inhibition of OB differentiation (1). The immature OBs provide a favourable environment for plasma cell growth (2). The immature OBs and stromal precursors produce high levels of RANKL (3) and in association with IL-1β and ET-1, promote angiogenesis (4), increase the recruitment of OC precursors and enhance the osteoclastic bone resorption. ET-1 directly inhibits bone formation by inhibiting OB-like cell differentiation (5). The increased bone resorption and decreased bone formation result in MM-mediated osteolytic bone disease (6).

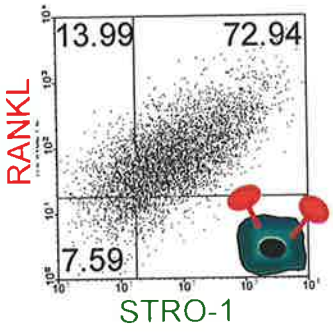

ET-1
6
Bone formation
Bone resorption
IL-1β,
IL-6, M-CSF,
PTHrP, RANKL,


4
RANK
IL-6, M-CSF,RANKL
OC
precursors
MSC
3
RANKL
OAFs:
TNFα, IL-6,
IL-1β, ET-1,
RANKL,
1
PC
OPG
RANKL
OB
2
5
New formed bone
OC
osteocyte


RANKL
STRO-1


44.6
37.3
16.2
CD38
RANKL


IL-1β
22.79
59.06
3.90
STRO-1
ALP


13.99
72.94
7.59
RANKL
STRO-1

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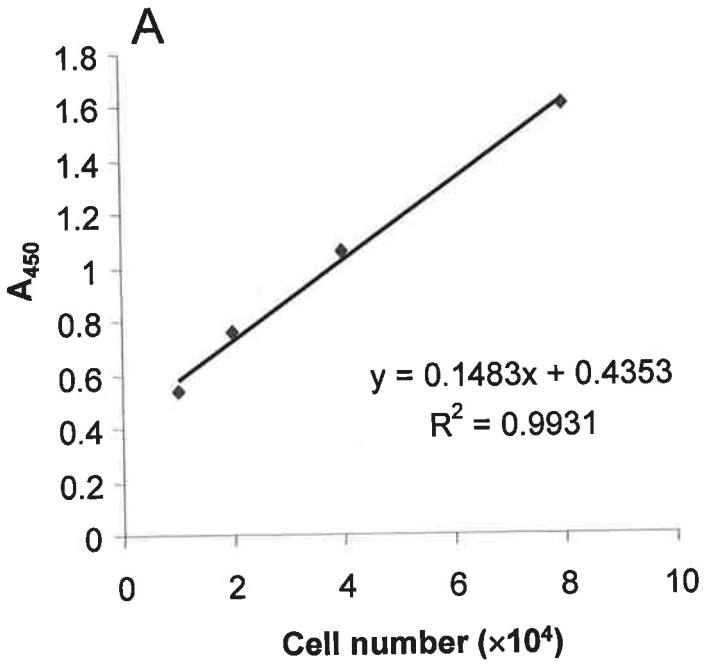
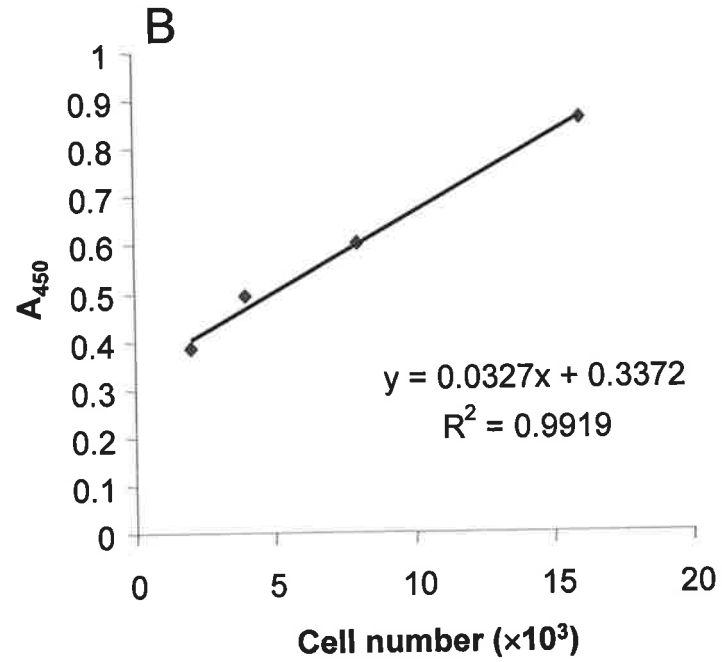
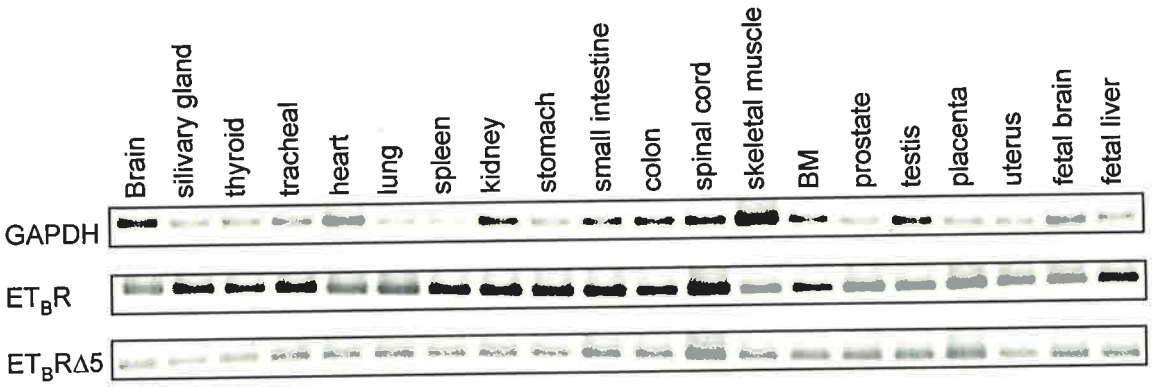
Appendix 1. The colorimetric dye, WST-1, provides a reliable method of cell number determination. The myeloma cells line, ARH 77, and human OB-like cells were seeded in 96-well plates at the indicated cell density. The cell number was quantitated using WST-1 as described in the methods (refer to section 2.2.2). The absorbance at 450nm wavelength ($A_{450}$) of WST-1 exhibited a linear correlation with the number of viable cells. Non adherent cell ARH-77 were found to be in the linear range of $A_{450}$ at cell density between $1\times10^4$ and $8\times10^4$(A). In contrast, adherent human OB-like cells were in the linear range of $A_{450}$ at cell density between $2\times10^3$ and $1.6\times10^4$(B).


A
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A450
y = 0.1483x + 0.4353
R2 = 0.9931
0
2
4
6
8
10
Cell number (×10^4)

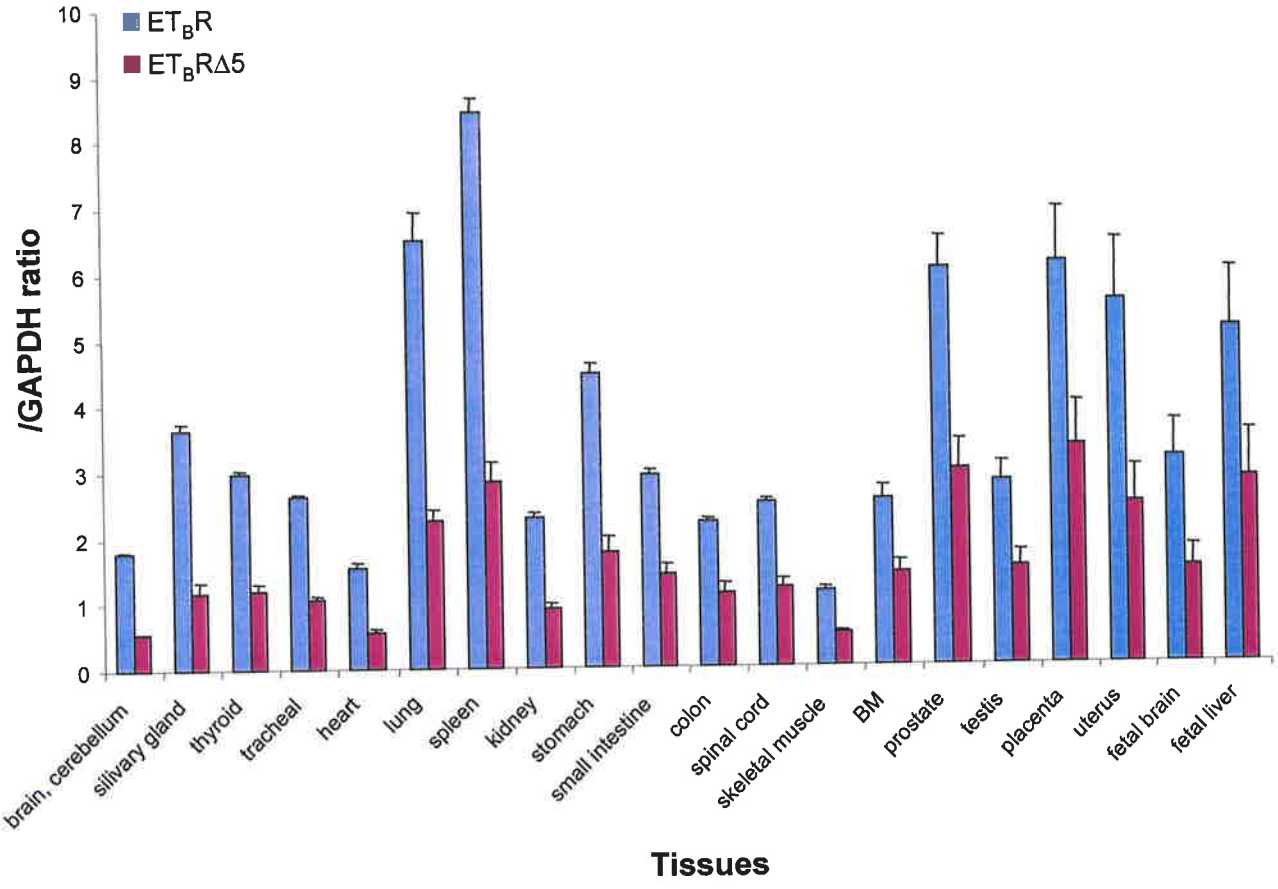

B
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A450
y = 0.0327x + 0.3372
R2 = 0.9919
0
5
10
15
20
Cell number (×10^3)

Appendix 2. Human tissues express both $ET_BR$ and its splice variant $ET_BR\Delta5$. Semi-quantitative RT-PCR was performed as described in the methods using human tissue RNA panel. The house-keeping gene, GAPDH was used as an internal control for mRNA integrity. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised using a Fluorimager at 532nm wavelength. All human tissues tested were found to express $ET_BR$ and its splice variant $ET_BR\Delta5$ (A). When the product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to the expression of GAPDH, it showed that spleen tissue expressed the highest level of $ET_BR$, with high levels in the lung, prostate, placenta, uterus and foetal liver (B). Interestingly, the level of $ET_BR\Delta5$ expression was positively associated with $ET_BR$ expression, e.g. when tissues expressed more $ET_BR$, they also expressed more $ET_BR\Delta5$.

A


Brain
silivary gland
thyroid
tracheal
heart
lung
spleen
kidney
stomach
small intestine
colon
spinal cord
skeletal muscle
BM
prostate
testis
placenta
uterus
fetal brain
fetal liver
GAPDH
ETBR
ETBRΔ5

B


ETBR
ETBRΔ5
/GAPDH ratio
0
1
2
3
4
5
6
7
8
9
10
brain, cerebellum
silivary gland
thyroid
tracheal
heart
lung
spleen
kidney
stomach
small intestine
colon
spinal cord
skeletal muscle
BM
prostate
testis
placenta
uterus
fetal brain
fetal liver
Tissues

Appendix 3. BLAST alignment analysis of human ET-1 cloned into retroviral vector pRUF(*neo*). The nucleotide sequence of cloned ET-1 was compared with sequences submitted to the GenBank database *via* standard "nucleotide-nucleotide BLAST ". Using pRUF sequence forward primer (A) and reverse primer (B) (refer to table 2.1), one base pair (797bp:G) was found to be different from the sequence (797bp:T) submitted to GenBank. This observation was consistent in two clones tested in which ET-1 cDNA was synthesised using mRNA isolated from human normal OB-like cells or myeloma cell line ARH-77.

A

>gi|21359861|ref|NM_001955.2| Homo sapiens endothelin 1 (EDN1), mRNA
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Score = 1481 bits (747), Expect = 0.0
Identities = 799/803 (99.5%), Gaps = 0/803 (0%)
Strand = Plus / Plus

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Query: 162 ctcatgattttctctctgctgtttgtggcttgccaaggagctccagaaacagcagtctta 221
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Query: 222 ggcgctgagctcagcgcggtgggtgagaacggcggggagaaacccactcccagtccaccc 281
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Query: 282 tggcggctccgccggtccaagcgctgctcctgctcgtccctgatggataaagagtgtgtc 341
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Query: 342 tacttctgccacctggacatcatttgggtcaacactcccgagcacgttgttccgtatgga 401
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Query: 402 cttggaagccctaggtccaagagagccttggagaatttacttcccacaaaggcaacagac 461
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Query: 462 cgtgagaatagatgccaatgtgctagccaaaaagacaagaagtgctggaatttttgccaa 459
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Sbjct: 516 cgtgagaatagatgccaatgtgctagccaaaaagacaagaagtgctggaatttttgccaa 575

Query: 522 gcaggaaaagaactcagggctgaagacattatggagaaagactggaataatcataagaaa 581
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```

A

```
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Query: 702 agcgtcaaatcatcttttcatgatcccaagctgaaaggcaagccctccagagagcgttat 761
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Sbjct: 935 catcctctgctggttcctgact 957
```

B

```
> gi|21359861|ref|NM_001955.2|  Homo sapiens endothelin 1 (EDN1), mRNA
          Length = 1334

 Score = 1469 bits (741), Expect = 0.0
 Identities = 798/804 (99%), Gaps = 1/804 (0%)
 Strand = Plus / Minus


Query: 87  agtcaggaaccagcagaggatgctcctgctctgatcccagccagggtggagagtgcagag 146
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Query: 147 tcggccacagggctctccgtggaggctatggcgtcagacaggccccgaaggtctgtcacc 206
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Query: 447 cagccctgagttcttttcctgcttggcaaaaattccagcacttcttgtctttttggctag 506
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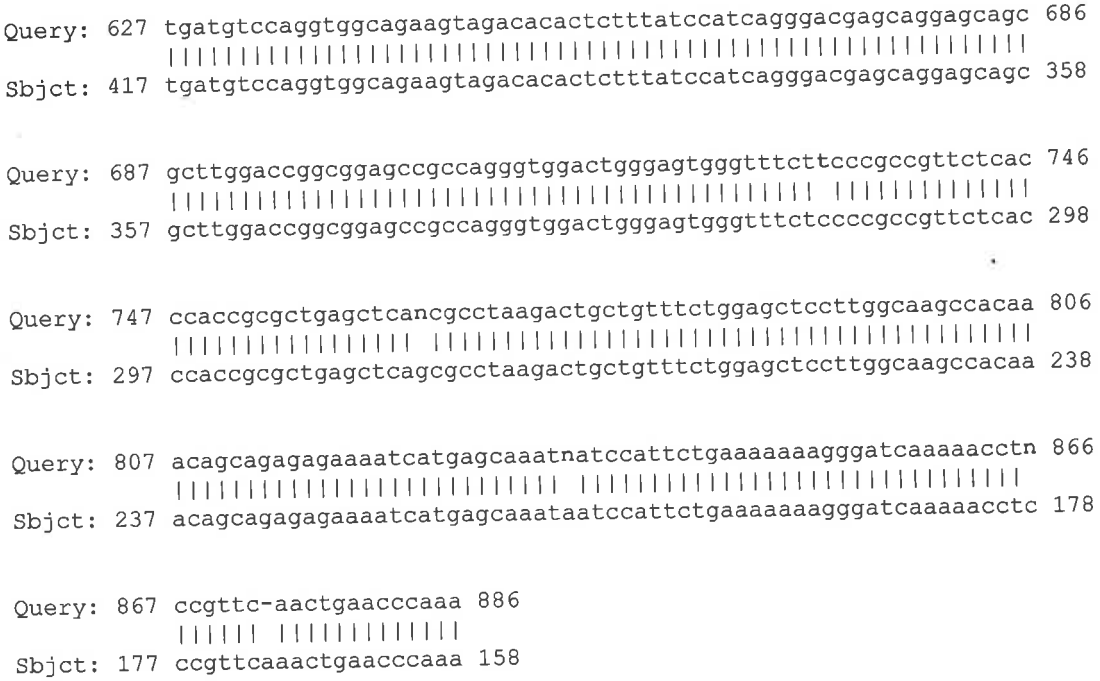
B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Query: 867 ccgttc-aactgaacccaaa 886
Sbjct: 177 ccgttcaaactgaacccaaa 158

Appendix 4. Nucleotide and deduced protein sequence of the ET-1clone. The protein sequence was deduced from the nucleotide using DNASIS software (Version 2.1, Hitachi Software Engineering Co., Ltd). Active ET-1 following cleavage is indicated in red letters. The base pair substitution at 797bp (from T to G) resulted in a substitution of Asn → Lys at amino acid position 198 as indicated in blue letters.

File : ET-1MR~2.TXT
Range : 204 - 842 Mode : Normal
Codon Table : Universal
Molecular Weight : 24423.9

```
                212        221        230        239        248        257
5' ATG GAT TAT TTG CTC ATG ATT TTC TCT CTG CTG TTT GTG GCT TGC CAA GGA GCT
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Met Asp Tyr Leu Leu Met Ile Phe Ser Leu Leu Phe Val Ala Cys Gln Gly Ala

                266        275        284        293        302        311
   CCA GAA ACA GCA GTC TTA GGC GCT GAG CTC AGC GCG GTG GGT GAG AAC GGC GGG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Pro Glu Thr Ala Val Leu Gly Ala Glu Leu Ser Ala Val Gly Glu Asn Gly Gly

                320        329        338        347        356        365
   GAG AAA CCC ACT CCC AGT CCA CCC TGG CGG CTC CGC CGG TCC AAG CGC TGC TCC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Glu Lys Pro Thr Pro Ser Pro Pro Trp Arg Leu Arg Arg Ser Lys Arg Cys Ser

                374        383        392        401        410        419
   TGC TCG TCC CTG ATG GAT AAA GAG TGT GTC TAC TTC TGC CAC CTG GAC ATC ATT
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Cys Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile

                428        437        446        455        464        473
   TGG GTC AAC ACT CCC GAG CAC GTT GTT CCG TAT GGA CTT GGA AGC CCT AGG TCC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Trp Val Asn Thr Pro Glu His Val Val Pro Tyr Gly Leu Gly Ser Pro Arg Ser

                482        491        500        509        518        527
   AAG AGA GCC TTG GAG AAT TTA CTT CCC ACA AAG GCA ACA GAC CGT GAG AAT AGA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Lys Arg Ala Leu Glu Asn Leu Leu Pro Thr Lys Ala Thr Asp Arg Glu Asn Arg

                536        545        554        563        572        581
   TGC CAA TGT GCT AGC CAA AAA GAC AAG AAG TGC TGG AAT TTT TGC CAA GCA GGA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Cys Gln Cys Ala Ser Gln Lys Asp Lys Lys Cys Trp Asn Phe Cys Gln Ala Gly

                590        599        608        617        626        635
   AAA GAA CTC AGG GCT GAA GAC ATT ATG GAG AAA GAC TGG AAT AAT CAT AAG AAA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Lys Glu Leu Arg Ala Glu Asp Ile Met Glu Lys Asp Trp Asn Asn His Lys Lys

                644        653        662        671        680        689
   GGA AAA GAC TGT TCC AAG CTT GGG AAA AAG TGT ATT TAT CAG CAG TTA GTG AGA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Gly Lys Asp Cys Ser Lys Leu Gly Lys Lys Cys Ile Tyr Gln Gln Leu Val Arg
```

```
                698                 707                 716                 725                 734                 743
GGA AGA AAA ATC AGA AGA AGT TCA GAG GAA CAC CTA AGA CAA ACC AGG TCG GAG
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
Gly  Arg  Lys  Ile  Arg  Arg  Ser  Ser  Glu  Glu  His  Leu  Arg  Gln  Thr  Arg  Ser  Glu

                752                 761                 770                 779                 788                 797
ACC ATG AGA AAC AGC GTC AAA TCA TCT TTT CAT GAT CCC AAG CTG AAA GGC AAG
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
Thr  Met  Arg  Asn  Ser  Val  Lys  Ser  Ser  Phe  His  Asp  Pro  Lys  Leu  Lys  Gly  Lys

                806                 815                 824                 833                 842
CCC TCC AGA GAG CGT TAT GTG ACC CAC AAC CGA GCA CAT TGG TGA 3'
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
Pro  Ser  Arg  Glu  Arg  Tyr  Val  Thr  His  Asn  Arg  Ala  His  Trp  ***
```