STROMAL PRECURSOR CELLS: PURIFICATION AND THE DEVELOPMENT OF BONE TISSUE

STAN GRONTHOS

BSc. M.App.Sc.

Matthew Roberts Laboratory, Hanson Centre for Cancer Research,

I.M.V.S., South Australia

Department of Orthopaedics Surgery and Trauma

Faculty of Medicine, The University of Adelaide,

South Australia

A thesis presented to The University of Adelaide in candidature

for the degree of Doctor of Philosophy

December, 1998

DEDICATION

This thesis is dedicated to my dear and good friend Evangelos Cotsaris (Cotty) who tragically passed away during his PhD. candidature.

TABLE OF CONTENTS

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institute and, to the best of my knowledge and belief, 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.

STAN GRONTHOS

ACKNOWLEDGMENTS

I am very grateful to both of my supervisors, Dr. Paul J. Simmons and Dr. Stephen E. Graves for their constructive advice and support throughout the project. Their continual encouragement was important in shaping the direction of this work. I would also like to thank them both for their helpful discussions and advice on the preparation of this thesis.

I enjoyed the stimulating discussions with Assoc. Prof. David Findlay and gratefully appreciate his critical review of my thesis.

I especially wish to thank my dear friends and colleagues who made this gruelling process enjoyable. To Dr. Andrew Zannettino for his tuition and help with the molecular and protein studies and for the occasional nicotine fix. To Dr. Shuichi Ohta for his advice and assistance with the bone cell studies. To Ms. Shelley Hay for providing me with the bone cell cultures and for her excellent technical assistance with the animal studies. To Ms. Silvana Niutta for keeping the laboratory and me running smoothly.

I greatly appreciate the technical assistance of Ms Sonia Schultz and Dr. Rebeca Mason for their help with the measurement of calcium and osteocalcin levels, Mr Kim Smith for his TEM analysis of our cell cultures, Mr. Peter McNeil and Mr. Hans Schoppe for processing and sectioning of the wax embedded material.

Last but not least I wish to express my gratitude and appreciation to my dear wife Tsambika Kakas for her unlimited support, help, patience and understanding.

This study was initially supported by grants from the Department of Orthopaedics and Trauma, The University of Adelaide and the National Health and Medical Research Council of Australia. In the final two years of my candidature I was supported by an Australian Postgraduate Award stipend and a University of Adelaide Medical Supplementary Scholarship .

ABSTRACT

Experiments were designed to identify and purify human bone marrow (BM) stromal precursor cells (SPC) by positive immunoselection, based on the cell surface expression of the VCAM-1 and STRO-1 antigens. The STRO-1^{bright}/VCAM-1^{+} BM cell fraction was found to contain all measurable SPC. One out of two cells expressing this phenotype formed a clonogenic cell cluster in vitro. Purified SPC were found to represent an early noncycling population, lacking expression of lineage specific markers of stromal cell commitment. In vitro, all SPC clones demonstrated an osteoblast-like phenotype with the ability to form a mineralized matrix. A proportion of these clones (38%) also developed clusters of lipid laden adipocytes. In vivo, all SPC clones produced a fibrous tissue where a proportion of clones (49%) were also shown to develop a calcified osteogenic tissue. These data infer that a proportion of human BM SPC are multipotential with the ability to develop into fibroblast cells, adipocytes and osteoblasts and suggests that these cell lineages share a common origin.

Normal human bone cells (NHBC) were found to express a range of β_1 integrin adhesion receptors which mediate NHBC binding to collagen and laminin and in part to fibronectin but not to vitronectin. These cells displayed a heterogeneous phenotype based on the cell surface expression of the STRO-1 and alkaline phosphatase (AP) antigens. NHBC expressing STRO-1 exclusively (STRO-1^{+}/AP^{-}), were found to represent osteoprogenitors both functionally by their reduced ability to form a mineralized bone-matrix over time and in the lack of their expression of various bone-related markers. The majority of the NHBC which expressed the STRO-1^{-}/AP^{+} and STRO-1^{-}/AP^{-} phenotypes appeared to represent fully differentiated osteoblasts, while the STRO-1^{+}/AP^{+} subset represented an intermediate pre-osteoblastic stage of development. The data presented in this study demonstrate a hierarchy of bone cell development in vitro.

PUBLICATIONS

Gronthos S, Zannettino ACW, Graves SE, Ohta S, Hay SJ, Simmons PJ (1999). Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J. Bone Miner. Res. 14(1): 48-57.

Gronthos S, Graves S., Simmons PJ (1998). Isolation, Purification And In Vitro Manipulation Of Human Bone Marrow Stromal Precursor Cells. In: Owen ME and Beresford JN (eds): Practical Animal Cell Biology. Cambridge University Press UK. p. 26-42.

Gronthos S, Stewart K, Graves SE, Hay S, Simmons PJ (1997). Integrin Expression and Function on Human Osteoblast-like Cells. Journal of Bone and Mineral Research, 12: 1189-1197.

Gronthos S and Simmons PJ (1995). The growth factor requirements of STRO-1^+ human bone marrow stromal precursors under serum-deprived conditions. Blood 85 (4): 929-940.

Gronthos S, Ohta S, Graves SE, Simmons PJ (1994). The STRO-1^+ fraction of adult human bone marrow contains the osteogenic precursors. Blood 84 (12): 4164-4173.

Gronthos S, Zannettino ACW, Graves SE, Hay SJ, Simmons PJ. Human Bone Marrow Stromal Precursor Cells: Purification And Developmental Potential. In preparation.

ABBREVIATIONS

A_{260}	Absorbence at 260 nm
α-MEM	Alpha Modification of Eagle's Medium
AP	Alkaline phosphatase
ASC-2P	L-ascorbic acid 2-phosphate
bFGF	Basic Fibroblast Growth Factor
bp	Base pair(s)
BFU-E	Burst forming unit-erythroid
BFU-Meg	Burst forming unit-megakaryocyte
BM	Bone marrow
BMMNC	Bone Marrow Mononuclear cells
BMP-2	Bone morphogenic protein-2
BSP	Bone sialoprotein
BSA	Bovine Serum Albumin
CAM	Cell adhesion molecule
CD	Cluster Designation or Cluster of Differentiation
cDNA	Complimentary DNA
CFU	Colony forming unit
CFU-F	CFU-Fibroblast
cm	Centimetre
Col-1	Collagen type I
cpm	Counts per minute
Cr^{51}	Sodium Chromate
CSF	Colony Stimulating Factor

DEX	Dexamethasone sodium phosphate
DNA	Deoxyribonucleic acid
E	Erythrocyte
ECM	Extracellular Matrix
EDX	Energy dispersive X-ray microanalysis
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor-Receptor
Eo	Eosinophil
EPO	Erythropoietin
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf Serum
FITC	Fluorescein isothiocyanate
FSC	Forward light scatter
G-CSF	Granulocyte-CSF
G418	Geneticin
GAG	Glycosaminoglycan
GM	Granulocytes and Monocytes
GM-CSF	Granulocyte/Macrophage-CSF
gp	Glycoprotein
GPI	Glycosyl-phosphatidylinositol
GS	Goat serum
HA	Hydroxyapatite
HBSS	Hank's balanced salt solution
HGF	Human growth factor

HLA	Human Leucocyte Antigen
HSC	Haemopoietic Stem Cell
HSPG	Heparan sulphate proteoglycan
h	Human
hr	Hour
INF	Interferon
i.u.	International Units
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IgG_1	Immunoglobulin gamma-1 isotype
IgG_{2a}	Immunoglobulin gamma-2a isotype
IgG_{2b}	Immunoglobulin gamma-2b isotype
IgG_3	Immunoglobulin gamma-3 isotype
IgM	Immunoglobulin M
IL-1 to 11	Interleukin-1 to 11
IMVS	Institute of Medical and Veterinary Science
IGF-1	Insulin-like growth factor-1
kDa	Kilodalton
LacZ	β-galactosidase gene
LAPSN	LTR-AP-SV40 -Neo
LFA	Leukocyte Function-Associated Antigen
LIF	Leukaemia inhibitory factor
Lin	Lineage
LNPOZ	LTR-Neo-PO-LacZ
LPL	Lipoprotein lipase

LTBMC	Long term bone marrow culture
LTR	Long terminal repeat
M	Molar
M-CSF	Macrophage-CSF
mab	Monoclonal Antibody
MACS	Magnetic activated cell sorting
Meg	Megakaryocytes
mg	Milli gram
MHC	Major Histocompatibility Complex
mIgG, M	Mouse immunoglobulin G or M
min	Minute
MIP-1 α	Macrophage inflammitory protein-1
ml	Milli litre
mm	Milli metre
mM	Milli Molar
mRNA	Messenger RNA
μl	Micro litre
μg	Micro gram
μm	Micro mitre
neo	Neomycin phosphotransferase gene
ng	Nano gram
nm	Nano mitre
NHBC	Normal human bone cells
NP-40	Nonidet P40
O/N	Overnight

OCN	Osteocalcin
OD	Optical density
ON	Osteonectin
OP	Osteopontin
PAGE	Polyacrylamide gel electrophoresis
PB	peripheral blood
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGF-R	Platelet derived growth factor-receptor
PE	Phyco-erythrin
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule-1
PI-PLC	Phosphatidylinositol-specific phospholipase C
PO	Poliovirus 5' nontranslated region
PO_{4i}	Inorganic phosphate
PPARγ2	Peroxisomal proliferator activated receptor γ2
PTH	Parathyroid hormone
PTH-R	Parathyroid hormone receptor
PTHrp	Parathyroid hormone related-protein
Rh123	Rhodamine 123
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Standard error of mean
SBA	Soy bean agglutinin
Sca-1	Stem cell antigen-1

SCF	Stem cell factor
SCID	Severe combined immunodeficient
SDM	Serum deprived medium
SDS	Sodium dodecyl sulphate
SPC	Stromal precursor cell
SSC	Side light scatter
SV40	SV40 early promoter and enhancers
TEM	Transmission Electrom Microscopy
TGFβ	Transforming Growth Factor-β
TNFα	Tumour Necrosis Factor-α
UV	Ultra violet
V-CAM-1	Vascular-Cell Adhesion Molecule-1
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
VLA	Very late acting antigen
WGA	Wheat germ agglutinin
w/v	Weight per volume

INDEX TO TABLES

(*) Page number of text preceding Table.

INDEX TO FIGURES

(*) Page number of text preceding Table.

CHAPTER ONE: GENERAL INTRODUCTION

1.0 INTRODUCTION

The organs of animals are generally comprised of two types of tissues, a supportive fibrous network of connective tissue, referred to as the stroma (Greek: mattress), and a specialised functional tissue called the parenchyma, which gives each organ its distinctive character. There appears to be a complex and interdependent relationship between stromal and parachymal cells which is necessary for normal function. In humans, the bone marrow organ forms the soft inner core of most bones at birth, and contains the sites of active haemopoiesis (red marrow). The development of haemopoietic cells in vivo occurs in intimate association with a heterogeneous population of connective tissue type cells and their associated biosynthetic products, where haemopoiesis is thought to be under the direct control of the underlying stroma [Weiss, 1976; Dexter, 1977; Lichtman, 1981; Allen et al, 1981; Bentley, 1982a; Tavassoli and Friedenstein, 1983; Simmons et al, 1987; Allen et al, 1990].

Traditionally, the bone and bone marrow stromal tissues have been studied as two distinct fields of research. There is increasing evidence that both tissues are interrelated both functionally in their regulation of haemopoiesis and through a common ontogeny [Owen, 1998]. During foetal development, the bone marrow begins to appear following the formation of new bone around cartilage rudiments. Bone marrow develops as a consequence of invading mesenchymal cells and associated blood vessels penetrating into the medullary cannals of the rudimentary bone, forming a reticular and sinusoid meshwork upon which haemopoietic stem cells can later seed and proliferate. The reticular cells of the pre-haemopoietic marrow stroma are thought to be derived from stromal precursor cells (SPC) found in the perichondrium of the developing osteogenic tissue which houses the bone marrow cavity [Bianco and Riminucci, 1998]. In adults, the bone marrow tissue is also believed to harbour SPC with the ability to develop into the different mature elements of the stroma including the bone forming cells referred to as

osteoblasts [Owen, 1985; Owen and Friedenstein, 1988]. The present thesis reviews the various stromal cell types which comprise the bone and bone marrow tissues. In addition, this thesis addresses a poorly understood aspect of stromal cell biology, namely the developmental relationships between the different bone and bone marrow stromal cell lineages. In particular, evidence for the existence of a multi-potential bone marrow stromal stem cell is discussed, and includes an examination of the cellular and molecular characteristics of this precursor cell population.

1.1 THE BONE MARROW MICROENVIRONMENT

Haemopoiesis occurs initially in the yolk sac during the initial stages of mammalian embryogenesis and later progresses to the liver and spleen during the development of the foetus. Towards the end of foetal life, haemopoiesis begins to settle in the bone marrow and evetually becomes the major site of blood cell formation in the post-natal organism. However, haemopoiesis also continues to occur in the spleens of rodents following birth [Moore and Metcalf, 1970; Migliaccio et al, 1986; Dommergues et al, 1992]. In human infants, all the bone marrow is haemopoietic, but during childhood there is progressive fatty replacement of marrow throughout the long bones until in adults, haemopoietic marrow is confined mainly to the vertebrae, ribs, sternum, skull, sacrum and pelvis, and proximal ends of the femur.

A specialised haemopoietic inductive microenvironment, with the capacity to support local haemopoiesis, must first form in each of the respective tissues during development before migrating haemopoietic stem cells (HSC) can seed and develop [Van Den Heuvel et al, 1987]. The existence of a specialised haemopoietic inductive microenvironment is supported by the observation that haemopoietic rescue, in recipients of allogeneic and autologous HSC transplants, only occurs once the infused HSC 'home' into the bone marrow via the circulation [Till and McCulloch, 1961; Robertson, 1979]. Spangrude and colleagues (1988), using fluorescence-activated cell sorting (FACS), demonstrated that a minor subpopulation of murine HSC which exhibit a phenotype of low levels of the Thy-1

antigen, lineage marker negative and stem cell antigen-1 positive (Thy-1^{low} Lin^- $Sca1^+$), have the capacity to reconstitute the lymphoid and myeloid compartments in lethally irradiated mice. Subsequent studies demonstrated that as few as thirty HSC, sorted on the basis of this Thy-1^{lo}/Lin^-/$Sca1^+$ phenotype in combination with the low retention of the fluorescent supravital dye rhodamine 123 ($Rh123^{dull}$), are able to completely repopulate the myeloid and lymphoid compartments of irradiated recipients for many months post-transplant [Szilvassy et al, 1989; Li and Johnson, 1992; Wolf et al, 1993; Uchida and Weissman, 1992].

FACS analysis of primate and human bone marrow has identified a rare population of non-cycling HSC characterised by their high expression of the CD34 antigen ($CD34^{high}$) [Andrews et al, 1986], and by their ability to completely reconstitute the haemopoietic system in lethally irradiated baboons and in humans treated with high-dose chemoradiotherapy [Berenson et al, 1988; Berenson et al, 1991]. In analogy with the murine haemopoietic system, subsequent studies have constructed a detailed composite phenotype of primitive multipotential human HSC: $CD34^+$/$CD38^-$/$Rh123^{dull}$/Thy-1^{lo}/lin^-/$HLA\text{-}DR^-$ [Haylock et al, 1995]. Differentiation of HSC into lineage restricted (committed) progenitors is accompanied by a reduced proliferative potential and the acquisition of lineage specific markers. The HSC are thought to maintain themselves by a process of self-renewal, where some of their progeny lose this ability and go on to differentiate into the committed progenitor cells of the myeloid and lymphoid cell lineages. This occurs in a well defined hierarchy of cellular differentiation which is under the influence of the surrounding stroma (Figure 1) [Siminovitch et al, 1963; Lewis and Trobaugh, 1964; Fowler et al, 1967, Prchal et al, 1978].

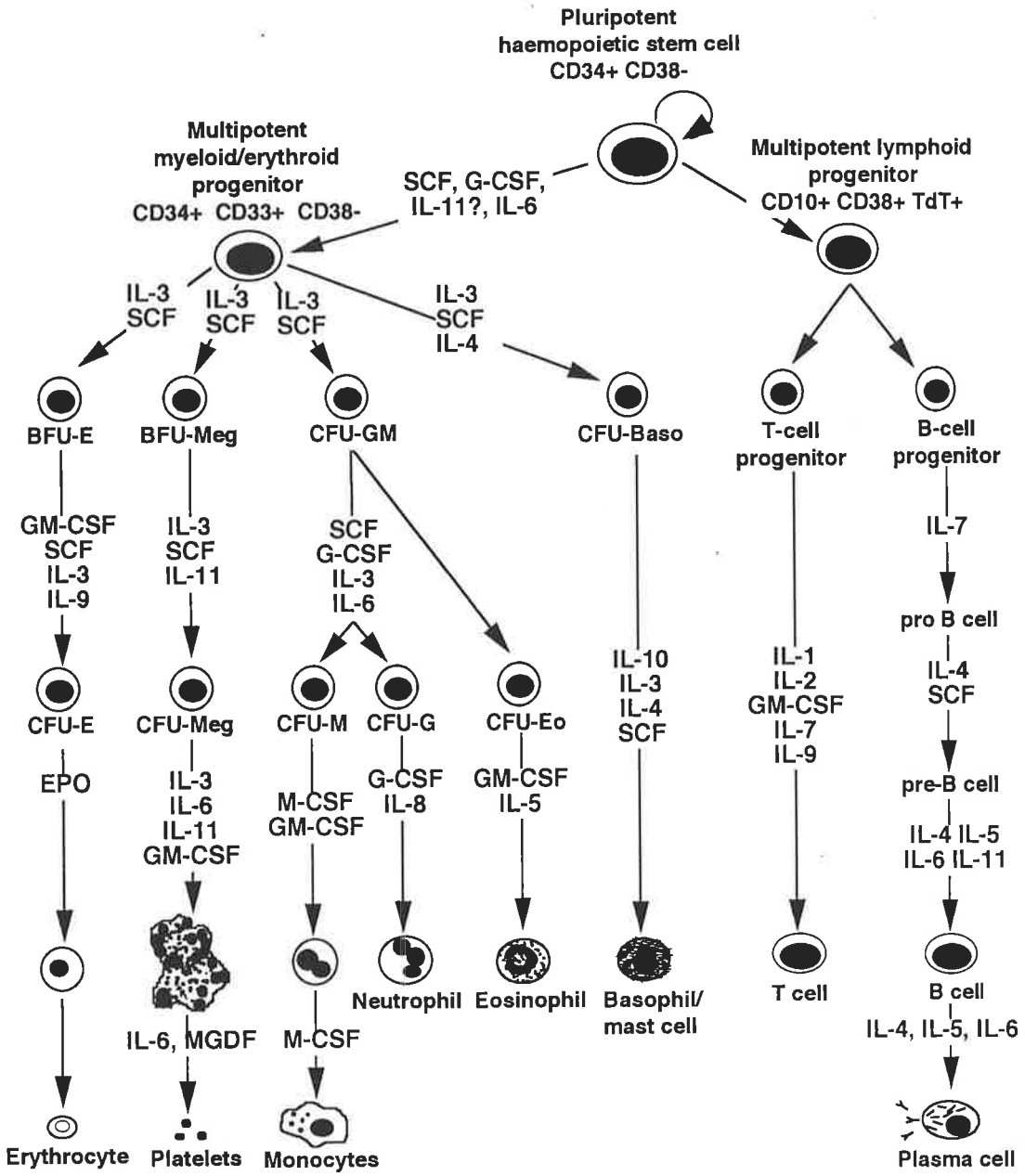
The regulation of haemopoiesis by stromal cells is thought to involve many complex interactions between haemopoietic progenitor cells, stromal cells and the products of these cells (summarised in Tables 1 and 2) including: the production of cytokines, which express a range of biological activities [reviewed in Dorshkind, 1990; Brizzi et al, 1991;

Table 1. Cytokines produced by bone marrow stromal cells that regulate the growth of haemopoietic and stromal cell growth and function[1].

Cytokine	Receptor (R)	Target Cells
IL-1	IL-1-R1	Myeloid & Lymphoid cells, Osteoclasts, Stromal & Endothelial cells
IL-3	IL-3-Rα, GM-CSF-Rβ	Myeloid & Lymphoid cells, Osteoclasts
IL-4	IL-4-R	Myeloid, Lymphoid & Stromal cells, Osteoclasts
IL-6	IL-6-Rα, gp130	Myeloid & Lymphoid cells, Osteoclasts, Stromal & Endothelial cells
IL-7	IL-7-R	Lymphocytes, Megakaryocytes
IL-8	IL-8-R	T-cells, Neutrophils
IL-11	IL-11-R, gp130	B-cells, Megakaryocytes
LIF	LIF-Rα, gp130	Osteoclasts,Stromal, Endothelial, Myeloid cells
M-CSF/CSF-1	c-fms	Monocytes/macrophage, Osteoclasts
G-CSF	G-CSF-R	Neutrophils
GM-CSF	GM-CSF-Rα & -Rβ	Myeloid cells, Osteoclasts
SCF	c-kit	Myeloid cells, Osteoclasts
TNF-α	TNF-Rα, TNF-Rβ	Ubiquitous
flt3 Ligand	flt3/ft2 TKR	Bone Marrow $CD34^+$ cells
$TGF\beta_1$	Type I, Type II Type III (Betaglycan)	Ubiquitous
BMP-2	ALK-2,-3-,6, T-ALK	Osteoblasts, Chondrocytes, Myoblasts
IFN-α	IFN-R	Haemopoietic & Stromal cells
bFGF (FGF-2)	FGFR1/flg, FGFR2/bek, K-Sam, flg-2, HSPG, CFR	Ubiquitous
IGF-1	Type I IGF-R, IGFBP	Lymphoid & Stromal cells
PDGF	$PDGF\text{-}R_\alpha$,-Rβ	Myeloid & Stromal cells
EGF	EGF-R	Stromal & Endothelial cells

(1) Adapted from [Dorshkind, 1990; Brizzi et al, 1991; Olsson et al, 1992; Miyajima et al, 1992; Massague and Pandiella, 1993; Metcalf, 1993; Bagby, 1994; Alexander, 1998; Rubinstein, et al, 1998]. For descriptions of abbreviations refer to "abbreviation" section.

Figure 1. A schematic representation of the development of haemopoietic tissue (adapted from Bagby, 1994; Morrison et al, 1995). The differentiation of haemopoietic stem cells ($CD34^+/CD38^-$) through different developmental lineages ultimately gives rise to functional mature blood cell types as shown. Clonogenic haemopoietic progenitor cells are referred to as: colony forming units (CFU); Burst forming unit (BFU). Haemopoietic cell lineages: B (B-lymphocyte); Baso (Basophil); E (Erythroid); Eo (Eosinophil); GM (Granulocyte/Macrophage); Meg (Megakaryocyte); T-cell (T-lymphocyte). Growth factors and cytokines involved in haemopoietic differentiation: Colony stimulating factors (CSF-G, -M, -GM); erythropoietin (EPO); stem cell factor (SCF); Interleukins (IL-1 to IL-11); megakaryocyte growth and differentiation factor(MGDF).


Pluripotent
haemopoietic stem cell
CD34+ CD38-
SCF, G-CSF,
IL-11?, IL-6
Multipotent
myeloid/erythroid
progenitor
CD34+ CD33+ CD38-
Multipotent lymphoid
progenitor
CD10+ CD38+ TdT+
IL-3
SCF
IL-3
SCF
IL-3
SCF
IL-3
SCF
IL-4
BFU-E
BFU-Meg
CFU-GM
CFU-Baso
T-cell
progenitor
B-cell
progenitor
GM-CSF
SCF
IL-3
IL-9
IL-3
SCF
IL-11
SCF
G-CSF
IL-3
IL-6
IL-7
pro B cell
IL-4
SCF
IL-10
IL-3
IL-4
SCF
IL-1
IL-2
GM-CSF
IL-7
IL-9
CFU-E
CFU-Meg
CFU-M
CFU-G
CFU-Eo
EPO
IL-3
IL-6
IL-11
GM-CSF
M-CSF
GM-CSF
G-CSF
IL-8
GM-CSF
IL-5
pre-B cell
IL-4 IL-5
IL-6 IL-11
Neutrophil
Eosinophil
Basophil/
mast cell
T cell
B cell
IL-6, MGDF
M-CSF
IL-4, IL-5, IL-6
Erythrocyte
Platelets
Monocytes
Plasma cell

Olsson et al, 1992; Miyajima et al, 1992; Massague and Pandiella, 1993; Metcalf, 1993; Bagby, 1994; Alexander, 1998; Rubinstein, et al, 1998]; and the expression of a broad repertoire of adhesion molecules which serve to mediate specific cell-cell and cell-matrix interactions [reviewed in Albelda and Buck, 1990; Long, 1992; Hynes, 1992; Carlos and Harlan, 1994; Simmons et al, 1994a; Clark and Brugge, 1995; Aplin et al, 1998; Humphries et al, 1998]. However, the precise role of individual stromal cell types in the support and regulation of haemopoiesis has yet to be determined.

1.2 CELLS OF THE BONE MARROW CONNECTIVE TISSUE

The supportive stroma in bone marrow comprises a network of loosely woven connective tissue laden with haemopoietic cell clusters and consisting of a extensive vascular supply made up of thinly walled sinuses derived from blood vessels that infiltrate through the cortical bone encasing the marrow (Figure 2). Haemopoiesis takes place in the extravascular spaces between the marrow sinuses. The developing blood cells must then pass through the sinus wall to enter the circulation [Weiss, 1970; Weiss, 1976; Lichtman, 1981]. In humans, the bone marrow of long bones, is also characterised by an extensive intrusion of trabecular bone. The unicellular layer that covers the surface of the bone is called the endosteum and has been proposed as one possible site for the development of osteogenic progenitor cells [Weiss, 1976].

The bone marrow microenvironment contains a variety of different cell types including; reticular cells, smooth muscle cells, adipocytes, and endothelial cells, macrophage and immature haemopoietic cells [Wiess, 1976; Lichtman, 1981; Bently, 1982a; Tavassoli and Friedenstein, 1983; Dexter et al, 1984; Allen et al, 1990]. A similarly diverse population of cells develops in vitro when BM aspirates are explanted under defined culture conditions [Dexter et al, 1977]. The stroma which forms in these long-term bone marrow cultures (LTBMC) possess the ability to support the development of haemopoietic progenitor cells over several months without the addition of exogenous growth factors. In contrast, cultured stromal cells from different adult organs such as lung, kidney, skin, liver and

Table 2. Summary of cell adhesion molecule superfamilies on stromal and/or endothelial cells [1].

INTEGRINS	LIGAND(S)
$\alpha_1\beta_1$ (CD49a/CD29)	Collagen, Laminin
$\alpha_2\beta_1$ (CD49b/CD29)	Collagen, Laminin
$\alpha_3\beta_1$ (CD49c/CD29)	Collagen, Laminin, Fibronectin, Epilligrin
$\alpha_4\beta_1$ (CD49d/CD29)	Fibronectin, VCAM-1
$\alpha_5\beta_1$ (CD49e/CD29)	Fibronectin
$\alpha_6\beta_1$ (CD49f/CD29)	Laminin
$\alpha_7\beta_1$ (CD49g/CD29)	Laminin
$\alpha_8\beta_1$ (CD49h/CD29)	?
$\alpha_v\beta_1$ (CD51/CD29)	Fibronectin
$\alpha_v\beta_3$ (CD51/CD61)	Vitronectin, Fibronectin, Fibrinogen, F VIII, Collagen, Laminin, Thrombospondin, Osteopontin, Bone sialoprotein
$\alpha_6\beta_4$ (CD49f/CD?)	Laminin
$\alpha_v\beta_5$ (CD51/CD?)	Vitronectin, Fibronectin

Immunoglobulin Superfamily	LIGAND(S)
ICAM-1 (CD54)	$\alpha_L\beta_2$ (CD11a/CD18), CD43
ICAM-2 (CDXX)	$\alpha_L\beta_2$ (CD11a/CD18)
ICAM-3 (CD50)	$\alpha_L\beta_2$ (CD11a/CD18)
VCAM-1 (CD106)	$\alpha_4\beta_1$ (CD49d/CD29)
PECAM-1 (CD31)	?
LFA-3 (CD54)	LFA-2
THY-1	?
c-kit	SCF

Continued....

Table 2. Summary of cell adhesion molecule superfamilies on stromal and/or endothelial cells continued.

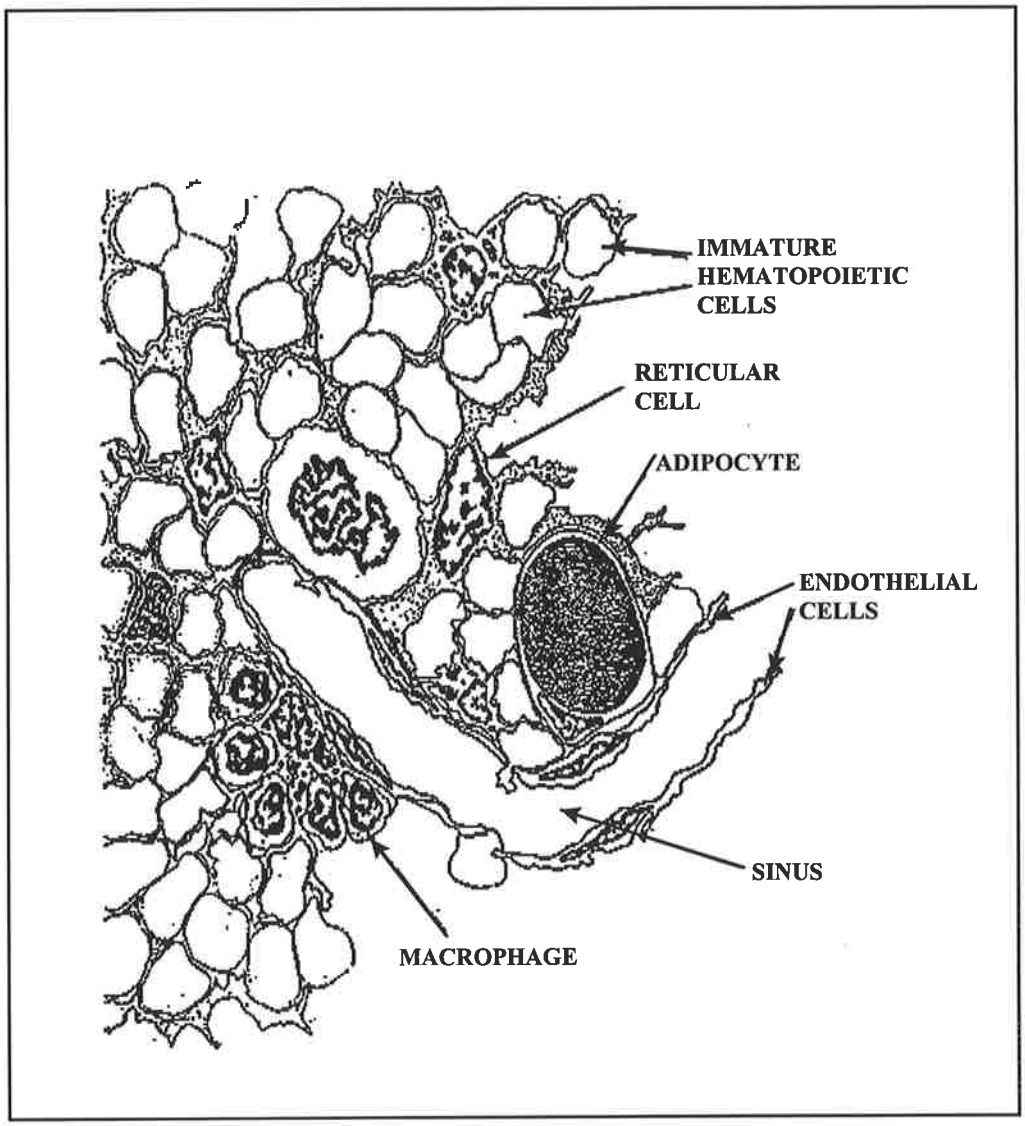
SELECTINS	LIGAND(S)
E-Selectin (CD62E)	sialyl Lewis-X , sialyl Lewis-a, PSGL-1
P-Selectin (CD62P)	sialyl Leiws-X, PSGL-1

SIALOMUCINS	LIGAND(S)
CD34	L-Selectin
GlyCAM-1	L-Selectin
MadCAM-1	L-Selectin

Miscellaneous	LIGAND(S)
CD44 (H-CAM)	Collagen, Fibronectin, MadCAM-1, hyaluronic acid proteoglycans, Syndecan,
Syndecans	CD44, Collagen, Fibronectin, FGF-2
CD36	Collagen, thrombospondin

(1) Adapted from [Albelda and Buck, 1990; Long, 1992; Hynes, 1992; Carlos and Harlan, 1994; Simmons et al, 1994a; Clark and Brugge, 1995; Aplin et al, 1998; Humphries et al, 1998]

Figure 2. A schematic representation of the bone marrow microenvironment in vivo (adapted from Allen et al, 1990). The reticular cells form the scaffolding, which supports the developing immature hematopoietic cells. Macrophages aid in the support of developing hematopoietic cells. Adipocytes represent adventitial reticular cells that have accumulated fat in their cytoplasmic vacuoles. Endothelial cells form the venous sinuses that infiltrate through the marrow connective tissue. The sinuses allow the passage of blood cells into the circulation.


IMMATURE
HEMATOPOIETIC
CELLS
RETICULAR
CELL
ADIPOCYTE
ENDOTHELIAL
CELLS
SINUS
MACROPHAGE

spleen can all form adherent stromal layers but have a limited or poor capacity to support haemopoiesis [Rios and Williams, 1990; Van Den Heuvel et al, 1991]. Depending on the culture conditions used, the same bone marrow stroma can support the growth of both myeloid and lymphoid progenitor cells [Whitlock and Witte,1982; Johnson and Dorshkind, 1986].

The adherent multicellular layers of human LTBMC consist of fibroblastic cells (60-70%), endothelial cells (10-20%), macrophage (10-20%) and adipocytes (5-10%) and extracellular matrix components such as fibronectin, laminin, collagen types I, III and IV and glycosaminoglycans [Gartner and Kaplan, 1980; Strobel et al 1986, Chen et al, 1991b]. Similar cell types have also been described in LTBMCs derived from murine marrow [Bentley et al 1981; Dexter, 1984a; Zuckerman et al, 1983]. Other cell types present in the adherent layers of LTBMC include smooth muscle cells and osteoblast-like cells [Charbord et al,1985; Kassem et al 1991]. Thus the LTBMC system provides an in vitro model system for studying the interactions between haemopoietic and stromal cells and for investigating the ontogeny of both cell populations.

1.2.1 Reticular Cells

Reticular cells have a large irregular morphology, with long cytoplasmic processes and are the predominant cell type of the bone marrow stroma. These cells secrete the structural matrix of reticular fibres which support the haemopoietic tissue [Weiss, 1965; Bessis, 1973]. The extracellular matrix secreted by the reticular cells is composed mainly of glycosaminoglycans attached to proteoglycans, fibronectin, and collagen types I, III and V [Bentley et al, 1981; Bentley et al, 1982a]. In situ, reticular cells have also been shown to express high levels of the nerve growth factor receptor, the endopeptidase, CD13, the cell adhesion molecule VCAM-1 and express alkaline phosphatase activity but were found to be negative for endothelial (Factor VIII and CD34), neural (CD56 and neurofilament) and haemopoietic markers (CD45 and CD68) [Westen and Bainton, 1979; Cattoretti et al, 1993; Jacobsen et al, 1996; Hempstead et al, 1991; Mallet et al, 1991; Orazi et al, 1992].

The expression of the osteoblast-related marker, alkaline phosphatase suggests an association with the bone forming cells. Indeed, it has been proposed that alkaline phosphatase positive reticular fibroblasts in the vicinity of bone surfaces may serve as a pool of osteogenic precursor cells [Bianco & Bonucci, 1991; Bianco and Boyde, 1993]. Those reticular cells that line the endothelium of the marrow sinuses are referred to as adventitial reticular cells. The cytoplasmic processes of these cells cover the outer wall of the sinus forming an adventitial sheath [Weiss, 1961; Weiss and Chen, 1975]. The adventitial reticular cells are also thought to help regulate the migration of blood cells into the circulation by varying their cover over the sinuses [Weiss, 1970]. The reticular cells have an intimate association with myeloid progenitor cells and in particular with granulopoiesis implicating a possible role in myeloid development [Westen and Bainton, 1979; Bianco and Riminucci, 1998].

1.2.2 Bone Marrow Adipocytes

The adipocytes found in the bone marrow microenvironment are found in proximity to the adventitial reticular cells that line the sinuses [Tavassoli, 1976]. Histological evidence suggests that marrow reticular cells may have the capacity to develop into fat cells [Weiss and Sakai, 1984; Bianco et al, 1988]. Therefore, it remains to be determined whether adipocytes represent a separate cell lineage. The bone marrow adipocytes are usually smaller in size and are characterised by the appearance of multiple fat droplets with a different fat triglyceride content and by an increase in the level of mitochondria, when compared to mature adipocytes in the nonhaemopoietic areas [Tavassoli et al, 1978]. Marrow adipocytes do not undergo lipolysis in response to acute starvation as do adipocytes in other fat sites and are sensitive to corticosteroids but are unaffected by the actions of insulin [Greenberger, 1978; Greenberger, 1989; Bathija et al 1979]. Adipocytes increase in number in areas of decreasing haemopoietic activity (yellow marrow) and may provide energy stores for cellular processes and/or serve as a mechanism to block the passage of blood cells into the sinuses [Tavassoli et al, 1974]. During skeletal maturation,

the red marrow in the central cavities of the long bones is slowly replaced by yellow marrow, consisting mainly of fat cells.

In addition to morphological criteria, adipocytes have also been characterised histochemically by the abundance of certain lipid metabolising enzymes including; lipoprotein lipase, the human adipocyte lipid binding protein and the obese gene product (leptin) [Zhang et al, 1994; MacDougald and Lane, 1995; Gimble et al, 1996; Flier, 1997]. Furthermore studies have shown that adipogenesis is dependent on the appropriate expression of the early transcriptional activating factors, the peroxisomal proliferator activated receptor $\gamma 2$ (PPAR$\gamma 2$) and the CCAAT/enhancer binding protein (C/EBPα) [reviewed in MacDougald and Lane, 1995]. This is analogous to the master regulatory gene MyoD (myocyte origin) whose expression is essential for the differentiation of myoblasts and the development of smooth muscle [Davis et al, 1987a].

1.2.3 Bone Marrow Endothelium

Endothelial cells form a complete covering of the inner surface of the sinuses. These cells are broad and flat with irregular edges which overlap with other endothelial cells forming the barrier between the circulation and the bone marrow [Weiss, 1961; Watanabe, 1966]. In blood vessels larger than capillaries, endothelial cells may form the intracellular rod shaped granules called Weibel-Palade bodies which contain the blood coagulation component Factor VIII (von Willbrand factor). Other markers have also been associated with an endothelial-like phenotype including the CD34 antigen [Watt et al, 1987], and members of the immunoglobulin superfamily involved in cell adhesion, homing and migration such as, the MUC-18 antigen (CD146) [Pickl et al, 1997; Bardin et al, 1996] and the VCAM-1 antigen [Jacobsen et al, 1996]. Vascular endothelial cells have also been characterised by their predominant production of the basement membrane constituents, laminin and collagen type IV. Functionally, vascular endothelial cells possess the ability to regulate the passage of maturing blood cells into the marrow sinuses and seem to have a significant role in the 'homing' of transplanted HSC to the marrow and lymphocytes to the

different lymphoid tissues [Weiss, 1978; Tavassoli and Hardy, 1990; Carlos and Harlan, 1991; Issekutz, 1991].

1.2.4 Smooth muscle cells and pericytes

Smooth muscle cells and pericytes, line the bone marrow capillaries which in turn are accompanied by both myelinated and non-myelinated nerve fibres [Lichtman, 1981]. Pericytes are fibroblast-like cells with long cytoplasmic processes that partly surround the endothelial cells. Smooth muscle cells and pericytes synthesise collagen type IV and laminin. The presence of myosin, actin and tropomysin in pericytes suggests that these perivascular cells may have a contractile function, similar to that of smooth muscle cells [D'Amore, 1990; Shepro and Morel, 1993; Schor et al, 1995]. Another proposed role for pericytes is that these cells may be a potential source of stromal progenitors with the capacity to develop into other stromal cell types including fibroblasts, smooth muscle cells and osteoblasts [reviewed in Schor and Canfield, 1998]. However, the proposal that pericytes are a pool of stromal stem cells remains to be proven. This is due in part to the lack of specific markers for pericytes, which makes it difficult for isolating pure populations of these cells for assessing their growth and developmental properties

1.2.5 Bone Marrow Macrophages

Bone marrow macrophages although haemopoietic in origin, seem to be present at different sites of the bone marrow microenvironment. They occur in association with haemopoietic clusters and at the sinuses where the adventitial reticular cells reside [Weiss, 1976]. The macrophages have a stellate morphology with extended cytoplasmic processes but are distinguished morphologically from reticular cells by the presence of large amounts of primary and secondary lysosomes and phagocytized materials. In addition, macrophages stain positive for acid phosphatase and non specific esterase and express Fc receptors [Westen and Bainton, 1979; Yoffey and Yaffe, 1980; Dexter et al, 1984] characteristics which also distinguish them from stromal elements. The cell surface antigen CD14 is expressed specifically by human monocytes/macrophage and provides a

means to discriminate between the macrophage and the different stromal elements in the BM [Bazil et al, 1989; Simmons et al, 1991a]. Macrophages present in the BM have an association with both developing erythrocytes and granulocytes, suggesting that macrophages may contribute to the maturation of these cells [Weiss, 1976; Westen and Bainton, 1979].

1.3 OSTEOGENIC TISSUE

Bone is an organic matrix composed mostly of collagen type I (approximately 95%) with the remainder of the matrix consisting of various other molecules such as proteoglycans, other collagen types and non-collagenous proteins [reviewed in Robey, 1996]. The main functions of bone are that: it provides the mechanical support for soft tissue; it functions as a lever for muscle action; it is the major organ for calcium homeostasis; it is the major site of haemopoiesis in adult humans. The strength of bone comes from the formation of calcium/ phosphate crystals (hydroxyapatite) deposited in the collagen matrix.

Structurally, there are two different kinds of bone which convey different functions [reviewed in Marks and Hermey, 1996]. Cortical bone is the dense outer layer covering most bones, while cancellous or trabecular bone, is the spongy porous bone within the hollow cavities of cortical bone. Cancellous bone has a loosely organised porous matrix made up of evenly layed lamellae forming a network of trabeculae, and is metabolically more active than cortical bone. The cortical bone has a more compact matrix consisting of organised tight bundles of collagen forming concentric lamellae, and provides mechanical support. The collagen bundles in adjacent lamellae are layed down at right angles to form multilayers surrounding a canal containing blood vessels, nerves and connective tissue, where the whole complex is called a Harversian system. Each Harversian system is surrounded by circumferential and interstital lamellae adding to the strength of the cortical bone. The outer bone surface is called the periosteum consisting of collagen fibres, fibroblastic cells and an inner layer of osteoprogenitor cells. Similarly, the connective tissue layer that lines the internal cavities of bone, also contains osteoprogenitor cells at

the bone surface and is called the endosteum. The growth of bone tissue results from the proliferation and differentiation of osteoprogenitor cells at different sites in osteogenic tissue.

During embryogenesis, the osteoblasts arise from two separate sources [reviewed in Caplan, 1988]. The first source of osteoblasts are derived from the mesoderm, which give rise to the majority of the bones in the skeleton [Hall, 1978]. Bone formation during embryogenesis occurs by a process termed endochondral ossification in which osteoblasts are responsible for the initial conversion of a cartilage template into bone. Cartilage templates are composed mainly of collagen type II that originally form during embryogenesis. This occurs when mesenchymal cells condense and then differentiate into the cartilage producing cells known as chondrocytes. Endochondral ossification in the post-natal organism also occurs in the epiphyseal growth plate and is responsible for the longitudinal growth of the long bones (Figure 3). The epiphyseal side of the cartilage is comprised of a zone of resting chondrocytes, followed by a zone of proliferating chondrocytes, which align themselves in columns parallel to the long axis of the bone. Below the proliferating zone lies the hypertrophic cartilage zone where chondrocytes undergo hypertrophy and are characterised by the production of collagen type X. The hypertrophic chondrocytes begin to degenerate and die, resulting in the mineralisation of the surrounding cartilage-matrix. This is preceded by the penetration of blood vessels and the invasion of osteoblasts and the bone resorbing cells referred to as osteoclasts. The combined activities of the bone cells eventually leads to osteoclastic breakdown of the calcified cartilage-matrix and the development of a new mineralized bone-matrix by osteoblasts.

The craniofacial skeleton develops from the second embryonal source of osteoblasts derived from the neural crest [Hall, 1978]. These osteoblasts produce bone by the process of intramembranous ossification which also contributes to the growth of short bones and the thickening of long bones in the post-natal organism (Figure 4). The starting

Figure 3. A schematic illustration of the growth of the long bones. The events during the growth of long bones occurs via endochondral ossification (adapted from Erlebacher et al, 1995), where proliferation and maturation of resting chondrocytes in the epiphyseal growth plate leads to the formation of a calcified-cartilage matrix by hypertrophic chondrocytes, which eventually undergo cell death. This matrix in turn provides the scaffolding for the formation of new trabecular bone in the metaphysis region as a result of the activities of osteoblasts generated from incoming osteoprogenitors and osteoclasts derived from the macrophage cell lineage. The cartilage above the growth plate in the epiphysis region is also replaced with bone and bone marrow.

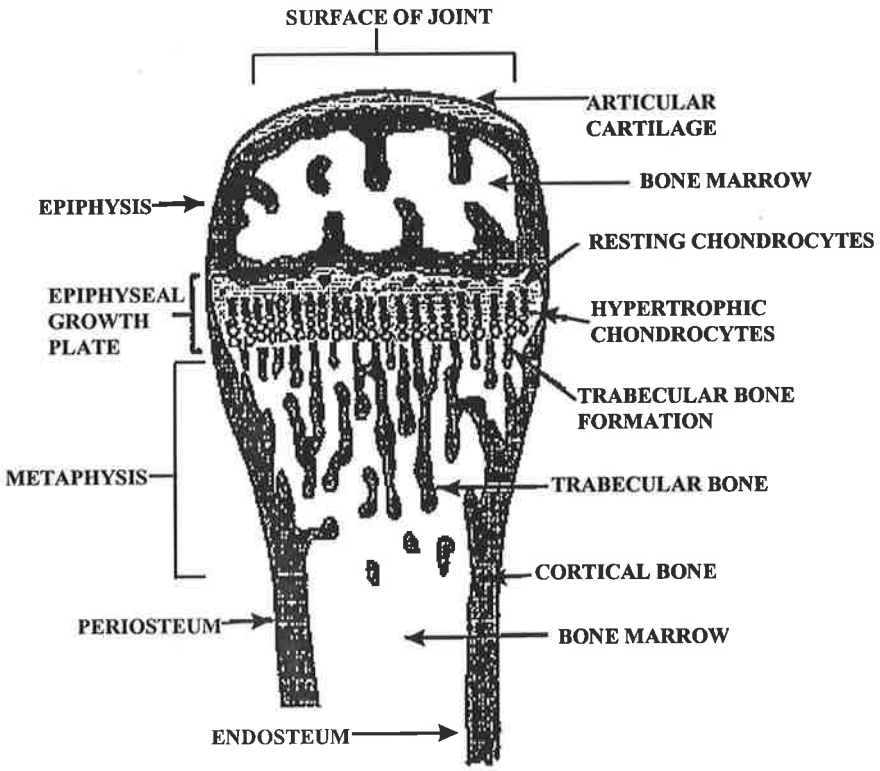
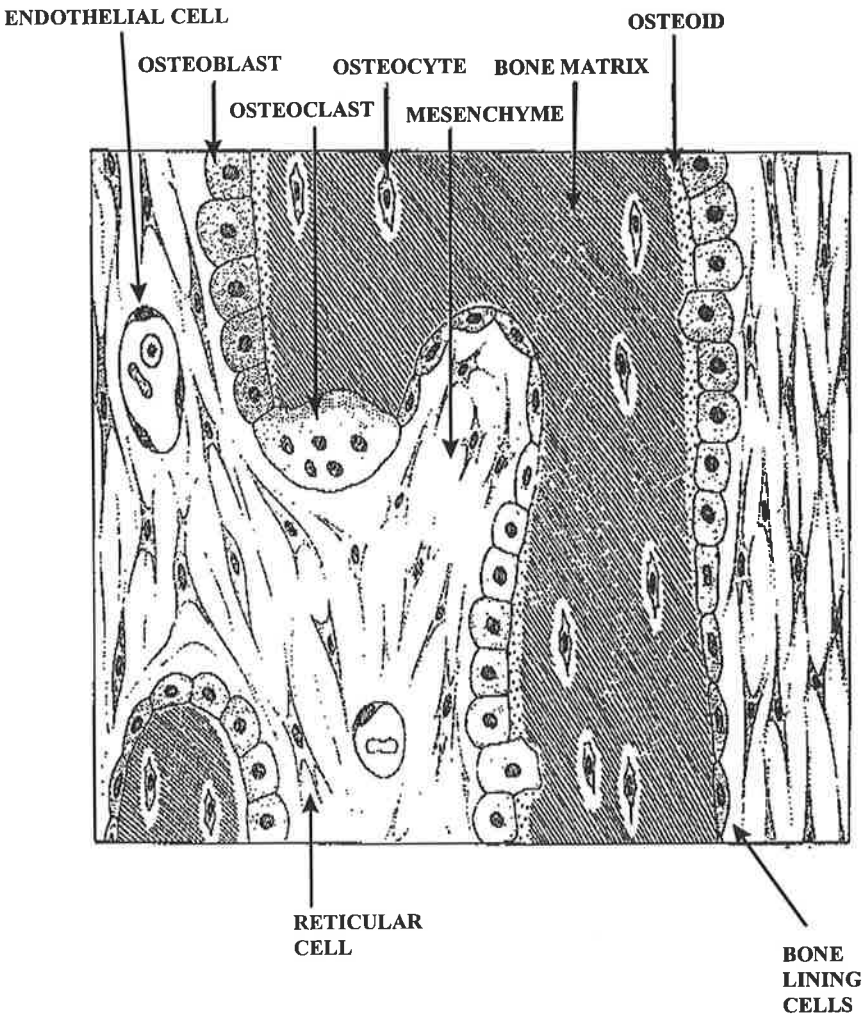

SURFACE OF JOINT
ARTICULAR CARTILAGE
BONE MARROW
EPIPHYSIS
RESTING CHONDROCYTES
EPIPHYSEAL GROWTH PLATE
HYPERTROPHIC CHONDROCYTES
TRABECULAR BONE FORMATION
METAPHYSIS
TRABECULAR BONE
CORTICAL BONE
PERIOSTEUM
BONE MARROW
ENDOSTEUM

Figure 4. A schematic illustration of the processes of intramembranous ossification (adapted from Junqueira et al, 1989). Pre-osteoblasts in the mesenchyme develop into functional osteoblasts that first form an osteoid composed mostly of collagen type I. The osteoblasts then begin to mineralise the surrounding matrix to form a calcified bone tissue. Those osteoblasts trapped inside the new bone, differentiate into osteocytes. Osteoclasts, which are derived from haemopoietic tissue, act in resorbing the older bone tissue as the new bone forms.


ENDOTHELIAL CELL
OSTEOBLAST
OSTEOCLAST
OSTEOCYTE
MESENCHYME
BONE MATRIX
OSTEOID
RETICULAR CELL
BONE LINING CELLS

point for the ossification is from the underlying condensed mesenchymal layer that gives rise to osteoprogenitors defined by their capacity to proliferate and differentiate into functional osteoblasts. Eventually a calcified matrix is formed resulting in the encapsulation of some of the osteoblasts in lacunae. Once completely surrounded by bone matrix the cells are termed osteocytes, which represent the terminally differentiated cells of the bone lineage.

1.4 CELLS OF BONE TISSUE

1.4.1 Osteoblasts

Cells of the osteoblast lineage are responsible for the production of the bone matrix constituents [Aubin and Liu, 1996]. Recent studies have demonstrated that osteoblasts can regulate haemopoiesis in vitro and enhance the engraftment of allogeneic HSC in co-transplantation studies [Taichman and Emerson, 1994; Taichman and Emerson, 1998; EIL-Badri et al, 1998]. This is perhaps not so surprising since osteoblasts in vitro are known to synthesise a similar range of cytokines and express many of the adhesion molecules detected in BM stromal cell cultures. Osteoblasts are identified morphologically by their cuboidal appearance and by their association with newly synthesised bone matrix (osteoid) at sites of active bone formation. These cells are characterised histochemically by their expression of high levels of alkaline phosphatase (AP) activity and biochemically by their ability to synthesise a wide range of bone-associated matrix proteins such as collagen type I (COL-1), osteocalcin (OCN), bone sialoprotein (BSP), osteonectin (ON) and osteopontin (OP) [Aubin and Liu, 1996; Robey et al, 1992; Robey et al, 1995; Robey, 1996]. These molecules such as AP, OCN, ON, BSP, and OP have been implicated as being important mediators of the mineralisation process (see section 1.5). Additionally, there is heterogeneity of the osteoblast phenotype according to the proliferative and functional state of the cell population [Aubin and Liu, 1996; Liu et al 1997].

Studies using in situ hybridization analysis have shown that differentiated osteoblasts express the receptor complex for the PTH and PTH-related protein [Lee et al, 1994]. Parathyroid hormone (PTH) stimulation of osteoblasts has long been known to trigger the activation of two second messenger systems [Abbou-Samra et al, 1992]. Activation of adenyl cyclase leads to the generation of cAMP, while activation of phopholipase C leads to the generation of inositol phosphates and diacylglycerol [Morris and Bilezikian, 1996]. PTH stimulates a variety of responses in osteoblasts including: an increase in the synthesis of collagen type I; a reduction in alkaline phosphatase activity; regulation of the transport of inorganic phosphate and calcium; the production of cytokines such as IL-11 and IGF-1 [Hall and Dickson, 1985; Simon et al, 1988; Schmid et al, 1994; Arao et al, 1994; Elias et al, 1995].

The recent discovery of the function of the DNA-binding protein CBFA1 (also known as AML3 and PEBP2αA) has provided insights into molecular mechanisms responsible for early osteogenic commitment. CBFA1 is a homologue of the runt family of transcription factors and has been specifically associated with osteogenic tissue in rodents [Ducy et al, 1997; Komori et al, 1997; Rodon and Harada, 1997]. The precise function of CBFA1 is unknown but it is thought to regulate the osteoblast specific gene osteocalcin by complexing with the osteocalcin promoter [Rodon and Harada, 1997]. Ultimately the differentiation of osteoblasts appears to be dependent on the appropriate expression of the CBFA1 transcription factor, as demonstrated in vivo by the development of a CBFA1 knockout mouse [Otto et al, 1997]. Future studies are still required to precisely define the role of CBFA1 in bone cell development

1.4.2 Bone Lining Cells

One of the least characterised bone cell types are the bone lining cells, which cover the majority of the bone surfaces. The bone lining cells have a flat, elongated morphology and either lack the expression or exhibit low levels of osteoblast-related markers such as alkaline phosphatase, bone sialoprotein and PTH/PTHrp-receptor [reviewed in Aubin and

liu, 1996]. Bone lining cells also appear to be functionally inactive in respect to bone formation or resorption. It has been proposed that bone lining cells may be stimulated to develop into active osteoblasts with the capacity to form new bone [Dobnig and Turner, 1995].

1.4.3 Osteocytes

Osteocytes are thought to be terminally differentiated osteoblasts that occupy a space or lacunae in an ordered pattern within the calcified bone-matrix. They represent the predominant cell type in bone tissue. The osteocytes have a stellate shape with long cytoplasmic processes, that extend through small canals (canaliculi) in the matrix to blood vessels, adjacent osteocytes and bone lining cells [Nijweide et al, 1996]. In situ hybridization and immunohistochemical studies have shown that otseocytes express a range of bone-matrix proteins including collagen type I, osteopontin and osteonectin [Aubin and liu, 1996]. Other bone cell-related markers such as alkaline phosphatase, bone sialoprotein, osteocalcin and PTH-PTHrp receptor have been shown to be either weakly expressed or absent in osteocytes [Van der Plas et al, 1994]. Functionally, osteocytes appear to have a limited capacity to synthesise new bone-matrix.

One of the main functions of osteocytes is that they collectively form a mechanosensory system which is sensitive to mechanical stress [Oster, 1989; Van der Plas and Nijweide, 1992]. Deformation or strain of bone tissue results from mechanical stress and leads to the flow of interstitial fluid through the osteocyte canalicular network from areas of high strain to areas of low strain. In vitro, fluid flow has been shown to greatly affect osteocyte metabolism even more that other cell types such as osteoblasts, fibroblasts and endothelial cells [Reich et al, 1990]. Osteocytes are affected through direct fluid shear stress and through the movement of ions over their cell surfaces, which modulates the production of signalling factors, such as nitric oxide and prostaglandins. These factors in turn act to stimulate other factors such as IGF-1 that regulate bone formation and bone resorption by osteoblasts and osteoclasts, respectively [Nijweide et al, 1996].

1.4.4 Osteoclasts

Another bone cell population, osteoclasts are large motile cells often displaying multi-nucleation with irregular cytoplasmic branches. Osteoclasts are derived from haemopoietic progenitor cells of the monocyte/macrophage lineage [Suda et al, 1996; Vaananen, 1996; Suda et al, 1997]. These cells are responsible for the resorption of bone during development, growth and repair. 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, (the integrin $\alpha_v\beta_3$) [Suda et al, 1997]. Bone resorption occurs when differentiated osteoclasts attach to calcified tissues where they develop a ruffled border and clear zone at the site of contact with the bone-matrix [Suda et al, 1996; Vaananen, 1996]. This process is then followed by the release of acids and lysosomal enzymes into the spaces beneath the ruffled border, resulting in the breakdown of the calcified matrix.

1.5 COMPONENTS OF THE BONE AND BONE MARROW EXTRACELLULAR CELL MATRIX

1.5.1 Collagen Fibrils

Bone is composed mainly of collagen type I with small traces of collagen types III and V, while collagen type III is the main collagen constituent of the BM extracellular matrix [Zuckerman and Wicha, 1983] and to a lesser extent collagen types I and V. These molecules are part of the fibrillar collagen family which includes the cartilage specific molecules, collagen types II and XI. The principal amino acids composing these collagens are glycine, proline and hydroxyproline. The collagens are characterised by their continuous triple helix structure. For example, collagen type I is composed of two $\alpha 1$ peptide chains and one $\alpha 2$ peptide chain which form a tropocollagen molecule [Rossert and de Crombrugghe, 1996; Eyre, 1996]. Aggregates of tropocollagen molecules result in the formation of collagen fibrils with unique transverse striations determined by the

overlapping arrangement of the tropocollagen molecules for each collagen type. The fibrils have a very high tensile strength and provide the structural frame-work for the bone and marrow connective tissues. Aggregation of collagen type I fibrils leads to the formation of collagen fibres and bundles [Rossert and de Crombrugghe, 1996; Eyre, 1996].

Collagen also plays a role in cell adhesion in the BM, as demonstrated by the ability of haemopoietic progenitors to bind to collagen type I in vitro [Koenigsmann et al, 1992]. The inhibition of collagen synthesis in LTBMCs by the addition of cis-4-hydroxyproline interferes with the development of the stromal layer and blocks the development of haemopoiesis [Zuckerman et al, 1985]. Similarly, in BM stromal cultures, stimulated with retinoic acid, cis-4-hydroxyproline inhibition of collagen type I production caused a decrease in the number of cells expressing the osteoblastic marker, alkaline phosphatase [Shi et al, 1996]. In addition, osteoclasts and osteoblasts are also known to bind to collagen in vitro [Grzesik et al, 1994; Helfrich et al, 1996]] which may contribute directly to the regulation of bone remodelling in vivo. Functional studies have shown that when pre-osteoblast cells were cultured onto collagen type I gels expression of the bone-related protein osteopontin was strongly upregulated [Traianedes et al, 1996]. Collagen has also long been known to be involved in the mineralization process. In vivo, hydroxyapatite crystals, the mineral identified in bone, dentine and calcified cartilage, are found aligned in an ordered pattern parallel to the collagen fibrils [Boskey, 1992]. The importance of collagen in bone development is demonstrated by the congenital defects in collagen type I synthesis that manifests as the various forms of osteogenesis imperfecta. This condition may be associated with bone deformity, osteoporosis and spontaneous fractures. The defect in synthesis is attributed to a genetic resulting from single point mutations in either one of the genes that encode for the $\alpha 1$ or $\alpha 2$ peptides of collagen type I [Shapiro et al, 1996].

1.5.2 Glycosaminoglycans and Proteoglycans

The amorphous intercellular ground substance which fills the spaces between cells and the fibres of the connective tissue is composed of mainly two components, glycosaminoglycans (GAGS) and structural glycoproteins. The GAGS (Hyaluronic acid, chondroitin sulfate, keratin sulfate, dermatan sulfate and heparin sulfate) are repeating units of polysaccharides usually made up of a uronic acid, either glucuronic or iduronic acid and a hexosamine, either glucosamine or galactosamine. These long chain polysaccharides are heavily sulfated and with the exception of hyaluronic acid, form proteoglycans by binding covalently to a protein core [Macrum et al, 1987; Ruoslahti, 1989; Ruoslahti, 1989; Gallagher, 1989; Jackson et al, 1991]. Hyaluronic acid is found in abundance in cartilage, and is known to be one of the ligands for the cell adhesion molecule (proteoglycan) CD44 [Aruffo et al, 1990]. The bone and bone marrow connective tissue contains mainly chondroitin sulfate (>70%) and to a lesser extent various levels of hyaluronic acid, heparin sulfate, dermatan sulfate and keratan sulphate (>30% in total) [Hunter et al, 1983; Wight et al, 1986; Waddington et al, 1989; Spooncer et al, 1991]. Due to their high viscosity, GAGS have a lubricating function in connective tissue but their main function appears to be keeping structural integrity by interacting with collagen fibrils [Jackson et al, 1991].

In addition, GAGS are able to bind exogenous growth factors synthesised by bone and marrow cells. It has been shown that heparan sulphate is able to bind a range of different cytokines such as GM-CSF, IL-3 and bFGF [Gordon et al, 1987; Roberts, 1988]. Heparan sulphate is bound covalently to extracellular matrix and stromal membrane bound proteoglycans and is also involved in the adherence of HSC to these proteins [Siczkowski et al, 1992]. The binding of GM-CSF and IL-3 to heparan sulfate and the presentation of them in a biologically active form to the haemopoietic progenitor cells may be one mechanism for the enhanced local regulation of haemopoiesis in the BM [Hattersley et al, 1991; Kerby et al, 1992]. Similarly the growth factor bFGF [Ruoslahti and Yamaguchi, 1991] becomes activated after complexing with its respective cell surface receptor and

with heparan sulfate, either on the surface membrane of target cells, as free heparin or in the form of a proteoglycan such as syndecan-1 [Kim et al, 1994]. Basic FGF is involved in the regulation of both haemopoietic and stromal cell differentiation, growth and function as well as having a role in blood vessel formation, cell migration, bone remodelling and tissue repair. Another cytokine, TGFβ also important in the regulation of haemopoiesis and bone remodelling, is known to bind to two proteoglycans, betaglycan and decorin through the protein core rather than the GAGs themselves [Lopez-Casillas et al, 1991; Santra et al, 1997]. Betaglycan helps TGFβ attach to its receptors, while decorin, which associates with collagen type I fibrils, acts to neutralise TGFβ activity. Recombinant decorin protein core has also been shown to inhibit cell adhesion and proliferation in vitro [Yamaguchi et al, 1990; Santra et al, 1997]. Proteoglycans may therefore provide long-term reservoirs of growth factors in the ECM by protecting the cytokines against degradation by proteolytic enzymes, in addition to acting as mediator components in regulating cell growth, function and cytokine activity in haemopoiesis and bone development.

1.5.3 Glycoproteins

Glycoproteins are molecules containing a protein moiety that undergo postranslational modification resulting in the addition of N- and O-linked oligosaccharides. The carbohydrates themselves can also be further modified by the addition of phosphate and/or sulfate. Several glycoproteins have been identified in the bone and marrow connective tissue which seem to be important in the interaction of neighbouring cells and in the adhesion of cells to their substrate.

The large glycoprotein laminin is composed of three subunits, one α-chain (400 kDa) and two β-chains (200 kDa) which form a cross-like structure with one long and three short arms [Timpl, 1989; Beck et al, 1990; Yurchenco and O'Rear, 1990]. Laminin is a major constituent of the basal laminae and is found in association with collagen type IV and heparan sulphate proteoglycans to provide adhesive and structural support between the

blood vessels and the bone marrow stroma. In vitro, laminin promotes cell adhesion, migration, differentiation and growth of a variety of different cell types. These interactions are governed by various integrin type cell surface receptors which utilise laminin as a ligand [Ruoslahti, 1991] (see Chapter 7).

Fibronectin is a large glycoprotein (440 kDa) found in serum and bone and BM connective tissue. This molecule is made up of two nonidentical peptide units and functions in cell adhesion, motility, proliferation, differentiation and migration [Hynes, 1985; Hynes, 1990]. In addition, fibronectin binds to fibrin, heparin, gelatin and collagen on different binding sites of the molecule [Pierschbacher and Ruoslahti, 1984; D'Souza et al, 1991]. Studies have shown that fibronectin, produced by bone marrow fibroblastic cells, provides anchorage for erythroid progenitors, whereas granulocyte-macrophage progenitors bind only weakly to fibronectin [Tsai et al, 1987]. Cell adhesion can be partially blocked by anti-fibronectin antibodies suggesting that other molecules are involved in the attachment of haemopoietic progenitor cells to stroma [Coulombel et al 1988]. Studies using anti-fibronectin antibodies specific for various regions of the fibronectin molecule, showed that different cell populations bound preferentially to different fibronectin fragments [Puleo and Bizios, 1992; Underwood et al, 1992; Dalton et al,1995]. Bone cells, endothelial cells and fibroblasts were all shown to attach to both the heparin binding site and cell binding site on fibronectin but not to the gelatin binding site. In addition, corneal epithelial cells only bound to the cell attachment site while melanoma cells bound preferentially to the heparin binding site. Alternate binding sites on molecules like fibronectin may induce a variety of cellular responses such as cell differentiation or migration by the activation of different signalling pathways.

Vitronectin is another serum glycoprotein, also found in BM and bone in low amounts, and is known to bind to plasminogen activator inhibitor enhancing its activity [Preissner, 1991]. Although, the exact role of vitronectin in haemopoiesis and bone formation is largely unknown, this molecule has been shown to mediate the adhesion of a variety of different

cell types such as osteoclasts through the classical vitronectin cell surface receptor, the integrin $\alpha_V\beta_3$ [Helfrich et al, 1992]. However, evidence suggests that osteoblasts use an alternative receptor to bind with vitronectin [Aarden et al,1996].

Alkaline phosphatase is a membrane bound glycoprotein anchored through a glycosyl-phophatidylinositol moiety covalently attached at the carboxylterminus of the polypeptide. In humans, four AP isoenzymes are encoded by four distinct genes [Millan, 1988; Harris, 1990; Moss, 1992]. Three of these AP isoenzymes demonstrate a tissue-specific pattern of expression and are therefore referred to as intestinal, placental and germ cell (placental-like) AP. The fourth AP isoenzyme has a wide tissue distribution, and is most abundant in bone, liver and kidney (Bone/liver/kidney AP or tissue-nonspecific AP). The different isoforms of bone/liver/kidney AP can be distinguished by differences in their physiochemical properties such as heat stability and electrophoretic mobility. These isoforms have the same polypeptide sequence, and differ only by postranslational modification involving different carbohydrate motifs [Harris, 1990]. The exact function of these molecules in bone and bone marrow tissues remains to be determined. AP may have a specific role in skeletal mineralization by stimulating calcium phosphate precipitation and by helping to orientate mineral deposition in osteoid [Moss, 1992, Whyte, 1994].

Osteonectin, also known as secreted protein rich in cysteine (SPARC), culture shock protein and basement membrane-40 protein (BM-40) [Mason et al, 1986a; 1986b], is a glycoprotein of 43 kDa that contains EF-Hand calcium binding domains [Swaroop et al, 1988]. Findlay et al (1988) demonstrated that osteonectin is highly conserved between species. The molecule was first identified as a major (5-10%) noncollagenous protein in bone [Termine et al, 1981]. Because osteonectin was initially purified from foetal bovine bone extract, based on its binding to hydroxyapatite and collagen, it was thought to play a major role in mineralization [Termine et al, 1981]. Osteonectin is also found on many non osteogenic cell types including platelets and endothelium [Villarreal et al, 1991]. Platelet

osteonectin has a larger molecular weight (3 kDa) than that found in bone and is known to interact with thrombospondin forming a complex that is thought to mediate platelet-platelet and platelet-endothelium adhesion, and may be involved in blood coagulation [Clezardin et al, 1988]. In vitro studies have shown that osteonectin is secreted by endothelial cells in response to certain types of injury, inducing changes in the adhesion of endothelial cells [Goldblum et al, 1994]. This study demonstrated that osteonectin regulates endothelial barrier function through F-actin-dependent changes in cell shape, causing the appearance of intercellular gaps, providing a pathway for the extravasation of macromolecules. The physiological function of osteonectin is not known but may involve mediation of cell adhesion and proliferation to facilitate repair of tissue damage and regulation of the extracellular matrix during bone remodelling.

Osteopontin (OP), also known as SPP1, 2ar, pp69 and bone sialoprotein-1 (BSP-1), is a 44 to 75 kDa glycophosphoprotein with high affinity for calcium ions and contains aspartic rich domains and an Arg-Gly-Asp (RGD) tripetide sequence also found in other ECM molecules such as bone sialoprotein (BSP-II), collagen, fibronectin and vitronectin [Butler, 1989]. Bone cells are believed to be the major site of synthesis and secretion of OPN [Mark et al, 1987]. Osteopontin is also found in many other sites outside of osteogenic tissue including the kidney, sensory epithelium of the ear, unidentified cells in the brain, the metrial gland of the placental decidua and in various body fluids such as milk and urine. These studies have identified OP synthesis in cells associated with osteogenic tissue including preosteoblasts, osteoblasts, bone lining cells, osteocytes, some fibroblastic cells of the marrow and hypertrophic chondrocytes [Yoon et al, 1987; Mark et al, 1987; Mark et al, 1988a; Nomura; 1988, Weinreb et al, 1990]. Osteoblast and osteoclast function may be mediated through their binding to OP, present predominantly in the osteoid, via integrin-RGD mediated adhesion [Grzesik and Robey, 1994; Ross et al, 1993]. Osteopontin is thought to negatively regulate the mineralization process by acting as an inhibitor of hydroxyapatite crystal formation and growth [Boskey et al, 1993; Hunter et al, 1994]. The synthesise of OP has also been shown to be enhanced in a wide variety

of transformed mammalian cells and may be related to the metastatic process [Senger, 1989; Craig et al, 1990].

Bone sialoprotein (BSP-II) is a 46 to 75 kDa glycoprotein that constitutes about 10-15% of the noncollagenous proteins found in the mineralized compartment of bone and is characterised by multiple polyglutamic acid sequences and a RGD sequence [Fisher et al, 1983; 1990]. Unlike OP, the tissue distribution of BSP seems to be restricted to mineralized tissues such as bone, dentin, cementum and calcifying cartilage of the growth plate [Fisher et al, 1983; Bianco et al, 1991; MacNeil et al, 1994]. However, trophoblasts of the developing placenta also express high levels of BSP where hydroxyapatite crystals have been associated with aging trophoblasts in late term human placentas [Bellahcene et al, 1994]. Breast cancer tumours also express BSP are known to metastasise to bone [Bellahcene et al, 1994]. Given the strong affinity of BSP for hydroxyapatite crystals and collagen the function of BSP is largely unknown but it is thought to be involved in the earliest events of the mineralization process. BSP is not expressed by preosteoblasts but is detectable in developing osteoblasts and is strongly expressed in fully differentiated osteoblasts and osteocytes [Bianco et al, 1991; Chen et al, 1991a]. Functionally, BSP can support cell attachment in a RGD-dependent and RGD-independent manner and may act to mediate cell shape and migration [Oldberg et al, 1988; Mintz et al, 1994]. One study has shown that BSP induces a dose-dependent increase in osteoclast resorption in pit assays in vitro [Raynal et al, 1996]. It has also been used as a marker of: bone turnover due to bone resorption [Seibel et al, 1996]; increased joint destruction measured in samples of synovial fluid [Saxne et al, 1995]; an indicator of the relative severity of multiple myeloma at presentation, pretreatment [Seibel et al, 1996]; a marker of breast cancer in relation to its ability to metastasise to bone [Bellahcene et al, 1994].

Osteocalcin (OCN) also known as bone gla protein (BGP) is a small protein (approximately 5.5 kDa) that constitutes 20% of the noncollagenous protein in bone and contains three highly conserved vitamin K-dependent γ-carboxylated glutamic acid (gla)

residues. Vitamin K is essential for its biosynthesis which is stimulated by 1,25-$(OH)_2$ D_3 [Hauschka et al, 1975; Price et al, 1976; Price and Baukol, 1980]. As described in section 1.4.1 the transcription factor CBFA1 binds to the OCN gene promoter and regulates its expression. Other ECM related gla proteins found in bone include matrix gla protein and protein S and are predominantly synthesised in cartilage and in the liver, respectively. Studies have shown that OCN is expressed by differentiated osteoblasts and osteocytes and is generally considered to be bone specific [reviewed in Ducy and Karsenty, 1996a]. The measurement of OCN levels in plasma is used as a biochemical indicator of bone turnover. However, there are several reports of OCN message in other cell types such as megakaryocytes [Thiede et al, 1994], and in adipocytic cell lines [Benayahu et al, 1997]. Studies have also demonstrated the presence of OCN protein in some hypertrophic chondrocytes and preosteoblasts [Mark et al, 1988b; Gerstenfeld and Shapiro, 1996]. OCN has a high affinity for hydroxyapatite where studies have shown that the growth of mineral crystal can be inhibited in the presence of osteocalcin [Romberg et al, 1987]. The exact role of OCN is not known, however knock out studies in the mouse have resulted in a phenotype which developed an increase in cortical bone thickness and density [Ducy et al, 1996b]. These studies concluded that OCN was a negative regulator of bone formation, however surprisingly osteoclastic function appeared to be unaffected in the knockout mouse. The ability of OCN to mediate osteoclast differentiation and/or function remains to be assessed.

The cellular and the extracellular matrix components of bone and marrow demonstrates the complex nature of these tissues which gives them the unique ability to support and regulate haemopoiesis and to maintain the structural integrity of the skeletal system. Given the extent to which these tissues have been studied in terms of their ultrastructure and function, it is still not clear of the precise origin of the different stromal cell lineages found in bone and marrow and the exact relationship between the various stromal cell types.

1.6 ORIGIN OF BONE AND BM STROMAL CELLS

1.6.1 Stromal Precursor Cells in Bone Marrow

Due to the diversity of the BM stromal cell population, attempts to characterise the biological properties of each cellular component have been complicated in part by the lack of lineage specific markers which might facilitate precise identification and isolation of each cell type. One of the major questions concerning stromal cell differentiation relates to the origin and developmental relationship between each component of the marrow stroma. Studies in rodents and in humans have demonstrated that BM stromal tissue is capable of extensive regeneration following a variety of insults ranging from radiation and cytotoxic drugs to mechanical disruption [Knopse et al, 1966; Tavassoli and Crosby, 1968; Patt and Maloney, 1975; Simmons et al, 1987]. Given the heterogeneous composition of the marrow stromal tissue, these studies suggest at least two possible scenarios. Firstly, each of the different stromal cell lineages may represent a separate self-maintaining population within the BM. Alternatively, the repair and turnover of the bone and BM connective tissue may result from the proliferation and differentiation of a population of multi-potential stromal precursor cells (SPC) with the capacity to give rise to each of the different stromal elements.

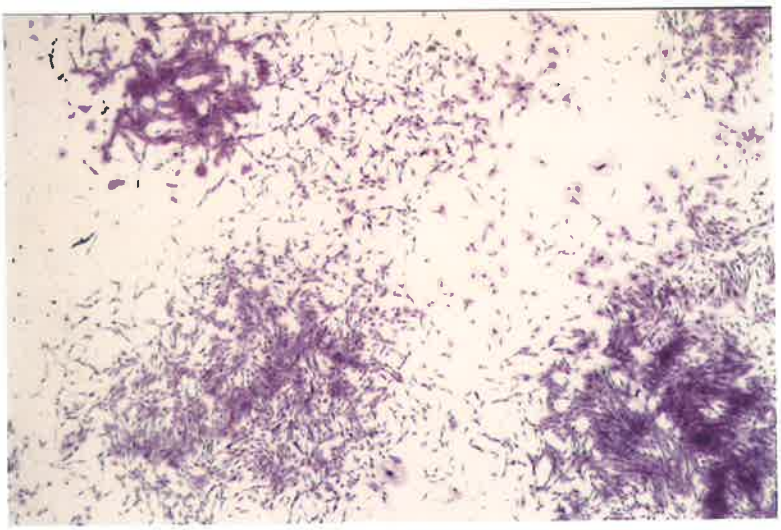
Putative stromal precursor cells isolated from rodent spleen, thymus, lymph node and bone marrow were first identified by their ability to form adherent colonies consisting of fibroblast-like cells (colony forming unit-fibroblastic; CFU-F) when grown in short-term liquid culture conditions [Friedenstein, 1970]. Subsequent studies identified BM CFU-F in a number of other species, including humans [Castro-Malaspina et al, 1980]. The CFU-F were shown to be clonal in nature using various techniques [Friedenstein, 1976; Friedenstein, 1970; Castro-Malaspina et al, 1980; Owen et al, 1987; Perkins and Fleischman, 1990]. In vivo, the majority of the BM CFU-F were found to be in a non-cycling state [Castro-Malaspina et al, 1980; Castro-Malaspina et al, 1981], analogous to

HSC which are known to be a population of quiescent cells [Sutherland et al, 1989; Brant et al, 1990].

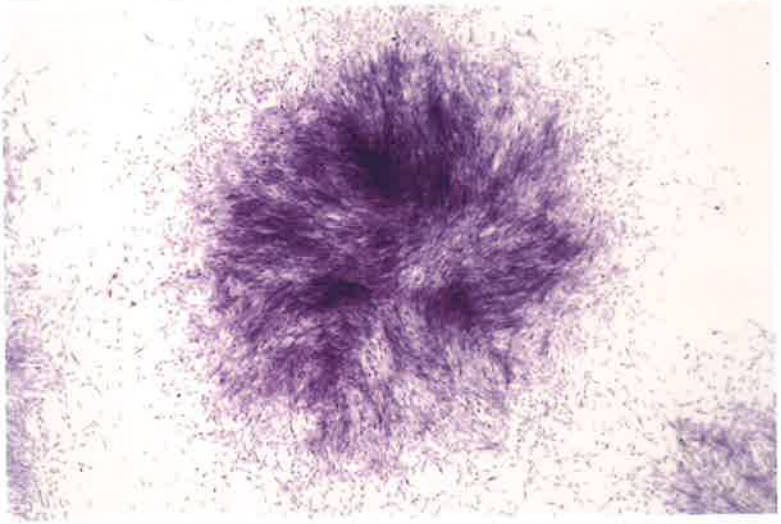
CFU-F colonies isolated from the BM of virtually all species, are heterogeneous in size and morphology (Figure 5), prompting the suggestion that they develop from clonogenic progenitors at various stages of differentiation [Owen, 1985]. A proportion of CFU-F also demonstrated an extensive replating potential after passaging [Friedenstein et al 1976; Simmons and Gronthos, 1991]. These observations led Friedenstein to propose that the CFU-F compartment contains SPC with the characteristics of stem cells. To test this theory, clones of CFU-F derived from mouse BM were grown and expanded in vitro and then transplanted beneath the renal capsules of syngeneic host animals to assess the developmental potential of each clone [Friedenstein et al, 1980]. Analysis of the transplanted material revealed that a minor proportion (approximately 15%) of the CFU-F clones produced a connective tissue containing all the different stromal cell types listed above, including bone cells and vascular cells. These stromal organs were also shown to have the capacity to support local haemopoiesis. A further 15% of the BM CFU-F clones produced a calcified bone-matrix only, while the remainder formed either a soft connective tissue or failed to give rise to any tissue [Friedenstein et al, 1980]. The blood cells contained within the stromal organs were identified as being derived from the host animal while the fibroblast-like cells were determined to be of donor origin using immunological and chromosome analyses. Similarly, when whole aspirates of rodent marrow from a donor animal were implanted into ectopic sites (such as renal, hepatic and muscular tissue) a stromal organ developed in the recipient animal prior to the establishment of haemopoiesis [Tavassoli, 1968; Tavassoli and Khademi 1980]. The ectopic stromal organs were shown by chromosome analysis to be of donor stromal origin while the haemopoietic cells associated with the stroma were shown to be derived from the host animal [Maniatis, 1971; Tavassoli, 1975; Tavassoli, 1980]. However, these early studies failed to determine the origin of the vascular cells which formed an extensive network within the stromal organs. Since angiogenesis originates from the mesoderm [Risau and

Figure 5. In vitro expanded BM colony forming units-fibroblastic (CFU-F). Light photomicrographs of day 14 BM CFU-F fixed in 2% paraformaldehyde and stained with 0.1% toluidine blue. Panel A depicts the variation in colony size of day 14 CFU-F (10X). Panel B represents a day 14 CFU-F with high proliferative potential containing many hundreds or thousands of cells (10X). Panel C depicts the typical morphology of individual fibroblast-like cells, present in an individual colony as either long bi-polar cells or flat irregular cells, (40X).

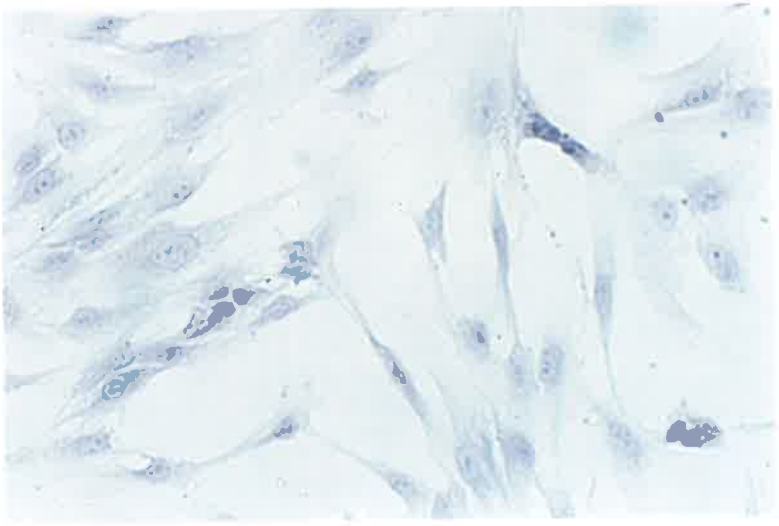
A



B



C

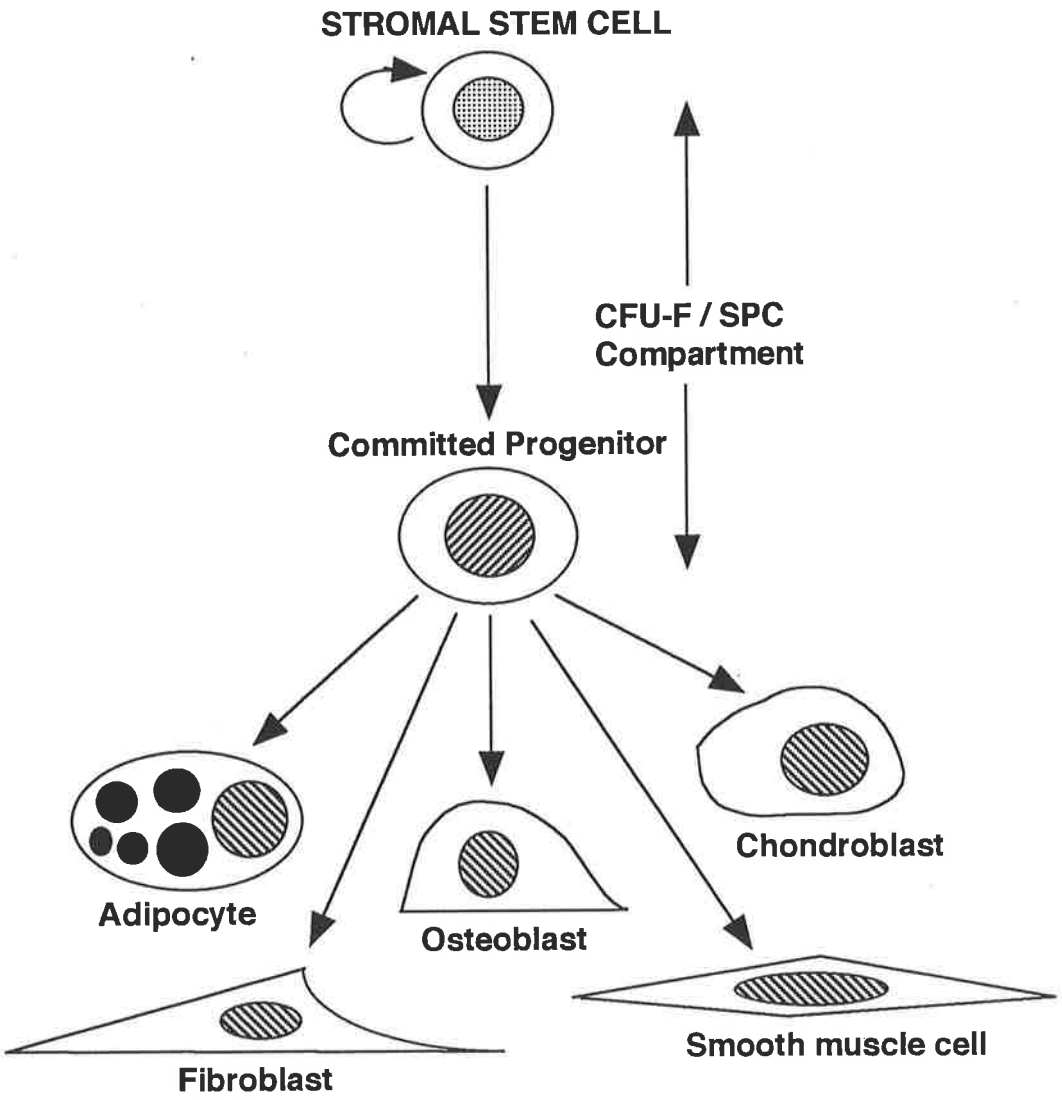


Flamme, 1995], it remains to be proven whether in the post-natal organism there exists a precursor cell common to endothelial cells and the other stromal cell types in the marrow microenvironment. Recent studies have identified embryonic stem cells isolated from chick embryonic bodies with the capacity to form haemopoietic cells and adherent stromal-like cells characteristic of endothelial cells in vitro [Choi et al, 1998]. However, there is no evidence for the existence of a common stem cell between haemopoietic cells, stromal cells and endothelial cells in the postnatal organism (see section 1.5.3).

Other investigators have demonstrated that a proportion of CFU-F derived from rodent marrow were induced to form adipocytes in the presence of the glucocorticoid hydrocortisone [Bennett et al, 1991]. These observations were consistent with those previously reported describing the induction of adipocytes by glucocorticoids in different stromal cell lines [Grigoriadis et al, 1988; Yamaguchi and Kahn, 1991; Dorheim et al, 1993]. Furthermore, approximately 40% of CFU-F clones comprising either fibroblastic or adipocytic cells were induced to form an osteogenic tissue when transplanted in vivo using diffusion chambers implanted into nude mice [Bennett et al, 1991]. These studies support the stromal stem cell hypothesis proposed by Owen and Friedenstein where by analogy with the haemopoietic system, there exists a hierarchy of cellular differentiation supported at its apex by a population of self-renewing, multi-potential stromal stem cells [Owen, 1985; Owen and Friedenstein 1988] (Figure 6). CFU-F which yielded BM organs were hypothesised to be derived from multi-potent stromal stem cells while those CFU-F which gave rise to only bone or soft connective tissue were proposed to be SPC of more restricted developmental potential (committed stromal progenitors).

Following on from the pioneering work of Freidenstien and Owen, various studies have supported the concept of a multi-potential stromal stem cell population in aspirates of BM using a variety of in vivo assays. Cultured stromal cells derived from rodent marrow have the capacity to develop into fibrous tissue, fat, bone and cartilage when transplanted in vivo using a variety of transplantation vehicles including, demineralized bone, gelatin and

Figure 6. Hypothetical hierarchy of stromal stem cell differentiation (adapted from Owen, 1985; 1988). The stromal precursor cell (SPC) compartment includes all cells with the ability to form clonogenic clusters (CFU-F) in vitro. These cells have the potential to develop into one or several of the mature stromal cell types listed using available in vitro or in vivo differentiation assays.


STROMAL STEM CELL
CFU-F / SPC
Compartment
Committed Progenitor
Adipocyte
Osteoblast
Chondroblast
Fibroblast
Smooth muscle cell

collagen gels, porous synthetic sponges, diffusion chambers, porous hydroxyapatite coated ceramic cubes, and when transplanted directly into ectopic sites of recipient animals [Ashton et al, 1980; Friedenstein et al, 1982; Bab et al, 1984; 1986; Mardon et al, 1987; Ohgushi et al, 1989; Goshima et al, 1991; Umezawa et al, 1992; Ohgushi et al, 1993; Benayahu et al, 1994; Cassiede et al, 1996; Krebsbach et al, 1997; Mizuno et al, 1997]. In contrast, initial experiments utilizing adult human marrow, failed to detect cells with osteogenic potential following the ectopic transplantation of BM stromal cells implanted into nude mice using diffusion chambers [Ashton et al, 1985; Davies, 1987]. To date, only a handful of studies have demonstrated the developmental potential of human BM stromal cells in vivo. One study has reported the formation of bone and cartilage in diffusion chambers implanted with marrow from young children but not from adult marrow [Bab et al, 1988]. Another such study found that bone formation in vivo was dependent on pretreating the human stromal cells in vitro with dexamethasone and ascorbate for several weeks prior to transplantation [Gundle et al, 1995]. The majority of the marrow samples in this study were also obtained from young subjects indicating that there may be age-related differences in the osteogenic potential of human bone marrow stromal cells. Using alternative modes of transplantation, studies have shown that cultured adult human stromal cells are capable of forming bone but not cartilage when transplanted subcutaneously into nude mice, using porous hydroxyapatite coated ceramic cubes and when injected directly into the calvaria or hind legs of immunodeficient (SCID) mice [Haynesworth et al, 1992a; James et al, 1996]. In parallel experiments, Haynesworth et al (1992a) failed to demonstrate bone formation in diffusion chambers, implying that osteogenic differentiation in human BM stromal cells is dependent on the type of transplantation model used. More recently, Kusnetsov et al (1997) have demonstrated that a proportion (58%) of individually expanded CFU-F derived from human marrow were capable of forming bone in vivo using HA cubes implanted into SCID mice. Comparative studies between different modes of transplantation and using different mouse strains as hosts, had a significant bearing on the frequency of stromal cells developing a mineralised bone-matrix [Krebsbach et al, 1997; Kuznetsov et al, 1997]. Collectively, these studies

highlight the need for further development and refinement of approaches to assess the full differentiation potential of human SPC in vivo.

1.6.2 Characterisation of SPC In Vitro

The majority of studies designed to investigate the properties of SPC in vitro have utilised unfractionated populations of bone marrow mononuclear cells to generate primary cultures of fibroblast-like cells generated from the proliferation of CFU-F colonies. Whole aspirates of marrow contain both haemopoietic accessory cells and committed stromal cells, which may in part explain the appearance of phenotypically distinct stromal colony types (macrophage-like, endothelial-like and fibroblast-like) in cultures derived from murine BM. Macrophage/monocyte type clusters were characterised functionally by their ability to phagocytose small particles and by their expression of the murine myeloid cell markers, F4/80, 7/4, Mac-1, and T200 [Wang and Wolf, 1990; Perkins and Fleischman, 1990; Pen et al, 1993]. In contrast, so called endothelial-like colonies contained rounded nonphagocytic cells which were shown by immunological staining to express collagen type IV and laminin but not collagen types I and III. Immunohistochemical analysis demonstrated that these cell clusters also stained with antibodies to the endothelial related markers, MECA-99, MECA-32, MJ7 and von Willebrand's factor [Wnag and Wolf, 1990; Perkins and Fleischman, 1990; Pen et al, 1993]. Fibroblast-like colonies exhibited a high proportion of alkaline phosphatase positive cells and were found to express collagen types I, III, IV, V, fibronectin and the smooth muscle-specific actin isoforms recognised by the mabs CGA-7 and HHF-35 [Wang and Wolf, 1990; Perkins and Fleischman, 1990; Van Den Heuvel et al; 1991; Pen et al, 1993]. Many of the colonies were also found to contain mixed populations of cells representative of all three phenotypes. Subsequent analysis of different CFU-F colonies derived from mouse chimeras, demonstrated that the macrophages in the mixed colonies were donor in origin while the stromal elements were derived from the host mouse strain [Perkins and Fleischman, 1990].

The generation of various cloned murine BM stromal cell lines displaying fibroblastic-, endothelial-, or preadipocytic-like phenotypes, have been shown to support both myelopoiesis and lymphopoiesis. This is in contrast to macrophage-like cell lines which have not been successful in supporting long-term haemopoiesis in vitro [Zipori et al, 1985; Brockbank et al, 1986; Kodama et al, 1986; Hunt et al, 1987; Itoh et al, 1989; Suzuki et al, 1992; Dorheim et al, 1993; Knospe et al, 1993]. However, the ECM analysis of most murine cell lines, regardless of their morphological features, is generally not predictive of their ability to support haemopoiesis [reviewed in Deryugina and Muller-Sieburg, 1993]. One study has shown that the fibroblastic-like murine BM stromal cell line KUSA-MTAg, has the capacity to support haemopoiesis both in vitro and in vivo, and can be induced in the presence of 5-azacytidine to develop into distinct stromal cell types including osteocytes, adipocytes and myotubes, and to form bone when transplanted subcutaneously into SCID mice [Umezawa et al, 1992]. Several other haemopoietic supporting stromal cell lines can also be induced to form differentiated osteoblast-like cells and or fat laden adipocytes in vitro [Gimble et al, 1990; Benayahu et al 1991; Knospe et al, 1993; Doreheim et al, 1993]. These data suggest that stromal cell lines characteristic of bi- or multi-potential stromal progenitor cells may be responsible for maintaining the primitive HSC population.

The in vitro characterisation of BM SPC in other species, such as rat and rabbit has illustrated the developmental potential of these cells using various assay systems. As described above, cultured CFU-F clones derived from rabbit marrow have the potential to develop clusters of adipocytes in the presence of hydrocortisone where a proportion of these cells have the potential to form an osteogenic tissue in vivo [Bennett et al, 1991]. Under similar culture conditions, CFU-F colonies were also found to have a marked increase in the expression of the osteoblast marker alkaline phosphatase [Owen et al, 1987]. A more recent study demonstrated the induction of a chondrocyte-like phenotype in culture expanded rabbit BM CFU-F [Johnstone et al, 1998]. Single cell suspensions were grown in aggregate cultures, in the presence of ascorbate and TGF-β1. Chondrogenesis

was assessed by the down regulation of collagen type I expression, the upregulation of AP, and by the appearance of the cartilage specific markers collagen types II and X [Johnstone et al, 1998]. Therefore according to the culture conditions used, rabbit BM CFU-F can display a range of phenotypes characteristic of different stromal cell lineages.

Culture expanded CFU-F colonies derived from rat bone marrow have been shown to express various fibroblastic and osteoblastic related markers such as collagen types I and III, OP, ON, and AP. However, other ostensibly specific bone markers such as OCN, BSP and PTH-R were not detected [Maniatopoulos et al, 1988; Leboy et al, 1991; Kasugai et al, 1991; Simmons et al, 1991; Kamalia et al, 1992a; Rickard et al, 1994]. In vitro, rat BM stromal cells were found to develop an osteoblast-like phenotype when cultured for several weeks in the presence of the glucocorticoid, dexamethasone [Maniopoulos et al, 1988; Tsuji T et al, 1990; Simmons et al, 1991; Kasugai et al, 1991; Leboy et al, 1991; Rickard et al, 1994]. Osteogenic differentiation in the BM stromal cultures was characterised by the expression of the bone related markers described above in addition to BSP and OCN, following stimulation with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Furthermore, small mineralized nodules formed throughout the cultures after a period of exposure to growth medium supplemented with ascorbate, dexamethasone and β-glycerophosphate [Maniopoulos et al, 1988; Tsuji et al, 1990; Kasugai et al, 1991]. The bone-like nodules were found to be positive for von Kossa staining and were shown to be composed of hydroxyapatite crystals identical to that observed in bone in association with collagen fibrils. Similar culture conditions have also been used to promote the development of an osteogenic-like phenotype, and the formation of a mineralized matrix, in cultures of stromal cells derived from BM aspirates of other species including humans [Howlett et al, 1986; Owen et al, 1987; Schoeters et al, 1992; Barling et al, 1989; Benayahu et al, 1991; Mathieu et al, 1992; Kamalia et al, 1992b; Thomson et al, 1993; Cheng et al, 1994; Gronthos et al, 1994; Beresford et al, 1994; Jaiswal et al, 1997]. However, the ability to form a calcified-matrix for certain species such as rat and human, was found to be dependent on the continuous presence of glucocorticoids in the culture

medium. This may reflect species differences in the frequency of early and committed progenitor cell populations in BM, where glucocorticoids may act to promote osteogenic differentiation in primitive multi-potential SPC.

Immunohistochemical studies have shown that cultured CFU-F derived from adult human marrow are AP positive, synthesise collagen types I and III and lack expression of the endothelial marker factor VIII antigen [Castro-Malaspina et al, 1980]. It has also been reported that stromal cells derived from human BM CFU-F clones constitutively express the adipocyte marker lipoprotein lipase and the bone-related markers AP, OP and ON [Vilamitjana-Amedee et al, 1993; Rickard et al; 1996]. In addition, the expression of the bone markers OCN and PTH-R could also be induced in CFU-F colonies following induction with 1,25-dihydroxyvitamin D_3 and dexamethasone, respectively. Thus the growth of BM CFU-F in the presence of serum seems to promote the commitment of their progeny towards a more differentiated phenotype characteristic of reticular cells, osteoblasts and adipocytes. However, when cultured under conditions permissive for mineral formation, human BM stroma produce sheets of mineral of varying densities across the whole adherent layer, unlike the nodule formation observed in rodent cultures.

The development of these in vitro differentiation assays has helped pave the way to identify and isolate cell lines derived from bone marrow representative of either distinct mature stromal cell lineages or expressing properties characteristic of multi-potential SPC. The generation and study of mesenchymal cell lines as models of stromal development, is an important step to understanding the processes of cellular differentiation and may facilitate the discovery of genes regulating commitment and differentiation within the bone and bone marrow stromal cell system. Collectively, these studies demonstrate the presence of cells in bone marrow cultures with the potential to form bone, fat, smooth muscle, cartilage and fibrous tissue. The question arises as to whether these progenitor cells are only committed to a particular stromal cell lineage or whether the different phenotypes generated can be induced in a more primitive stromal cell precursor

population. In analogy with haemopoietic progenitor cells, a stromal precursor population may contain a hierarchy of primitive multi-potent SPC and lineage restricted bi- and uni-potent progenitor cells.

1.6.3 Characterization of BM SPC In Vivo

The incidence of clonogenic stromal precursor cells in unfractionated BM is in the range of one CFU-F per five thousand to ten thousand BM mononuclear cells (BMMNC) plated [Castro-Malaspina et al 1980; Simmons and Torok-Storb, 1991a; 1991b; Falla et al, 1993; Waller et al, 1995a]. The rarity of the SPC population is a major limitation to their study, a problem compounded until recently by a shortage of information regarding the immunologic phenotype of these cells and a lack of efficient enrichment procedures.

To date, there has only been a few studies which describe the phenotypic properties of SPC in aspirates of rodent BM. SPC were identified in 5-fluoracil (5-FU) treated aspirates of murine marrow by fluorescence-activated cell sorting (FACS) based upon the composite immunophenotype; lymphoid and myeloid cell lineage (Lin) negative/ Stem cell antigen-1 (Sca-1) positive/ wheat germ agglutinin (WGA) bright ($lin^-/Sca\text{-}1^+/WGA^{bright}$) [Falla et al, 1993; Van Vlasselaer et al, 1994]. This cell population was shown to possess the potential to develop a calcified bone-matrix in vitro in the presence of ascorbate and β-glycerolphosphate. Under these culture conditions there was an increase in alkaline phosphatase activity and the production of osteocalcin following treatment with $1,25(OH_2)D_3$. Although this phenotype is similar to that previously reported for murine haemopoietic stem cells, these studies were able to distinguish the two populations based on differences in their light scatter characteristics: osteoprogenitors were found to have a higher forward and perpendicular light scatter than candidate HSC. In addition, the stromal precursor cells lacked the haemopoietic surface markers Thy1.2 and c-kit characteristic of primitive murine HSC but were found to express the endothelial cell markers, Sab-1 and Sab-2 and the fibroblastic marker KM16 [Van Vlasselaer et al, 1994].

Studies of human foetal BM based on FACS analysis, have shown that stromal precursor cells express the CD34 antigen at high levels but were distinguished from the bulk of haemopoietic progenitors by their lack of CD38 and HLA-DR [Hang and Terstappen, 1992; Waller et al, 1995a]. Single SPC isolated by FACS from foetal human BM were claimed to have the potential for differentiation into multiple stromal cell lineages including bone and cartilage [Huang and Terstappen, 1992]. While clearly an important study, these data must however be interpreted with caution since for several stromal elements (particularly the osteogenic and chondrogenic lineages), cell type specific, immunohistologically defined markers of differentiation were not employed, their identification being based on morphological criteria only. It was further claimed that within this $CD34^{+}/CD38^{-}/HLA\text{-}DR^{-}$ subpopulation were so-called "common stem cells" with the potential to develop into both haemopoietic and stromal cell progeny [Huang and Terstappen, 1992]. This conclusion was subsequently retracted by the authors based on further analysis of this heterogenous population which demonstrated that haemopoietic progenitors were contained within a $CD50^{+}$ (ICAM-3) subset of the $CD34^{+}/CD38^{-}/HLA\text{-}DR^{-}$ cells while SPC were restricted to a $CD50^{-}$ subset [Waller et al, 1995b].

Prior to these studies, other researchers have also proposed that BM stromal cells and haemopoietic cells are derived from a common stem cell [Keating et al, 1982]. However, the majority of reports investigating the 'common stem cell theory' have concluded that these represent two distinct stem cell populations in the postnatal organism [Maniatis et al, 1971; Tavassoli et al, 1975; Tavassoli and Khademi 1980, Fialkow et al, 1977; Friedenstein et al, 1978; Golde et al, 1980; Bentley et al, 1982b; Lim et al, 1986; Simmons et al, 1987]. Although the term 'mesenchymal stem cell' has become popular of late [Bruder et al, 1994; Caplan and Dennis, 1996], this nomenclature infers the existence of a common precursor for all mesenchymal tissue. With no substantial evidence to support this view, particularly in the postnatal organism, the alternative historical acronym 'CFU-F' or the preferred term 'stromal precursor cell' are both used throughout this thesis to described those cells in the BM which can give rise to clonogenic cell clusters in vitro.

Consistent with the observations in foetal human BM, antibodies to CD34 were subsequently shown to bind to CFU-F in adult BM [Simmons and Torok-Storb, 1991b]. However, unlike the high level of CD34 antigen expression characteristic of foetal BM SPC, adult BM SPC express CD34 but at considerably lower levels, at least 10-fold less than that exhibited by haemopoietic progenitors in the same sample. In addition, not all CFU-F are recovered in the $CD34^+$ fraction which might reflect either inefficient capture of SPC as a result of low CD34 antigen density or heterogeneity of CD34 expression within the SPC compartment [Simmons and Torok-Storb, 1991b]. For these reasons, and the fact that the $CD34^+$ cell fraction represents a mixed progenitor cell population, the selection of CFU-F based on the use of CD34 antibody does not represent the method of choice for isolating these cells from adult human BM.

Differences in the cell surface expression of haemopoietic and stromal cells have been observed in various studies providing a means to isolate the two cell populations. For example, stromal precursor cells can be recovered from human BM aspirates based on their binding of the lectin, soybean agglutinin which does not bind at detectable levels to haemopoietic progenitors [Ebell et al, 1985; Sutherland et al, 1989; Simmons and Torok-Storb, 1991b]. Stromal precursor cells can also be partially enriched from unfractionated human bone marrow aspirates based on their lack of expression of cell surface antigens characteristic of myeloid, erythroid and lymphoid cells [Simmons and Torok-Storb, 1991a; 1991b; Simmons et al, 1994b; Van Vlasselaer et al, 1994; Waller et al, 1995b; Rickard et al, 1996]. The isolation and purification of the CFU-F population in the absence of accessory cells and mature stromal cells becomes a crucial issue to establish whether all these cells or only a proportion exhibit a phenotype characteristic of multi-potential stromal stem cells. The development of antibody reagents with restricted specificity for SPC would therefore be a significant advance for identifying and isolating pure populations of human BM SPC.

1.6.4 Novel Markers Identified on Human BM SPC

The murine mab STRO-1, previously developed by Simmons and Torok-Storb (1991a), reacts with as yet an unidentified antigen expressed on approximately 10% of adult human BMMNC. Within this minor population are essentially all assayable CFU-F. STRO-1^+ BMMNC are a heterogeneous population, the majority (approximately 90%) of which comprise late stage glycophorin A^+ erythroblasts, together with a minor proportion of $CD19^+$ B-cells. CFU-F are restricted to the minor population of STRO-1^+ cells which lack glycophorin A [Simmons and Torok-Strob,1991a]. There was no detectable binding of the STRO-1 mab to either haemopoietic progenitors (CFU-GM, BFU-E, BFU-Meg, CFU-GEMM) or to their precursors (pre-CFU) [Simmons and Torok-Storb 1991a; Gronthos and Simmons unpublished observations]. The STRO-1 mab was found to bind to the human erythroleukaemia cell line HEL-DR^+ and to Epstein-Barr virus-immortalised B-cell lines but was not reactive with a range of myeloid and T-lymphoid cell lines including, KG1, KG1a, HL60, U937, CEM and Jurkat.

Adult BM cells sorted on the basis of STRO-1 expression have previously been shown to develop into an adherent stromal layer when grown under LTBMC conditions with an increased capacity to support haemopoiesis in comparison to stroma derived from unfractionated BM [Simmons and Torok-Storb, 1991a]. The adherent layers derived from STRO-1^+ BM consist of a number of phenotypically distinct stromal cell types including fibroblasts, smooth muscle cells and adipocytes but lacked the presence of macrophage and endothelial cells which are readily detected in LTBMC derived from unfractionated BM [Simmons and Torok-Storb, 1991a].

The murine mab 6-19 (IgG2a) was found to be reactive with an 80 kDa protein expressed on human neuroblastoma but not with cells in unfractionated blood or bone marrow, using immunofluorescence and flow cytometric analysis [Abboud et al, 1986; Iyer et al, 1990]. 6-19 mab was found to bind to human bone marrow stroma, endothelial cells, foreskin and lung fibroblasts. Studies were performed where unfractionated human bone marrow was

incubated with 6-19 and complement before the cells were cultured in erythroid (BFU-E), myeloid (CFU-GM) and stromal (CFU-F) progenitor cell assays. The growth of haemopoietic progenitors was unaffected by the 6-19 dependent complement mediated cytotoxicity. In contrast almost 100% of the CFU-F were killed, indicating that 6-19 reacted with stromal precursors. Furthermore, bone marrow cells treated with 6-19 and complement were unable to establish an adherent stromal layer under LTBMC conditions. The function and identity of the 6-19 antigen is not known.

The murine mab HOP-26 (IgM) reacts with an unknown antigen with a restricted distribution pattern during bone formation [Joyner et al, 1997]. In sections of human foetal limb HOP-26 binds to cells close to new bone formation in periosteum and between bone trabeculae. Similarly, in adult trabecular bone tissue, HOP-26 was found to react with a minor population of cells within the bone marrow spaces but was not expressed on osteoblasts, adipocytes, fibrous tissue, skin, muscle, appendix, brain, tonsil, or the osteosarcoma cell lines, MG63 and SAOS. In addition, the mab HOP-26 reacted with a minor population of unfractionated BM both at the cell surface and intracellularly. Immunopanning experiments demonstrated that the CFU-F population was able to be isolated from unfractionated BM using HOP-26 coated dishes. The frequency of CFU-F was increased by 46 fold following immunopanning when compared to unfractionated BM with approximately 70% recovery of the expected total CFU-F population.

A panel of murine mabs (SH-2, SH-3 and SH-4) was previously generated following immunisation with cultured BM stromal cells [Haynesworth et al, 1992b]. All three mabs were shown to react with human stromal elements both in vivo and in vitro but were unreactive with BM derived haemopoietic cells. In sections of various tissues all three mabs identified unknown components in the extracellular matrix but fail to react with active osteoblasts and differentiated osteocytes in sections of bone. The mabs SH-3 and SH-4 were also found to react with chondrocytes from articular and rib cartilage. However, the identity of the type of cells that these mabs react with is unknown. Reactivity with SH-

2, SH-3 and SH-4 with CFU-F in aspirates of BM has not be determined but might be predicted given that all three mabs identify antigens constitutively expressed on BM stromal cells in vitro.

The development of the mab SB-10 was based on its reactivity to an antigen identified on undifferentiated alkaline phosphatase negative stromal elements in the periosteum of foetal mouse bone [Bruder et al, 1997]. Immunostaining of various tissues with SB-10 showed reactivity with epithelium of the oesophagus, intestinal serosa, the epidermis of the skin and the capsule of the adrenal gland. Primary cultures of BM stromal cells, bone cells derived from trabecular bone chips, and the osteosarcoma cell lines MG63 and U2OS were all found to constitutively bind SB-10. It remains to be determined whether the mab SB-10 can identify CFU-F in aspirates of human bone marrow. Peptide sequence analysis was performed following purification of the SB-10 antigen (approximately 99 kDa) and was subsequently used to clone the cDNA encoding the SB-10 antigen [Bruder et al, 1998a]. Analysis of the sequence data demonstrated homology with human activated leukocyte-cell adhesion molecule (ALCAM). The ALCAM molecule is a member of the Ig superfamily and is a ligand for the CD6 antigen expressed on lymphoid cells and therefore may play a role in cell migration and/or homing of haemopoietic cells.

1.6.5 Osteogenic Progenitors in Bone Tissue

Various methods have been developed for the isolation and culture of cells from osteogenic tissue at different stages of skeletal development, for a variety of different species including humans [Beresford et al, 1984; 1986; Auf'mkolk et al, 1985; Ashton et al, 1985; Robey and Termine 1985; Bellows et al, 1986; Koshihara et al, 1987; Gerstenfeld et al, 1987; Lomri et al, 1988; Marie et al, 1989; Owen et al, 1990; Johansen et al, 1992; Yao et al, 1994; Boss et al, 1996; Gallagher et al, 1996]. Collectively, these studies have demonstrated that cells isolated from bone display an osteoblast-like phenotype, under various culture conditions, characterized by the expression of different bone-related markers such as COL-1, AP, ON, OP, BSP, PTH-R. However, bone cell

cultures are thought to be comprised of a mixed population of osteogenic cell types, representative of different developmental stages. The identification of osteogenic precursor cell populations in these cultures has been difficult mainly due to the choice of an appropriate model system. To complicate matters, differences in the properties and behaviour of osteogenic cells have also been observed due to age-, species-, and sex-related differences, and in their responsiveness to hormones and growth factors in vitro [Tsuji et al, 1990; Fedarko et al, 1992; Pfeilschifter et al, 1993; Shigeno and Ashton, 1995; Quarto et al, 1995; Gerstenfeld et al, 1996; Davis et al, 1997; Depollack et al, 1997; Ishida and Heersche, 1997]. There is also evidence that even within the same organism osteogenic cells isolated from different anatomical sites may not be functionally equivalent [Kasperk et al, 1995; Rawlinson et al, 1995].

In analogy with the marrow system, studies have identified osteoprogenitor cells isolated from bone fragments by their capacity to develop an osteogenic tissue when transplanted into ectopic sites of recipient animals [Simmons et al, 1982; Yamamoto et al, 1991; Nakahara et al, 1991; Gundle et al, 1995]. In addition, bone derived osteoprogenitors have the ability to form a mineralized bone-matrix following stimulation for several weeks in the presence of ascorbate, β-glycerophosphate with or without the addition of dexamethasone [Sudo et al, 1983; Ecarot-Charrier et al, 1983; Nefussi et al, 1985; Bellows et al, 1986; 1987; 1991; Koshihara et al, 1987; Gerstenfeld et al, 1987; Beresford et al, 1993; Gallagher et al, 1996]. The in vitro formation of a three-dimensional mineralized matrix throughout the cultures was found to resembled new or woven bone in vivo and was shown to be associated with collagen fibrils [Bellows et al, 1987; Bhargava et al, 1988; Ecarot-Charrier et al, 1988; Gerstenfeld et al, 1988]. Nodule formation in rodent bone cultures was also comparable to the development of mineralized nodules in cultures of rodent BM stromal cells as described above [Maniopoulos et al, 1988]. The nodules were shown to contain a small number of viable cells (approximately 100 cells) resembling osteoblast-like cells at the surface and osteocyte-like cells inside the nodules. In addition, each nodule is believed to be derived from the proliferation and differentiation

of a single osteoprogenitor cell [Bellows et al, 1986; 1987; Bhargava et al, 1988]. The authors in one study were able to measure the number of osteoprogenitors in a given cell population isolated from foetal rat calvaria, by demonstrating a linear relationship between the number of cells plated and the number of bone nodules formed. Using Poisson distribution statistics, it was calculated that there is approximately 1 osteoprogenitor per 300 cells of the total cell population in cultures derived from foetal rat calvaria under standard growth conditions [Bellows and Aubin, 1989]. To date, the osteoprogenitor cells in rodent and human bone cell cultures have yet to be fully characterized.

Subsequent studies demonstrated that there exists at least two populations of nodule forming osteoprogenitors in primary cultures derived from foetal rat calvaria. The predominant osteoprogenitor population was found to express the cell surface marker AP as demonstrated by immunoselection using mabs specific for AP [Bellows et al, 1990; Turksen and Aubin, 1991; Herbertson and Aubin, 1997]. The majority of these cells were capable of forming nodules in vitro in the absence of glucocorticoids such as dexamethasone. A second osteoprogenitor population lacked expression of AP was also identified where nodule formation was found to be dependent on the presence of dexamethasone. In addition, the proliferative and differential potential of nodule forming osteoprogenitor cells was found to decrease with successive passages, demonstrating that this cell population has a limited capacity for self-renewal in vitro [McCulloch et al, 1991]. These studies are therefore consistent with the existence of a hierarchy of osteogenic differentiation in bone cell cultures: according to the expression of AP, where the AP negative osteoprogenitors appear to be the more primitive progenitor population. Comparative studies have shown that rat bone cultures contain more AP positive osteoprogenitors when compared to rat bone marrow cultures, demonstrating differences in the frequency of early osteoprogenitor cells at different anatomical sites [Bellows et al, 1990; Turksen and Aubin, 1991; Herbertson and Aubin, 1997]. Overall, osteogenic precursor cells therefore appear to display similar characteristics and cellular properties to osteoprogenitors isolated from bone marrow aspirates.

In analogy with the capacity of various mouse embryonic and bone marrow cell lines to form bone, cartilage, fat and muscle in the presence of 5-azacytidine [Constantinides et al, 1977; Taylor and Jones 1979; 1982; Umezawa et al, 1992;], cloned rodent cell lines derived from osteogenic tissue could also be induced to form muscle, fat, cartilage and bone in vitro [Grigoriadis et al, 1988; Yamaguchi and Kahn, 1991]. Together these data provide circumstantial evidence that there exists multi-potential progenitors within osteogenic and non osteogenic tissues with the capacity to differentiate into various functional mature stromal cell types. Recently, one study has identified a stromal progenitor population present in stromal cultures prepared from foetal rat periosteum. FACS analysis demonstrated that these cells had low perpendicular and forward light scatter properties and were found to be in the G_1 and S phases of the cell cycle [Zohr et al, 1997]. The putative SPC population, referred to as S-cells, lacked the expression of the differentiation markers; collagen types I, II, III, osteopontin, alkaline phosphatase and the adhesion molecule CD44 [Zohr et al, 1997]. Furthermore, when the S-cells were cultured under conditions permissive for osteogenic differentiation, they were found to express all the above markers and demonstrated an enhanced ability to develop von Kossa positive mineralised nodules in vitro, in comparison to other cell fractions. In addition, the S-cells demonstrated the ability to develop cartilage, adipocyte and smooth muscle phenotypes in vitro, based upon histochemical and immunological criteria. However, the presence of bi- or multi-potential stromal progenitors in cell cultures derived from human osteogenic tissue remains to be determined.

1.7 OBJECTIVES

Thus far this thesis has reviewed studies which have focussed in large part on the phenotypic characteristics and properties of bone and bone marrow stromal cells and their precursors. There is clearly a need to develop more specific reagents and cell isolation procedures in order to aid in the identification and purification of stromal precursor cells in order to properly study their biology. The present thesis examines the characteristics of the STRO-1 antigen and examines the utility of the STRO-1 antibody reagent as a means of isolate and purify human BM SPC. The thesis also aims to determine the properties of SPC in particular: the growth requirements of SPC in vitro; the morphological features and immunophenotype of SPC; the gene expression pattern of stromal lineage-restricted markers; the developmental potential of purified SPC in respect to their ability to form bone and other stromal tissues both in vitro and in vivo.

The second series of studies in this thesis were designed to characterise normal human bone cells (NHBC) in vitro, by firstly examining their integrin expression patterns and adhesive properties to various extracellular matrix proteins. Moreover, the feasibility of using cultured bone cells as an in vitro model of human bone cell development was investigated by identifying NHBC at various stages of differentiation, based on the cell surface expression of the STRO-1 and alkaline phosphatase antigens. Isolation of phenotypically distinct bone cell subpopulations would be subjected to further analysis of their osteogenic commitment, based both on their expression of bone-associated matrix proteins and the osteogenic transcription factor CBFA1 and by the ability of each subpopulation to form a mineralized bone matrix in vitro.

CHAPTER TWO: MATERIALS AND METHODS

2.1 SUBJECTS AND SPECIMENS

2.1.1 Bone Marrow Specimens

Fresh bone marrow (BM) aspirates from the iliac crest and the sternum and peripheral blood (PB) samples were obtained from normal adult volunteers (20-35 years old) with their informed consent on the established normal donor program of the Leukaemia Research Unit in the Hanson Centre for Cancer Research, Adelaide, South Australia. Bone marrow mononuclear cells (BMMNC) and peripheral blood mononuclear cells (PBMNC) were routinely obtained on a Lymphoprep gradient (S.G. 1.077 g/ml) (Nycomed, Oslo, Norway) by centrifugation at 400 x g for 30 min. The light density interface cells were removed and then washed twice in IF buffer (section 2.5.1a), by centrifugation at 400 x g for 5 min at $4^{o}C$. The cell pellet was resuspended in fresh IF buffer and left to incubate on ice before being used for cell culture and/or immunological studies.

2.1.2 Normal Human Bone Cells

Pieces of trabecular bone from the tibia and the femoral head of normal individuals (30-75 years old) were generously supplied by Dr. S.E. Graves during routine knee and hip replacements performed in the Department of Orthopaedic Surgery and Trauma at the Royal Adelaide Hospital, South Australia. The bone chips were cut with a sterile scalple into small pieces of approximately 4 mm^2 then washed vigorously three times in PBS (section 2.2.1b) to remove the non-adherent hematopoietic component. The bone chips were then cultured as described in section 2.2.2d.

2.2 CELL CULTURE

All tissue culture was performed in Class 2 "biohazard" laminar flow hoods (Gelman Sciences). 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 by incubating the cells in 0.4% trypan blue (w/v) in phosphate buffered saline (PBS) and then counting the cells using a light microscope.

2.2.1 General Buffers and Basal Media

Tissue culture solutions were made with Milli-Q water prepared by further 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). Solutions and buffers were subsequently sterilised by autoclaving 130^{o}C for 20 min or filter sterilisation as indicated or filter sterilized using either a sterivex GS 0.22 mm filter system (Millipore Corp.), bottle filter (Corning, USA), Spin-X filter, (Costar, MA, USA) or by 0.22 mm ministart filters (Sartorius, Germany). Following buffer preparation, all solutions were stored at 4°C, -20°C or -80°C as indicated.

(a) Hank's Balanced Salt Solution (HBSS)

Single strength Hank's balanced salt solution (HBSS: Ca^{2+}- and Mn^{2+}-free), consisted of 0.14 M NaCl (BDH Chemicals), 5 mM KCl, 0.3mM $Na_2HPO_4.12H_20$, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$ (BDH), 5.5 mM glucose, 1% Phenol Red in 0.1 M NaOH (M&B, UK) dissolved in Milli-Q water and buffered with 20 mM N-2-Hydroxyethylpiperazine N'-2-ethaneslphonic acid pH 7.2 (HEPES: Boehringer-Mannheim, Australia). The HBSS was subsequently autoclaved-sterilised as above.

(b) Phosphate Buffered Saline (PBS)

Single strength PBS (Ca^{2+}- and Mn^{2+}-free) comprised 0.14 M NaCl (BDH Chemicals), 3 mM KCl (BDH Chemicals), 8 mM $Na_2HPO_4.12H_20$ (BDH Chemicals), and 1 mM KH_2PO_4 (BDH Chemicals) dissolved in Milli-Q water. The pH was adjusted to 7.4 with 1 M HCl prior to sterilisation by autoclaving.

(c) Roswell Park Memorial Institute Medium (RPMI-1640)

Single strength RPMI-1640 (Life Technologies, Glen Waverley, Victoria, Australia) was prepared by dissolving one RPMI-1640 sachet into 970 mls of Milli-Q water. The medium was supplemented with penicillin (100 i.u./ml) (CSL, Melbourne, Australia), streptomycin (100 μg/ml) (GIBCO BRL) and buffered with 10 mM HEPES and 27 mls of 7.5% (w/v) $NaHCO_3$ and subsequently filter-sterilised and stored at 4^oC

(d) Dulbecco's Modified Eagles Medium (DMEM)

Single strength DMEM (GIBCO BRL, USA) was prepared by dissolving one sachet of DMEM powder into 940 mls of Milli-Q water. This was supplemented with penicillin (100 i.u./ml), streptomycin (100 μg/ml) and buffered with 10 mM HEPES and 52 mls of 7.5% (w/v) $NaHCO_3$ (BDH). The medium was then filter-sterilised and stored at 4^oC.

(e) Alpha Modification of Eagle's Medium (α-MEM)

Single strength a-MEM (with glutamine, without sodium bicarbonate; Flow Laboratories, Irvine, Scotland) was prepared by dissolving one sachet into 960 mls of Milli-Q water supplemented with penicillin (100 i.u./ml), streptomycin (100 μg/ml) and buffered with 26 mls of 7.5% (w/v) sodium bicarbonate. The medium was filter-sterilised and stored at 4^oC.

2.2.2 Cell Culture Of Primary Tissues

Serum lots of foetal calf serum (FCS: batch 593; GIBCO BRL, Victoria, Australia), bovine serum albumin (BSA: Cohn fraction V; Sigma, St Louis, MO) and horse serum (HS, batch 8100500-3; Cytosystems) were selected following extensive batch testing for optimal clonal growth of haemopoietic progenitor cell colonies and proliferation of primary stroma precursors (CFU-F).

(a) BM CFU-F Clonogenic Assay (Serum Replete)

Details of this procedure have been described elsewhere [Gronthos and Simmons, 1995; Gronthos et al, 1998]. Single strength α-MEM was supplemented with 20% (v/v) FCS, 2

mM L-glutamine (Life Technologies) and β-mercaptoethanol (5×10^{-5} M). The medium was filter sterilised and stored at 4°C. BMMNC or STRO-1$^+$ sorted cells (0.1 to 1.0 x 10^5 cells/ml) were cultured in triplicate wells (6-96-well plates; NUNC, Intermed, Roskilde, Denmark) and incubated at 37°C in 5% CO_2 for 14 days. Day 14 cultures were washed twice with warm RPMI-1640 and then fixed for 20 min in 1% (w/v) paraformaldehyde in PBS (BDH Chemicals). Once fixed the cultures were stained with 0.1% (w/v) toluidine blue (in 1% paraformaldehyde solution) for 1hr then rinsed out in tap water. 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). Colony size was also determined by measuring the diameter (mm) of day 14 CFU-F with the use of a graded monocular eye piece and a standard graticule.

(b) BM CFU-F Clonogenic Assay (Serum Deprived)

This method is a modification of the serum deprived medium (SDM) developed for the growth of haemopoietic progenitor cells [Migliaccio et al, 1988; Lansdorp and Dragowska, 1992] and was adapted for the growth of stromal progenitors [Gronthos and Simmons, 1995]. Single strength α-MEM was supplemented with 10μg/ml bovine pancreas derived insulin (Sigma), 2% (w/v) BSA, 4 μg/ml human low density lipoprotein (LDL: Sigma, L121139), 200 μg/ml iron saturated human transferrin, 2 mM L-glutamine, dexamethasone sodium phosphate (10^{-8} M) (DEX: David Bull Laboratory, Sydney, Australia), 100 μM L-ascorbic acid-2-phosphate (ASC-2P: Novachem, Melbourne, Australia), β-mercaptoethanol (5×10^{-5} M), 10 ng/ml platelet derived growth factor-BB (PDGF: Pepro Tech Inc., Rocky Hill, NJ) and 10ng/ml epidermal growth factor (EGF: Sigma). The SDM was made fresh as required and was filter sterilised prior to its use. Cell culture vessels (6 to 96-well plates; NUNC) were pre-coated with purified fibronectin (Boehringer Mannheim, Germany) diluted in PBS to give a final coating concentration of 5 μg/cm^2 surface area for 90 min at RT. Following this the fibronectin solution was pipetted off, and the vessels were then rinsed once with PBS. BMMNC or STRO-1$^+$ sorted cells (0.1 to 1.0 x 10^5 cells/ml) were cultured in triplicate and incubated at 37°C in 5% CO_2 for

14 days. Day 14 cultures were washed twice with warm RPMI-1640 and then fixed for 20 min in 1% paraformaldehyde (in PBS). After fixation the cultures were stained with 0.1% toluidine blue (in 1% paraformaldehyde solution) for 1hr then rinsed out in tap water. 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). Colony size was also determined by measuring the diameter (mm) of day 14 CFU-F with the use of a graded monocular eye piece and a standard graticule.

Other growth factors/ cytokines used in this study include: Recombinant Human (rHu-) basic fibroblast growth factor (bFGF: GIBCO BRL); rHu-long R^3 insulin-like growth factor-1 (IGF-1: Gro Pep Pty Ltd, Adelaide, Australia); interleukin-1 beta (IL-1b), tumor necrosis factor alpha (TNFα), vascular endothelial cell growth factor (VEGF), stem cell factor (SCF) (Pepro Tech Inc., Rocky Hill, NJ); rHu-interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-9 (IL-9), interleukin-11 (IL-11), granulocyte-macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), transforming growth factor beta (TGFβ), macrophage inflammitory protein-1 alpha (MIP-1α), leukaemia inhibitory factor (LIF), bone morphogenic protein-2 (BMP-2) (Kindly donated by Dr. S. Clark, Genetics Institute); rHu-interleukin-4 (IL-4), interleukin-7 (IL-7), interferon gamma (IFNγ), Human growth factor (HGF), Leptin (Genzyme Corp., Cambridge, MA); rHu-granulocyte-colony stimulating factor (G-CSF) (R & D Systems, Minneapolis, MN); rHu-interleukin-8 (IL-8: Dr W. Smith, Human Immunology, H.C.C.R.); rHu-interferon alpha 2a (IFNα 2a: Roche Ltd.); purified mouse nerve growth factor (NGF: Kindly donated by Dr. B. Rush, Flinders Medical Centre, Adelaide, Australia).

(c) Osteo-induction of BM CFU-F In Vitro

This method is a modification of two different procedures [Maniatopoulos et al 1988; Matsumoto et al 1991] adapted and optimised for human bone marrow cells [Gronthos et al, 1994]. STRO-1^+ sorted BMMNC (1×10^3 cells/cm^2 culture flask; Corning, USA) were cultured in standard CFU-F growth medium (2.4.1) with ASC-2P (100 μg/ml) at 37^oC, 5%

CO_2. After 7 days the culture medium was switched to osteo-inductive medium consisting of single strength a-MEM supplemented with 10% FCS, 2 mM L-glutamine, DEX (10^{-8} M), 100 μg/ml ASC-2P, 1.8 mM KH_2PO_4 (BDH Chemicals) to give a final phoshate concentration of 2.9 mM, and 10 mM Hepes. The medium was filter sterilised and stored at 4°C. Cultures were fed three times a week for up to six weeks and then sacrificed for gene expression studies, osteocalcin measurements, electron microscopy and light microscopy studies.

(d) Culture of Normal Human Bone Cells (NHBC)

Explants of trabecular bone from normal individuals were cultured as described previously [Gronthos et al, 1997]. Bone chips prepared as described in section 2.1.2 were suspended in α-MEM supplemented with 10% foetal calf serum, 2 mM L-glutamine and 100 μM ASC-2P and distributed (10-12 pieces/flask) into each of six 75 cm^2 tissue-culture flasks (Corning) per sample. Cultures were incubated at 37°C in 5% CO_2 with complete medium changes every 7 days for two weeks followed by complete medium changes three times a week for a further 2 to 4 weeks. Cells were harvested by collagenase/dispase digestion (Section 2.3.2).

(e) Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC were generously provided by Dr J. Gamble, (Division of Human Immunology, Hanson Centre for Cancer Research, Adelaide, South Australia) as single cell suspensions. Primary cultures of HUVEC were grown in M199 medium suplemented with 10% FCS, 2 mM L-glutamine, heparin sulfate (0.08 units/ ml) and EGF (20 ng/ml). The medium was filter sterilized and stored at 4°C and the HS and GF were added prior to culture. The cultures were fed twice a week by complete media changes. Cells were harvested by trpsin digest (Section 2.3.1).

2.2.3 Maintenance of Cell Lines

Prior to use, all cell lines used in these studies were shown to be mycoplasma-free using the Gen-Probe Mycoplasma TC Rapid Detection System (Gen-Probe Inc., San Diego, CA, USA). Adherent and non-adherent cell lines were subcultured, every 2 to 3 days, to ensure maintenance of a logarithmic phase of growth, approximately $2x10^5$ cells per ml for non-adherent lines and $2x10^5$ cells per 25 cm^2 flask for adherent lines after trypsin digestion (Section 2.3.1).

(a) Retroviral Packaging Cell Line (PA317)

The murine fibroblastic, amphotropic virus-packaging cell line, PA317 was obtained from the American Type Culture Collection (ATCC: Rockville, MD), and was maintained in DMEM supplemented with 10% FCS and 2mM L-glutamine. Cultures were harvested by trypsin detachment (Section 2.3.1). The medium was filter sterilized and stored at 4^oC until required.

(b) Adherent Stromal Cell Lines

The human osteosarcoma cell lines (MG63, HOS and SAOS) and the murine bone marrow stromal cell line (BMS2) were kindly provided by Dr. Karina Stewart (Bath Institute of Rheumatic Diseases, Bath, U.K.). The human foreskin fibroblast cell line (HFF-2) was generated by Dr. P.J. Simmons and the mouse osteoblast-like cell line (ST2) was kindly provided by Dr. David Findlay (Department of Orthopaedic Surgery and Trauma, Adelaide University, Adelaide, S.A., Australia). All cell lines were maintained in α-MEM medium supplemented with 10% FCS, 2 mM L-glutamine and 100 μM ASC-2P. The medium was filter sterilised and stored at 4^oC. Cells were harvested by trypsin detachment.

2.3 ENYMATIC RELEASE OF ADHERENT CELLS

2.3.1 Trypsin-EDTA Digest

Trypsinisation solution consisted of 0.05% (w/v) trysin (Difco, USA) and 0.5 mM EDTA in sterile PBS. The trypsin was firstly pre-warmed in a 37^{o}C water bath. During this period the medium from the adherent cell cultures was removed and the cells rinsed once in sterile PBS. After aspirating off the PBS, the trypsin solution was added to the cells (1 ml per 25 cm^{2} surface area). The cultures were placed in a 37^{o}C incubator for 5 min to detach the cells. The trypsin was inactivated in the presence of FCS by washing the cells twice in IF buffer.

2.3.2 Collagenase/Dispase Digest

Adherent cell cultures were washed in PBS as described in section 2.4.1. The cells were then incubated in a pre-warmed solution of collagenase (3 mg/ml in PBS) (Collagenase Type I; Worthington Biochemical Co., Freehold, NJ) and dispase (4 mg/ml in PBS) (Neutral Protease Grade II; Boehringer Mannheim, GMBH, W. Germany) (1 ml per 25 cm^{2} surface area) for 60 min at 37^{o}C. Single cell suspensions were then washed twice in IF buffer.

2.4 CRYOPRESERVATION

2.4.1 Cryopreservation of Cells

Primary cells and cell lines were cryopreserved in the presence of 10% analytical grade dimethyl sulphoxide (DMSO: BDH Chemicals) to prevent the crystallisation and fracturing of the cell membranes. Cells were harvested at log phase and resuspended at 0.5-1x10^{7} cells/ml in cold IF buffer. Adherent cell lines were enzymatically digested as described above to generate a single cell suspension. The cells were suspended in a volume of neat FCS to give a cell concentration of 2 X 10^{7}/ml, then left to incubate on ice for 15 min. Immediately prior to freezing, an equal volume of freezing mix (20% DMSO, 80% FCS),

pre-cooled on ice, was added dropwise to the cells with mixing over approximately 2-5 min to give a final cell concentration of 1 x 10^7/ml. The cell mixture was aliquoted into cryopreservation ampoules (Nunc, Intermed, Roskilde, Denmark) and control-rate cryopreserved in a control rate freezer (KRYO 10 series) at 1°C/min from 5°C to -12°C, 4°C/min from -12°C to -20°C, 0°C for 5 min, at 1°C/min from -20°C to -40°C and 3°C/min from -40°C to -80°C. The vials were then transferred to liquid nitrogen storage (-196°C).

2.4.2 Thawing Cryopreserved Samples

The appropriate medium for the cell type was prewarmed to 37°C. Following rapid thawing in a 37°C water bath, the sample was subsequently transferred to a 50 ml polypropylene tube (Falcon; Becton Dickinson, Linkon Park, NJ), containing the appropriate medium pre-cooled at 4°C. The sample was then washed twice by centrifugation at 200 x g for 5 min at 4°C to remove any residual DMSO prior to culturing.

2.5 IMMUNOLOGICAL STUDIES

2.5.1 General Buffer and Fixatives

(a) Immunofluorescence Buffer (IF Buffer)

Single strength HBSS was suplemented with 5% FCS. The buffer was filter sterilized and stored at 4°C.

(b) Blocking Buffer For Flow Cytometric Analysis

Single strength HBSS was supplemented with 10% w/v BSA, 10% (v/v) normal human serum (NHS: Red Cross, Adelaide, South Australia) and 5% FCS. The buffer was filter sterilised and stored at 4°C.

(c) Magnetic Activated Cell Sorting Buffer (MACS)

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

(d) Flow Cytometry Fixative (FACS FIX)

Single strength PBS was supplemented with 1% (v/v) formalin (formaldehyde, ACE Chemical Company, Adelaide, Australia), 2% (w/v) g D-glucose (AJAX Chemical Company), 0.01% sodium azide (Sigma). The buffer was stored at 4°C.

2.5.2 One-Colour Flow Cytometric Analysis using Indirect Immuofluorescence

This procedure has been reported previously [Gronthos et al 1998]. The following method describes the dection of cell surface antigens and can be applied to non-adherent cells and single cell suspensions of adherent cells detached by enzymatic digestion. Cell suspensions were resuspended in blocking buffer and incubated for 30 min on ice to block non-specific binding to Fc receptors. Approximately 2 x 10^7 cells (for cell sorting) and 2 x 10^5 cells (for phenotypic analysis) were pelleted in 12 ml and 4 ml polypropylene tubes (NUNC) respectively and resuspended in 50-200μl of the monoclonal antibody (Mab) supernatant or 20 μg/ml of purified antibody for 1hr on ice. Control tubes, (2 to 5 x10^5 cells/tube) were reacted with the appropriate mouse monoclonal isotype negative control antibodies (Table 3) under the same conditions. Following this, the cells were washed in IF buffer and the second layer antibodies were added in a final volume of 100 μl consisting of either goat anti-mouse IgM (μ-chain specific) or IgG (γ-chain specific) conjugated to either fluorescein isothiosyanate (FITC) or phycoerythrin (PE) (1/50 dilution) (Southern Biotechnology Associates, Birmingham, AL). The cells were then incubated for 45 min on ice, and subsequently washed twice in IF buffer. Cells stained for flow cytometric analysis only were resuspended in 500 μl of FACS FIX solution. Flow cytometric analysis was performed using the EPICS-PROFILE II (Coulter, Hialeah, FL) flow cytometer while fluorescence activated cell sorting was conducted using the

Table 3. List of antibodies used in this study.

ANTIBODY: ISOTYPE (Dilution)	SPECIFICITY	SOURCE
STRO-1: mIgM (1/2)	Erythroid & Stromal cells, CFU-F, Endothelial cells	Dr. P. Simmons [1]
6-19: mIgG_2 (1/50)	Neuroblastoma, CFU-F, Stromal & Endothelial cells	Dr. C Frantz[2]
Leu-4: mIgG_1 (1/10)	T-cell receptor complex (CD3)	BD[3]
J5-RD1: mIgG_{2a} (1/10)	Endopeptidase (CD10)	Coulter[4]
MY7: mIgG_1 (1/10)	Aminopeptidase (CD13)	Coulter
Leu-M3: mIgG_{2b} (1/10)	gp55/monocytes (CD14)	BD
AntiCD18: mIgG_1 (1/100)	Integrin β_2 (CD18)	Dr. P Batty[5]
Leu12: mIgG_1 (1/10)	gp/95 B-cells (CD19)	BD
B1:mIgG_1 (1/10)	Bp35, B-cells (CD20)	BD
P4C10: mIgG_1 (1/4)	Integrin $\beta 1$ (CD29)	Dr. W. Carter[6]
61.2C4: mIgG_1 (1/50)	Integrin $\beta 1$ (CD29)	Dr.J. Gamble[7]
B2B1: mIgG_1 (1/4)	PECAM-1 (CD31)	Dr. L. Ashman[8]
Leu-M9: mIgG_1 (1/10)	gp67, Myeloid (CD33)	BD
QBEN-10: mIgG_1 (1/10)	Sialomuscin CD34	Serotec[9]
Leu-17: mIgG_1 (1/10)	gp45, Lymphocytes (CD38)	BD
PIG12: mIgG_1 (1/4)	H-CAM (CD44)	Telios[10]
KC56: mIgG_1 (1/10)	Leukocyte antigen (CD45)	Coulter
HB245: mIgG_1 (1:4)	Integrin CD49a/CD29	ATCC[11]
AC11: mIgG_1 (1/50)	Integrin CD49b/CD29	ATCC
P1B5: mIgG_1 (1/4)	Integrin CD49c/CD29	Dr. E. Waner[12]
P4G9: mIgG_1 (1/4)	Integrin CD49d/CD29	Telios
PHM2: mIgG_1 (1/500)	Integrin CD49e/CD29	Prof. R. Atkins[13]
4F10: mIgG_{2b} (1/4)	Integrin CD49f/CD29	Serotec
P3G8: mIgG_1 (1/4)	Integrin CD51	Dr.J. Gamble
2C36: mIgG_1 (1/4)	Integrin CD51/CD61	Serotec
PH59: mIgG_1 (1/4)	Integrin $\alpha_V\beta_5$	Serotec
Y2/51: mIgG_1 (1/4)	Integrin β_3 (CD61)	DAKO[14]

continued....

Table 3. List of antibodies used in this study, continued.

ANTIBODY: ISOTYPE (Dilution)	SPECIFICITY	SOURCE
HLA-DR: mIgG-PE (1/10)	MHC Class II antigen	BD
HCC-1 : mIgM (1/2)	CD59	Dr. P. Simmons
GMP-140: mIgG$_1$ (1/4)	P-Selectin (CD62P)	Dr. M. Berndt[15]
Dreg-56: mIgG$_1$ (1/4)	L-Selectin (CD62L)	Dr. J Gamble
Leu-8 : mIgG$_1$ (1/10)	E-Selectin (CD62E)	BD
T9 : mIgG$_1$ (1/10)	Transferrin-R (CD71)	DAKO
F15.42 : mIgG$_1$ (1/100)	THY-1 (CD90)	Immunotech[16]
6G10: mIgG$_1$ (1/4)	VCAM-1 (CD106)	Dr. B. Masinovski[17]
YB5-B8 : mIgG$_1$ (1/4)	c-kit (CD117)	Dr. L. Ashman
7H6: mIgG$_1$ (1/4)	Stem Cell Factor	Amgen[18]
AA6: mIgG$_1$ (1/4)	Glycophorin-A	Dr. L Ashman
12.2D6.2C7: rIgG (1/20)	CSF-1-Receptor, c-fms	Dr. C. Sherr[19]
Ab-1: mIgG$_1$ (1/5)	IGF-1-Receptor	Oncogene Science[20]
ME20-4: mIgG$_1$ (1/10)	NGF-Receptor	Amersham[21]
AntiPDGF-AB : gIgG (50µg/ml)	hPDGF-AA, -AB, -BB	UBI[22]
AntiPDGF-R: mIgG$_1$ (1/50)	PDGF-Receptor-α	Genzyme[23]
AntiEGF: gIgG (50µg/ml)	hEGF	UBI
AntiEGF-R: mIgG$_1$ (1/50)	EGF receptor	Genzyme
K35-1H11-9-7: mIgG$_1$ (1/5)	Leptin Receptor	Dr. D.J. Hilton[24]
M-38: mIgG$_1$ (1/100)	Collagen (pro) type I	DSHB[25]
CIIC1 : mIgG$_{2a}$ (1/100)	Collagen type II	DSHB
WVID1 (9C5): mIgG$_{2a}$ (1/100)	Bone sialoprotein II	DSHB
AON-1: mIgG$_3$ (1/100)	Osteonectin	DSHB
8B6: mIgG$_{2a}$ (1/10)	Placental Alk. Phos.	DAKO
B4-78: mIgG$_1$ (1/100)	Bone/liver Alk. Phos.	DSHB
S-endo 1: mIgG$_1$ (1/10)	MUC-18 (CD146)	Dr. J Sampol[26]
Factor VIII: rIgG (1/1000)	Von Willebrand Factor	DAKO
QBEN-40: mIgG$_1$ (1/10)	Thrombomodulin	Serotec

Continued.....

Table 3. List of antibodies used in this study, continued.

ANTIBODY: ISOTYPE (Dilution)	SPECIFICITY	SOURCE
1A4: mIgG$_{2a}$ (1/100)	α-smooth muscle actin	Sigma[28]
Ki-67: mIgG$_1$ (1/10)	Cell cycle antigen (G_1, S, M, & G_2)	DAKO
Negative Control Mabs		
3D3: mIgG$_1$ (1/4)	Anti-Salmonella	Dr. L Ashman
1A6.11: mIgG$_{2b}$ (1/4)	Anti-Salmonella	Dr. L Ashman
1A6.12: mIgM (1/4)	Anti-Salmonella	Dr. L Ashman
1D4.5: mIgG$_{2a}$ (1/4)	Anti-Salmonella	Dr. L Ashman
mIgG$_3$ (1/10)	Anti-Aspergillus	DAKO
mIgG$_1$-FITC (1/10)	Anti-Aspergillus	DAKO

(1) Dept. Haematology, H.C.C.R., I.M.V.S, Adelaide South Australia; (2) Dept. Medicine, University of Rochester, NY USA; (3) Becton Dickinson, Knoxfield, Melbourne, Victoria Australia; (4) Coulter Immunology, Hialeah, Florida, USA; (5) Fred Hutchinson Cancer Research Center, Seattle WA USA; (6) Fred Hutchinson Cancer Research Center, Seattle WA USA; (7) Dept. Human Immunology, H.C.C.R., I.M.V.S, Adelaide South Australia; (8) Dept. Haematology, H.C.C.R., I.M.V.S, Adelaide South Australia; (9) Serotec, Oxford England; (10) Telios Pharmaceuticals Inc., San Diego CA USA; (11) American Type Culture Collection Rockville, MD USA; (12) University of Minnesota Medical School, St. Paul MN USA; (13) Monash Medical Centre, Melbourne Victoria, Australia; (13) Dept. Human Immunology, H.C.C.R., I.M.V.S, Adelaide South Australia; (14) DAKOPATTS A/S, Glostrup, Denmark; (15) Baker Research Institute, Melbourne Victoria; (16) Immunotech, Coulter Corp., Miami FL USA; (17) FCOS Corp., Seattle WA USA; (18) Amgen Inc., Thousand Oaks CA USA; (19) St. Jude Children's Research Hospital, Memphis, TN USA; (20) Oncogene Science Inc., Manhasset, NY USA; (21) Amersham, Birmingham England; (22) Upstate Biotechnology Inc., Lake Placid NY USA; (23) Genzyme Corp., Cambridge, MA USA; (24); The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria Australia; (25) Developmental Studies Hybridoma Bank, University of Iowa, IA USA; (26) CRTS, Marseilles, France; (27) Dept. Haematology, Westmead Hospital, NSW Australia; (28) Sigma Chemical Co., St. Louis MO USA.

FACStar^{PLUS} (Becton Dickinson, Sunnyvale, CA) flow cytometer in the Department of Haematology of the Hansen Centre for Cancer Research, Adelaide, South Australia. Cells were maintained at 4^{o}C throughout the cell sorting procedures. Positive expression was defined as the level of fluorescence greater than 99% of the corresponding isotype matched control antibody.

2.5.3 Two-Colour Flow Cytometric Analysis using Indirect Immuofluorescence

This procedure has been reported previously [Gronthos et al, 1988]. Briefly BMMNC were resuspended in blocking buffer and incubated for 30 min on ice. Approximately 2x10^{7} cells (for cell sorting) and 2 x 10^{5} cells (for phenotypic analysis) were pelleted in polypropylene tubes and resuspended in 200 μl of primary antibody cocktail for 1 hr on ice. The primary antibody cocktail consisted of saturating concentrations of the mouse IgM monoclonal antibody STRO-1 and a mouse IgG monoclonal antibody for each tube (Table 3). The mouse isotype IgM and IgG negative control Mabs were treated under the same conditions. After the cells were washed in IF buffer, the second labels were added in a final volume of 100 μl consisting of goat anti-mouse IgM μ-chain specific-FITC (1/50 dilution) and goat anti-mouse IgG γ-specific-PE (1/50 dilution). The cells were incubated for 45 min on ice, then washed twice in IF buffer before being analysed as described above. Laser compensation was adjusted for each fluorochrome independently according to the level of fluorescence emitted for each Mab isotype incubated with the corresponding opposing isotype control Mab.

2.5.4 Three-Colour Flow Cytometric Analysis using Indirect Immuofluorescence

Cells were incubated in blocking buffer then resuspended and incubated in primary Mab cocktail as described above. The Mab cocktail consisted of saturating concentrations of the mouse IgM Mab STRO-1 and the mouse IgG$_1$ Mab B4-78. The appropriate mouse monoclonal isotype IgM, and IgG$_1$ negative controls (Table 3) were treated under the same conditions. After the cells were washed in IF buffer, the second labels were added in a final volume of 100 μl consisting of goat anti-mouse IgM μ-chain specific-PE (1/50

dilution) and goat anti-mouse IgG_1 γ-specific-tri-color (TC) (1/50 dilution), and incubated for 45 min on ice. The cells were then washed twice in IF buffer and analysed as decribed above then fixed for 10 min with cold ethanol (70%) on ice. Following this, the cells were washed thrice with PBS and then incubated in blocking buffer for 15 min. The monoclonal antibody Ki-67 conjugated to FITC was added directly to the cells (1/10 dilution) in blocking buffer for 45 min on ice. The mouse IgG_1-FITC conjugate negative control antibody was treated under the same conditions. Dual-colour flow cytometric analysis was performed as described in section 2.5.2.

2.5.5 Magnetic Activated Cell Sorting (MACS)

This procedure has been described previously [Gronthos and Simmons, 1995, Gronthos et al, 1998]. Briefly, BMMNC (1×10^8 cells) were suspended into saturating levels of mouse IgM or IgG Mab supernatant in a 14 ml polypropylene tube and incubated on ice for 60 min. The cells were then washed two times in IF buffer and resuspended in 1 ml of buffer containing the appropriate second layer isotype specific goat anti-mouse IgM (μ-chain specific) or anti-mouse IgG (γ-chain specific) biotinylated antibody (1/50 dilution) (CALTAG Laboratories), for 45 min on ice. Following this the cells were washed twice in MACS buffer and resuspended in 900μl of MACS buffer to which 100μl of streptavidin microbeads (Miltenyi Biotec; Bergisch Gladbach, F.R.G.) were added (10μl of microbeads/10^7 cells in 9μl) for 15 min. Strepavidin-FITC or -PE conjugate (1/50) (CALTAG Laboratories, San Francisco, CA) was added directly to the same suspension at the end of the incubation time for a further 5 min. The cells were then washed twice in MACS buffer and a small aliquot of cells was removed for flow cytometric analysis while the remaining cells were passed through a Mini MACS magnetic column (column capacity 10^7 cells, Miltenyi Biotec) according to the manufacturers specifications. The eluate was collected as the negative cell fraction. To obtain the bead-positive cells the column was removed from the magnet and then flushed with MACS buffer into a separate tube. Small samples from each cell population were taken for flow cytometric analysis to determine the antigen expression in the bulk, bead-positive and bead-negative cell fractions.

2.5.6 Detection of Antigen Expression In Situ

Adherent cell cultures were digested as described above and sub-cultured into 8-chamber slides (Nunc, Inc, Naperville, IL). Non-adherent cells were prepared as cytospins on gelatin coated slides. Sections of 5μm were cut from frozen specimens using a 1720 Digital Kryostat (Wild Leitz; W. Germany) and placed onto gelatin coated glass slides. Prior to immunostaining, specimens were washed thrice with PBS and then fixed in acetone/methanol 1:1 at -20^{o}C for 15 min. Paraffin embedded samples were prepared as 5 μm thick sections on glass, slides pre-coated with Cell-Tak (Collaborative Sciences) according to the manufacturer's specifications. To remove the wax, the sections were treated with xylene for 5 min, then rehydrated through graded (100%, 90%, 70%, 50%) ethanol solutions for 5 min in each solution and finally rinsed in water.

After washing three times in PBS the specimens were left to block in either 5% normal goat serum (NGS: for the rabbit anti-human and mouse anti-human antibodies) or 5% normal rabbit serum (NRS: for the goat anti-human antibodies) for 1 hr at room temperature. The blocking serum was removed and saturating levels of primary antibodies (Table 3) were added for 2 hr at RT. The appropriate negative control mouse antibodies, purified pre-immune rabbit serum and normal goat sera were used under the same conditions. Following this, the slides were washed thrice in PBS and then incubated with the respective secondary antibodies (goat anti-rabbit biotin conjugated, goat anti-mouse biotin conjugated and rabbit anti-goat biotin conjugated; Vector Laboratories, Burlingame CA) diluted 1/200, for a further 45 min. The cells were then washed three times in PBS and were left to block in 1% BSA for 20 min. Streptavidin conjugated to FITC (Caltag Laboratories South San Francisco CA) was then added to the cells at a dilution of 1/50 for a further 20 min. The specimens were then washed thrice in PBS and mounted using 'Gurr' aqueous solution (Uvinert, BDH). The labeled specimens were examined using an Olympus BH2-RFCA fluorescence microscope. Photographs were taken on Ektachrome 400 Kodak colour film and with TMAX 400 Kodak black and white film.

Alternatively, antigen expression was also detected by peroxidase activity following reactivity of the cells with biotin conjugated second labels using a peroxidase Vectorstain ABC kit (PK-4010; Vector Laboratories) and a peroxidase substrate kit AEC (SK-4200; Vector Laboratories). After staining, the specimens were washed thrice in water and then counter stained with Mayer's haematoxylin (1/10 dilution) for 2 min. Specimens were then washed with tap water and mounted as described above then analysed using an Olympus IMT-2 inverted light microscope. Photographs were taken using Ektachrome 64 T colour Kodak film.

2.5.7 Analysis of Cell Cycling Status of STRO-1$^+$ Cells by Ki-67 Reactivity

Single cell suspensions of MG63 cells were stained for STRO-1 using a goat anti-mouse IgM-PE congugated second label as described in section 2.5.5. STRO-1$^+$ BMMNC were isolated by MACS as described above and then incubated with streptavidin PE for 15 min on ice. After staining, the cells were washed twice with PBS and then fixed for 10 min with cold ethanol (70%) on ice. Following this, the cells were washed thrice with PBS and then incubated in blocking buffer for 15 min. The monoclonal antibody Ki-67 conjugated to FITC was added directly to the cells (1/10 dilution) in blocking buffer for 45 min on ice. The mouse IgG_1-FITC conjugate negative control antibody was treated under the same conditions. Dual-colour flow cytometric analysis was performed as described in section 2.5.2..

2.6 CELL ADHESION ASSAY

This method is a modification of a previously described adhesion assay [Levesque et al, 1995]. Non-tissue culture grade 48-well polystyrene plates (Nunc) were pre-coated overnight at 4^{o}C with various purified extracellular matrix proteins (ECM) at a concentration of 5 $\mu g/cm^2$; collagen type I (rat), collagen type III (mouse), collagen type IV (mouse), collagen type V (human), vitronectin (human) (Collaborative Research Inc, Two Oak Park, Bedford, MA), laminin (human), fibronectin (human) (Boehringer Mannheim

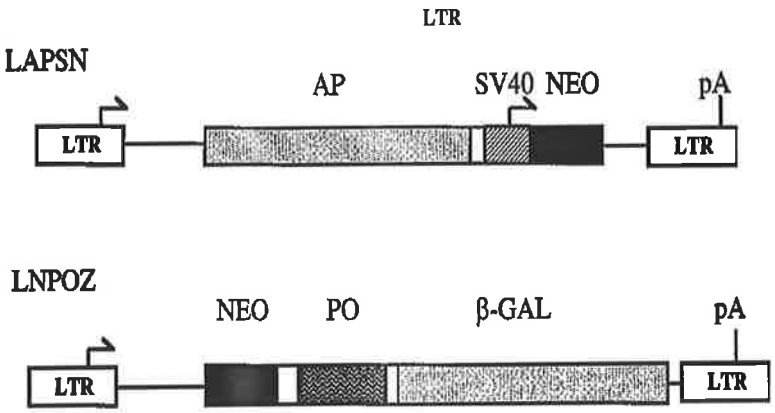
GmbH, Germany) and BSA (Cohn fraction V, Sigma). The plates were then washed once with adhesion buffer (HBSS supplemented with 20mM Hepes, 0.1% BSA, 1mM $CaCl_2$, 1mM $MgCl_2$, 0.2mM $MnCl_2$) and blocked at 37°C for 2 hr with 1.0% BSA in PBS. Adherent bone cells from primary cultures were detached by collagenase/dispase treatment, washed in adhesion buffer and resuspended in a final concentration of 10^6 cells/ml in the presence of 100μCi ^{51}Cr (sodium chromate; Dupont-NEN, Wilmington, DE) for 1 h at 37°C. The ^{51}Cr-labelled bone cells were washed thrice in adhesion buffer then plated at 10^5 cells/well in triplicate per matrix protein for 90 min at 37°C to permit cell attachment to occur. Following this, the plates were washed six times in adhesion buffer to remove all the unbound cells. The bound ^{51}Cr-labelled bone cells were then lysed by the addition of 0.5% SDS/0.1M NaOH. Radioactivity associated with the lysates was quantitated by liquid scintillation counting. Cell attachment for the adhesion studies on different ECM matrix proteins was calculated as a percentage of the input population. For the inhibition studies cell attachment (cpm) for each condition was calculated as a percentage of the corresponding wells containing isotype matched control antibody.

2.7 RETROVIRAL TRANSFECTION

The amphotropic retroviral packaging cell line PA317 was transfected with two different LXSN retroviral constructs [Miller and Buttimore, 1986; Adam et al, 1991; Miller et al; 1993] LAPSN and LNPOZ (Figure 7), and was generously obtained from Dr. Hairong Peng Department of Virology and Infectious Diseases. I.M.V.S., South Australia. The PA317-derived culture supernatant (containing the amphotropic retroviral particles) were harvested from either the LAPSN or LNPOZ transfected PA317 cells in log phase and filtered through a 0.45 mm filter (Sartorius, Germany). Aliquots of 10 ml were used immediately to infect subconfluent BM stromal cultures in 25 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. Stromal cultures were incubated with fresh viral-supernatant three times over a 72 hr period. Following infection, the stromal cells were washed twice in PBS, harvested by trypsin digest, then transferred to 25 cm^2 tissue

Figure 7. A schematic illustration of the retroviral vectors used in this study. (adapted from Miller and Buttimore, 1986; Adam et al, 1991; Miller et al; 1993). Abbreviations: LTR, retroviral long terminal repeat; AP, human placental alkaline phosphatase; SV40, SV40 early promoter and enhancers; NEO, neomycin phosphotransferase gene; pA, polyadenylation signal; PO, poliovirus 5' nontranslated region; LacZ gene or β-GAL, β-galactosidase. The vector names are acronyms based on the order of genetic elements within each vector: LAPSN; L (LTR), AP, S (SV40), N (NEO); LNPOZ; L, N, PO, Z (LacZ/β-GAL).

Retroviral Vectors



culture flasks at low density. Transfected cells were selected with G418 at a final concentration of 600 μg/ml for one week in regular α-MEM stromal growth medium. Stable LAPSN and LPOZN transfectants were assesed for alkaline phosphatase (human placental-AP, levamisole resistant) and β-galactosidase activity, respectively by cytochemical staining (see section 2.14). The G418 resistant stromal cell lines were maintained with regular growth media supplemented with 200 μg/ml G418.

2.8 TRANSPLANTATION OF STROMAL CELLS IN VIVO

All procedures described below were performed after obtaining approval from the Animal Ethic Committees of the University of Adelaide and the Royal Adelaide Hospital/I.M.V.S, South Australia for the duration of the project. The animals used were either six week old SCID mice and were housed in sterile microisolator cages in the sterile barrier room located in the animal facility of the University of Adelaide. All surgical procedures were performed in a non-biohazard laminar flow cabinet using sterile instruments. Prior to operating the mice were anaethetised 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 anaethesia with a reduced air flow with 1-2% halothane.

2.8.1 Kidney Capsule

(a) Preparation of Cells

Confluent stromal cultures grown in 25 cm^2 flasks were washed twice with PBS then gently teased off as a whole skin using a sterile scraper prior to transplantation.

(b) Ectopic Implantation of Cell Preparations

Six week SCID mice were firstly anaethetised as descried above and then shaved on the left hand dorsal side and was swabbed with Betadine. A 1 cm long incision was made laterally along the skin using small curved Metzenbaum scissors. The skin on each side of the cut was teased away from the underlying tissue. The skin flaps were separated and

kept open to expose the underlying muscle by clamping and gently stretching the skin flaps in opposite directions using spencer wells. Following this, a 1 cm lateral incison in the muscle layer was made using a pair of pointy scissors. Two blunt forceps were then inserted into the cavity, gently lifting out the kidney and allowing it to rest outside of the incison. A small pointy pair of forceps was then used to pinch a small area of the kidney capsule where a surgical gauge 19 needle (16.75 inch cancer implant needle; Popper & Sons Inc, New Hyde Park NY) containing the stromal cell skin was inserted under the renal capsule and the stromal cells injected out to from a blister underneath the capsule. Sterile cotton pads were used to stop any bleeding from the wound. Two blunt forceps were then used to replace the kidney into the peritoneal cavity. Using a tapered needle (Vicryl 4-0), three interupted stiches were used to bring the muscle facis layers together and 2 sterile 9 mm wound clips (Clay Adams; Becton Dickinson, Sparks MD) were used to suture the skin. The wound was washed liberally with Betadine and the mice were allowed to recover in clean microisolator cages.

2.8.2 Diffusion Chambers

(a) Preparation of Diffusion Chambers and Cells

Diffusion chambers were assembled form commercially available componenets (Millipore Corporation, Bedford, Massachusetts, USA). Plastic rings with the dimmensions of 13 mm external diameter, 9 mm internal diameter and 2 mm thick were covered on each side by membrane filters (HAWP 0.45 mm) using cement glue (MF Cement#1). The chambers were then gas sterilized at the Royal Adelaide Hospital Srerilization Unit using ethylene oxide.

Confluent stromal cultures grown in 25 cm^2 flasks were washed twice with PBS then trypsinised as described above. Single cell suspensions were washed twice in growth medium and resuspended into 200 µl of medium for each flask. The cells were injected into the diffusion chambers through a hole in the external ring which was then sealed with

a plastic plug. The diffusion chambers were left in growth medium and then thoroughly rinsed in PBS prior to transplantation.

(b) Transplantation of Diffusion Chambers

Six week SCID mice were firstly anaethetised as described above and then shaved on the ventral side below the diaphram where the site was subsequently swabbed with Betadine. A pair of forceps was used to pinch the skin and a 1 cm long longitudinal incision was then made along the skin using small curved Metzenbaum scissors. The skin on each side of the cut was teased away from the underlying tissue using the same scissors. The skin flaps were separated and kept open to expose the underlying muscle by clamping and gently stretching the skin flaps in opposite directions using spencer wells. Following this, a 1 cm longitudinal incison in the muscle layer was made using a pair of pointy scissors. One diffusion chamber was transferred into the peritoneal cavity of the abdomen. The wound was then sutured and sterilized as described above.

2.8.3 Transplantation of Polyvinyl Sponges

(a) Preparation of Sponges and Cells

Disks were made from 2mm sheets of polyvinyl alcohol sponge (Kanebo, PVA, Rippey Co., Santa Clara, CA) using a 10 mm cork borer. Membrane filters (Millipore, 0.45 μm) of the same diameter were glued to both sides of each disk using MF Cement#1 glue leaving the outer circumference of the disks open. The completed disks were sterilised with ethylene oxide, and then soaked for 5 days in sterile PBS with daily changes of PBS. Following this, the disks were incubated in 25 μg/ml of fibronectin for two hr at RT. Excess fibronectin was then drained off prior to the addition of cells to the disks.

Single stromal cell suspensions were prepared as described above. Cells were resuspended into 100 ml of medium and then injected into the disks. The cells were

allowed to settle in the disks for 30 min before the addition of growth medium. The disks were thoroughly rinsed in PBS prior to transplantation.

(b) Transplantation of Polyvinyl Disks

Six week SCID mice were firstly anaethetised as descried above and then shaved on the dorsal side parallel to the shoulders where the site was subsequently swabbed with Betadine. A 1 cm long longitudinal incision was then made along the skin using small curved Metzenbaum scissors. The skin on the lateral side of the opening was teased away from the underlying tissue using blunt scissors to form two pockets behind each shoulder. Following this, the sponge disks were rinsed in sterile PBS and then one disk was inserted into each of the 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.

2.8.4 Transplantation of Porous Ceramic Hydroxyapatite Cubes

(a) Preparation of Cells

Ceramic blocks of porous hydroxyapatite/TCP phosphate (Zimmer Corp., Warsaw, IN USA) were used to cut 27 mm^3 cubes using a diamond wafering blade on a Buehler. The cubes were gas sterilized with ethylene oxide and then incubated in a solution of 25 μg/ml of fibronectin for 2 hr under vacuum to eliminate air pockets. Prior to adding the cells, the blocks were blotted to remove excess fluid.

Single stromal cell suspensions were prepared as described above. The cells were resuspended into 100 μl of medium and then injected into the cubes. The cells were allowed to settle in the cubes for 30 min before the addition of growth medium. The cubes were thoroughly rinsed in PBS prior to transplantation.

(b) Transplantation of Hydroxyapatite Cubes

Six week SCID mice were firstly anaethetised as descried above and then shaved on the dorsal side parallel to the shoulders where the site was subsequently swabbed with Betadine. A 1 cm long longitudinal incision was then made along the skin using small curved Metzenbaum scissors. The skin on the lateral side of the opening was teased away from the underlying tissue using blunt scissors to form two pockets behind each shoulder. Following this, the HA cubes were rinsed in sterile PBS and then one cube was inserted into each of the 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.

2.9 Analysis of Cell Surface Antigens by Western blot

2.9.1 Buffers And Reagents

(a) Tris-Saline-EDTA (TSE)

50 mM Tris-HC1, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium azide, pH 8.0.

(b) 1% NP40-TSE

1% (v/v) Nonidet P40 (NP40, Sigma-Aldrich, Castle Hill, NSW, Australia), 50 mM Tris-HC1, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium azide, in water, pH 8.0.

(c) 0.1% NP40-TSE

0.1% (v/v) Nonidet P40, 50 mM Tris-HC1, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium azide, in water, pH 8.0.

(d) 100 x Protease Inhibitor Cocktail

To prepare a 100 x protease inhibitor cocktail, 40 mg of each of the following protease inhibitors (all purchased from Sigma Chem. Co., USA) were dissolved in 10 ml of

dimethylformamide (DMF); phenyl methyl sulphonyl fluoride (PMSF), L-[tosylamido-2-phenyl] ethyl chloromethylketone (TPCK), 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), p-nitrophenyl-p'-guanidino-benzoate-HCl (NPGB). The inhibitor cocktail was stored at -20^{o}C.

(e) Reducing Sample Buffer

1 ml 0.5 M Tris-HCl, pH 6.8, 800 ml glycerol, 1.6 ml 10% SDS, 400 µl β-mercaptoethanol, 200 µl 0.05% bromophenol blue and 4 ml Milli-Q water.

(f) Non-Reducing Sample Buffer

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

(g) Electrophoresis "Running" Buffer

0.3% Tris-HCl, 1.44% Glycine (ICN Biomedicals Inc., OH) , 0.1% SDS, pH 8.3 in water

(h) Transfer Buffer

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

(i) Separating Buffer

1.5 M Tris-HCl, pH 8.8

(j) Stacking Buffer

0.5 M Tris-HCl, pH 6.8

2.9.2 Preparation of Cell Lysates For Analysis by Western Blotting

Cell surface lysates were prepared essentially as described by Cole et al (1987). Routinely, single cell suspensions Single cell suspensions of adherent cell populations were washed thrice in PBS and resuspended to 2 x 10^{8} cells per ml and then placed on

ice. Lysates were prepared by adding an equal volume of cold 1% NP40-TSE and a 1/100 volume of protease inhibitor cocktail. The mixture was immediately chilled on ice, for 30 min with occasional gentle mixing. The lysates were then microfuged at 14,000 x g for 15 min and were then used immediately for Western blotting.

To analyse cell surface antigens by Western blotting, protein derived from 1-2 x 10^6 cell equivalents (20 µl) resuspended with 20 µl of 2X reducing or non-reducing sample buffer where first boiled for 3 min and the proteins were separated on a 10% acrylamide SDS-PAGE gel prepared according to the following formulation: 20 ml distilled water + 16.6 ml 30% acrylamide + 12.5 ml 1.5 M Tris pH 8.8 + 0.5 ml 10% SDS. TEMED (20 µl) and 10% ammonium persulphate (0.5 ml) were added to the gel solution immediately before pouring. The size of the immunoprecipitated proteins were determined by comparison with pre-stained molecular weight standards (Seablue: NOVEX; San Diego, CA). The gel was electrophoresed at 20 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 membrane (Amersham)Amersham, Poole, UK) overnight at 20 mA using a blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). The membrane was then cut into strips representing individual sample lanes. The filters were subsequently blocked for 1 h at RT in 5% (w/v) membrane blocking reagent (Vistra Systems, Amersham) in PBST. The blocked filters were washed in PBST for 1 x 15 min and 2 x 10 min periods at RT, then incubated with 3 ml of mab in the form of hybridoma tissue culture supernatant in 1% BSA/PBS. The membrane was washed 4 times in PBST, 5 min per wash at room temperature as and then incubated in 3 ml of 1:10,000 dilution of goat-anti mouse IgG+IgM (H+L) conjugated to alkaline phophatase (Vistra Systems, Amersham) for 1 h at RT. Following the immunolabelling, the membranes were washed as above and then reacted with 1 ml of Vistra ECF substrate (Vistra Systems, Amersham) according to

the manufacturer's recommendation. Immunoreactive proteins were detected directly on the PVDF membrane by fluorescence at 570 nm using a Molecular Dynamics FluorImager.

2.10 ENZYMATIC MODIFICATION OF CELL SURFACE ANTIGENS

2.10.1 Treatment of STRO-1$^+$ MG63 Cells with Proteases

To investigate whether the STRO-1 antigen was a protein sensitive to enzymatic digestion single cell suspensions of trypsinised MG63 cells were washed in serum-free RPMI and then incubated at 10^6/ml for 1 hr at 37°C in the same medium containing the following proteases: bromelain, chymopapain, chymotrypsin, collagenase, dispase, elastase, papain, pronase, proteinase K, and thermolysin at a final concentration of 20 mg/ml, except for dispase (8 mg/ml) collagense (6mg/ml) and proteinase K (1 mg/ml). All enzymes were obtained from Boehringer Mannheim (Mannheim, Germany) with the exception of papain (Sigma, USA), chymopapain (Boots, UK), collagense (Worthington) and elastase (Calbiochem., USA). Following protease treatment, cells were washed twice in assay medium and assayed for their reactivity to the STRO-1 mab by single-colour flow cytometric analysis.

2.10.2 Treatment of MG63 Cells with Phosphatidylinositol-Specific Phospholipase C (PI-PLC)

Loss of antigen expression following treatment with the enzyme PI-PLC, is characteristic of glycosyl-phosphatidylinositol (GPI)-linkage of membrane proteins. Single cell suspensions of MG63 cells in log phase were washed several times in α-MEM supplemented with 2% FCS, and resuspended at a final concentration of 5×10^6 cells per ml. To this, 50 mU/ml of PI-PLC (Boehringer Mannheim) was added, and the cells were incubated at 37°C for 2 hours (mock-treated cells were incubated in an equivalent manner in the absence of PI-PLC). Following this, the MG63 cells were washed in ice-cold IF buffer in preparation for single-colour FACS analysis.

2.10.3 Treatment of MG63 Cells with Tunicamycin

To determine whether the STRO-1 antigen was an N-linked carbohydrate MG63 cells were incubated in the presence of tunicamycin (Boehringer Mannheim). Single cell suspensions of MG63 cells were seeded into 96-well plates at a density of 5 x 10^3/well in a-MEM supplemented with 10% FCS, 2 mM L-glutamine and 100 μM ASC-2P and incubated overnight at 37^{o}C in 5% CO_2. On the following day the cultures were fed with fresh medium containing various concentrations of tunicamycin (0.0, 0.1, 0.3, 1.0, 3.0, and 10 μg/ml) then left to incubate for a further 48 hr. Following this, the cultures were processed for in situ immunofluorecence as described in section (2.5.6).

2.11 ISOLATION AND ANALYSIS OF RNA

Sterile, disposable plasticware was considered to be essentially free of RNases, and was used for the preparation and storage of RNA without pretreatment. General laboratory glassware however, was treated by baking at 180°C for a minimum of 8 hours or alternatively filled with a solution of 0.1% (v/v) diethyl pyrocarbonate (DEPC, Sigma, USA), a potent inhibitor of RNases. The DEPC-filled glassware was allowed to stand at 37°C for 2 h, rinsed several times with sterile water and autoclaved for 30 min. The autoclave treatment, removes all traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation. Equipment such as gel tanks, combs, and perspex plates were alkali-treated prior to use by washing the column with a solution of 0.5 M NaOH. To remove residual alkali, the columns were subsequently washed with several column volumes of DEPC-treated water.

2.11.1 Buffers and Solutions

All solutions were prepared in RNase-free glassware, using DEPC-treated and autoclaved water. Alternately, where possible, solutions were treated with 0.1% DEPC for a minimum of 12 h at 37°C prior to autoclaving as above.

(a) 1X TAE Buffer

The buffer was comprised of 40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.2

(b) 1 x TBE Buffer

The buffer was comprised of 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3

(c) 10 x Running Buffer

The buffer was comprised of 20 mM 3-[N-Morpholino]propane-sulfonic acid (MOPS) (Sigma), 1 mM EDTA, 5 mM sodium acetate, pH 5.5. The pH was subsequently adjusted to 7.0 with the addition of NaOH pellets.

(d) Loading Buffer

The buffer was comprised of 0.25% bromophenol blue (BDH), 0.25% xylene cyanol FF (Sigma) and 20% Ficoll (Type 400, Pharmacia, Sweden) in DEPC-treated water.

(e) 1.5% Agarose Gel

To prepare 100 mls of 1.5% agarose gels, 1 g of molecular biology grade, RNase-free agarose (IBI, Connecticut, USA) was dissolved in 72 ml of DEPC-treated water.

(f) 1% RNA Agarose Gel

To prepare 100 mls of 1% agarose gels, 1 g of molecular biology grade, RNase-free agarose was dissolved in 72 ml of DEPC-treated water. After cooling to approximately 56°C, 10 ml of 10 x Running buffer, and 18 ml of 35% formaldehyde solution (final concentration 2.2 M; BDH, AnalR, Victoria, Australia) was added. Gels were subsequently electrophoresed in 1 x Running buffer containing 2.2 M formaldehyde.

(g) Deionised Formamide

To prepare deionised formamide, 20% (w/v) Analytical Grade mix bed resin AG 501-X8 (Bio-Rad) was added to formamide solution (BDH) and stirred for several hours at room temperature. Mix bed resin was subsequently removed by filtration through Whatman No. 1 paper, aliquoted and stored at -20°C.

(h) Sample Buffer

RNA sample buffer was prepared by adding together 100 ml 10 x Running buffer, 500 ml deionised formamide, 178 µl 35% formaldehyde solution and 222 µl of DEPC-treated water to make a final volume of 1 ml.

(i) 20 x Sodium Saline Citrate buffer (SSC)

A stock of 20 x SSC was prepared by dissolving 175.3 g of NaCl and 88.2 g of sodium citrate in DEPC-treated water. The pH was subsequently adjusted to 7.0 and the volume made up to 1 litre.

(j) 100 x Denhardt's Reagent

A stock of 100 x Denhardt's reagent was prepared by dissolving 10 g Ficoll Type 400 (Pharmacia, Sweden), 5 g polyvinylpyrrolidone (Sigma) 5 g BSA in 500 ml DEPC-treated water.

(k) Prehybridisation Solution

The prehybridisation solution contained the following: 10 ml deionised formamide; 5 ml of 20 x SSC; 1 ml 100 x Denhardt's reagent; 200 µl 10% SDS; 2 ml of 100 mM HEPES, pH 7.1; 200 µl of 100 mM EDTA, pH 7.5; 200 µl of 200 mM sodium pyrophosphate, pH 7.0; 200 µl of 10 mg/ml, sheared and boiled salmon sperm DNA (Sigma) and 30 µl of 10 mg/ml tRNA (Gibco BRL) to make up a final volume of 20 ml. Routinely 5 ml of prehybridisation solution was used per 100 cm^2 membrane in a Hybaid Oven hybridisation bottle.

(l) Reverse Transcription (RT) Reaction Buffer

The buffer contained 40 mM KCl, 50 mM Tris-HCl pH 8.3, 8 mM $MgCl_2$, 0.5 mM deoxyribonucleotide triphosphate mix (dNTP: Pharmacia/LKB, Sweden), 10 units of RNasin (Promega), and 100 units of Superscript MMLV reverse transcriptase (Life Technologies, Australia) per 20 µl reaction.

(m) Polymerase Chain Reaction (PCR) Buffer

A 10 x PCR buffer mix contained: 670 mM Tris HCl pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5% Triton-X100, 2 µg/ml gelatin, 20 mM $MgCl_2$, 2 mM dNTP mix and 2.5 units of Amplitaq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA).

2.11.2 Isolation Of Total RNA: "The RNAzol^{TM} B Method"

Total RNA was extracted from tissues and cultured cell lines using the RNAzol^{TM} B Method (Biotecx Laboratories, Inc., Houston, TX, USA) which represents a modification of guanidinium isothiocyanate procedure described by Chomczynski and Sacchi (1987). For the isolation of RNA, single cell suspensions in a 1.5 ml Eppendorf tube were washed twice in cold PBS then pelleted and left on ice. The cells were then lysed by the addition of 0.2 ml of RNAzol^{TM} B per 10^6 cells and the RNA solubilised by passing the lysate several times through a pipette tip. The RNA was extracted by the addition of 1/10 volume of chloroform, mixed by vortexing and allowed to stand on ice for 5 min. The samples were subsequently centrifuged at 12,000 x g for 5 min at 4^{o}C after which the aqueous phase was removed and transferred to a new 1.5 ml Eppendorf tube. The total RNA was then precipitated by the addition of an equal volume of isopropanol, and incubated on ice for 20 min. The RNA was pelleted by centrifugation at 12,000 x g for 15 min at 4°C, washed in 75% (v/v) ethanol and dried under vacuum for approximately 10 min. The RNA pellet was resuspended in Milli-Q water and the absorbance values at 260 nm and 280 nm of each RNA sample was determined on a Beckman UV spectrophotometer. The A_{260}/A_{280} ratios of the RNA samples were consistently in the range of 1.6-2.0. The

relationship of one A_{260} unit equal to 40 μg/ml RNA was used in the calculation of RNA concentrations. The RNA was used immediately, or stored at -20°C following the addition of 1/10 volume 3M sodium acetate and 2.5 volumes absolute ethanol.

2.11.3 Synthesis and Purification of Oligonucleotides

The following synthetic DNA primers were synthesised "in house" on an Applied Biosystems 391 DNA synthesiser, in the Molecular Pathology Unit, Division of Haematology, I.M.V.S., Adelaide, South Australia. Oligonucleotides were cleaved from the synthesis columns by treating with ammonium hydroxide 30% (v/v) (BDH/Merck) for 20 min at RT. This was repeated three times and the oligonucleotides deprotected by the overnight incubation at 56°C. The ammonium hydroxide was removed by vacuum desiccation and the oligonucleotide pellet was dissolved in 200 μl of Milli-Q water and the concentration determined by spectrophotometry at an OD of 260 nm assuming that an A_{260} of 1.0 represents 33 μg/ml of DNA. The oligonucleotides (Table 4) were stored at -20°C at a concentration of 100 ng/μl.

2.11.4 Detection of Osteocalcin mRNA by Northern Blot Analysis

Routinely, 20 μg of total RNA was pelleted by centrifugation at 12,000 x g, 4°C for 15 min. The RNA was subsequently washed in 70% ethanol, vacuum dried and denatured in the presence of 12 μl of "sample buffer" and 2 μl of 20 μg/ml ethidium bromide. Samples were heated to 60°C for 5 min prior to loading on a denaturing 1% agarose gel in TBE followed by electrophoresis in "running" buffer. The gels were subsequently washed for 30 min in water and then ethidium bromide-stained. The integrity of the RNA was visualised and photographed under ultraviolet illumination. The electrophoresed RNA was subsequently transferred overnight by capillary action in 10 x SSC onto a nylon membrane (Shuleicher & Schuell, Dassel, Germany). Following transfer, the filters were washed in 2 x SSC, air-dried and the RNA covalently cross-linked to the filters by exposure to 0.4 J/cm^2 of UV radiation in a Stratagene UV Stratalinker™ 1800. Filters were prehybridised for 4-16 hours at 42°C in a prehybridisation solution. After prehybridization, the blots were

Table 4. Oligonucleotides used in this study.

Transcript [Reference]	Primer Set Upper Primer (UP) Lower Primer (LP)	Product Size (base pairs)
hGAPDH	UP: TTGACCTCAACTACATGG	992
[Tokunaga et al, 1987]	LP: GTGAAAACTTCGGTTGCTGG	
hOsteopontin	UP: TTGCTTTTGCCTCCTAGGCA	430
[Crosby et al, 1995]	LP: GTGAAAACTTCGGTTGCTGG	
hCBFA1	UP: GTGGACGAGGCAAGAGTTTCA	632
[Ogawa et al, 1993]	LP: TGGCAGGTAGGTGTGGTAGTG	
hCollagen type I	UP: AGGGCTCCAACGAGATCGAGATCCG	222
[Chu et al, 1985]	LP: TACAGGAAGCAGACAGGGCCAACGTCG	
hParathyroid	UP: AGGAACAGATCTTCCTGCTGCA	571
hormone receptor	LP: TGCATGTGGATGTAGTTGCGCGT	
[Bilbe et al, 1996]		
hBone sialoprotein	UP: TCAGCATTTTGGGAATGGCC	657
[Kim et al, 1996]	LP: GAGGTTGTTGTCTTCGAGGT	
hOsteonectin	UP: ATGAGGGCCTGGATCTTCTT	576
[Villarreal et al, 1989]	LP: CTGCTTCTCAGTCAGAAGGT	
hOsteocalcin	UP: ATGAGAGCCCTCACACTCCTC	289
[Fleet and Hock, 1994]	LP: CGTAGAAGCGCCGATAGGC	
hLipoprotein lipase	UP: ATGGAGAGCAAAGCCCTGCTC	564
[Wion et al, 1987]	LP: GTTAGGTCCAGCTGGATCGAG	
Human-adipocyte lipid	UP: AGTCAAGAGCACCATAACCTTAGA	170
binding protein	LP: CCTTGGCTTATGCTCTCTCATAA	
[Vidal-Puig et al, 1997]		
hPPARγ2	UP: AACTGCGGGGAAACTTGGGAGATTCTCC	
[Vidal-Puig et al, 1997]	LP: AATAATAAGGTGGAGATGCAGGCTCC	341
hObese gene product	UP: ATGCATTGGGAACCCTGTGC	
[Masuzaki et al, 1995]	LP: GCACCCAGGGCTGAGGTCCA	492

continued.

Table 4. Oligonucleotides used in this study, continued.

Transcripts [Reference]	Primer Set (5'-3') Upper Primer (UP) Lower Primer (LP)	Product Size (base pairs)
hCollagen type II	UP: ATGATTCGCCTCGGGGCTCC	390
[Baldwin et al, 1989]	LP: CATTACTCCCAACTGGGCGC	
hCollagen type X	UP: AGCCAGGGTTGCCAGGACCA	387
[Bonaventure et al, 1994]	LP: TTTTCCCACTCCAGGAGGGC	
hAggrecan	UP: CACTGTTACCGCCACTTCCC	184
[Doege et al, 1991]	LP: ACCAGCGGAAGTCCCCTTCG	

Universal Primers (pGEM-4Z plasmid):

SP6 RNA polymerase promoter	5'-ATTTAGGTGACACTATAG-3'
T7 RNA polymerase promoter	5'-TAATACGACTCACTATAGGG-3'

Probes

hOsteocalcin probe [Celeste et al, 1986]	5'-CCACTCGTCACAGTCCGGATTGAGCTCACACACCTCCCT-3'
poly dT_{17} adapter [Frohman et al, 1988]	5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'

hybridized in 50% formamide to a synthetic oligonucleotide probe for human osteocalcin (Table 4), complementary to the mRNA sequence coding for amino acids 20-32 of mature osteocalcin [Celeste et al, 1986] at 42^{o}C for 16 hours. The probe was end labeled with (γ-^{32}P)-ATP (Bresatec, Adelaide, Australia). Filters were then washed in 2X SSC at RT, followed by washings at 42^{o}C for 1 hr. The membrane was exposed to Kodak X-Omat film (Eastman, Kodak, Rochester, NY) at -80^{o}C for 72 h with a Cronex intensifying screen (du Pont, Wilington, DE).

2.11.5 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Amplification of RNA

(a) Synthesis of Complementary DNA (cDNA)

Total cellular RNA was prepared with RNAzol, as described above. Routinely, 2 μg of total RNA was washed once in 70% ethanol and vacuum dried for 10 min then reverse transcribed into single-stranded cDNA at 37°C for 120 min in 50 μl of RT reaction buffer containing 100 ng of poly (dT)$_{17}$ adaptor (Table 4).

(b) Polymerase Chain Reaction Amplification of cDNA

This method is a modification of a previously described protocol [Sambrook et al, 1989]. A volume of 5 μl of each cDNA synthesis reaction was utilised as template DNA in each PCR. Routinely, the cDNA mixture was added to a 500 μl microcentrifuge tube, (Perkin Elmer), containing 5 μl of 10 x PCR amplification buffer followed by the addition of 100ng of the appropriate "forward" and "reverse" primer sets (Table 4) and sterile Milli-Q water to a final volume of 50 μl. Reaction mixes were overlayed with mineral oil and amplification achieved by incubation in a Perkin-Elmer/Cetus thermal cycler. Primer design enabled typical cycling conditions of 94°C/(2 min), 60°C/(30 sec), 72°C/(1 min) for 40 cycles, with a final 10 minute incubation at 72°C. To control for the integrity of the various RNA preparations, the expression of GAPDH was also assessed. Following amplification, 10μl of each reaction mixture was analysed by 1.5% agarose/TAE gel electrophoresis, and

visualised by ethidium bromide staining. The molecular weight of the PCR products (size in kilobases) was estimated visually by comparisons with the 1kb ladder markers (Life Technologies, Glen Waverley, Victoria, Australia).

2.12 IN SITU HYBRIDIZATION FOR THE HUMAN SPECIFIC DNA ALU SEQUENCE

2.12.1 Buffers and Reagents

(a) Polymerase Chain Reaction (PCR) Buffer

A 1X PCR buffer mix contained: 67 mM Tris HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.5% Triton-X100, 0.2 μg/ml gelatin, 2.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 1.9 mM dTTP, 0.1 mM digoxygenin-11-dUTP (Boehringer Mannheim), and 0.25 units of Amplitaq DNA Polymerase.

(b) Prehybridization Solution

The prehybridization solution consisted of 50% deionized formamide containing 4X SSC.

(c) Hybridization Solution

The hybridization solution was prepared by adding 1ng/μl digoxigenin-labeled probe in 1X Denhhardt's solution, 5% dextrane sulfate, 0.2mg/ml, salmon sperm DNA, 4X SSC, 50-% deionized formamide. A volume of 100 μl was added to every slide.

(d) Buffer 1

The buffer consisted of 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl and was filter sterilized.

(e) Buffer 2

The buffer consisted of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$. The solution was filter sterilized.

(f) Blocking solution

The blocking solution consisted of 0.1% Triton X-100 in PBS supplemented with 2% normal goat serum.

(g) Alkaline Phophatase Substrate Solution

The substrate-colour solution was prepared by adding 45 ml of nitroblue tetrazolium solution (75 mg NBT/ml in 70% dimethylformamide) and 35 µl of 5-bromo-4-chloro-3-indolyl-phosphate solution (50 mg BCIP/ml in 100% dimethylformamide) into 10 ml of buffer 2.

2.12.2 Preparation of Human Alu DNA Probe

The human specific alu sequence (pBLUR8; ATCC) was subcloned into the BamH1 restriction site of a pGEM-4Z plasmid (Promega) and was generously supplied by Dr. A. Zannettino. The digoxigenin-labeled alu specific sequence was prepared by PCR containing 1 X PCR buffer supplemented with 100 ng of SP6 and T7 primers (Table 4) and 1 ng of plasmid DNA. Reaction mixes were overlayed with mineral oil and amplification achieved by incubation in a Perkin-Elmer/Cetus thermal cycler. Cycling conditions were performed as follows: 94°C for 3 min, then 94^{o}C/(1 min), 60°C/(30 sec), 72°C/(1 min) for 40 cycles, with a final 7 min incubation at 72°C. The reaction mix was then stored at -20^{o}C.

2.12.3 In Situ Hybridization

The HA ceramic cubes were recovered 8 weeks post transplant and prepared for paraffin embedding as described in section 2.5.6. To determine the origin of the cells within the implants in situ hybridization analysis was performed using a DNA pobe specific to the unique human repetitive alu sequence [Tokunaga et al, 1996; Kuznetsov et al,1997]. Sections of 5 µm thickness on Cell-Tak coated slides were deparaffinized with xylene for 5 min, then rehydrated through graded (100%, 90%, 70%, 50%) ethanol solutions for 5

min in each solution and finally rinsed in water. The rehydrated sections were treated with 0.2N HCl for 7 min at RT and then washed in water for 5 min after which the sections were incubated in 1 mg/ml pepsin (Sigma) in 0.1N HCl for 10 min at 37^{o}C. After washing twice for 5 min in PBS, the sections were treated with 0.25% acetic acid containing 0.1M triethanolamine (pH 8.0) for 10 min at RT. Following this, the sections were prehybridized for 15 min at 37^{o}C. The hybridization solution was then added to the sections for denaturation at 95^{o}C for 3 min followed by hybridization at 45^{o}C for 3 hr. The sections were then washed twice with 2X SSC for 10 min at RT and once in 0.1X SSC for 10 min at 42^{o}C.

The digoxigenin-labeled DNA was detected by immunohistochemistry. Sections were firstly washed twice with buffer 1 for 10 min at RT and then covered with blocking solution for 30 min at RT. The sections were then incubated with an antidigoxigenin alkaline phosphatase-conjugated Fab fragments (1/500 dilution in blocking solution) (Boehringer Mannheim) for 2 h at RT. Following this, the slides were washed twice in buffer 1 for 10 min at RT then once in buffer 2 for a further 10 min at RT. Following incubation with the substrate, the cells were washed in distilled water and mounted in Uvinert. The alkaline phosphatase activity was assessed using an Olympus IMT-2 inverted light microscope. Photographs were taken using Ektachrome 64 T colour film.

2.13 DETECTION OF OSTEOCALCIN PROTEIN BY RADIOIMMUNOASSAY

This method is a modification of that described by Lajeunesse et al (1991) and was adapted for serum deprived conditions. Six week old mineralized bone marrow cultures and controls were washed twice in PBS then switched to SDM (without dexamethasone) supplemented with $CaCl_2$-$2H_2O$ (2mM) (Sigma), KH_2PO_4 (1.8 mM), Menadione sodium bisulfite (10^{-8}M) (Vitamin K_3; Sigma), and Calcitriol (10^{-7}M) (1,25-dihydroxyvitamin D_3; Donated by F. Hoffmann-La Roche Ltd) for 48 hours at 37^{o}C, 5% CO_2. After incubation, the media was removed and the osteocalcin concentration in the medium was measured by a specific radioimmunoassay (Department of Endocrinology of the I.M.V.S., Adelaide,

South Australia) using a polyclonal antibody raised against bovine osteocalcin (>90% homologous to human osteocalcin). In preliminary experiments, media supplemented with 1% FCS contained approximately 3ng of osteocalcin per ml, in contrast to SDM which contained less than the detection limit of the assay of 0.4 ng of osteocalcin/ml. The results were expressed as the mean values from duplicate cultures of osteocalcin ($ng/10^6$ cells/48 hr induction with 1,25-dihydroxyvitamin D_3).

2.14 CYTOCHEMISTRY

2.14.1 Alkaline Phosphatase Activity

Single cell suspensions prepared as cytospins or cultures of adherent cell populations were washed twice in RPMI and then fixed with 95% ethanol for 10 min at RT. The cells were washed thrice with PBS then stained for alkaline phosphatase activity using a Sigma in vitro diagnostic kit 85L-2. Once stained, the cultures were washed in distilled water, counter stained with Mayer's haematoxylin then rinsed in tap water and mounted in Uvinert. Alkaline phosphate activity was assessed using an Olympus IMT-2 inverted light microscope. Photographs were taken using Ektachrome 64 T and Technical PAM Kodak black and white film.

2.14.2 Von Kossa Staining of Bone Derived Mineral

Six week old bone mineral inductive cultures were washed twice in PBS where the confluent cells were gently teased off as a single layer using a plastic pipette. The cell layers were fixed in 95% ethanol for 60 min before being infiltrated and embedded in 50% glycol methacrylate (Tokyo Kasei; Tokyo, Japan) and 50% methyl methacrylate (BDH Chemicals) over one week at RT. Transverse sections (5nm) of the cell layers were cut using a glass knife on a Jung K motorised sledge microtome (Reichert, W. Germany). Alternatively, live cultures were rinsed twice with PBS then fixed in situ with 10% neutral formalin for 30 min. Staining for von kossa was performed according to the method of Pearse (1972). Sections or culture wells were washed twice in distilled water and then

stained in 5% aqueous $AgNO_3$ (May & Baker Laboratory Products, West Footscray, Victoria) for 60 min under ultraviolet light. After staining with $AgNO_3$, the sections were washed twice with distilled water and then placed in 5% sodium thiosulphate (BDH Chemicals) for 1 min. The sections and culture plates were then washed in distilled water, counter stained with Mayer's haematoxylin and mounted in Uvinert. Von Kossa staining was analysed using an Olympus SZ-PT light microscope. Photographs were taken using Ektachrome 64 T and Technical PAM Kodak film.

2.14.3 Detection of β-Galactosidase Activity

Single cell suspensions prepared as cytospins or cultures of adherent cell populations were washed twice in RPMI and then fixed with 0.5% glutaraldehyde (EM Grade) for 20 min at 4°C. The cells were subsequently washed thrice in PBS then incubated with Xgal solution (50 mM NaH_2PO_4 pH7.3, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6.3H_20$, 100 mM NaCl, 2 mM $MgCl_2$, in distilled water + 0.1% Xgal) for 2-4 hr in a humidified incubator at 37°C. Following incubation with the substrate, the cells were washed in distilled water and mounted in Uvinert. The β-galactosidase activity was assessed using an Olympus IMT-2 inverted light microscope. Photographs were taken using Ektachrome 64 T and Technical PAM Kodak black and white film.

2.15 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Six week old mineralized bone marrow cultures were washed in 0.05M sodium cacodylate buffer and then fixed in 2.5% glutaraldehyde (EM Grade) in 0.05M sodium cacodylate buffer for two hours at room temperature. After washing with 0.05M cacodylate buffer, the cultures were were postfixed with 2% osmium tetroxide (VIII) (BDH Chemicals) in cacodylate buffer for 1 hour at RT and then washed in cacodylate buffer. Following this, the cultures were dehydrated with graded alcohol (70%, 90%, 100% ethanol solutions) for two 15 min changes, for each alcohol grade. Epoxy resin (TAAB Laboratories; Berkshire, England) was then used to infiltrate the cultures overnight at 37°C. Polymerization of the resin was carried out at 60°C for 24 hr under vacuum. Ultrathin sections were cut on a

LKB 8800 Ultrotome II (Broma, UK) and mounted on copper grids. Sections were then examined using a JEOL 1200 EX II (Tokyo, Japan) transmission electron microscope. Photographs were taken using ILFORD EM Technical film. Mineral deposits were analyzed using a Tracor-Northern Series II EDX System (EDX: Energy Dispersive X-ray microanalyser). Electron diffraction analysis was performed using a camera length of 100 cm, on the mineralized bone cultures and on a commercially available hydroxyapatite standard (Hydroxyapatite type 1; Sigma).

2.16 STATISTICAL ANALYSIS

2.16.1 Analysis of the Growth of CFU-F Colonies In Vitro

Analysis of variance (ANOVA) was employed to determine differences in CFU-F colony numbers and in colony size between the different groups of cytokine combinations and the control cultures with serum. The level of statistical difference between the groups was estimated by the Fisher Limited significance difference (FLSD) test at $p \leq 0.05$. The paired t-test was also used to determine any differences (at $p \leq 0.05$) in the mean number of CFU-F colonies over a range of plating concentrations between the serum containing and serum deprived cultures.

2.16.2 Analysis of Osteocalcin Levels Following Mineralization In vitro

The paired t-test ($p \geq 0.05$) was employed to compare the levels of osteocalcin protein detected in BM stromal cells cultured in the presence of ASC-2P, DEX and PO_{4i} for six weeks with osteocalcin levels in tthe unstimulated control cultures. Osteocalcin levels were examined in all cultures following exposure to 1,25-$(OH)_2D_3$ for 48 h in serum deprived medium.

2.16.3 Analysis of the Interactions between Bone Cells and ECM proteins

One-way ANOVA was used to determine any differences in the binding of NHBC cells to various purified matrix proteins in serum deprived conditions. Statistical significance ($p \leq$

0.05) in the cell attachment between different matrix proteins was determined using the Fisher PLSD test. The paired t-test ($p \leq 0.05$) was employed to compare the cell attachment between the different ECM protein coated wells and their corresponding control wells containing BSA for each antibody combination.

2.16.4 Analysis of Matrix Calcium Levels in NHBC Cultures

One-way ANOVA was used to determine any differences in the calcium concentrations in the cell matrix between the different STRO-1/AP NHBC subsets, following stimulation with ASC-2P, DEX and PO_{4i} for up to seven weeks. Statistical significance ($p \leq 0.05$) in the level of calcium detected between the different STRO-1/AP subgroups at each time point was determined using the Fisher PLSD test.

2.16.5 Analysis of the Proliferation rates between different Bone Cell populations.

Statistical significance ($p \leq 0.05$) in the cell proliferation rates between different STRO-1/AP NHBC subsets was determined using the paired t-test ($p \leq 0.05$).

CHAPTER THREE: DETERMINATION OF THE GROWTH FACTOR REQUIREMENTS NECESSARY TO SUPPORT THE CLONOGENIC GROWTH OF HUMAN BONE MARROW STROMAL PRECURSORS IN VITRO.

3.0 INTRODUCTION

In vivo, haemopoiesis occurs in association with a phenotypically and probably functionally diverse population of vascular and connective tissue type cells that constitute the bone marrow microenvironment. A considerable body of evidence derived from immunological and functional studies both in vivo and in vitro, demonstrate the existence of pluri-potential haemopoietic stem cells with the capacity to self-renewal and to develop into committed progenitors. Haemopoietic progenitors have been identified by their ability to form clonogenic colonies in semi-solid cultures consisting of mature erythroid and myeloid cell populations. The in vitro proliferation and differentiation of haemopoietic progenitors is well defined, where the development of colonies representing different mature blood cell lineages is dependent on the presence of specific combinations of exogenous growth factors (Figure 1) [Clark and Kamen, 1987; McNiece et al, 1991; Haylock et al, 1992; Metcalf, 1993]. The cytokines are produced by a wide range of cell types including, vascular endothelial cells, monocytes/macrophages, lymphocytes and stromal cells, where a particular cytokine may stimulate the growth of haemopoietic progenitor cells and also modulate cell function and survival of mature blood cells [Dexter, 1987]. Conversely, the effects of these cytokines on the development and function of bone and marrow stromal cell populations is largely unknown. Bone marrow stromal precursor cells (SPC) have been identified by their ability to form clonogenic clusters of adherent fibroblast-like cells (CFU-F), when cultured in the presence of 10% to 20% foetal calf (FCS) serum [Friedenstein, 1970; Castro-Malaspina et al, 1980]. In contrast to haemopoietic progenitor cells, whose growth requirements and responses to a wide variety of growth factors have been studied in great detail, little is known about the growth requirements of BM SPC.

Studies in rodents have demonstrated that the marrow stroma has the capacity to regenerate as a response to the bone marrow cavity being mechanically excavated [Tavassoli and Crosby, 1968; Knopse et al, 1972; Patt and Maloney, 1975]. Other studies have shown that removal of BM from the tibia of rats causes an increase in systemic bone and cartilage formation [Bab et al, 1985; Gazit et al, 1990]. Similar effects are observed when the bone marrow microenvironment has been previously damaged through disease such as aplastic anaemia, or through exposure to cytotoxic agents such as bulsulfan or radiation [Knopse et al 1966; Fried et al 1976; Fried et al, 1977; Appelbaum et al 1980; Ershler et al, 1980; MacManus and Weiss 1984; Simmons et al, 1987]. The ability of the stromal tissue to regenerate is attributed to a population of SPC characterised by a high proliferative and differentiation capacity with the ability to reconstitute the BM microenvironment and to form osteogenic tissue in vivo [Owen, 1985; Owen and friedenstein, 1988]. Marrow ablation has been shown to result in an increase in the number of stromal progenitor cells isolated from nonablated sites that may occur due to the release of cytokines into the circulation with the ability to stimulate stromal cell proliferation and differentiation [Gerasimov and Chailakhyan, 1979].

Megakaryocytes are known to stimulate the proliferation of bone marrow fibroblasts in vitro [Castro-Malaspina et al 1981], where the α-granules of platelets are a major source of both EGF and PDGF. Previous studies have implicated PDGF as the major growth factor in serum, responsible for stimulating the clonal growth of SPC in vitro (CFU-F assay) [Hirata et al 1985; McIntyre and Bjornson, (1986); Kimura et al 1988; Wang et al, 1990]. In contrast, it has been proposed that PDGF and EGF are not responsible for the growth of SPC in vitro, and is attributed to an undefined factor(s) released by haemopoietic cells [Latsinik et al, 1990; Freidenstein et al, 1992]. Another study also found that the continued presence of EGF in culture had no bearing on the number of colonies formed compared to control cultures, but EGF was shown to increase the average size of individual colonies [Owen et al, 1987]. However, in all these studies cultures contained unfractionated BM cells plated at high densities that promote the

affects of autocrine growth or inhibitory factors. The unfractionated BM cells also contain accessory cells, such as macrophage, T-cells, and megakaryocytes, capable of producing cytokines that inhibit or enhance the response of SPC to exogenously added growth factors. In addition, all the studies used medium supplemented with either foetal calf serum, horse serum or human plasma which contained varying amounts of hormones, co-factors, growth factors and inhibitors. The use of serum and whole BM fractions has resulted in conflicting evidence regarding the role of any one cytokine in the development of CFU-F.

The clonogenic growth of SPC from different normal BM samples has been shown in our laboratory to be highly variable in the presence of FCS serum. Often there is also variability between different serum batches in respect to the number of colonies formed and the proliferation or size of individual colonies from the same marrow sample. We therefore explored the possibility of increasing the cloning efficiency of CFU-F colonies by developing a serum deprived medium to define those growth factors directly responsible for the proliferation of SPC in vitro. In a previous report it was shown that the STRO-1 mab binds to minor population of BMMNC which contains all detectable CFU-F as measured under standard serum-containing conditions [Simmons and Torok-Storb 1991a]. Importantly, the STRO-1 antibody shows no detectable binding to monocytes, megakaryocytes or T-lymphocytes [Simmons and Torok-Storb 1991a; 1991b]. To circumvent the problem of accessory cell contamination, all CFU-F assays were performed using cells selected for STRO-1 antigen expression.

3.1 Establishment of a Clonogenic Assay for CFU-F Under Serum-Deprived Conditions: A Role for Glucocorticoids and Ascorbate

Due to the rarity of the CFU-F population and the constraints of a serum based culture system, colony formation in vitro is dependent on cell density where colony growth is not linear at high or low cell concentrations. Before an attempt could be made to isolate a homogeneous population of CFU-F in order to properly study their biology an alternative

to the serum-based CFU-F assay was developed to support the growth of individual CFU-F at low plating densities. This assay was based on a standard serum deprived medium (SDM) used to culture HSC [Migliaccio et al, 1988; Lansdorp and Dragowska, 1992]. To exclude the influence of accessory cells, STRO-1^+ bone marrow cells which contain all the assayable CFU-F were isolated using magnetic cell sorting (see section 4.2) in order to obtain large numbers of purified stromal progenitors. In initial experiments, the STRO-1^+ cells were plated in serum-deprived medium (SDM) comprising α-MEM supplemented with insulin, transferrin, bovine serum albumin and human LDL as a lipid source. In all experiments, culture plates were coated with fibronectin to promote the attachment of stromal progenitor cells in the absence of foetal cal serum (FCS). Based on previous studies [Rosenfeld et al, 1985; Hirata et al 1985; McIntyre and Bjornson, (1986); Kimura et al 1988; Wang et al, 1990; Huang and Terstappen, 1992], a combination of EGF, PDGF, IGF-1 and bFGF each at 10ng/ml were added to provide a likely stimulus for stromal colony growth. Under these conditions however, development of CFU-F colonies did not occur. However, after addition of the glucocorticoid dexamethasone (DEX) at 10^{-8}M and the long acting derivative of ascorbate, L-ascorbate-2-phosphate (ASC-2-P) 100μM to the SDM, colony growth was observed to the level of or greater to the FCS-stimulated control cultures (Figure 8). Importantly, CFU-F growth did not occur in cultures established in SDM/DEX/ASC-2P alone but was absolutely dependent upon an exogenous source of growth factors.

3.2 The Contribution of EGF, PDGF, IGF-1 and bFGF to CFU-F Growth Under Serum Deprived Conditions

The growth factors EGF, PDGF, IGF-1 and bFGF, used in the initial experiments, were examined to determine which factor(s) are responsible for supporting colony growth under serum-deprived conditions. The factors were added at a concentration of 10ng/ml, alone and in each of the possible 2- and 3-factor combinations. As shown in Figure 9A, the clonogenic growth of CFU-F occurred only in response to EGF or PDGF. bFGF failed to stimulate colony growth over a concentration range of 1 to 100ng/ml either alone or in the

Figure 8. The growth of BM CFU-F in serum-deprived conditions. The data indicate the mean number of CFU-F colonies ± SE of triplicate cultures, expressed as a percentage of the control cultures grown in 20% FCS. Serum-deprived medium (SDM: α-MEM supplemented with 10μg/ml bovine pancreas derived insulin, 2% w/v bovine serum albumin, 4 μg/ml human low density lipoprotein, 200 μg/ml iron saturated human transferrin, 2 mM L-glutamine, β-mercaptoethanol 5×10^{-5} M); L-ascorbic acid 2-phosphate (ASC-2P, 100μM); dexamethasone (DEX, 10^{-8}M); growth factors (GFs: PDGF+EGF+IGF-1+bFGF, 10ng/ml).

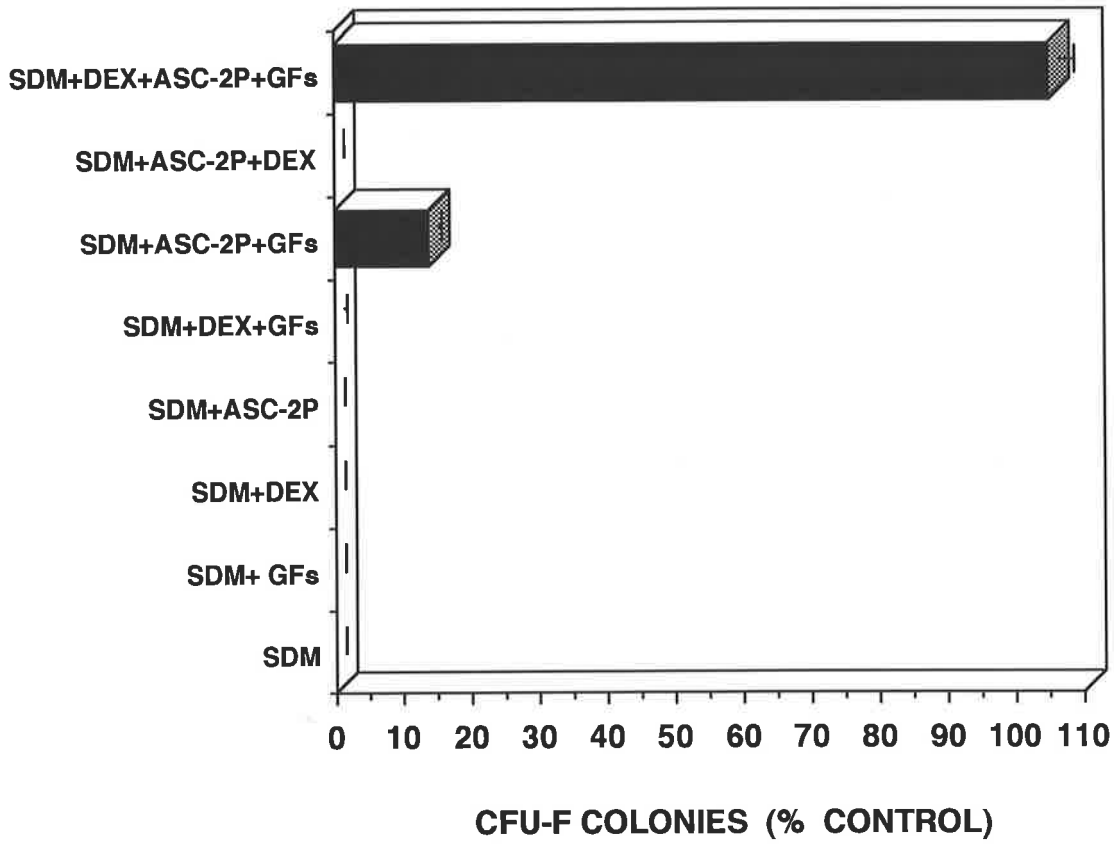
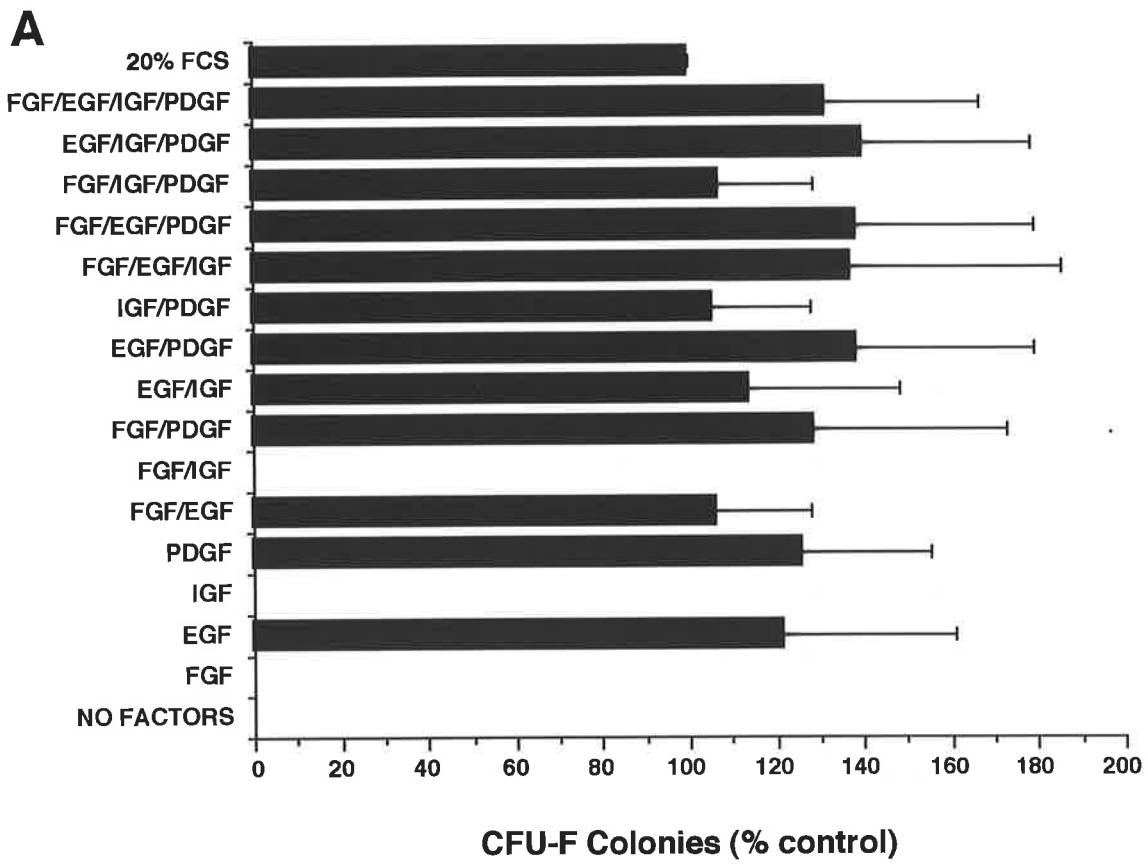
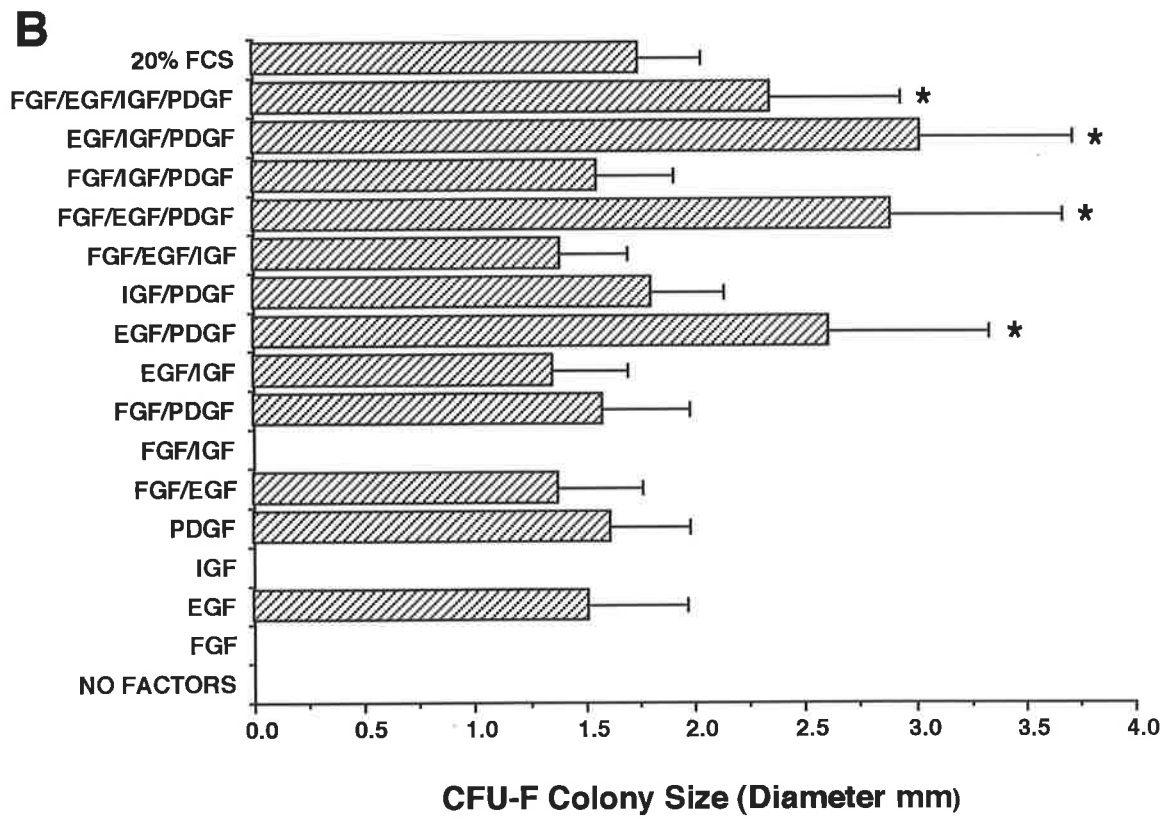

SDM+DEX+ASC-2P+GFs
SDM+ASC-2P+DEX
SDM+ASC-2P+GFs
SDM+DEX+GFs
SDM+ASC-2P
SDM+DEX
SDM+ GFs
SDM
0 10 20 30 40 50 60 70 80 90 100 110
CFU-F COLONIES (% CONTROL)

Figure 9. Cytokines which stimulate CFU-F development in serum deprived conditions. STRO-1$^+$ BMMNC were grown in serum deprived media supplemented with PDGF, EGF, IGF-1 and bFGF as single factors (10ng/ml) and in all possible 2- and 3-factor combinations. (A) CFU-F colony number. The data are expressed as the mean number of CFU-F ± SEM in triplicate cultures as a percentage of the number in control cultures grown in 20% FCS . (B) CFU-F colony size. The data are expressed as the mean colony diameter (mm) ± SEM derived from measurements of all colonies (approximately 25 colonies per well) in triplicate cultures. Colonies stimulated by all factor combinations containing both PDGF and EGF (*) were found to be significantly larger ($p \leq 0.05$; FLSD) than those stimulated by either factor alone or by 20% FCS.


A
20% FCS
FGF/EGF/IGF/PDGF
EGF/IGF/PDGF
FGF/IGF/PDGF
FGF/EGF/PDGF
FGF/EGF/IGF
IGF/PDGF
EGF/PDGF
EGF/IGF
FGF/PDGF
FGF/IGF
FGF/EGF
PDGF
IGF
EGF
FGF
NO FACTORS
0
20
40
60
80
100
120
140
160
180
200
CFU-F Colonies (% control)

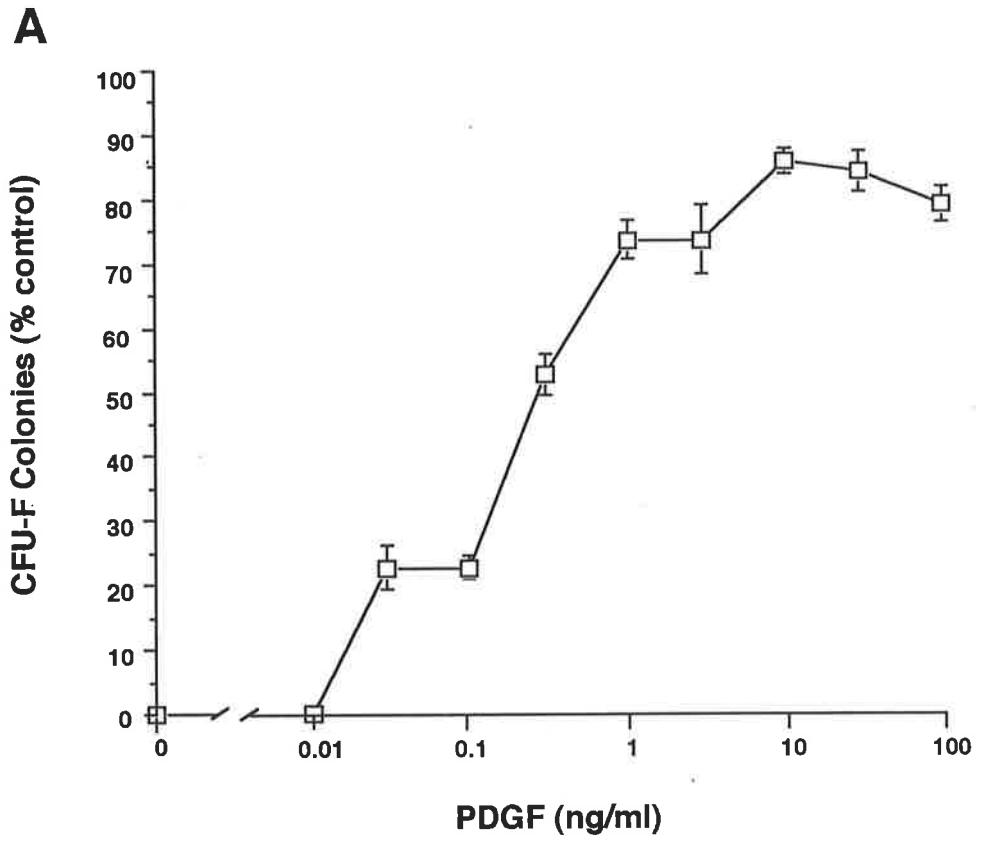

B
20% FCS
FGF/EGF/IGF/PDGF
EGF/IGF/PDGF
FGF/IGF/PDGF
FGF/EGF/PDGF
FGF/EGF/IGF
IGF/PDGF
EGF/PDGF
EGF/IGF
FGF/PDGF
FGF/IGF
FGF/EGF
PDGF
IGF
EGF
FGF
NO FACTORS
*
*
*
*
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
CFU-F Colony Size (Diameter mm)

presence of heparin at 50μg/ml (data not shown). IGF-1 over the same concentration range was similarly without activity in this assay regardless of whether insulin was included or omitted from the SDM (data not shown). EGF and PDGF either as single factors or in combination stimulated the development of equivalent numbers of CFU-F colonies (Figure 9A). Average colony size was however much increased in cultures stimulated with the combination of the two factors compared to either factor alone ($p \leq 0.05$) (Figure 9B). In contrast to the additive interaction between EGF and PDGF, analysis of CFU-F colony growth in response to each of the other possible combinations between the four growth factors did not reveal statistically significant differences in either colony number or size stimulated by combinations of factors involving EGF and/or PDGF with either IGF-1 and/or bFGF (Figure 9).

Collectively, these data demonstrate that the major stimuli for CFU-F growth under serum-deprived conditions are EGF and PDGF. To optimise growth conditions, dose-responses were performed for both factors (Figure 10). Plateau growth of CFU-F in response to EGF and to PDGF occurred over the concentration range 1 to 100ng/ml. Half-maximal colony formation in response to either factor occurred at approximately 0.2ng/ml. Maximal colony size was attained at concentrations of either factor in excess of 10ng/ml and for this reason subsequent experiments were performed (except where stated) using EGF and PDGF at a concentration of 10ng/ml.

To investigate the nature of the interaction between EGF and PDGF on CFU-F colony size, CFU-F assays were performed in which STRO-1^+ cells were plated at the optimal concentration of either factor (10ng/ml) and the second factor titrated in over the concentration range 0.01 to 100ng/ml. The assays were then scored with regard to colony number (Figure 11) and size (Figure 12) based on a comparison with the mean colony sizes obtained with EGF and PDGF alone at the 10ng/ml concentration. Consistent with our previous observations, CFU-F colony number did not change in response to the addition of EGF and PDGF (Figure 11) but colony size was increased in a dose

Figure 10. The dose-dependency of CFU-F colony formation under serum-deprived conditions in response to PDGF (A) and EGF (B). The data are expressed as the mean number of CFU-F colonies ± SE in triplicate cultures, expressed as a percentage of the number in control cultures grown in 20% FCS alone.


A
CFU-F Colonies (% control)
100
90
80
70
60
50
40
30
20
10
0
0
0.01
0.1
1
10
100
PDGF (ng/ml)

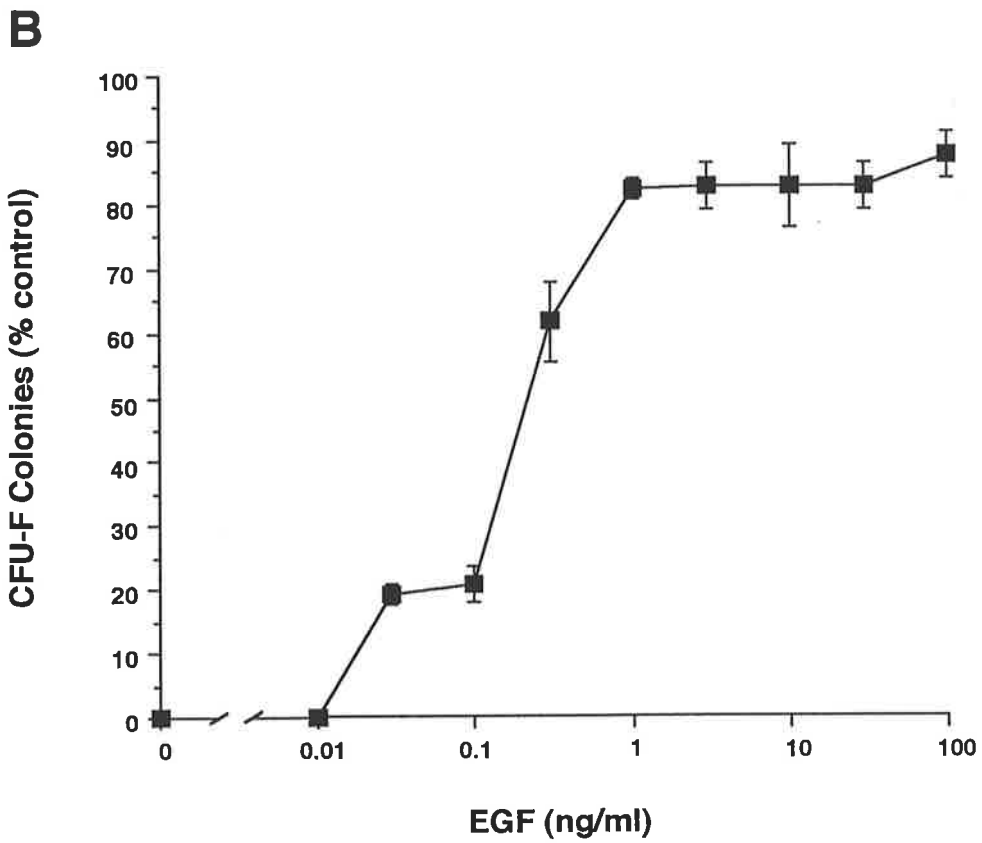
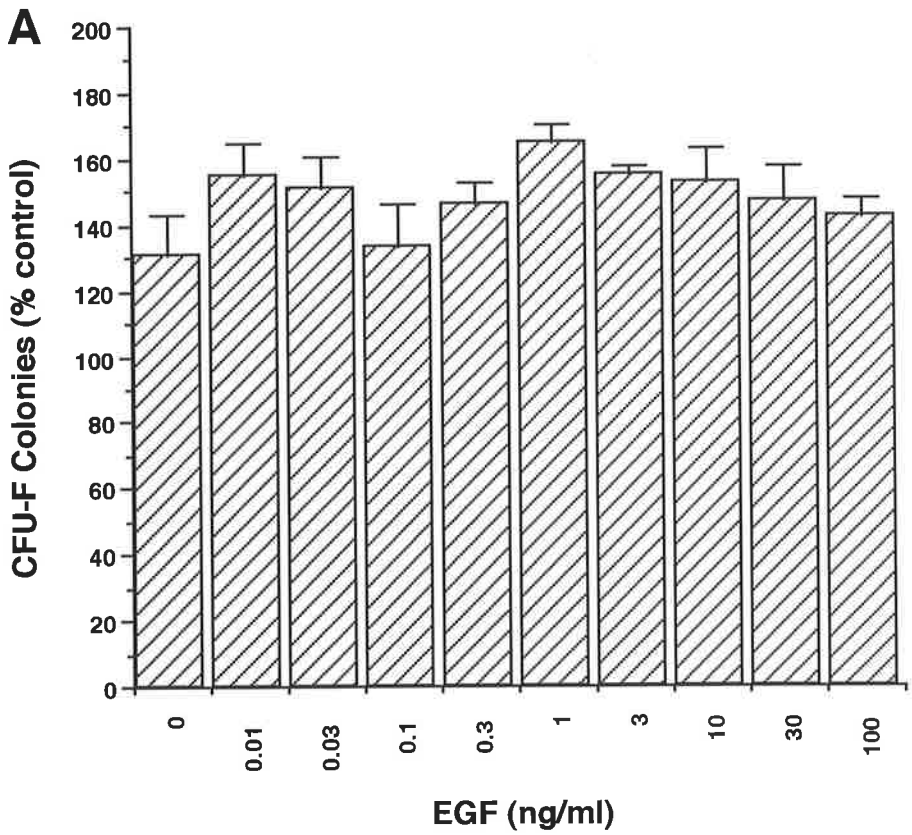

B
CFU-F Colonies (% control)
100
90
80
70
60
50
40
30
20
10
0
0
0.01
0.1
1
10
100
EGF (ng/ml)

Figure 11. Effects of PDGF and EGF on CFU-F colony number. STRO-1$^+$ BMMNC were cultured in SDM supplemented with either PDGF at 10ng/ml (A) or EGF at 10ng/ml (B), in the presence of the second factor over the concentration range 0.01-100ng/ml. Triplicate cultures were assayed for colony number at day 14. There was no significant difference in colony number over the entire concentration range used for either EGF (A) or PDGF (B).


A
CFU-F Colonies (% control)
200
180
160
140
120
100
80
60
40
20
0
0
0.01
0.03
0.1
0.3
1
3
10
30
100
EGF (ng/ml)

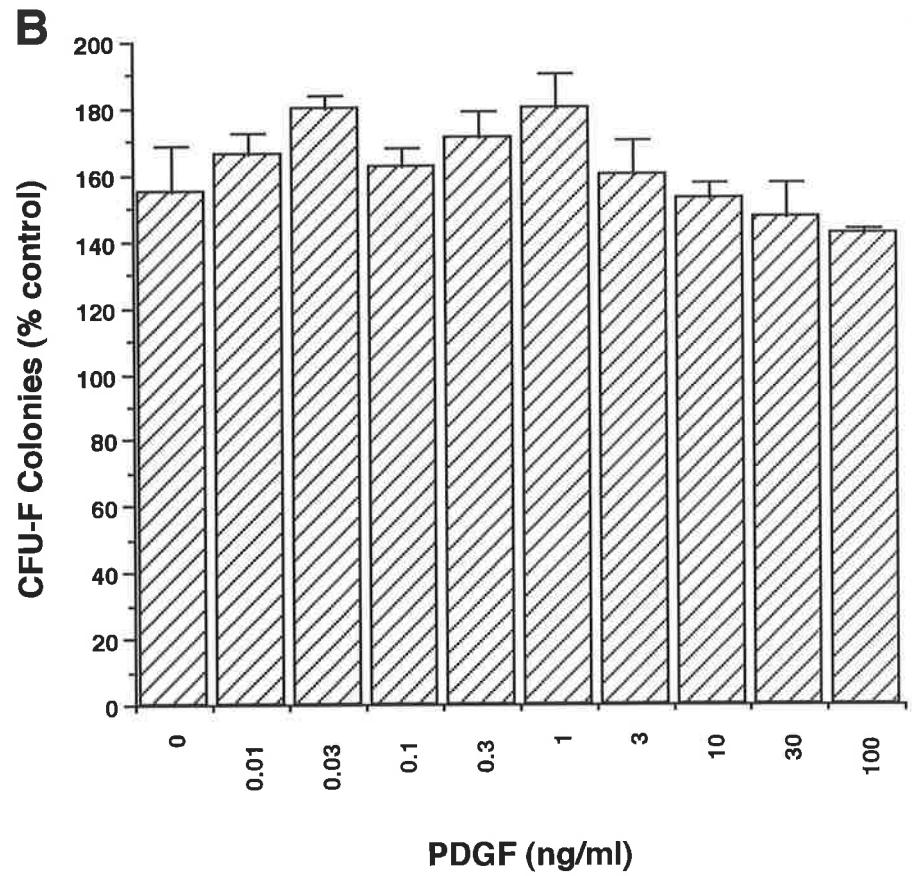
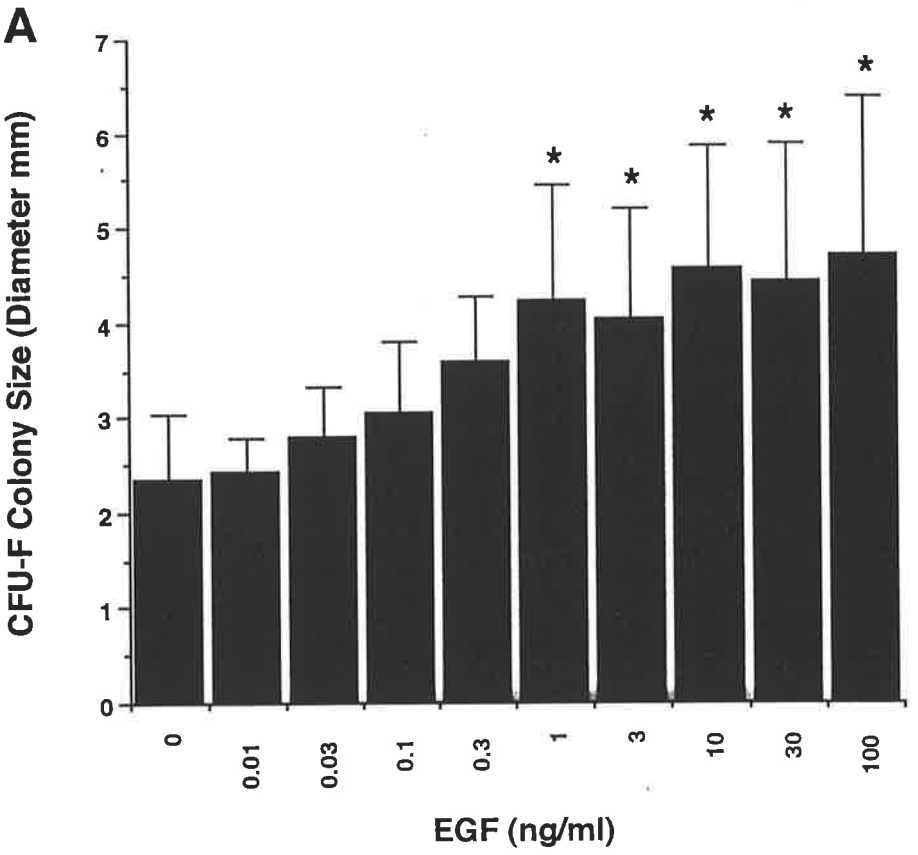
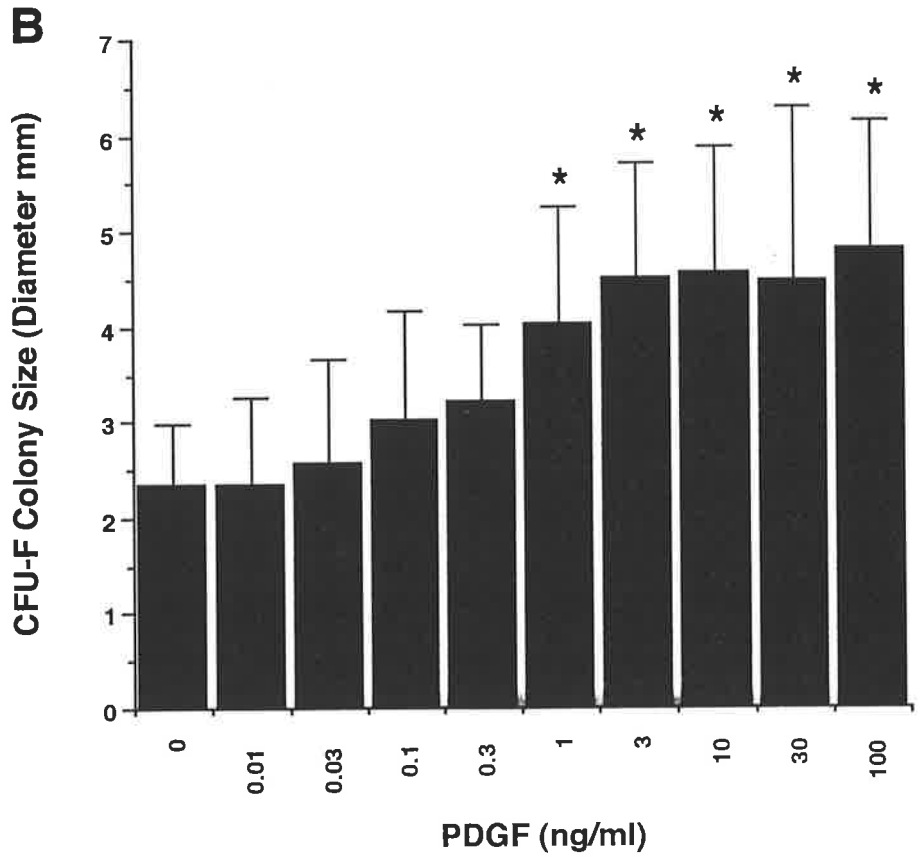

B
CFU-F Colonies (% control)
200
180
160
140
120
100
80
60
40
20
0
0
0.01
0.03
0.1
0.3
1
3
10
30
100
PDGF (ng/ml)

Figure 12. PDGF and EGF interact to promote increases in CFU-F colony size. STRO-1$^+$ cells were cultured in SDM supplemented with either PDGF at 10ng/ml (A) or EGF at 10ng/ml (B), in the presence of the second factor over the concentration range 0.01-100ng/ml. Triplicate cultures were assayed for colony size at day 14. There was a significant increase (*, $p \leq 0.05$; FLSD) of colony size in a dose-dependent manner at concentrations of EGF (A) or PDGF (B) in excess of 1ng/ml.


A
CFU-F Colony Size (Diameter mm)
0
1
2
3
4
5
6
7
*
0
0.01
0.03
0.1
0.3
1
3
10
30
100
EGF (ng/ml)


B
CFU-F Colony Size (Diameter mm)
0
1
2
3
4
5
6
7
*
0
0.01
0.03
0.1
0.3
1
3
10
30
100
PDGF (ng/ml)

dependent manner (Figure 12). Maximum colony diameters were attained at concentrations of EGF or PDGF in excess of 1ng/ml when added to cultures stimulated with the reciprocal factor at 10ng/ml. In addition, the proliferation of clonogenic cells was also found to be completely inhibited in the presence of saturating levels of functional blocking polyclonal antibodies to both PDGF-BB and EGF (Figure 13). However, the polyclonal antibodies to EGF could not completely inhibit the growth of EGF stimulated CFU-F colonies alone.

3.3 CFU-F Express Cell Surface Receptors for EGF, PDGF and IGF-1

Previous studies have demonstrated that various cultured stromal cell types express functional receptors for both PDGF and EGF [Carpenter and Zendegui, 1986; Tingstrom et al, 1992]. Expression of these receptors by primary clonogenic stromal precursor cells (CFU-F) in fresh aspirates of bone marrow has not been demonstrated. It is possible, therefore, that CFU-F in vivo do not constitutively express receptors for EGF or PDGF and that their response to these factors may represent some type of in vitro artefact, in part due to the induction of these receptors in culture. In addition, lack of response to a given factor may represent either absence of the appropriate receptor on CFU-F, failure to induce the receptor or inappropriate down-regulation of the receptor in vitro. To address these issues, FACS was employed to directly examine the expression of receptors for EGF, PDGF and IGF-1 on CFU-F isolated from freshly prepared BMMNC. The BMMNC were stained by 2-colour indirect immunofluorescence with STRO-1 in combination with monoclonal antibodies to the EGF-receptor (EGF^R), $PDGF^R$ α-chain and to the $IGF\text{-}1^R$. Given previous data demonstrating expression of STRO-1 by essentially all CFU-F, the BMMNC samples were sorted into $STRO\text{-}1^+/Receptor^+$ and $STRO\text{-}1^+/Receptor^-$ sub-populations. Each cell fraction was then assayed for CFU-F content under serum replete conditions. The data from three experiments demonstrated that a high proportion (consistently >90%) of CFU-F were recovered in the minor $STRO\text{-}1^+/Receptor^+$ sub-population (Table 5) indicating constitutive expression of all three growth factor receptors by CFU-F. In parallel experiments, BMMNC were double stained with STRO-1 and

Table 5. Cell surface markers expressed by stromal precursor cells and their progenitors. Immunophenotype of CFU-F was performed using two-colour FACS analysis based on the co-expression of various cell surface molecules with STRO-1. Cells from different STRO-1 subfractions were cultured for day 14 CFU-F. The number of CFU-F in the STRO-1$^+$/Marker$^+$ subfraction are shown. Primary cultures of stromal cells established from STRO-1$^+$ BM were trypsinised and assessed for antigen expression by immonfluorescence flow cytometric analysis. All of the results are expressed as the mean ± S.E (n=3 different BM aspirates).

Cell Surface Molecule	Number of CFU-F Colonies (% of Total STRO-1 Fraction)	Primary BM Stroma (Mean % Fluorescence)
STRO-1	100	4.2 ± 0.6
3D3	0.0	0.3 ± 0.3
1D4.5	0.0	0.4 ± 0.1
1A6.12	-	0.3 ± 0.1
1A6.11	0.0	0.5 ± 0.3
LFA-3	81.3 ± 11.1	0.1 ± 0.1
THY-1	100	97.1 ± 2.0
VCAM-1	100	76.7 ± 5.2
ICAM-1	66.8 ± 9.0	1.7 ± 0.2
PECAM-1	70.8 ± 9.5	0.3 ± 0.2
P-SELECTIN	63.0 ± 6.4	0.1 ± 0.1
E-SELECTIN	0.0	0.2 ± 0.1
L-SELECTIN	48.7 ± 9.9	0.1 ± 0.1
CD49a/CD29	100.0	96.1 ± 4.1
CD49b/CD29	93.7 ± 2.9	93.3 ± 1.1
CD49c/CD29	5.3 ± 5.3	87.3 ± 3.4
CD49d/CD29	50.3 ± 7.8	0.2 ± 0.2
CD49e/CD29	98.3 ± 0.9	92.1 ± 3.4
CD49f/CD29	95.1 ± 3.1	47.7 ± 5.3
CD29	98.0 ± 2.0	97.8 ± 5.2

Continued.

Table 5. Cell surface markers expressed by stromal precursor cells and their progenitors continued.

Cell Surface Molecule	Number of CFU-F Colonies (% of Total STRO-1 Fraction)	Primary BM Stroma (Mean % Fluorescence)
CD18	36.0 ± 8.7	0.7 ± 0.3
CD61	81.0 ± 8.0	23.0 ± 2.5
CD51	100	86.1 ± 8.3
CD51/CD61	100	78.6 ± 5.6
CD51/β_5	98.0 ± 4.7	65.6 ± 2.2
CD3	0.0	0.2 ± 0.1
CD10	100	32.3 ± 5.4
CD13	100	94.9 ± 2.9
CD14	0.0	0.3 ± 0.2
CD19	0.0	0.2 ± 0.1
CD33	0.0	0.5 ± 0.3
CD34	77.3 ± 6.4	0.9 ± 0.6
CD38	0.0	0.1 ± 0.1
CD44	5.1 ± 4.9	96.5 ± 2.9
CD45	0.0	0.3 ± 0.3
CD59	ND	96.3 ± 7.1
Glycophorin-A	0.0	0.1 ± 0.1
HLA-DR	0.0	0.2 ± 0.2

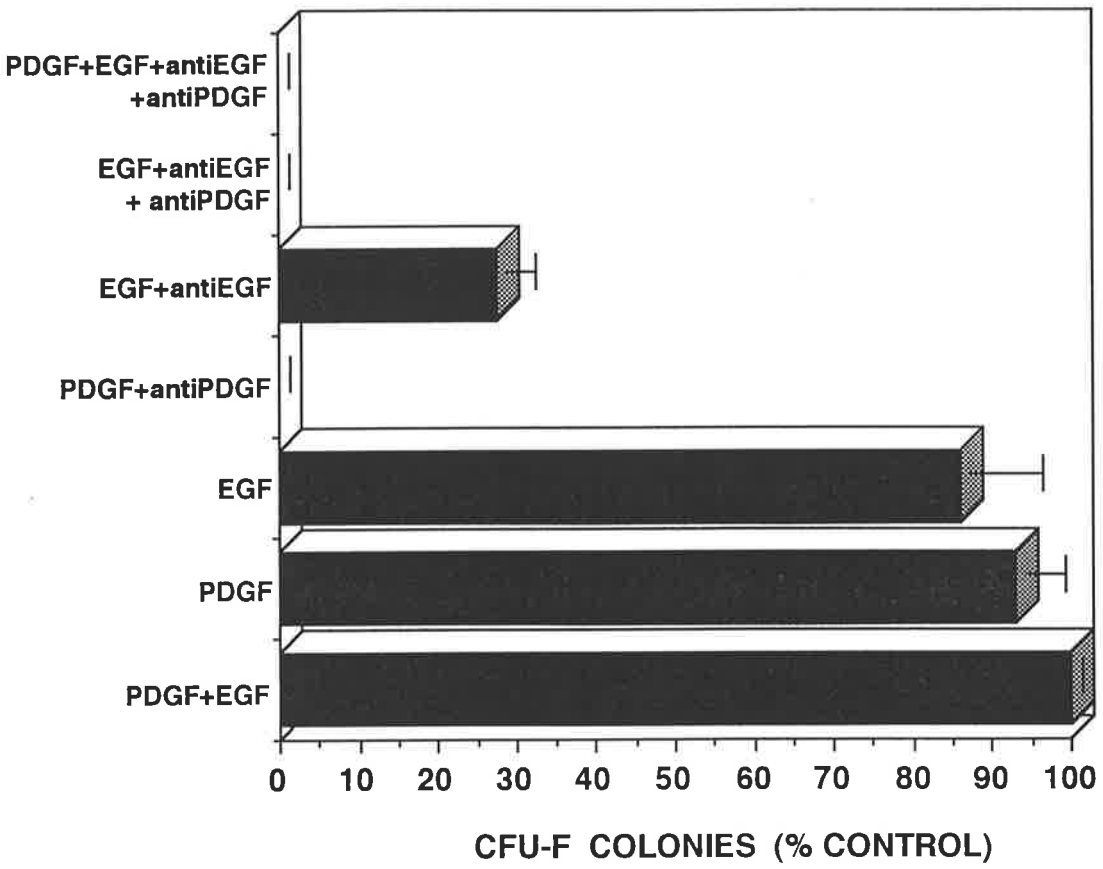
Continued

Table 5. Cell surface markers expressed by stromal precursor cells and their progenitors continued.

Cell Surface Molecule	Number of CFU-F Colonies (% of Total STRO-1 Fraction)	Primary BM Stroma (Mean % Fluorescence)
c-kit	4.5 ± 2.5	0.2 ± 0.1
c-fms	3.0 ± 1.7	0.1 ± 0.1
SCF	63.0 ± 6.5	4.8 ± 1.1
NGF-Receptor	99.3 ± 0.3	7.7 ± 1.5
IGF-1-Receptor	97.3 ± 1.0	7.3 ± 1.9
PDGF-aReceptor	90.7 ± 7.4	12.1 ± 1.8
EGF-Receptor	95.7 ± 1.1	76.9 ± 4.9
Leptin-Receptor	71.0 ± 5.2	99.6 ± 2.3
Trombomodulin	94.0 ± 2.7	0.5 ± 0.4
6-19	100	97.6 ± 1.1
MUC-18	89.6 ± 8.0	85.4 ± 7.7
Bone/Liver-Alkaline Phosphatase	93.2 ± 4.1	26.0 ± 3.8

(ND): Not done.

Figure 13. Inhibition of CFU-F growth in vitro by antibodies to PDGF and EGF. MACS isolated STRO-1$^+$ BMMNC were cultured in SDM in the presence of: PDGF and/or EGF (10ng/ml) with saturating concentrations (50μg/ml) of antisera to PDGF (antiPDGF) and/or antisera to EGF (antiEGF). The data are expressed as the mean number of CFU-F ± SE (n=5 replicate cultures) as a percentage of the control cultures stimulated with both PDGF and EGF (10ng/ml) in the presence of the isotype matched control antibody. The results demonstrated a complete inhibition of colony formation in the presence of antibodies to PDGF and EGF.


PDGF+EGF+antiEGF
+antiPDGF
EGF+antiEGF
+ antiPDGF
EGF+antiEGF
PDGF+antiPDGF
EGF
PDGF
PDGF+EGF
0
10
20
30
40
50
60
70
80
90
100
CFU-F COLONIES (% CONTROL)

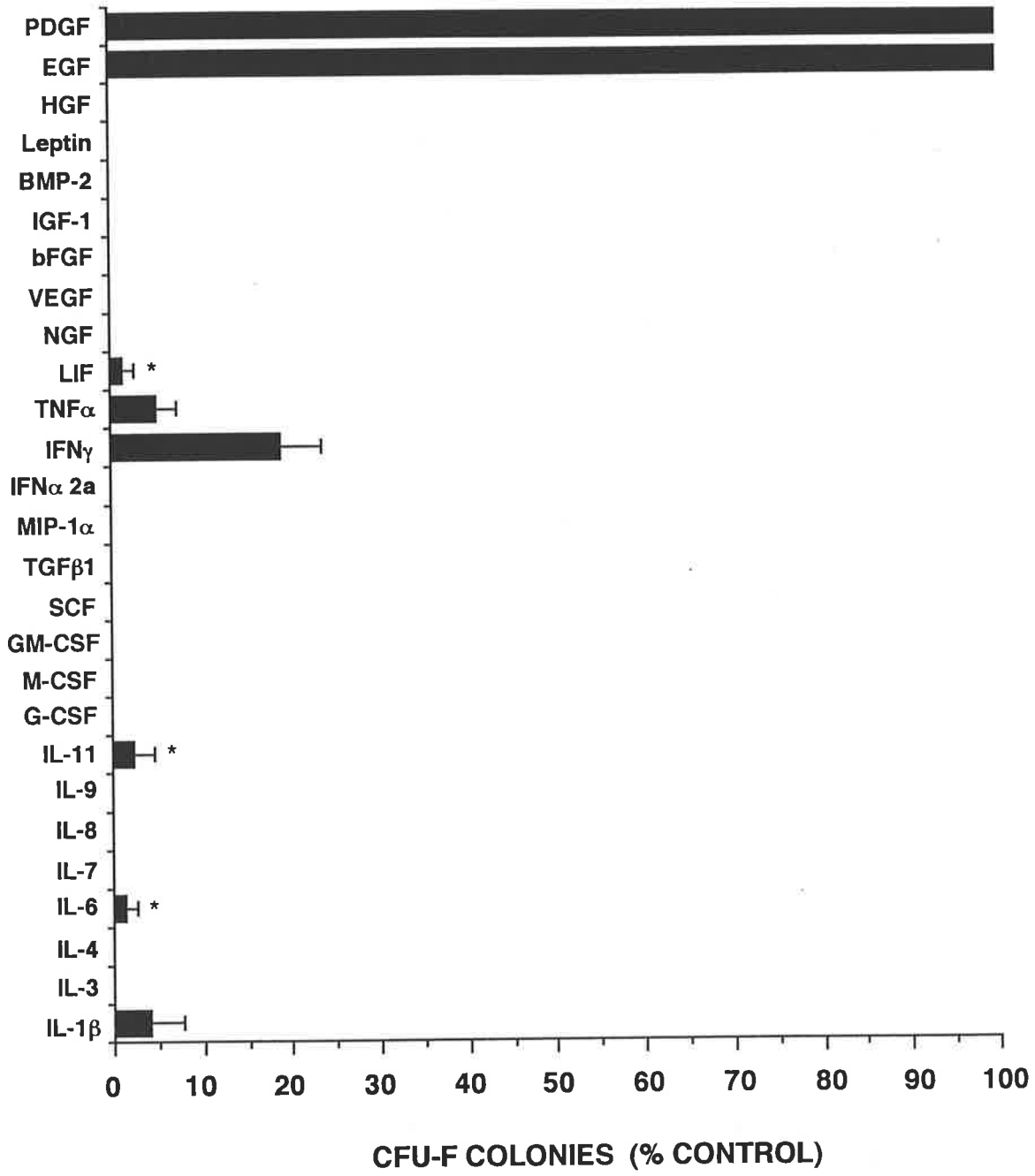
monoclonal antibodies to other receptor tyrosine kinases, c-kit and c-fms. In these cases, no expression of either receptor could be detected on CFU-F (Table 5).

To investigate the possibility of inappropriate receptor down-modulation as a consequence of in-vitro culture conditions, CFU-F assayed in serum-replete medium and in SDM supplemented with EGF and PDGF were stained in-situ at day 14 of culture by indirect immunofluorescence using antibodies to the above receptors. Expression of EGF^R, $PDGF^R$ α-chain, $IGF\text{-}1^R$ and NGF^R was maintained in culture on the progeny of the CFU-F while c-kit and c-fms remained undetectable (Table 5). However, $PDGF^R$ α-chain, and $IGF\text{-}1^R$ demonstrated low levels of expression on the total stromal cell population in vitro in contrast to the high EGF^R expression.

3.4 Clonogenic Growth of CFU-F Does Not Occur in Response to a Wide Variety of Cytokines and Growth Factors

Given the complete dependence upon an exogenous source of cytokines in this serum-deprived CFU-F assay (Figure 8) we next investigated the response of CFU-F to a broad range of growth factors. For these experiments, each of the growth factors was added at a single concentration at or above the dose determined in previous studies to have optimal activity in clonogenic assays of haemopoietic progenitors. Of a total of 25 purified recombinant growth factors assayed, only 5 demonstrated any capacity to stimulate CFU-F growth in SDM: EGF, PDGF, IFNγ, TNFα and IL-1 (Figure 14). Clonogenic growth was poor in response to IFNγ, TNFα and IL-1 with colony numbers approximately 19%, 5% and 4% respectively of those stimulated in control, serum replete cultures. Three factors, LIF, IL-6 and IL-11, only stimulated the development of small (10-20 cell) clusters of stromal cells. A number of the above growth factors have been shown in assays of haemopoietic progenitors to have no intrinsic colony stimulating activity when added as single factors. However, in combination with other growth factors, cytokines such as IL-1 and SCF demonstrate potent synergistic activity [Muench et al, 1993]. We therefore performed a series of experiments in which the activity of each of the above factors was

Figure 14. Assay of BM CFU-F colony growth in response to a range of recombinant growth factors under serum-derived conditions. STRO-1$^+$ BMMNC were cultured in SDM in the presence of various cytokines including: IL-1β (1ng/ml); IL-3 to IL-11 (10ng/ml); G-CSF (10ng/ml); GM-CSF (10ng/ml); M-CSF (10ng/ml); SCF (50ng/ml); TGFβ (3ng/ml); MIP-1α (10ng/ml); IFNα 2a (3000 i.u./ml); IFNγ (3000 i.u./ml); TNFα (100 u/ml); LIF (50ng/ml); NGF (10ng/ml); VEGF (10ng/ml); bFGF (10ng/ml); IGF-1 (10ng/ml); BMP-2 (10ng/ml); Leptin (50 ng/ml); HGF (10ng/ml); PDGF (10ng/ml); EGF (10ng/ml). Data represent the mean ± SE of colony growth in triplicate cultures per cytokine, presented as a percentage of CFU-F colonies elicited in response to PDGF + EGF (10ng/ml). Clonogenic growth occured only in response to 5 cytokines: PDGF; EGF; IFNγ; TNFα and IL-1β. Asterisks indicate formation of clusters (10-20 cells) only.


PDGF
EGF
HGF
Leptin
BMP-2
IGF-1
bFGF
VEGF
NGF
LIF
*
TNFα
IFNγ
IFNα 2a
MIP-1α
TGFβ1
SCF
GM-CSF
M-CSF
G-CSF
IL-11
*
IL-9
IL-8
IL-7
IL-6
*
IL-4
IL-3
IL-1β
0
10
20
30
40
50
60
70
80
90
100
CFU-F COLONIES (% CONTROL)

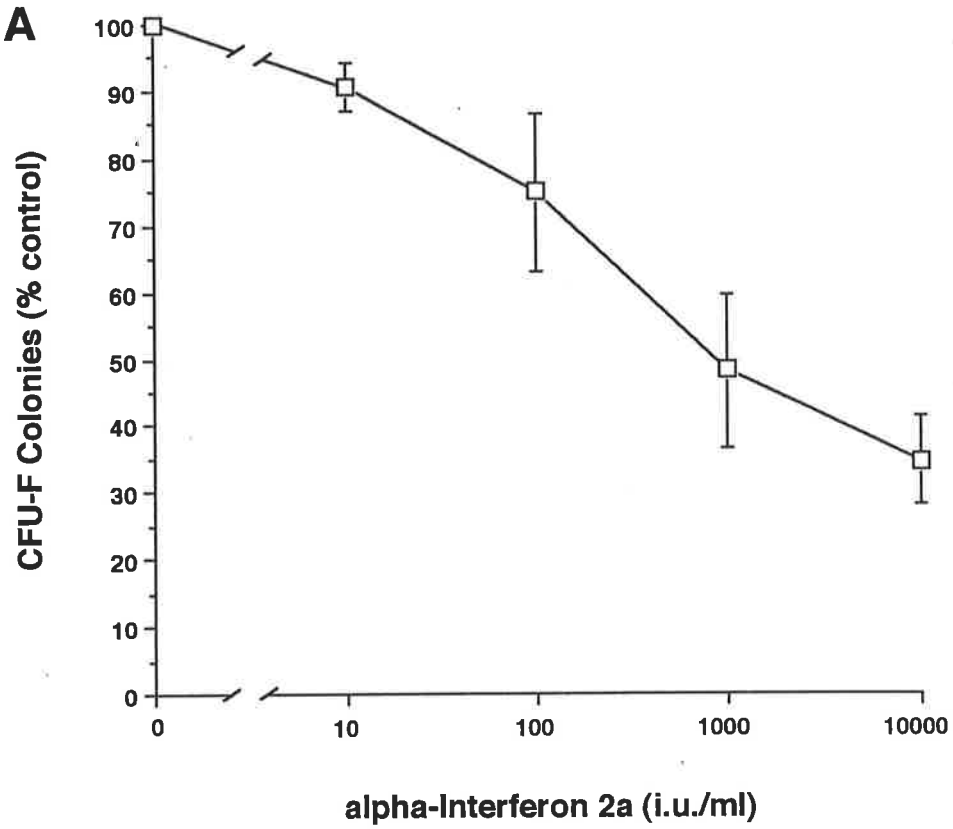
assessed by plating STRO-1$^+$ cells in SDM supplemented with EGF and PDGF at 0.03ng/ml. This concentration was based on the dose response of each factor (Figure 10) and represented the lowest concentration of either factor with activity in the assay. At this threshold dose of EGF/PDGF we were still unable to demonstrate any CFU-F colony-stimulating potential of these factors, including IGF-1 and bFGF (data not shown).

Notably, two factors, IL-4 and IFNα demonstrated inhibition of CFU-F colony formation. This was demonstrated by experiments in which each factor was titrated into the assay with EGF and PDGF at the optimum concentration of 10ng/ml (Figure 15). Other growth factors with inhibitory activity on primitive haemopoietic progenitors, TGFβ and MIP-1α were assayed in an identical fashion but failed to inhibit the in vitro growth of CFU-F (data not shown). Having established and optimised culture conditions for assay of CFU-F under serum deprived conditions studies were then performed comparing the plating efficiency of CFU-F in the serum replete and serum deprived assays. Based on the data in Figure 10, the serum deprived assay was performed using a combination of EGF and PDGF, both at 10ng/ml. A representative experiment is shown in Figure 16. Both assays demonstrated a linear relationship between the number of cells plated and the number of colonies formed at all cell densities except in the case of the serum replete cultures at the lower cell concentrations. The plating efficiency of CFU-F in SDM was found to be significantly higher than in FCS at all cell concentrations examined (t-test; $p \leq 0.05$) particularly at lower cell plating densities.

3.4 DISCUSSION

The response of bone marrow derived fibroblast colony-forming cells (CFU-F) to a variety of cytokines and growth factors in vitro under defined, serum-deprived conditions was examined. All assays were established using a purified, phenotypically defined population of BMMNC expressing the STRO-1 antigen to eliminate the affects of accessory cell populations. The STRO-1$^+$ cells were also cultured at a low plating density to minimise the stimulation of autocrine growth factor or inhibitor production. In regard to this, previous

Figure 15. Inhibition of PDGF and EGF stimulated CFU-F growth in vitro. MACS isolated STRO-1$^+$ BMMNC were cultured in SDM in the presence of both PDGF and EGF (10ng/ml) with varying concentrations of either alpha-interferon 2a (A) or interleukin-4 (B). The data are expressed as the percentage of CFU-F colony growth stimulated by PDGF plus EGF alone and represent the mean ± SE of triplicate cultures for each concentration of alpha-interferon 2a and interleukin-4.


A
CFU-F Colonies (% control)
100
90
80
70
60
50
40
30
20
10
0
0
10
100
1000
10000
alpha-Interferon 2a (i.u./ml)

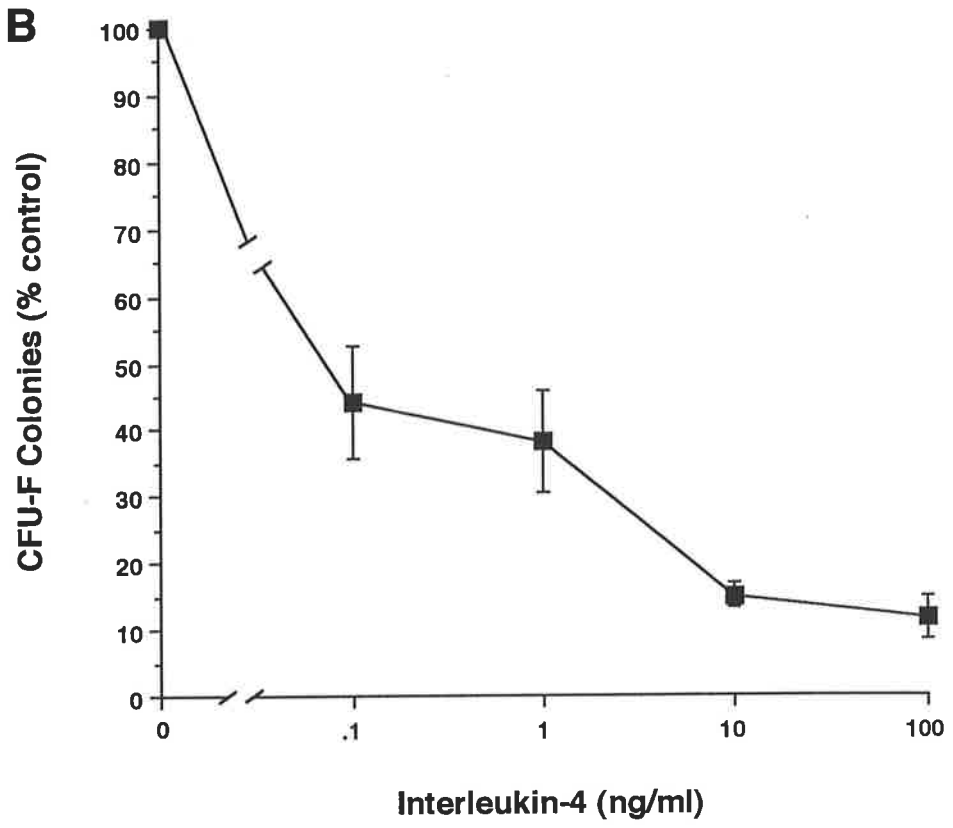
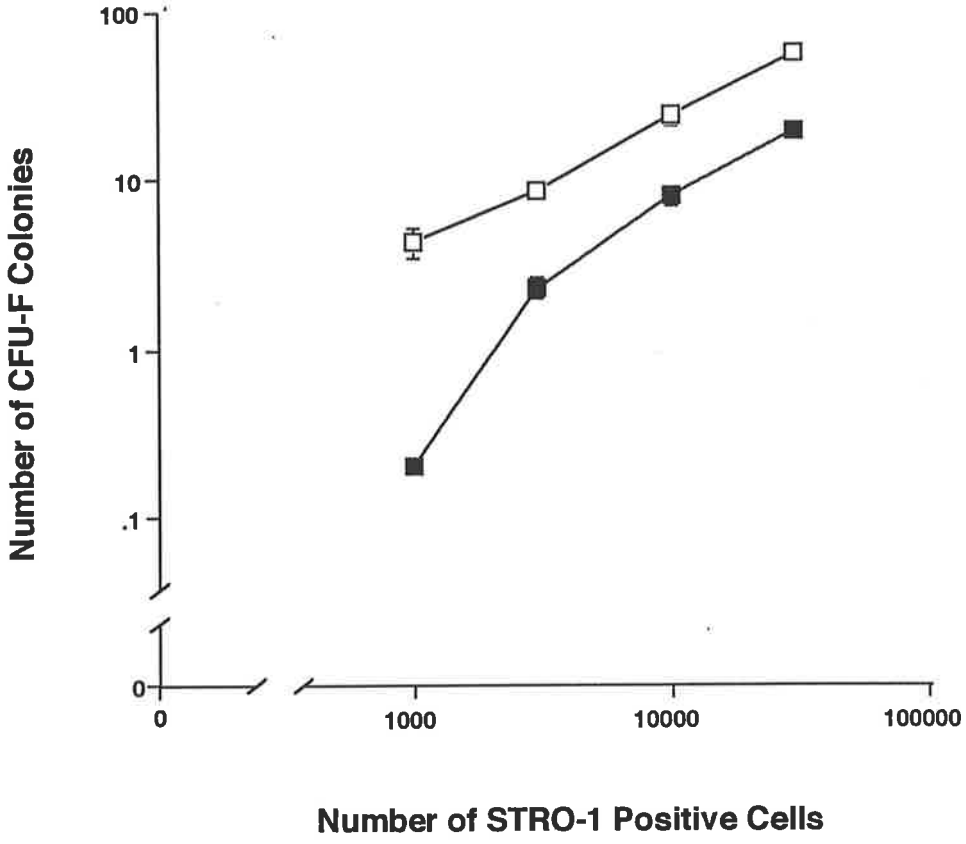

B
CFU-F Colonies (% control)
100
90
80
70
60
50
40
30
20
10
0
0
.1
1
10
100
Interleukin-4 (ng/ml)

Figure 16. A comparison of CFU-F in serum replete and optimized serum deprived medium. MACS isolated STRO-1$^+$ BMMNC were grown in either SDM in the presence of both PDGF and EGF (10ng/ml) (open square) or in standard CFU-F culture conditions in 20% FCS (closed square). The data are expressed as the mean number of CFU-F colonies ± SE from triplicate cultures. In both culture conditions there was a linear correlation between the number of STRO-1$^+$ cells plated and the number of day 14 CFU-F colonies formed. However, colony formation was significantly more efficient in SDM than in FCS at all cell concentrations ($p \leq 0.05$; t-test).


100
10
1
.1
0
0
1000
10000
100000
Number of CFU-F Colonies
Number of STRO-1 Positive Cells

studies of CFU-F growth factor responsiveness in human and rodent systems have, in virtually all instances, performed assays using whole bone marrow or passaged marrow stromal cells plated at high cell density [Castro-Malaspina et al 1981; Rosenfeld et al, 1985; Hirata et al 1985; McIntyre and Bjornson, (1986); Kimura et al 1988; Wang et al, 1990; Friedenstein et al, 1992]. Moreover, these studies were performed in medium supplemented with FCS. A major source of the mitogenic activity of serum is contributed by factors such as EGF and PDGF [Walthall and Ham, 1981] which together with other possibly inhibitory activities may therefore alter or otherwise mask the response of CFU-F to purified growth factors added to the cultures.

For this reason, it was necessary to establish in vitro culture conditions for clonogenic assay of CFU-F in the absence of FCS. A serum-deprived assay was developed which demonstrated absolute dependence on an exogenous source of growth factors for colony growth [Gronthos and Simmons, 1995]. The glucocorticoid dexamethasone and a source of ascorbate were found to be critical components of the SDM. Previous studies analysing the growth requirements of diploid human fibroblasts in serum-free culture have documented a requirement for glucocorticoid [Walthall and Ham, 1981; Chen and Rabinovitch, 1989]. Hydrocortisone has been found to be an important ingredient in long-term marrow cultures [Gartner and Kaplan, 1980; Suda and Dexter, 1981] although the precise role of steroid in this system remains to be determined. CFU-F colonies assayed from rabbit bone marrow were shown to increase in number in the presence of hydrocortisone [Owen et al, 1987] although a similar study of human marrow CFU-F failed to demonstrate a response to this steroid and may reflect differences between species [Hirata et al, 1985]. Similarly, the importance of ascorbic acid in the assay of CFU-F under serum-deprived conditions as described in this study is unclear. Several studies have reported that ascorbate stimulates the biosynthesis of extracellular matrix components such as collagen and increases the cell proliferation of murine and human mesenchymal cells in culture [Hata and Senoo, 1989; Harada et al, 1991]. The addition of ascorbate has

also been shown to facilitate cell proliferation by upregulating IGF-1 receptors on mouse MC3T3-E1 cells [Harada et al, 1990].

IGF-1 in the present serum-deprived system was unable to support colony formation by CFU-F at any concentration over a 3-log dose range, even in SDM lacking insulin which at supraphysiological concentrations can compete with IGF-1 for receptor binding. This lack of response to IGF-1 is not due to the absence of IGF-1^R on stromal precursors which were shown to be constitutively expressed by CFU-F and to be maintained on their progeny in vitro but at lower levels to that observed in the progenitor population. In serum replete cultures, failure to respond to IGF-1 may be explained by the presence of serum IGF binding proteins [Ross et al, 1989]. It is conceivable that trace amounts of these proteins may be in the BSA used in the present serum deprived culture system. However, the recombinant form of IGF-1 employed in this study lacks the region of the molecule which interacts with the binding proteins [Szabo et al, 1988] and thus cannot be inactivated even if binding proteins were present. An alternative explanation may be that CFU-F produce IGF-1 themselves thereby negating the effects of exogenously added factor. In accord with this, human fibroblasts have been shown to secrete IGF-1 into the culture medium constitutively [Clemmons and Shaw, 1983] and in response to PDGF [Clemmons et al, 1981]. Moreover, marrow stromal cells have been demonstrated to produce IGF-1 [Witte et al, 1993] therefore it remains to be shown whether IGF-1 is being produced endogenously by CFU-F under the serum deprived culture conditions described in the present study. Therefore, these data do not preclude a role for IGF-1 in regulating CFU-F growth in this serum-deprived assay. The potential importance of IGF-1 is suggested by data from other studies performed under serum-free conditions in which IGF-1 has been shown to regulate the rate of exit from G_1 into S phase in human diploid fibroblasts without affecting the cycling fraction [Chen and Rabinovitch, 1989], implying a mode of interdependence between IGF-1 and EGF/PDGF in regulating activation and cell cycle progression [Zumstein and Stiles, 1987].

Basic FGF similarly failed to stimulate CFU-F growth under the serum-deprived conditions described in this report. The expression by CFU-F of receptors for bFGF was not examined in the current study but clearly warrants investigation. In order to bind to its receptor and produce a biological response, bFGF needs to complex with a sulphated proteoglycan such as heparin [Olwin and Rapraeger, 1992; Roghan and Moscatelli, 1992]. The addition of heparin, however, failed to elicit colony formation in SDM supplemented with bFGF alone or added in combination with a suboptimal concentration of PDGF and EGF. However, a recent study has shown that even though cultured human bone marrow stromal cells express the FGF receptor 1, stimulation with bFGF failed to effect stromal cell proliferation and had little effect on tyrosine phosphorylation [Samatomura et al, 1998]. In contrast, Wang et al (1990) using unfractionated mouse bone marrow cell preparations demonstrated augmentation of CFU-F colony growth in response to bFGF, but only in the presence of high concentrations of FCS. At low FCS concentrations bFGF failed to show activity even in the presence of PDGF suggesting that FCS may contain an activity(ies) (other than PDGF) which synergizes with bFGF which is unlikely to be present in the SDM conditions employed here. Another possible explanation for the failure of bFGF to stimulate colony growth in culture system may, as in the case of IGF-1, be due to possible autocrine production of the factor by stromal cells themselves [Oliver et al, 1990; Brunner et al, 1993].

In vivo, more than 90% of CFU-F are non-cycling [Keiliss-Borok et al, 1971; Epichina and Latzinik, 1976; Castro-Malaspina et al 1981; Kaneko et al, 1982] but rapidly begin to proliferate in vitro when cultured in serum containing medium. In the present study, a total of 27 purified recombinant growth factors were tested in the serum-deprived assay, where only EGF and PDGF demonstrated the ability to support CFU-F colony growth. Platelet derived growth factor is found in three different forms made up of two disulphide-linked polypeptide chains: PDGF-AA, consisting of two covalently linked A chains; PDGF-BB, consisting of two covalently linked B chains; and PDGF-AB, a covalently linked heterodimer of one A and one B chain [Ross, 1989]. Epidermal growth factor is a small

polypeptide with a molecular weight of 6 kDa where the mouse and human EGF exhibit 70% homology and are highly species cross reactive [Carpenter and Zendegui, 1986]. A variety of cell types have been shown to produce PDGF in vitro, including; fibroblast cells, platelets, macrophages, smooth muscle cells, endothelial cells and several tumour cell lines [DiCorleto and Bowen-Pope, 1983; Nilsson et al, 1985; Martinet et al, 1986; Heldin et al, 1990; Abboud, 1993]. In the present study, the functional blocking antibodies to EGF could not completely inhibit colony formation in CFU-F cultures stimulated with EGF alone. In contrast, no growth was observed in replicate cultures incubated with antibodies to both PDGF and EGF. Further analysis is required to demonstrate the possible induction of PDGF production by primary CFU-F colonies following stimulation with EGF.

A recent report which examined the growth regulation of human diploid fibroblasts in a serum-free, chemically defined system similar to the one reported here, demonstrated that EGF and PDGF affect similar cell kinetic parameters, regulating the proportion of cells capable of entering the cell cycle from the quiescent state [Chen and Rabinovitch, 1989]. Receptors for both PDGF and EGF have been previously found on a variety of cell types including fibroblasts, smooth muscle cells, glial cells, endothelial cells, epithelial cells and various cell lines [Cross and Dexter, 1991; Tingstrom et al, 1992]. In accord with this, CFU-F could be isolated from fresh aspirates of bone marrow following immunostaining and FACS with monoclonal antibodies to the alpha-chain of the $PDGF^R$ and to the EGF^R [Downward et al, 1984]. All three isoforms of PDGF bind to a cell surface receptor of approximately 170-180 kDa [Glenn et al, 1982]. There are two different PDGF receptor types (α and β), existing as one of three non-covalently associated complexes: $(\alpha)_2$; $(\alpha)_1$ $(\beta)_1$; and $(\beta)_2$ [Heldin et al, 1988; Hart et al, 1988; Matsui et al, 1989]. The PDGF β-receptor has high affinity for PDGF-BB and a lower affinity for PDGF-AB, whereas the PDGF α-receptor shows high affinity binding to all three isoforms of PDGF [Heldin et al, 1988; Hart et al, 1988]. The PDGF receptor has a protein tyrosine kinase intracellular domain and is activated by ligand binding after which the PDGF-receptor complex is internalised [Nilsson et al, 1983; Rosenfeld et al, 1984]. Similarly, EGF binds to a specific

cell surface receptor, characterised as a transmembrane glycoprotein with a molecular weight of 170 kDa [Downward et al,1984]. The receptor exists as a monomer with low affinity for EGF. When EGF binds to a monomer receptor, a non-covalent associated complex forms between two monomers leading to a high affinity dimer receptor. The receptor has a tyrosine kinase intracellular domain analogous to the PDGF receptor [King and Cuatrecassas, 1982; Ulrich et al, 1984; Boni-Schnetzer and Pilch, 1987]. A recent study has confirmed the presence of the EGF receptor and demonstrated the presence of the PDGF β-receptor in cultures of human bone marrow stromal cells [Satomura et al, 1998]. In accord with the findings in the present study, Satomura et al, (1998) also showed that PDGF and EGF stimulated stromal cell growth in a dose-dependent manner in vitro, and induced tyrosine phosphorylation of intracellular molecules, including the EGF and PDGF receptors themselves.

Based on the data in the current study, EGF and PDGF may play a similar role in triggering the cycling of CFU-F. Very comparable numbers of colonies were initiated by the two factors alone or when added in combination suggesting a functional redundancy in the regulation of cycling of CFU-F. Consistent with this, studies using fibroblast cell lines have shown that PDGF and EGF both stimulate the expression of c-fos, c-myc and other early G_1 genes [Kelly et al, 1983; Kruijer et al,1984], show a similar time dependence of induction of commitment to DNA synthesis [Westermark and Heldin, 1985] and result in phosphorylation at tyrosine residues of a similar array of intracellular proteins [Cooper et al, 1982]. Thus a common intracellular pathway for PDGF and EGF signal processing has been proposed [Westermark and Heldin, 1985]. Although EGF and PDGF in combination failed to stimulate increases in total colony number, colony size was significantly enhanced. Similar observations were reported by Kimura et al (1988). The mechanism underlying the interaction between these two factors remains to be determined but is clearly of importance given the large increase in cell number which results from culture in the presence of both factors. Our observations are all the more intriguing given the data from studies in other systems which demonstrate decreased

cycling in response to a combination of EGF and PDGF [Chen and Rabinovitch, 1989; Wrann et al, 1980]. It is unlikely that the data presented here are explained by receptor modulation since it has been reported that PDGF can down regulate EGF^R expression [Wrann et al, 1980] or receptor affinity [Bowen-Pope et al, 1983], although the converse is not true [Bowen-Pope et al, 1983].

With the development of a factor-dependent, serum deprived assay for CFU-F, we examined an additional 25 recombinant cytokines for their ability, as single factors, to initiate colony formation. Only three cytokines: IL-1β, TNF-α and IFN-γ, demonstrated a minor stimulatory activity. Caution should however be exercised in interpreting this data. Primitive haemopoietic progenitors require multiple growth factors in order to initiate proliferation and development, some of which like IL-1 and IL-6 have no direct colony-stimulating activity but act in synergism with other factors [McNiece et al, 1991; Moore, 1991; Johnson and Li, 1992]. By analogy, therefore, it remains possible that a number of the growth factors examined in this study which failed to promote the growth of stromal progenitors as single factors could, when added in appropriate combinations, demonstrate stimulatory activity. A more detailed examination of the response of CFU-F to combinations of multiple growth factors will be required to address this point. Moreover, future investigations will survey the ability of these and other cytokines to promote cellular differentiation under serum deprived conditions, given that several studies have demonstrated the ability of single factors or combinations of factors such as; $TGF\beta_1$, $TGF\beta_2$, PDGF-BB, EGF, bFGF, BMP-2, IGF-1 and PTH, to stimulate osteogenic or adipocytic differentiation in cultured stromal cells [Canalis, 1993; Hauner et al, 1995; Cassiede et al, 1996; Isogai et al, 1996; Handa et al, 1997; Martin et al, 1997; Pri-Chen et al, 1998; Fromigue et al, 1998].

Notably, interferon-α 2a (IFNα) and interleukin-4 (IL-4) were found to be potent inhibitors of CFU-F colony formation stimulated by the EGF/PDGF combination at optimal concentration. Previous studies have shown that IFN-α [Wang et al, 1992] and interleukin-

2 (IL-2) [MacDonald et al, 1990] suppress the growth of CFU-F in vitro but the effect of IL-4 on CFU-F growth has not previously been reported. The inhibitory effects of IFNα, IL-2 and IL-4 may therefore have potential use in the treatment of conditions of altered stromal cell growth such as marrow myelofibrosis. High affinity receptors for IL-4 have been demonstrated on cloned murine bone marrow stromal cell lines [Lowenthal et al, 1988]. In addition, IL-4 has been shown to up-regulate the expression of the adhesion molecule VCAM-1 on human marrow stromal cells [Simmons et al, 1992]. By contrast, TGFβ and MIP-1α, well documented inhibitors of haemopoietic progenitor cell proliferation [Broxmeyer et al, 1990; Hooper, 1991] showed no inhibitory activity on CFU-F colony formation.

In conclusion, we have developed a simple, reproducible serum deprived clonogenic assay for CFU-F which has allowed us to stringently examine the growth requirements of these stromal progenitors in vitro. Our data demonstrate the lack of response of CFU-F to a wide range of factors active on haemopoietic progenitors and, conversely, the very specific stimulatory effects of EGF and PDGF on these SPC. These experiments represent an essential prerequisite for future studies of the role of cytokines in the regulation of stromal cell proliferation, differentiation and development. This work even may lead to the further development of more sophisticated culture systems with the capacity to measure multi-potential human stromal stem cells and may also have implications for the pathogenesis and possible treatment of conditions of altered stromal cell growth such as marrow myelofibrosis.

CHAPTER FOUR: IDENTIFICATION AND PURIFICATION OF HUMAN BONE MARROW STROMAL PRECURSOR CELLS

4.0 INTRODUCTION

The bone marrow (BM) connective tissue is currently thought to arise from a pool of multi-potential stromal precursor cells (SPC) [Owen, 1985; Owen and Friedenstein, 1988] with the ability to develop into the phenotypically diverse population of stromal cell lineages found in the bone and marrow tissues. Following the initial description of putative BM SPC (CFU-F assay) by Friedenstein and colleagues (1970), subsequent studies have documeted variations in the morphology and proliferative capacity of different BM CFU-F clones expanded in vitro [Friedenstein et al, 1970; 1976; Castro-Malaspina et al, 1980; Owen et al, 1987; Bennett et al, 1991; Simmons and Gronthos, 1991]. The heterogeneous nature of the BM SPC population was further demonstrated in studies where individually expanded CFU-F clones displayed different developmental potentials both in vitro and in vivo [Friedenstein et al, 1980; Owen et al, 1987; Bennett et al, 1991]. In analogy with the haemopoietic system of cellular differentiation, these studies provide circumstantial evidence to support the concept of a stromal cell hierarchy, where different mature functional stromal cell lineages are derived from a heterogeneous population of bi- and uni-potential stromal progenitor cells that are ultimately derived from a common self-replicating multi-potential stromal stem cell.

The characterization of the bone marrow stromal precursor compartment has only recently been explored. Given the extensive literature regarding the existance of BM SPC in the rodent, little has been published concerning their phenotypic characteristics. This is due in part to the low incidence of CFU-F in aspirates of marrow (0.05% to 0.001%) [Castro-Malaspina et al 1980; Simmons and Torok-Storb, 1991a; 1991b; Falla et al, 1993; Waller et al, 1995a], and because of the paucity of antibody reagents that allow for the precise identification, isolation and functional characterization of the SPC population. Van Vlasselaer and colleagues (1994) have isolated BM SPC from murine BM following 5

days of 5-fluoracil (5-FU) treatment, identified by fluorescence-activated cell sorting (FACS) as $lin^-/Sca\text{-}1^+/WGA^{bright}$. This immunophenotype was ostensibly similar to that previously reported for murine haemopoietic progenitor cells [Spangrude et al, 1988]. However, further analysis demonstrated that the SPC population differed from their haemopoietic counterparts based on differences in their light scatter characteristics and by the absence on stromal precursors of haemopoietic surface markers such as Thy1.2 and c-kit [Van Vlasselaer et al, 1994]. Ironically, more is known of the cell surface phenotype of SPC in human haemopoietic tissues. Terstappen and colleagues have conducted a similar phenotypic survey of the antigens expressed by SPC in foetal BM [Hang and Terstappen, 1992; Waller et al, 1995a, 1995b]. Initial studies claimed that both primitive haemopoietic and stromal cells expressed the same composite immunophenotype ($CD34^+CD38^-HLA\text{-}DR^-$), suggesting that these two progenitor cell populations shared a common ontogeny [Huang and Terstappen, 1992]. However, subsequent immunophenotyping of the respective progenitor cell populations has revealed differences in the cell surface expression between SPC ($CD50^-$) and HSC ($CD50^+$) [Waller et al, 1995a, 1995b].

In accord with the findings in foetal BM, haemopoietic and stromal progenitor populations derived from aspirates of adult human BM were both found to express the CD34 antigen [Simmons and Torok-Storb, 1991b]. Further immunophenotypic analysis demonstrated that HSC and SPC represent two distinct cell populations, where CFU-F express low levels of the CD34 antigen and exhibit binding sites for lectin SBA [Ebell et al, 1985; Simmons and Torok-Storb, 1991b, Simmons et al, 1994]. In contrast, HSC express high levels of CD34 antigen and demonstrate no binding to SBA [Sutherland et al, 1989; Simmons and Torok-Storb, 1991b; Simmons et al, 1994]. These studies concluded that antibody reagents that react with the CD34 antigen are not specific for SPC and exhibit a low efficiency for isolating purified populations of SPC from adult human BM using a positive selection strategy. Therefore, SPC can be partially enriched from unfractionated human bone marrow aspirates based on binding to SBA or by using a negative selection

process based on their lack of expression of various cell surface antigens restricted to the myeloid, erythroid and lymphoid cell lineages [Simmons and Torok-Storb 1991a; 1991b; Simmons et al, 1994; Rickard et al, 1996].

Recent advances in the study of stromal stem cell biology have been attributed to the development of novel mabs which recognise antigens on BM SPC that are correspondingly not reactive with haemopoietic progenitors. Thus far, only two mabs namely STRO-1 and HOP-26, have been reported to be effective reagents for obtaining partially purified populations of CFU-F from fresh aspirates of human bone marrow based on positive immunoselection [Simmons and Torok-Storb, 1991a; 1991b; Joyner et al 1997]. Another novel mab 6-19, has been shown to react with BM CFU-F, based on antibody dependent complement mediated cytotoxicity, without effecting the growth of haemopoietic progenitors in vitro [Abboud et al, 1986; Iyer et al, 1990]. Immunohistochemical studies, have also identified several other mabs such as SH-2, SH-3, SH-4 [Haynesworth et al, 1992b] and SB-10 (ALCAM) [Bruder et al, 1997; 1998a], which demonstrate a unique reactivity with primitive elements in sections of osteogenic tissue but lack reactivity with more mature bone cell types and haemopoietic cells. Therefore, these mab reagents provide essential tools for further analyses of the cellular properties and functions of the BM SPC population.

One of the major objectives of this thesis was to identify and isolate homogeneous populations of SPC by means of positive immunoselection based on the unique specificity of the STRO-1 mab for stromal progenitor cells in aspirates of BM in order to properly study their biology. Studies were therefore designed to isolate SPC from a heterogeneous population of unfractionated bone marrow cells using a combination of cell isolation procedures in order to maximise the recovery and purity of the SPC population.

4.1 Partial Characterization of the STRO-1 Antigen

Preliminary studies were conducted to confirm the ability of the STRO-1 mab to identify BM CFU-F. The STRO-1 mab reacted with a minor population (mean 6.5% ± 0.73 SEM, n=20 individual BM samples from different donors) of human BMMNC cells derived from normal individuals (Figure 17). To examine the reactivity of the STRO-1 mab with CFU-F, BMMNC were isolated using two-colour FACS analysis based on the cell surface expression of both STRO-1 and the erythroid marker, glycophorin-A as described in the methods (Figure 18). The results demonstrated that BM CFU-F were found exclusively in the STRO-1^+ fraction of the BMMNC population (Table 6) in accord with the initial findings of Simmons and Torok-Storb (1991a). Furthermore, there was a 15.6 fold enrichment in the incidence of CFU-F when selected on the basis of STRO-1^+/glycophorin-A^- expression, in comparison to unfractionated BMMNC and to cells sorted based on the STRO-1^+/glycorphorin-A^+ phenotype (Table 6).

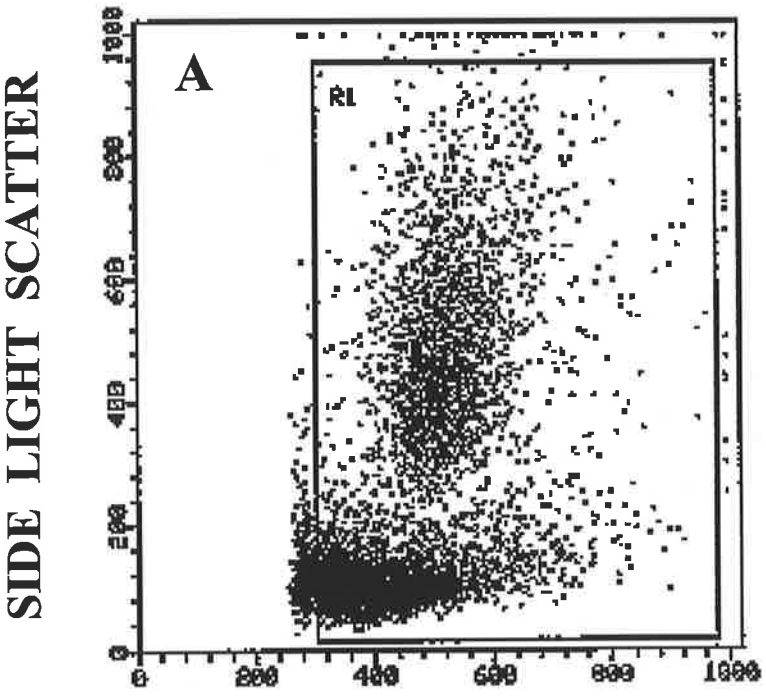
A survey of various stromal cell lines was conducted in order to identify those lines expressing high levels of the STRO-1 antigen. Single cell suspensions of each cell line were incubated with the STRO-1 mab and then analysed using single-colour immunofluorescence as described in the methods. The majority of the cell lines failed to react with the STRO-1 mab. However, the human osteosarcoma cell line, MG63 and the mouse BM stromal cell line BMS2 were found to exibit high levels of the STRO-1 antigen while the human foreskin fibroblast line, HFF-2 exibited intermediate levels of STRO-1 expression (Figure 19). The cell lines, MG63, BMS2 and HFF-2 were subsequently utilized in studies designed to identify and characterise the STRO-1 antigen.

The STRO-1 antigen was shown to be resistant to a wide range of proteolytic enzymes including trypsin (data not shown), with the exception of protease K, which slightly perturbed STRO-1 expression on MG-63 cells (Figure 20C). Enzyme activity was confirmed by demonstrating complete suppression of the protease K sensitive antigen CD71 on the same cells (Figure 21). Furthermore, many molecules such as CD59 are

Table 6. Human CFU-F are STRO-1$^+$/Glycophorin-A$^-$. BMMNC were selected by FACS then cultured under standard clonogenic conditions. The data represents the mean number of day 14 CFU-F per 10^5 cells plated ± S.E. (n=3 different BM aspirates).

Bone Marrow Cell Fraction	Frequency of CFU-F (CFU-F/10^5 Cells)	Enrichment (Fold Increase)
Unfractionated BMMNC	10.0 ± 0.8	1.0
STRO-1$^+$/AA6$^-$	156 ± 25.6	15.6
STRO-1$^+$/AA6$^+$	10.7 ± 1.0	1.3
STRO-1$^-$/AA6$^+$	0.0	0.0
STRO-1$^-$/AA6$^-$	0.0	0.0

Figure 17. The immunoreactivity of the mab STRO-1 with adult human bone BMMNC. A representative dot plot frequency histogram demonstrates the heterogeneous nature of the BMMNC population based on the side light scatter (granularity) versus the forward light scatter (cell size) properties (A). All subsequent studies using FACS analysis were conducted by gating on the total BMMNC population (quadrant R1). A frequency histogram is shown representing the flow cytometric analysis of BMMNC immunoreacted with the mab STRO-1 coupled to FITC (B). The data is expressed as the relative cell count (y axis) versus the fluorescence intensity (log scale) of the STRO-1 mab coupled to FITC (x axis) where 2×10^4 events were collected as listmode data. The horizontal bar (region M1) represents the level of fluorescence <1.0% of the isotyped matched control mab (1A6.12) coupled to FITC. Typically, the STRO-1 mab was found to react with approximately 6.5% of the BMMNC population in the designated region.


A
R1
SIDE LIGHT SCATTER
FORWARD LIGHT SCATTER

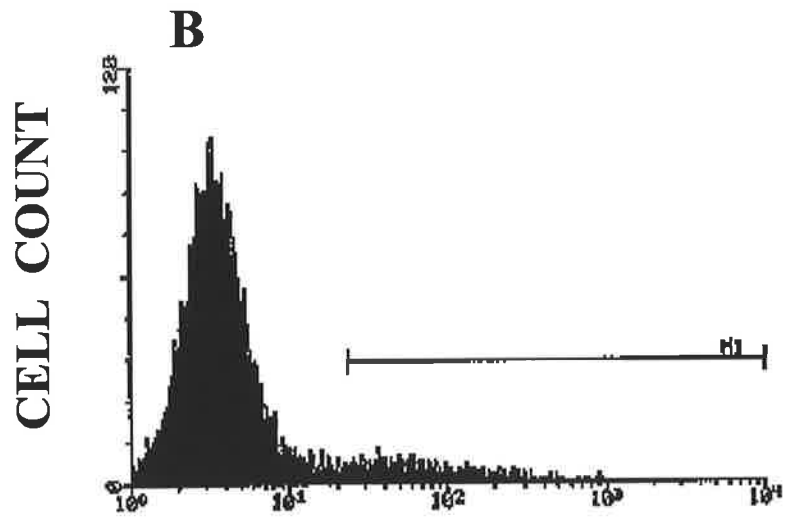
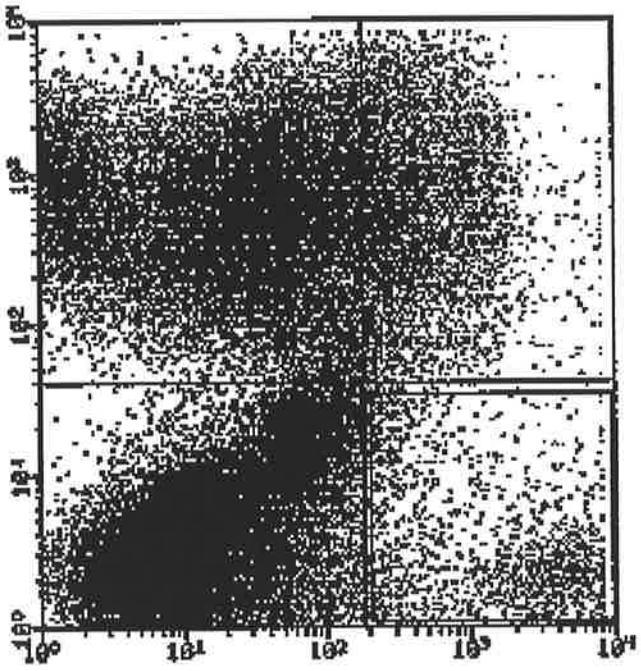

B
R1
CELL COUNT
STRO-1 FITC

Figure 18. Dual-colour immunofluorescence flow cytometric analysis of BMMNC immunoreacted with the mab STRO-1 coupled to FITC (x axis) and the mab AA6 (anti-glycophorin A) coupled to PE (y axis). The dot plot histogram represents 5 x 10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% mean fluorescence obtained with the isotype-matched control antibodies, 3D3 (PE) and 1A6.12 (FITC). The results demonstrate that the majority of the STRO-1 population (90%) co-expressed glycophorin-A (upper right quadrant) while the remaining STRO-1^+ cells failed to react with the mab AA6 (lower left quadrant). Cells isolated by FACS analysis from all four quadrants were subsequently assayed for the incidence of CFU-F (Table 6).

GLYCOPHORIN-A PE



STRO-1 FITC

Figure 19. STRO-1 expression on different stromal cell lines. Frequency histograms, representing the immunofluorescence flow cytometric analysis based on the immunoreactivity of the mab, STRO-1 with single cell suspensions of: the human osteosarcoma cell line, MG-63 (A); the murine bone marrow stromal cell line, BMS2 (B); and the human foreskin fibroblast line, HFF-2 (C). The data is expressed as the relative cell count (y axis) versus the fluorescence intensity (log scale) of the cell-bound STRO-1 mab coupled to PE (x axis) and 2×10^4 events were collected as listmode data. The horizontal bar (region D) depicts the level of fluorescence <1.0% of the isotyped matched control mab, 1A6.12 coupled to PE. The majority of the cell populations in panels A and B exhibited high expression of STRO-1 while only a subpopulation of cells in panel C expressed the STRO-1 antigen.

RELATIVE CELL COUNT

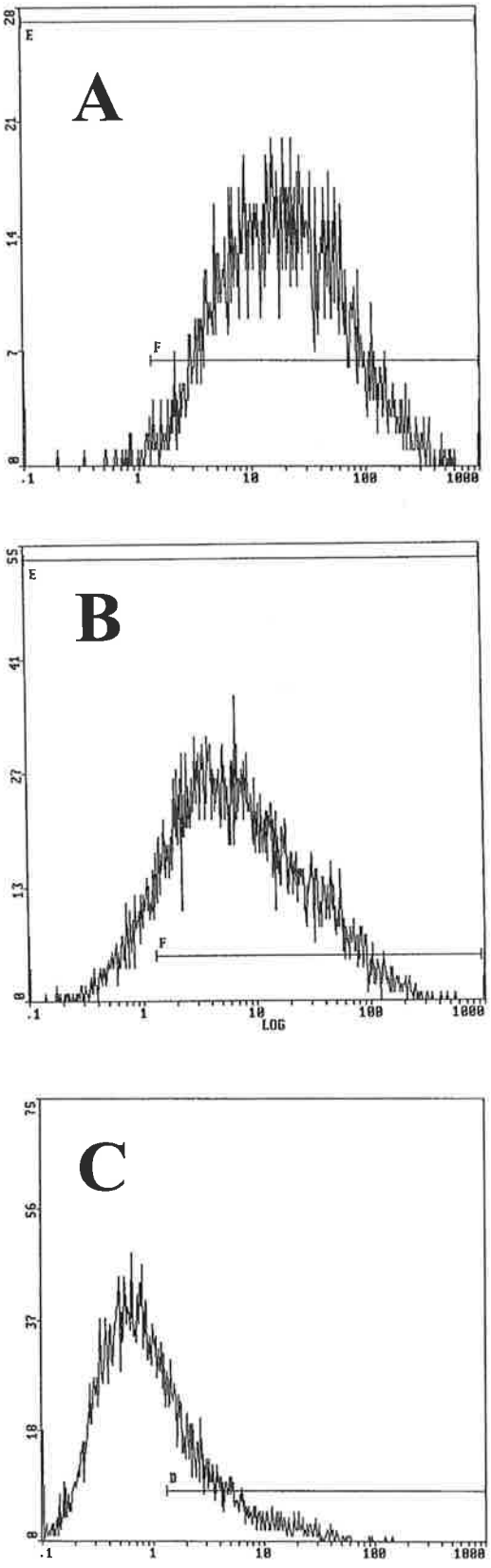


Figure 20. STRO-1 expression in response to PI-PLC and protease K activity. Frequency histograms, representing the immunofluorescence flow cytometric analysis of the STRO-1 expression on single cell suspensions of: untreated MG-63 cells (A); MG-63 cells pre-treated with 50 mU/ml PI-PLC for 2 h at 37^{o}C (B); MG-63 cells pre-treated with 1mg/ml protease K for 1 h at 37^{o}C (C). The data is expressed as the relative cell count (y axis) versus the fluorescence intensity (log scale) of the mab STRO-1 coupled to PE (x axis) where 2 x 10^{4} events were collected as listmode data. The horizontal bar (region F) depicts the level of fluorescence <1.0% of the isotyped matched control mab, 1A6.12 coupled to PE. There was no difference in the mean fluorescence between panels A and B. A decrease of 17% in the mean fluorescence was observed in panel C when compared to the control (A) in the designated region.

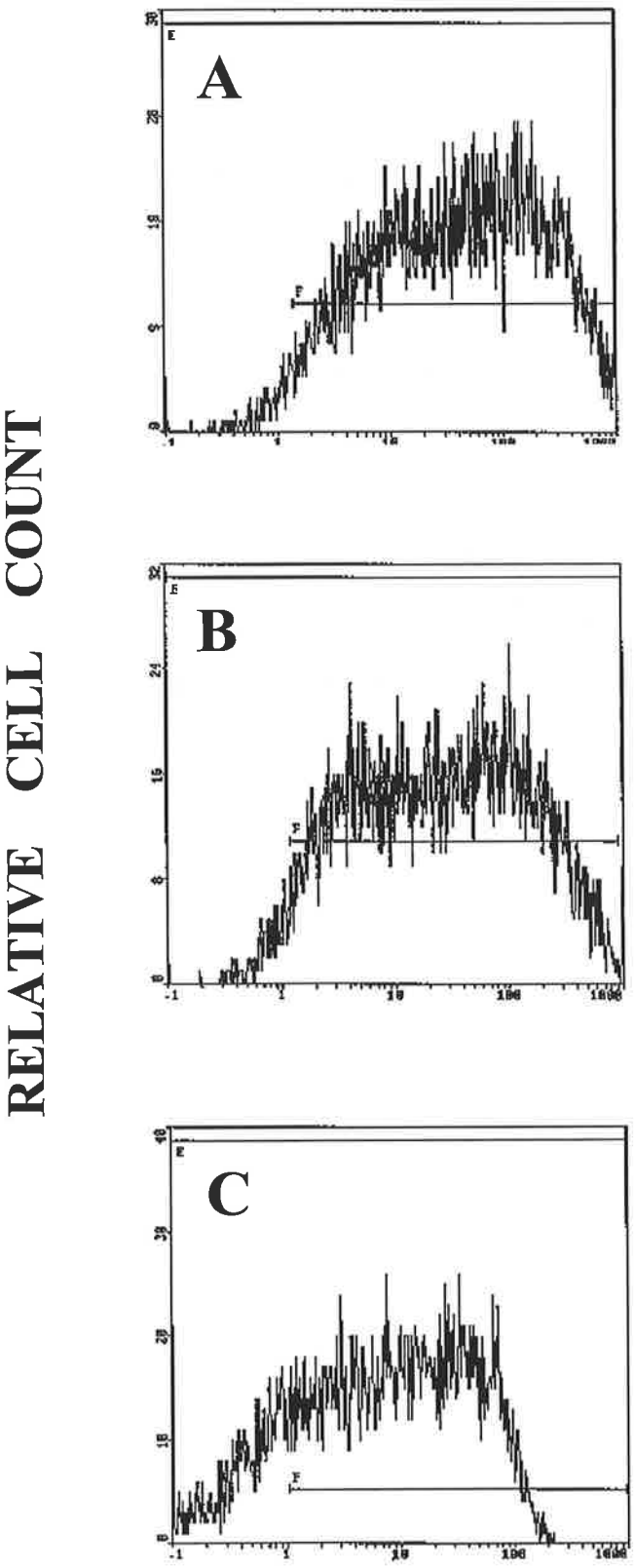
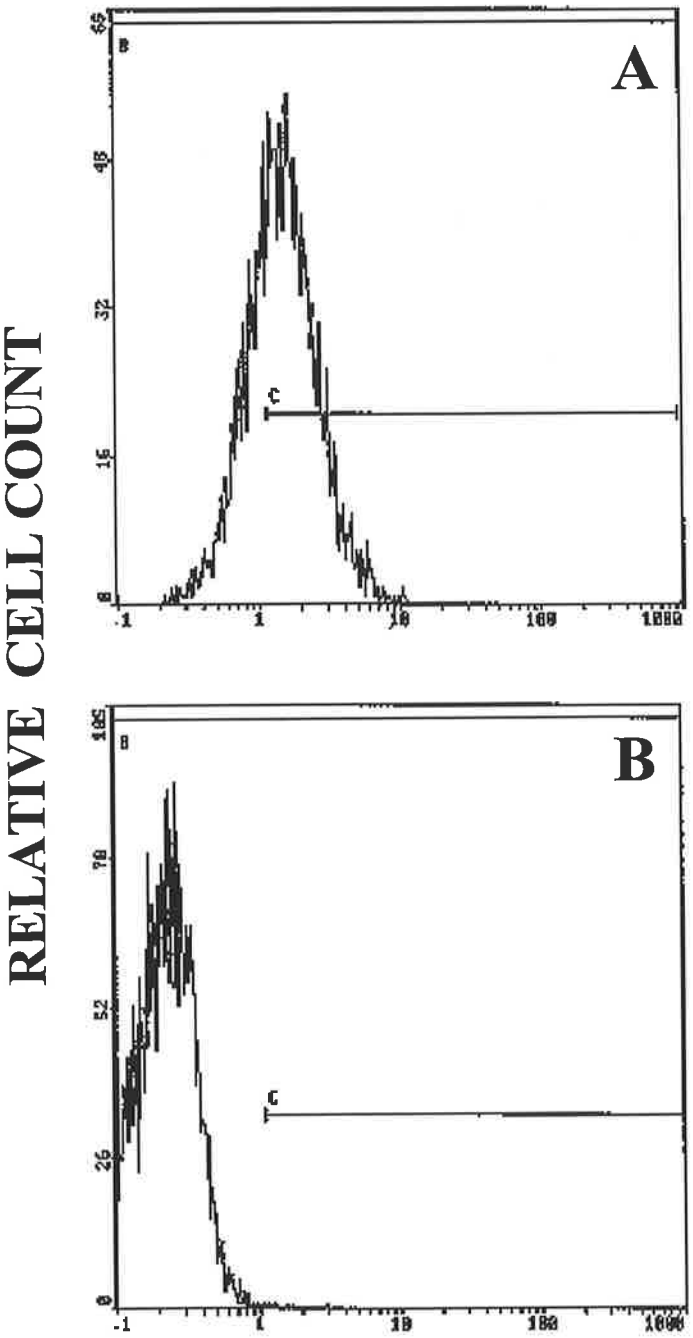

A
B
C
RELATIVE CELL COUNT
STRO-1 PE

Figure 21. CD71 expression in response to protease K activity. Frequency histograms, representing the immunofluorescence flow cytometric analysis of single cell suspensions of: untreated MG-63 cells (A); MG-63 cells pre-treated with 1mg/ml protease K for 1 h at $37^{o}C$ (B). The data is expressed as the relative cell count (y axis) versus the fluorescence intensity (log scale) of the mab T9 (anti-CD71) coupled to PE (x axis) where 2×10^{4} events were collected as listmode data. The horizontal bar (region C) depicts the level of fluorescence <1.0% of the isotyped matched control mab, 3D3 coupled to PE. The results demonstrated that there was a complete loss in the expression of CD71 in the presence of protease K (B).

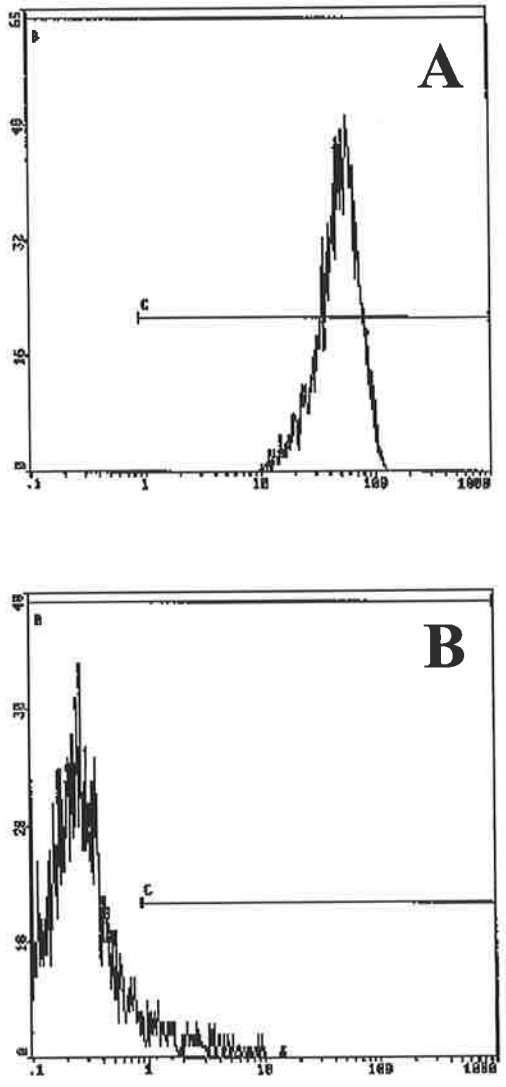

A
B
RELATIVE CELL COUNT
CD71 PE

known to be anchored to phosphatidylinositol on the cell membrane and are cleaved by the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) (Figure 22). Treatment of MG63 cells with PI-PLC failed to alter the expression of STRO-1 suggesting that the STRO-1 antigen was not attached to the cell surface via a glycosyl-phosphatidylinositol (GPI)-link (Figure 20B). To determine whether the STRO-1 antigen was glycosylated, MG63 cells were incubated in the presence of varying concentrations of tunicamycin and then immunoreacted with the STRO-1 mab in situ. The results indicated that the STRO-1 expression did not alter in the presence of tunicamycin and therefore did not recognise an N-linked glycosylated epitope (Figure 23A). In contrast, the known N-linked glycosylated CD59 epitope recognised by the HCC-1 mab was found to be sensitive to treatment with tunicamycin (Figure 23).

The relative molecular weight of the putative polypeptide chain recognised by the STRO-1 mab has been determined using extracts of cell membranes isolated from single cell suspensions of MG63, BMS2 and HFF-2 cell lines and processed for SDS-PAGE and western blotting as described in the methods. Following the immunolabelling with either the STRO-1 mab or the control mabs, the immunoreactive proteins were detected using a Molecular Dynamics FluorImager. The STRO-1 mab consistently reacted with a protein having an approximate molecular weight of a 60 kDa in all the different cell lysates used under both reduced and non-reduced conditions (Figure 24). However, the STRO-1 mab failed to immunoprecipitate the antigen using biotinylated or iodinated cell lysates generated from the same cell lines. In addition, repeated attempts to identify the cDNA encoding for the STRO-1 antigen were unsuccessful. Despite the limited knowledge concerning the nature of the STRO-1 antigen, the mab nevertheless exhibits a unique reactivity with BMMNC and is an ideal reagent for identifying and isolating potentially pure populations of bone marrow stromal precursor cells.

Figure 22. CD59 expression in response to PI-PLC activity. Frequency histograms, representing the immunofluorescence flow cytometric analysis of single cell suspensions of: untreated MG-63 cells (A); MG-63 cells pre-treated with 50mU/ml PI-PLC for 2 h at 37^{o}C(B). The data is expressed as the relative cell count (y axis) versus the fluorescence intensity (log scale) of the mab HCC-1 (anti-CD59) coupled to PE (x axis) where 2×10^{4} events were collected as listmode data. Region C depicts the level of fluorescence <1.0% of the isotyped matched control mab, 1A6.12 coupled to PE. The results demonstrate that there was a complete loss in the expression of CD59 on MG-63 cells in the presence of PI-PLC (B).

RELATIVE CELL COUNT



HCC-1 PE

Figure 23. STRO-1 expression in response to tunicamycin treatment. In situ immunofluorescence staining of: cultured MG-63 cells pre-treated with between 0.1 to 10 μg/ml tunicamycin for 48 h at 37°C then immunoreacted with the mab STRO-1 coupled to FITC (A); untreated cultured MG-63 cells immunreacted with the mab HCC-1 (anti-CD59) coupled to FITC (B); cultured MG-63 cells pre-treated with tunicamycin for 48 h at 37°C then immunoreacted with the mab HCC-1 coupled to FITC (C) (40X). The STRO-1 antigen was found to be unaffected by treatment with tunicamycin even at the highest dose used (A), and demonstrated the same fluorescence intensity when compared to the untreated cultures. In contrast, CD59 expression was lost in a dose-dependent manner in the presence of varying concentrations of tunicamycin (0.1 to 10mg/ml) and was completely diminished at the highest concentration (C).

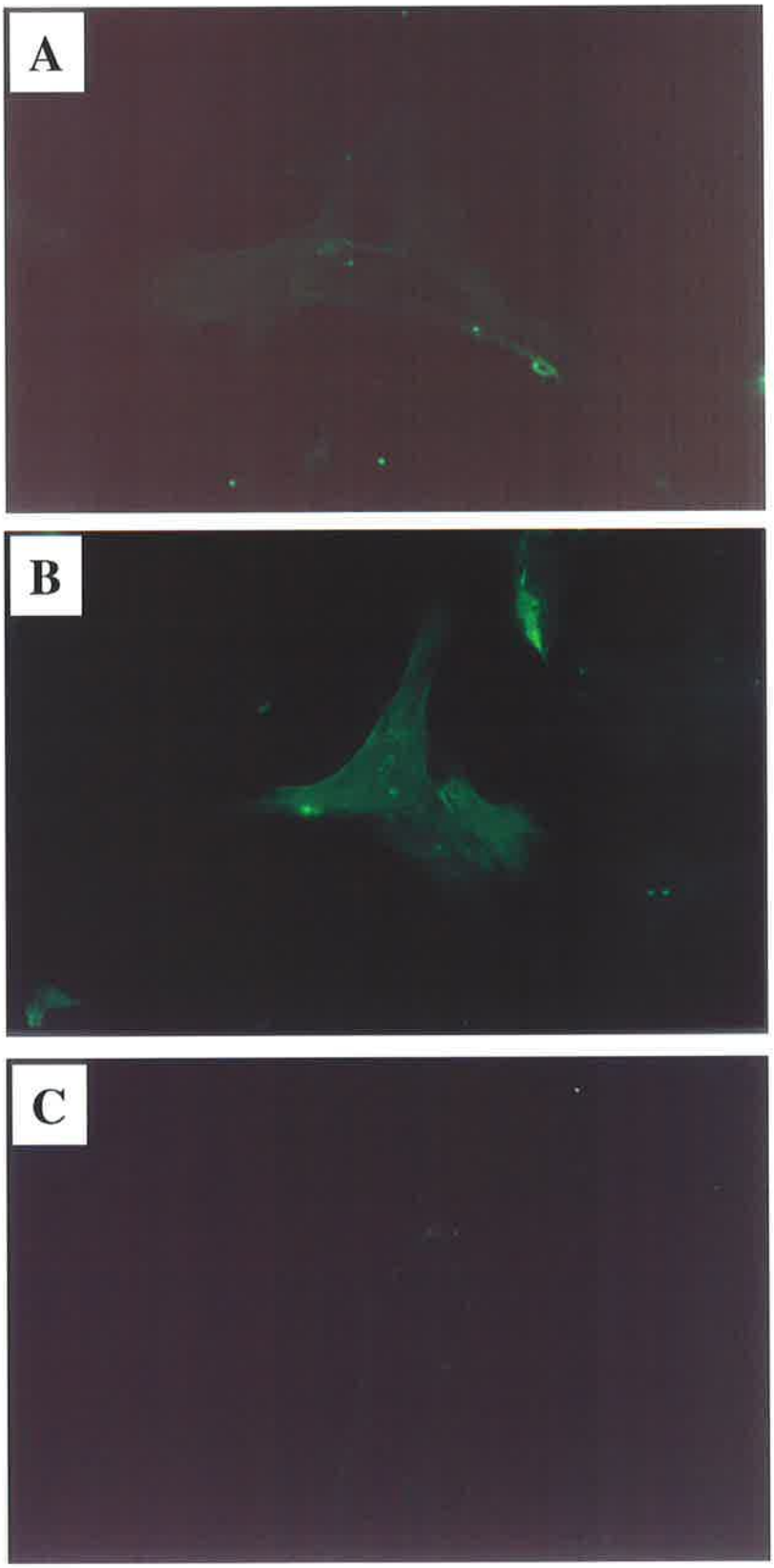
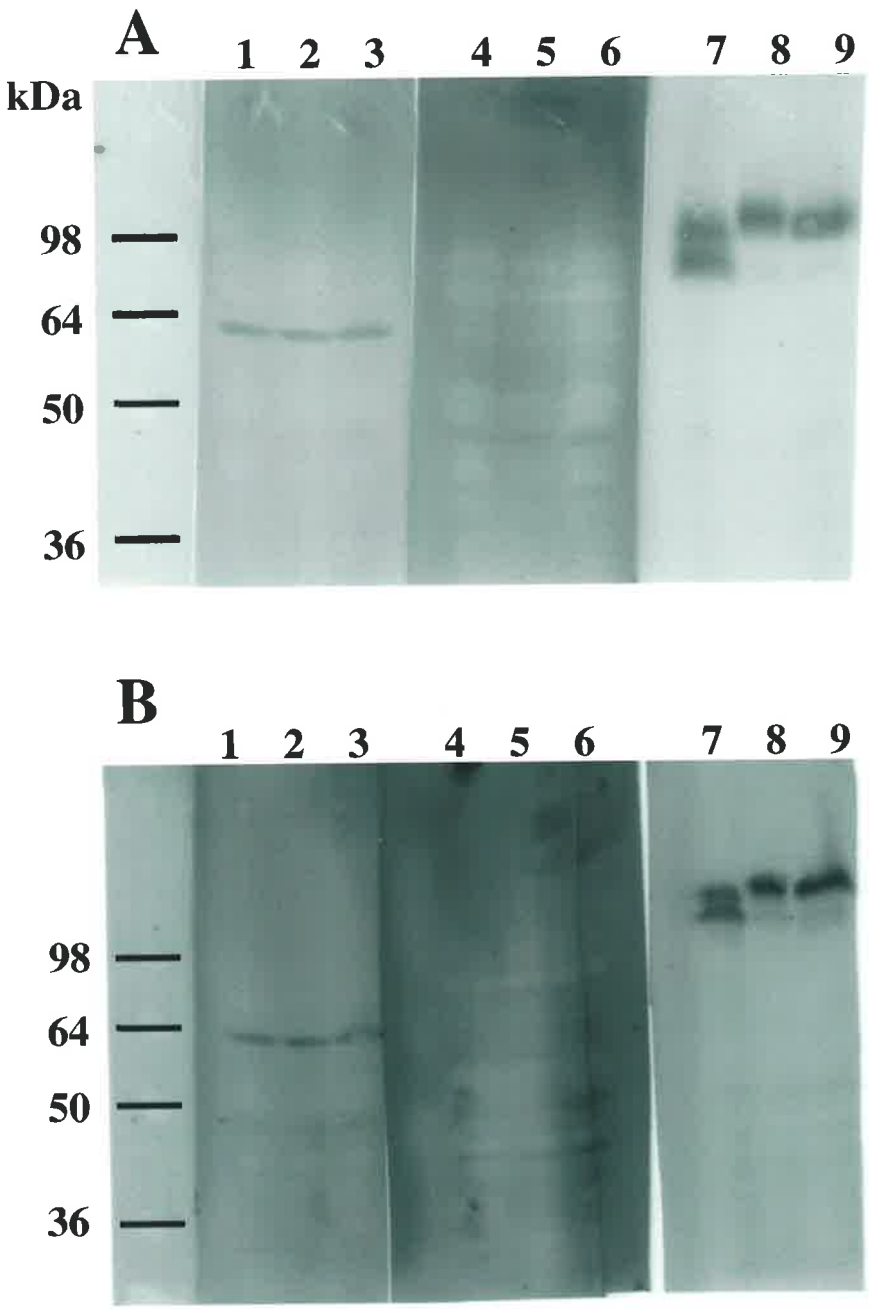

A
B
C

Figure 24. Determination of the molecular mass of the antigen identified by the mab STRO-1 using western blot analysis. Membrane preparations of: MG-63 cells (Lanes 1, 4 & 7); BMS2 cells (Lanes 2, 5, & 8); HFF-2 cells (Lanes 3, 6 & 9) were separated by 10% SDS-polyacrylamide gel electrophoresis under non-reducing (A) and reducing (B) conditions. The gels were transferred onto nitrocellulose and were subsequently immunoreacted with the mabs: STRO-1, Lanes 1, 2 & 3; HCC-1 (antiCD59), Lanes 4, 5 & 6; 61.2C4 (antiCD29), Lanes 7, 8 & 9. The immunoreactive proteins were detected by incubating the membranes with a goat-anti mouse Ig conjugated to alkaline phophatase, followed by exposure to the Vistra ECF substrate solution and then analysed by fluorescence using a FluorImager. The results demonstrate that the STRO-1 mab identified an antigen with a molecular mass of approximately 60 kDa in lysates prepared from MG-63 cells (Lane 1), BMS2 cells (Lane 2), and HFF-2 cells (Lane 3) under non-reduced (A) and reduced (B) conditions. No bands were detected in the negative control lanes 4, 5 & 6 for all conditions. The positive control lanes 7, 8 & 9 demonstrated immunoreactivity with the mab 61.2C4 corresponding to the CD29 antigen with the approximate molecular mass of 110 kDa in the non-reduced gel (A) and 130 kDa in the reduced gel (B). Note that membrane preparations from MG-63 cells yielded two distinct bands immunoreactive with the mab 61.2C4.


A
1 2 3 4 5 6 7 8 9
kDa
98
64
50
36
B
1 2 3 4 5 6 7 8 9
98
64
50
36

4.2 Isolation of STRO-1$^+$ Cells by Magnetic-Activated Cell Sorting (MACS)

We have demonstrated the effectiveness of MACS to isolate and enrich for CFU-F from aspirates of human BM based on the cell surface expression of the STRO-1 antigen (Figure 25) [Gronthos 1995; Gronthos et al, 1998]. This isolation procedure yields enrichments of approximately 11 fold in the incidence of CFU-F in the STRO-1$^+$ fraction compared to unfractionated BMMNC with 62% recovery of the total CFU-F population from the STRO-1$^+$ cell fraction. (Table 7). This was comparable to the enrichment (15 fold) in the frequency of BM CFU-F isolated by single-colour FACS using STRO-1. However, the recovery of CFU-F isolated by FACS was only 23% of the estimated total number of CFU-F present in the BMMNC samples.

Flow cytometric analysis of the STRO-1$^+$ cells isolated by MACS demonstrated a heterogeneous pattern of expression spanning over four decades in fluorescence intensity (Figure 26). Single-colour FACS was subsequently employed to subdivide the STRO-1$^+$ BMMNC fraction into three subsets; STRO-1^{dull}, STRO-1^{intermediate} and STRO-1^{bright}. Due to the STRO-1^{bright} population being several orders of magnitude greater in fluorecence intensity than the STRO-1^{dull} cells, it was found necessary to reduce the laser power in order to clearly observe the STRO-1^{bright} population. As a consequence, the STRO-1^{dull} cells in this and subsequent analyses now appear to completely lack expression of STRO-1. FACS was then used to separate the STRO-1$^+$ BMMNC isolated by MACS into STRO-1^{dull}, STRO-1^{intermediate}, STRO-1^{bright} subpopulations. Each fraction was subsequently assayed for CFU-F. These assays demonstrated that the majority of the CFU-F were contained within the STRO-1^{bright} cell fraction. There was a 950 fold increase in the incidence of CFU-F in the STRO-1^{bright} population when compared to unfractionated BMMNC (Table 8) demonstrating that BM CFU-F contained a high copy number of the STRO-1 antigen on their cell surface. The recovery of the CFU-F population in the STRO-1^{bright} fraction was 74% relative to the estimated total number of CFU-F in the BMMNC sample pre-MACS.

Table 7. Isolation of BM CFU-F: Comparison of two different cell separation techniques. STRO-1$^+$ BMMNC were isolated either by FACS or by MACS and then cultured under standard clonogenic conditions. The data represents the mean number of day 14 CFU-F per 10^5 cells plated ± SE.

Cell Fraction	Number of CFU-F/10^5 Cells (CFU-F Recovery % Total)	
	MACS, n=4 BM samples	FACS, n=6 BM samples
BMMNC	11.5 ± 2.5 (100)	14.0 ± 3.3 (100)
STRO-1$^+$	127 ± 6.8 (62)	212 ± 17.1 (23)
STRO-1$^-$	0.0	0.0

Table 8. BM CFU-F express high levels of the STRO-1 antigen. STRO-1$^+$ BMMNC were first isolated by MACS and then sorted according to the level of STRO-1 expression, using FACS. The different FACS sorted STRO-1 fractions were then cultured under standard clonogenic conditions. The data represents the mean number of day 14 CFU-F per 10^5 cells plated ± S.E. (n=5 different BM samples).

Cell Fraction	No. of CFU-F/10^5 Cells	CFU-F Recovery % Total
BMMNC	9.4 ± 2.3	100
STRO-1^{bright}	8,930 ± 415	74
STRO-1^{intermidiate}	48.0 ± 7.5	3.2
STRO-1^{dull}	0.0	0.0

Figure 25. STRO-1 expression following MACS isolation. Frequency histograms representing the immunofluorescence flow cytometric analysis of BMMNC isolated by MACS, on the basis of STRO-1 expression: unsorted BMMNC fraction (A); STRO-1^+ cell fraction (B); STRO-1^- cell fraction (C). Each histogram is based on 10^4 events collected as list mode data. The horizontal bar (region M1) depicts the reactivity levels of <1.0% mean fluorescence obtained with the isotype-matched control antibody 1A6.12 (FITC).

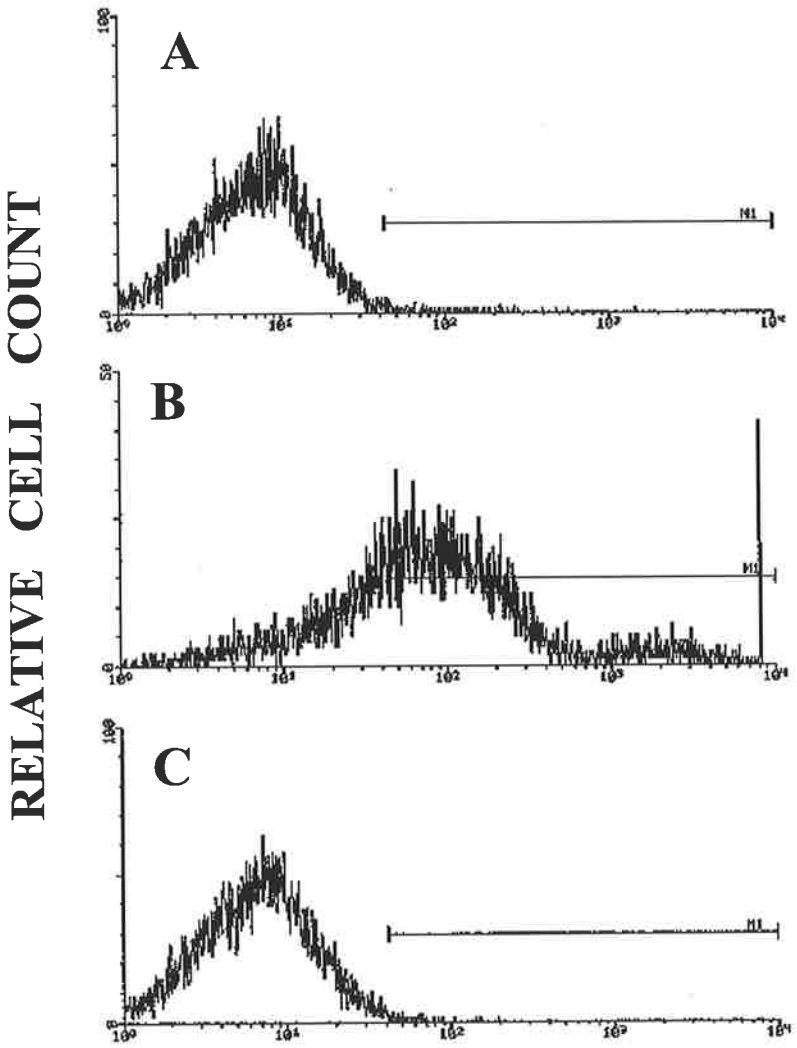
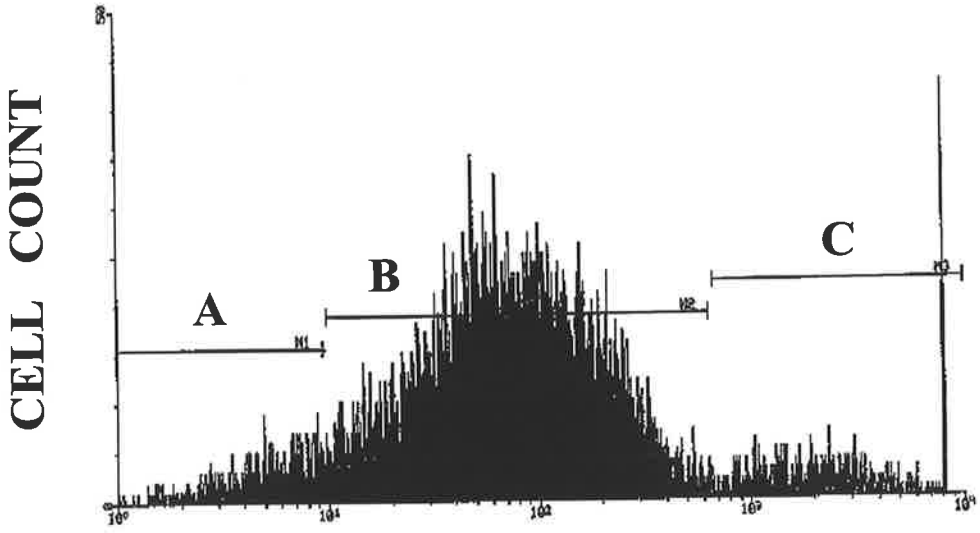

A
B
C
M1
RELATIVE CELL COUNT
STRO-1 FITC

Figure 26. Heterogeneous expression of STRO-1 following MACS isolation. Frequency histogram representing the immunofluorescence flow cytometric analysis of the total STRO-1^{+} BMMNC population isolated by MACS: STRO-1^{dull} cells (A, horizontal bar M1); STRO-1^{intermediate} cells (B, horizontal bar M2); STRO-1^{bright} cells (C, horizontal bar M3). The histogram is based on 10^{4} events collected as list mode data.


CELL COUNT
A
B
C
M1
M2
M3
STRO-1 FITC

4.3 Immunophenotyping of BM SPC using Dual-Colour FACS

A large panel of mabs specific for a variety of cell surface molecules were used to immunophenotype BM SPC in BM aspirates by FACS analysis inorder to find potential markers able to subset the STRO-1$^+$ BM cell fraction. The cell surface molecules examined were selected on the basis of previous in vitro studies which demonstrated the presence of these cell surface molecules on either bone marrow stroma, endothelial cells or on day 14 CFU-F [Abboud et al, 1986; Favaloro et al, 1990; Soligo et al, 1990; Aye et al, 1992; Chen et al, 1991b; Conforti et al, 1992; Irlin and Peled, 1992; Long 1992; Long et al, 1992; Simmons et al, 1992; Conway and Nowakowski, 1993]. An extensive immunophenotyping of the BM SPC population using dual-colour FACS analysis was compiled [Simmons et al, 1994]. Collectively, these studies revealed that SPC in fresh marrow aspirates, expressed a wide range of known leukocyte, vascular endothelial cell, and stromal cell surface antigens such as; cytoadhesion molecules, cytokines and cytokine receptors. The reactivity of these markers is summarised in Table 5. FACS analysis demonstrated that the cell surface markers; 6-19, VCAM-1, Thy-1, CD10, CD13, integrins (β_1, $\alpha_1\beta_1$,$\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, α_v, $\alpha_v\beta_3$, $\alpha_v\beta_5$), thrombomodulin, PDGF-receptor, EGF-receptor, IGF-1-receptor and nerve growth factor-receptor, were expressed on essentially all assayable CFU-F. Other markers such as; LFA-3, ICAM-1, PECAM-1, P-selectin, L-selectin, CD18, CD61, and SCF, were only present on a proportion (>10% but <80%) of SPC, while other cell surface molecules, such as CD44 and $\alpha_3\beta_1$, were either not expressed on SPC or were expressed on <10% of stromal progenitors respectively.

In vitro, the progeny of CFU-F demonstrated a similar immunophenotype to that observed for BM SPC in vivo, however there was a marked up regulation in the expression of the two markers CD44 and $\alpha_3\beta_1$, by cultured stromal cells. Conversely, various markers found to be expressed by all or a proportion of CFU-F (STRO-1, LFA-3, PECAM-1, P-Selectin, L-selectin, $\alpha_4\beta_1$, CD34, SCF, NGF-R, PDGF-R, IGF-1-R, and thrombomodulin) were found to be down regulated in vitro, where some markers were virtually undetectable in primary stromal cultures. When BMMNC were sorted based on the co-expression of

STRO-1 and either one of several markers (6-19, VCAM-1, Thy-1, CD10, CD13, β_1, $\alpha_1\beta_1$,$\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, α_v, $\alpha_v\beta_3$, $\alpha_v\beta_5$, thrombomodulin, PDGF-receptor, EGF-receptor, IGF-1-receptor and NGF-receptor), a level of greater than 100 fold enrichment of CFU-F was achieved in comparison to the frequency of CFU-F observed in unfractionated BMMNC or the STRO-1$^+$ fraction alone. A representative example is given demonstrating the level of enrichments achieved for BM CFU-F, using two-colour FACS (Table 9) based on the expression of STRO-1 and VCAM-1 antigens (Figure 27).

Although ideally suited as an analytical tool, FACS suffers from a number of significant limitations. Due to the low frequency of SPC present in human BMMNC (mean 11.8 CFU-F ± 1.14 SEM per 10^5 BMMNC, n=20 different individual BM samples, or approximately 0.01% of the total BMMNC population) FACS analysis was found to be technically limiting for recovering sufficient numbers of STRO-1$^+$ cells, required for large complex culture experiments or clinical studies. The isolation of rare cell types from heterogeneous populations using FACS often sacrificed cell recovery for cell purity. Therefore MACS was used in preference to FACS for the initial isolation of STRO-1$^+$ BMMNC because of its capacity to process high cell numbers.

4.4 Purification of the CFU-F population

To purify the CFU-F population to homogeneity a more accurate discrimination of the MACS isolated STRO-1^{bright} subset was required in order to differentiate those cells from the bulk of the STRO-1 population. This was achieved by incubating the total STRO-1$^+$ cells isolated by MACS, with a mab reactive with the stromal cell surface antigen VCAM-1 (Figure 28). VCAM-1 is part of the immunoglobulin superfamily of cell adhesion molecules and was chosen due to its restricted reactivity to a minor fraction of the BMMNC population including stromal precursor cells, and because the VCAM-1 molecule was not expressed on hematopoietic progenitor cells [Simmons et al, 1992]. Dual colour-FACS was then employed to identify and isolate the STRO-1^{bright}/VCAM-1$^+$ BMMNC fraction. Limiting dilution analysis by FACS was performed using the FACStar^{PLUS} automated cell

Table 9. Enrichment of human BM CFU-F by dual-colour FACS analysis based on the co-expression of the cell surface markers STRO-1 and VCAM-1. FACS sorted cells were cultured under standard clonogenic conditions. The data represents the mean number of day 14 CFU-F per 10^5 cells plated ± SE (n=4 different BM aspirates).

Bone Marrow Cell Fraction	Frequency of CFU-F (CFU-F/10^5 Cells)	Enrichment (Fold Increase)
Unfractionated BMMNC	12.0 ± 1.8	1.0
STRO-1$^+$/VCAM-1$^+$	1,834 ± 271	153
STRO-1$^+$/VCAM-1$^-$	0.0	0.0
STRO-1$^-$/VCAM-1$^+$	0.0	0.0
STRO-1$^-$/VCAM-1$^-$	0.0	0.0

Figure 27. STRO-1 and VCAM-1 expression on BMMNC. Dual-color immunofluorescence flow cytometric analysis of BMMNC immunoreacted with the mab STRO-1 coupled to FITC (x axis) and the mab 6G10 (anti-VCAM-1) coupled to PE (y axis). The dot plot histogram represents 5×10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% mean fluorescence obtained with the isotype-matched control antibodies, 3D3 (PE) and 1A6.12 (FITC). The results demonstrate that a small proportion of the STRO-1^+ population (approximately 2%) co-expressed VCAM-1 (upper right quadrant). Cells isolated by FACS analysis from all four quadrants were subsequently assayed for the incidence of CFU-F (Table 9).

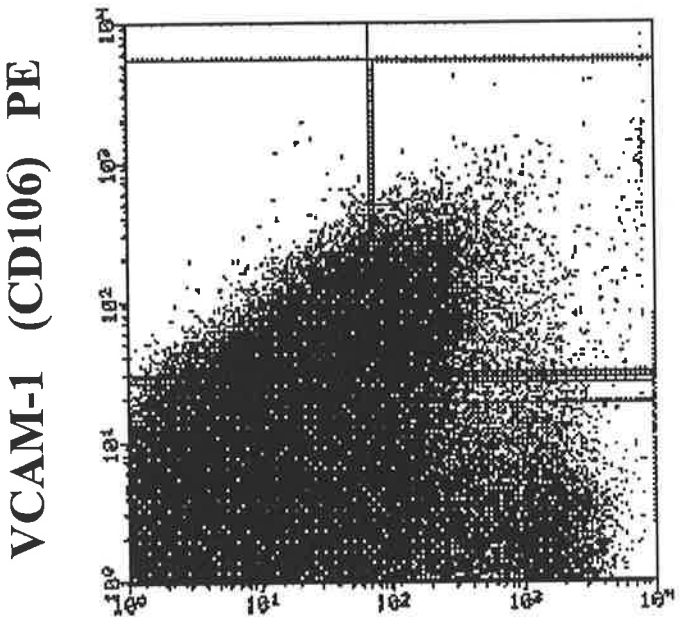
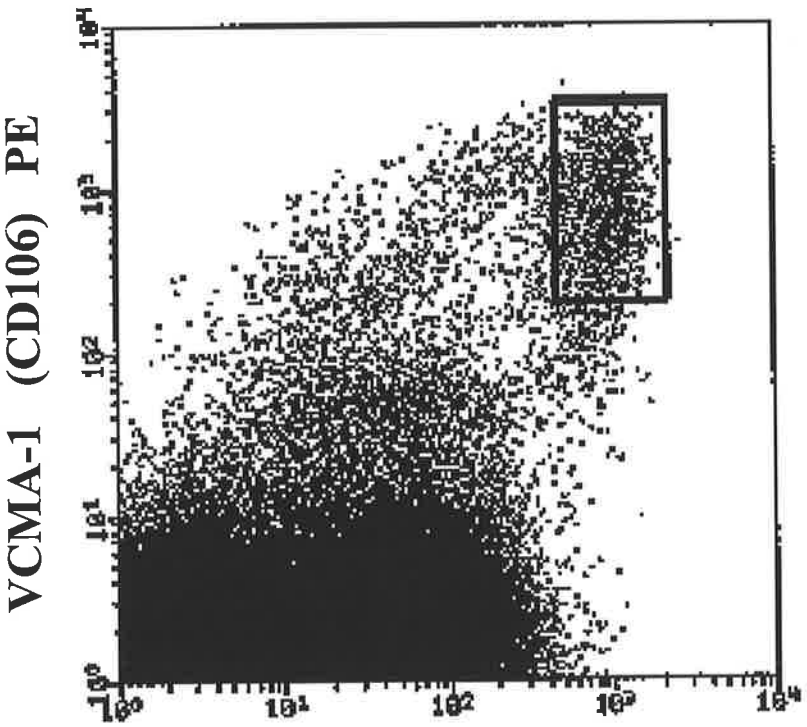

VCAM-1 (CD106) PE
STRO-1 FITC

Figure 28. Discrimination of the STRO-1^{bright} BM cell population by VCAM-1. Two-colour immunofluorescence cytometric analysis of MACS isolated STRO-1^{+} BMMNC incubated with the VCAM-1 mab, 6G10. The dot plot histogram represents 5 x 10^{4} events collected as listmode data. The STRO-1^{bright}/VCAM-1^{+} cells (rectangle) were sorted by FACS and represented less than 1.0% of the total STRO-1^{+} population.


VCMA-1 (CD106) PE
STRO-1 FITC

deposition unit to seed STRO-1^{bright}/VCAM-1^{+} cells at various plating densities under serum deprived conditions and then assayed for clonogenic growth. The data were analysed according to Poisson distribution statistics, previously used to quantitate the frequency of haemopoietic and osteogenic progenitors [Bellows and Aubin, 1989; Sutherland et al, 1990; Falla et al, 1993]. According to these analyses, the mean incidence (n=6 different marrow aspirates) of day 10 CFU-F colonies (≥50 cells) was determined to be 1 CFU-F per 3 STRO-1^{bright}/VCAM-1^{+} cells plated (Figure 29A). Furthermore, the incidence of clonogenic cells (clusters ≥10<50 cells + colonies) was found to be 1 per 2 STRO-1^{bright}/VCAM-1^{+} cells plated (Figure 29B). The combined MACS/FACS purification technique effectively achieved a 4.3 x 10^3 fold enrichment of the CFU-F population when compared to unfractionated BMMNC (1 CFU-F colony per 8.5 x 10^3 BMMNC).

The physical properties of the CFU-F population were assessed by either light microscopy of cytospin preparations stained with Wright's Geimsa or by TEM analysis of freshly sorted STRO-1^{bright}/VCAM-1^{+} cells. The morphological studies revealed that the BM CFU-F were a homogeneous population of large cells containing many long cytoplasmic processes and a large nucleus characterised with an open chromatin structure (Figure 30). No specific structures such as the endothelial specific Weibel-Palade bodies could be detected. This analysis demonstrates for the first time the morphological characteristics of BM SPC. A proportion of isolated CFU-F (approximately 25%) were also found to adhere to haematopoietic cells. The adhesion of leukocytes and or platelets to CFU-F may in part explain why some of the haemopoietic markers only identified a proportion of CFU-F in BMMNC preparations using FACS. In addition, cytospin preparations were fixed and permeabilised with acetone and methanol as described in the methods, then incubated with antibodies specific to smooth muscle actin and Factor VIII. Both antibodies failed to react with STRO-1^{bright}/VCAM-1^{+} cells (data not shown) suggesting that this cell population was not characteristic of differentiated smooth muscle cells or endothelial cells.

Figure 29. The incidence of SPC in the STRO-1^{bright}/VCAM-1^{+} BMMNC fraction. Limiting dilution analysis of the mean number of day 14 BM CFU-F colonies ± SE (A) and the mean number of colonies + cell clusters ± SE (B) from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC (n=6 different marrow donors). Colonies represented aggregates of ≥50 cells while clusters represented aggregates of ≥0<50 cells. The frequency of clonogenic cells was determined using Poisson distribution statistics by plotting the percentage of wells with no clonogenic growth against the number of cells plated per well (24 replicates per cell concentration).

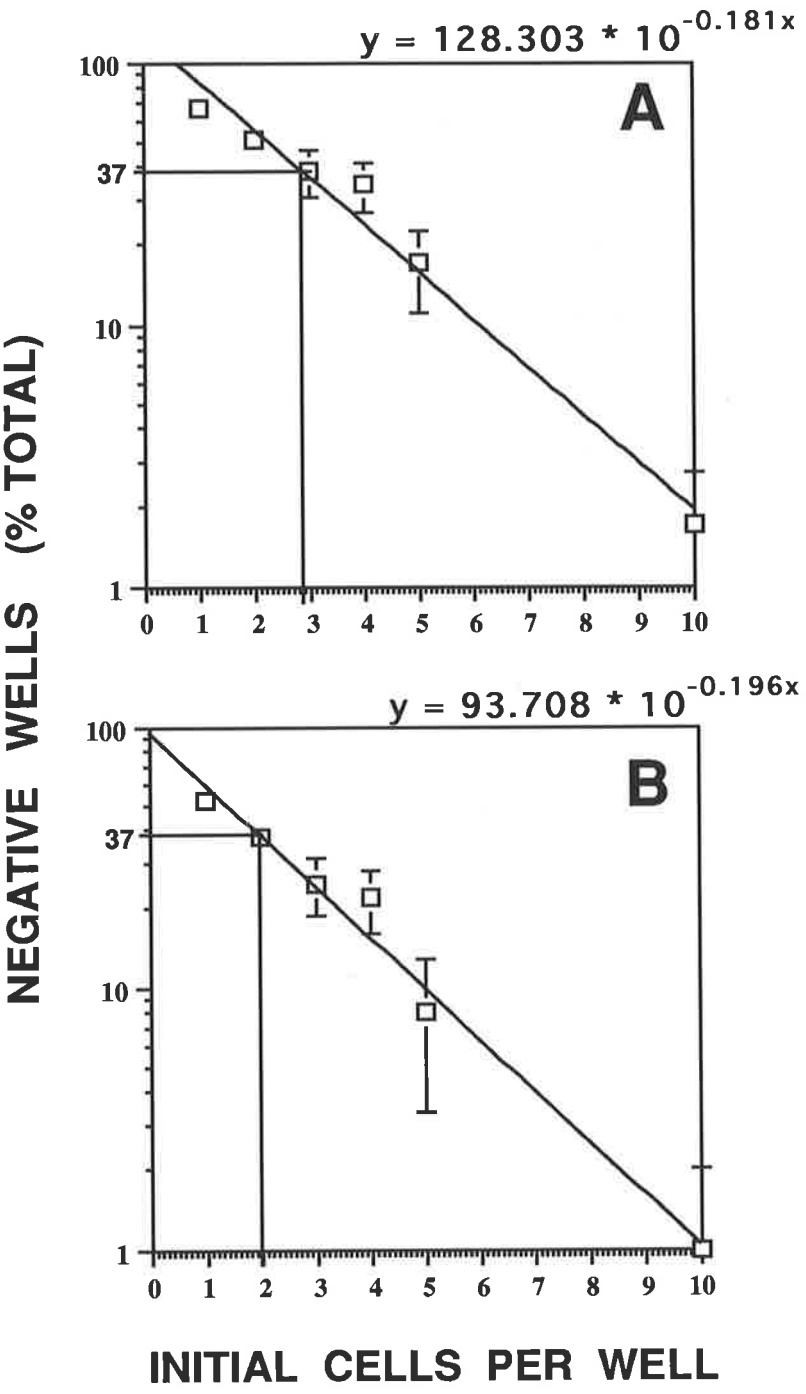
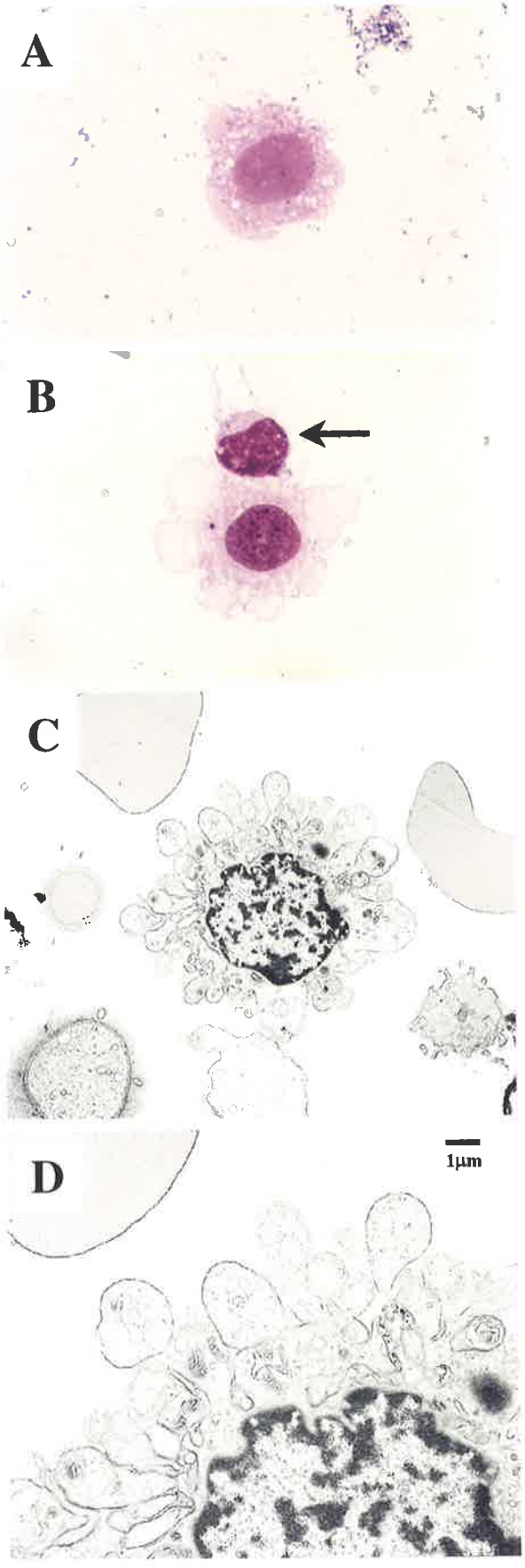
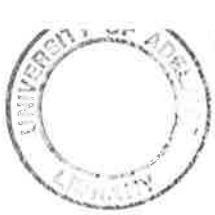

y = 128.303 * 10^-0.181x
A
100
37
10
1
0 1 2 3 4 5 6 7 8 9 10
y = 93.708 * 10^-0.196x
B
100
37
10
1
0 1 2 3 4 5 6 7 8 9 10
NEGATIVE WELLS (% TOTAL)
INITIAL CELLS PER WELL

Figure 30. Morphology of purified human SPC. Cells were isolated from bone marrow by MACS/FACS selection, based on the immunophenotype STRO-1^{bright}/VCAM-1^{+}. Panel A: depicts a photomicrograph representing a typical SPC prepared on a cytospin from freshly isolated STRO-1^{bright}/VCAM-1^{+} BMMNC and stained with Geimsa (100X). Panel B: represents a SPC on the same slide adhered to a haemopoietic cell (arrow) (100X). Panel C: shows an electron micrograph of a typical SPC from freshly isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, fixed in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer then prepared for transmission electron microscopy (2000X). Panel D: depicts the same cell at a higher magnification (15,000X). In vivo, BM SPC in vivo appear to be large cells of approximately 10 to 12 μm in diameter with numerous cytoplasmic processes and a large nucleus with an open chromatin structure.


A
B
C
1μm
D



Further analysis was conducted in order to define the molecular phenotype of the purified BM SPC population.

4.5 Gene Expression Pattern of BM SPC In Vivo

Total RNA obtained from STRO-1^{bright}/VCAM-1^{+} cells was used to generate full-length first-strand cDNA as described in the methods. RT-PCR analysis revealed the presence of various bone cell markers including bone sialoprotein, osteonectin, and collagen type I. However, there was an absence in the expression of osteopontin, the parathyroid hormone-receptor, and the more specific bone cell markers osteocalcin and the transcription factor CBFA1 (Figure 31). RT-PCR analysis of various fat-related markers revealed that lipoprotein lipase and the adipocyte human lipid binding protein were expressed by the STRO-1^{bright}/VCAM-1^{+} population but there was no detectable expression of other adipocyte restricted markers such as the obese gene product (leptin) and the early transcription factor PPARγ2 in these cells (Figure 32). Similarly, the cartilage specific markers collagen type II and aggrecan were not expressed by the CFU-F population using RT-PCR (Figure 33). However, the STRO-1^{bright}/VCAM-1^{+} population was found to express collagen type X, a marker normally associated with hypertrophic chondrocytes residing in the growth plate region of long bones. Overall the STRO-1^{bright}/VCAM-1^{+} population appeared to represent an early precursor population not yet fully committed to any particular stromal cell lineage. Furthermore, protein expression was also determined for some of these markers by means of flow cytometry. According to this analysis STRO-1^{bright} BMMNC were shown to co-express collagen type I, bone sialoprotein and osteonectin (Figure 34).

4.6 BM SPC are a Quiesent Cell Population In Vivo

Initial studies were conducted to examine any association between the expression of the STRO-1 antigen and the proliferation-associated human nuclear antigen, identified by the mab Ki-67 [Gerdes et al, 1983; 1984], in vitro using the STRO-1^{+} stromal cell lines MG63 and BMS2. Dual-colour immunofluorescence flow cytometry demonstrated that there was

Figure 31. BM SPC expression pattern of bone-related markers. Representative experiments of the expression of bone-related transcripts, as assessed by RT-PCR, using RNA derived from: primary cultures of normal human bone cells (NHBC) grown as explants from pieces of trabecular bone (positive control group) (A); purified human SPC isolated from marrow by MACS/FACS selection based on the immunophenotype STRO-1^{bright}/VCAM-1^{+} (B). The various bone-related markers examined included: CBFA1 (2); collagen type I (3); bone sialoprotein (4); osteopontin (5); osteonectin (6); osteocalcin (7); parathyroid hormone receptor (10). Molecular weight markers are shown in lanes 1 and 9. Water controls are represented in lane 8. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. The results indicated that SPC from freshly isolated STRO-1^{bright}/VCAM-1^{+} BMMNC lacked expression of the transcripts; CBFA1, osteopontin, osteocalcin and parathyroid hormone receptor, following 40 PCR cycles of amplification.

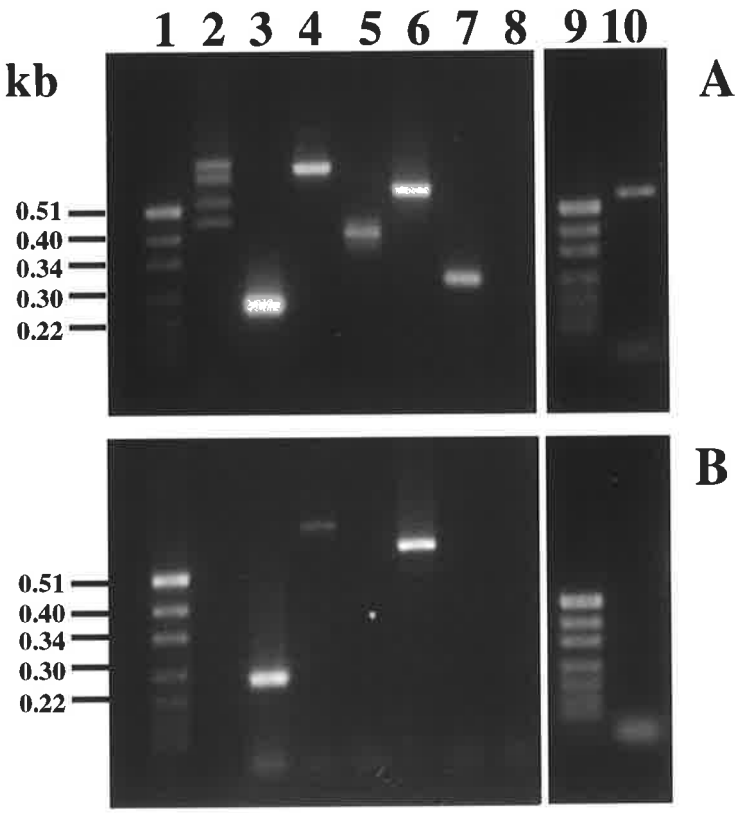

1 2 3 4 5 6 7 8 9 10
kb
A
0.51
0.40
0.34
0.30
0.22
B
0.51
0.40
0.34
0.30
0.22

Figure 32. BM SPC expression pattern of fat-related markers. Representative experiments of the expression of adipocyte-related transcripts, as assessed by RT-PCR, using RNA derived from: purified human SPC isolated from marrow by MACS/FACS selection based on the immunophenotype STRO-1^{bright}/VCAM-1^{+} (A); primary cultures of human marrow stromal cells grown in the presence of dexamethasone (10^{-8} M) for 6 weeks to induce adipogenesis (positive control group) (B). The various adipocyte-related markers examined in Panel A included: lipoprotein lipase (3); obese gene product (4); the transcription factor PPARγ2 (5); human-adipocyte lipid-binding protein (6). Molecular weight markers are shown in lane 1 and the water controls are represented in lane 2. The various adipocyte related markers examined in Panel B included: lipoprotein lipase (3); obese gene product (4); human-adipocyte lipid-binding protein (5); PPARγ2 (6). Molecular weight markers are shown in lane 2 and the water controls are represented in lane 1. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. The results indicated that SPC from freshly isolated STRO-1^{bright}/VCAM-1^{+} BMMNC lacked expression of the transcripts, obese gene product and PPARγ2, following 40 PCR cycles of amplification.

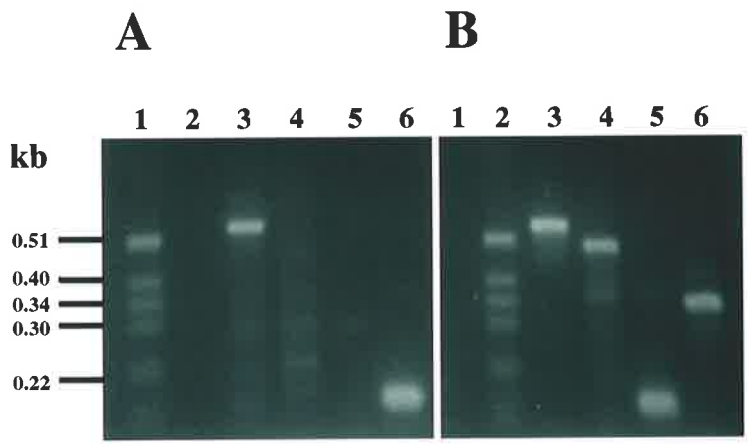

A
B
1 2 3 4 5 6 1 2 3 4 5 6
kb
0.51
0.40
0.34
0.30
0.22

Figure 33. BM SPC expression pattern of cartilage-related markers. Representative experiments of the expression of cartilage-related transcripts as assessed by RT-PCR using RNA derived from: a chondrosarcoma cell line and was used as a positive control group (A); purified human SPC isolated from marrow by MACS/FACS selection based on the immunophenotype STRO-1^{bright}/VCAM-1^{+} (B). The various cartilage-related markers examined included: aggrecan (2); collagen type II (3); collagen type X (4). Molecular weight markers are shown in lane 1 and the water controls are represented in lane 5. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. The results indicated that SPC from freshly isolated STRO-1^{bright}/VCAM-1^{+} BMMNC lack expression of the transcripts; aggrecan and collagen type II following 40 PCR cycles.

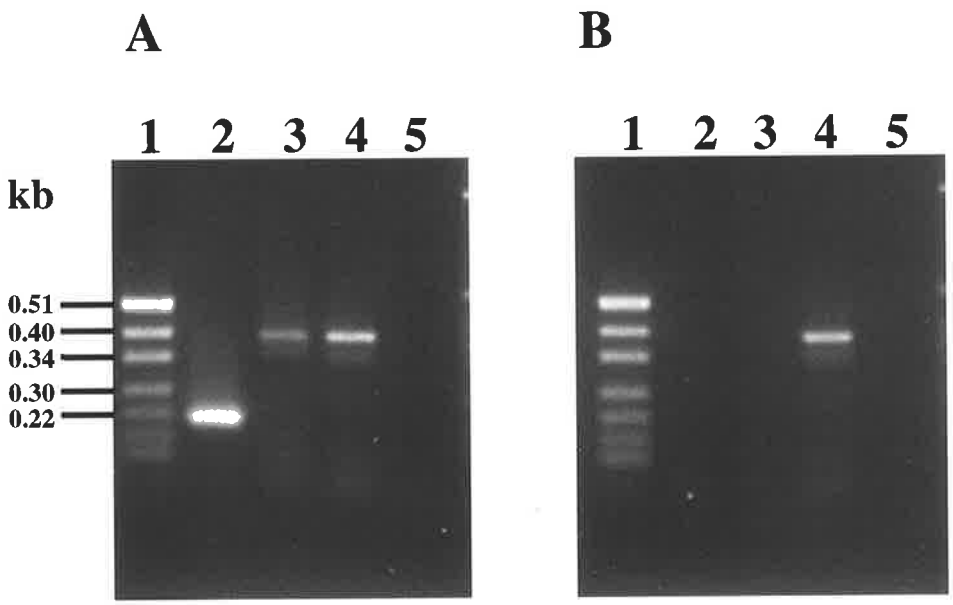

A
B
1 2 3 4 5
1 2 3 4 5
kb
0.51
0.40
0.34
0.30
0.22

Figure 34. STRO-1^{bright} BMMNC protein expression of bone-related markers. Dual-colour immunofluorescence flow cytometric analysis of STRO-1^{+} BMMNC isolated by MACS then fixed in 70% ethanol (-20^{o}C) for 15 min on ice and subsequently immunoreacted with the mabs: WVID1 (9C5) (antibone sialoprotein II, BSP); M-38 (anticollagen (pro) type I, COL-1); AON-1, (antiosteonectin, ON). The mabs WVD1 (9C5), M-38 and AON-1 were coupled to PE (y-axis) and the mab STRO-1 was coupled to FITC (x-axis) where the data was expressed as fluorescene intensity (log scale). The dot plot histogram represents 2 x 10^{4} events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% mean fluorescence obtained with the respective isotype-matched negative control antibodies, 3D3 (PE), 1D4.5 (PE), IgG_3 (PE) and 1A6.12 (FITC). The results demonstrated that freshly isolated STRO-1^{bright} BMMNC co-expressed BSP, COLL-1 and ON.

BSP PE
COLL-1 PE
ON PE
STRO-1 FITC

an even distribution in the number of STRO-1$^+$ MG63 (Figure 35A) and BMS2 cells (Figure 35B) which were either non-dividing cells (STRO-1$^+$/Ki67$^-$) or were in an active cycling state (STRO-1$^+$/Ki67$^+$). Therefore there was no correlation with the expression of the STRO-1 antigen and the cycling state of the cells examined.

Similar studies were performed to determine the cell cycling status of the SPC population in vivo by firstly obtaining the MACS isolated STRO-1$^+$ BMMNC fraction and then further incubating these cells with the Ki-67 specific mab as described in the methods. Two-colour flow cytometric analysis revealed that the majority of the STRO-1^{bright} cells which contained the CFU-F population lacked co-expression with the Ki-67 antigen demonstrating that these cells were a non-dividing population in vivo (Figure 36).

4.7 DISCUSSION

The present study investigated the possiblity of isolating homogeneous populations of SPC by means of a series of purification strategies designed to maximize the purity and the recovery of the CFU-F population. We and others have had success in isolating SPC based on their expression of the STRO-1 antigen either by FACS or by using immunomagnetic particles, such as Dynabeads [Tamayo et al, 1994] or by magnetic-activated cell sorting (MACS) [Gronthos et al, 1995 and 1998]. The latter was used initially to provide a reproducible technique for isolating BM derived CFU-F with the capacity to process high cell numbers. To date it has not been possible to characterise the cDNA encoding for the STRO-1 antigen that identifies a 60 kDa polypeptide. However, the Mab STRO-1 proved to be an ideal reagent for isolating SPC from adult BM because of its lack of reactivity to haemopoietic progenitors [Simmons and Torok-Storb, 1991a] yielding a clean separation between SPC and haemopoietic progenitors in adult BM. Moreover, the antigen identified by STRO-1 was found in the present study to be expressed at particularly high copy number by CFU-F, which may in part account for the high efficiency and recovery of BM CFU-F observed. These studies identified the minor STRO-1^{bright} subset of the total STRO-1$^+$ BMMNC fraction to contain the CFU-F population. However

Figure 35. The cell cycling status of STRO-1^+ MG-63 and BMS2 cells. Dual-colour immunofluorescence flow cytometric analysis of single cell suspensions of MG-63 cells (A) and BMS2 cells (B) immunoreacted with the STRO-1 mab coupled to PE (y-axis) then fixed in 70% ethanol (-20^{o}C) for 15 min on ice and subsequently immunoreacted with the mab Ki-67 coupled to FITC (x-axis). The dot plot histogram represents 2 x 10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% mean fluorescence obtained with the isotype-matched control antibodies, IgG_1 (FITC) and 1A6.12 (PE). The results demonstrated that there was an even distribution of cells expressing STRO-1 alone (upper left quadrant) in comparison to cells expressing both STRO-1 and Ki-67 (upper right quadrant).

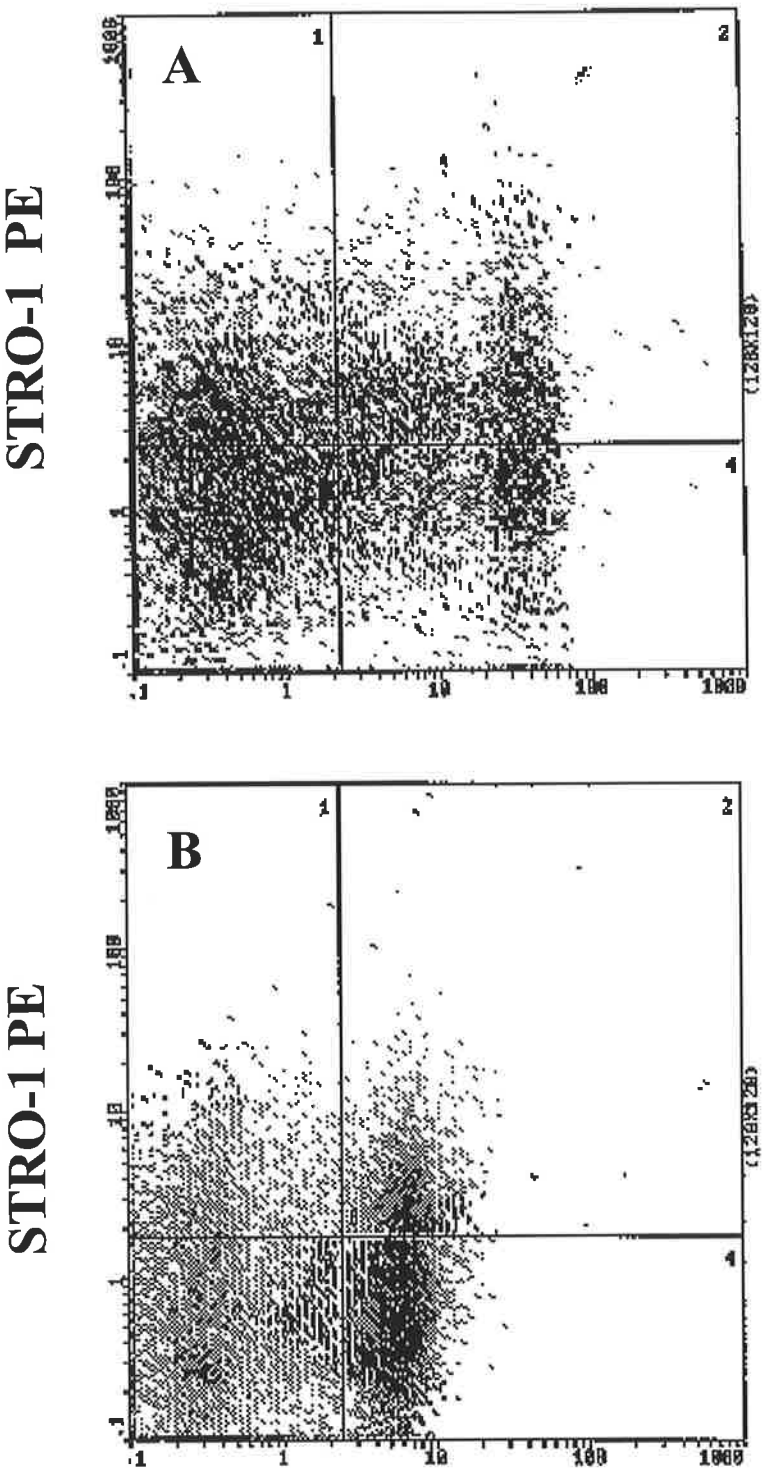
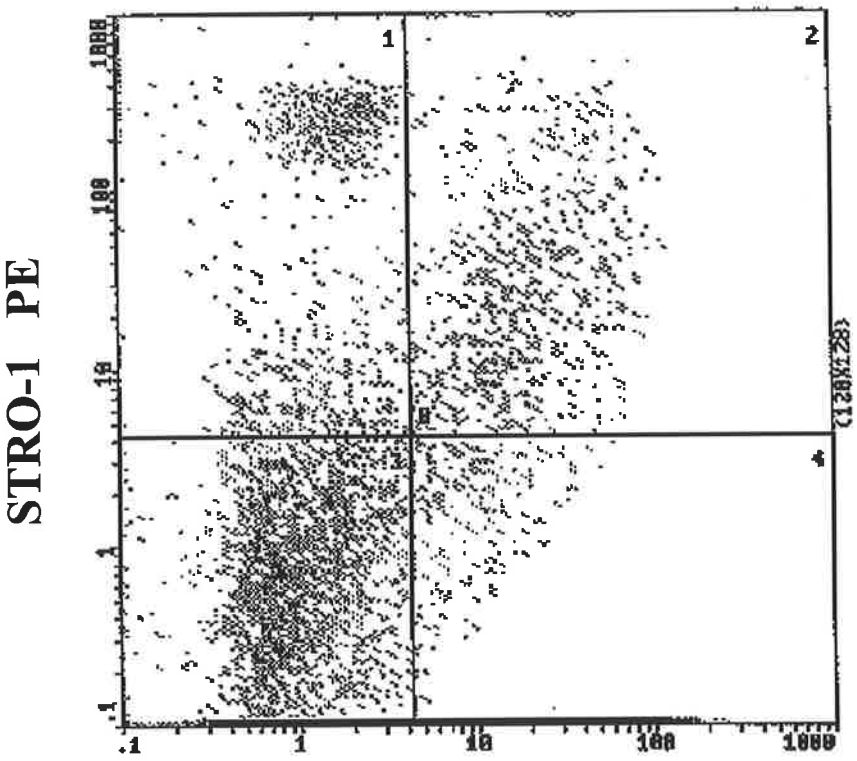

A
B
STRO-1 PE
STRO-1 PE
Ki-67 FITC

Figure 36. The cell cycling status of STRO-1^+ BMMNC. Dual-color immunofluorescence flow cytometric analysis of STRO-1^+ BMMNC isolated by MACS then fixed in 70% ethanol (-20^{o}C) for 15 min on ice and subsequently immunoreacted with the mab Ki-67. The mab Ki-67 was coupled to FITC (x-axis) and the mab STRO-1 was coupled to PE (y-axis) and the data is presented as fluorescence intensity (log scale). The dot plot histogram represents 2 x 10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% mean fluorescence obtained with the isotype-matched negative control antibodies, IgG_1 (FITC) and 1A6.12 (PE). The results demonstrated that freshly isolated STRO-1^{bright} BMMNC (upper left quadrant) lacked expression of the Ki-67 antigen.


STRO-1 PE
Ki-67 FITC
1
2
4
1000
100
10
1
.1
1
10
100
1000

the resulting post MACS STRO-1^{bright} cell population was only partially enriched for CFU-F.

To further increase the incidence of CFU-F in the STRO-1 selected fraction, additional purification steps were required. Characterisation of the SPC population involved the systematic analysis of the cell surface molecules expressed by CFU-F by means of two-colour FACS and by clonogenic assay of CFU-F in the sorted STRO-1 subpopulations. Antigens restricted to haemopoietic cells (CD3, CD14, CD19, CD33, CD38, CD45, glycophorin-A, HLA-DR) were not expressed at detectable levels on CFU-F in vivo or in vitro. The peptidase enzymes identified by CD10 (neutral endopeptidase) and CD13 (aminopeptidase) were expressed by virtually all CFU-F and their progeny, suggesting the possibility that they may be required to modify neighboring cell surface proteins (such as growth factors) or participate in the hydrolysis of other regulatory peptides in the immediate vicinity of the stromal precursor. These molecules are also found on haemopoietic cells [Ship and Look, 1993], as are immunoglobulin superfamily members including; PECAM-1 (CD31; Albelda et al, 1991), LFA-3 (CD58; Saeland et al, 1992), ICAM-I (CD54; Saeland et al, 1992; Arkin et al, 1991), and Thy-1 [Baum et al, 1992]. Immunoglobulin superfamily molecules such as LFA-3, ICAM-1, VCAM-1 and Thy-I are also present on cultured marrow stromal cells [Teixido et al, 1992; Caligaris-Cappio et al, 1991], as is N-CAM (CD56; Kincade et al, 1989) and have been shown to participate in adhesive interactions with haemopoietic progenitors [Arkin et al, 1991; Simmons et al, 1992]. Studies involving the cross-linking of ICAM-1 and VCAM-1 on the surface of stromal cells such as osteoblasts demonstrate an increase in the synthesis of the cytokine IL-6 [Tanaka et al, 1995] known to stimulate osteoclast differentiation and function. The expression by CFU-F of a wide range of members belonging to the integrin family of cell adhesion molecules on CFU-F may reflect the requirement for adhesion of SPC and their progeny to various components of the extracellular matrix (reviewed in Chapter 7). This data is in accord with previous studies demonstrating the presence of various integrins on culture expanded human BM CFU-F in vitro [Soligo et al, 1990].

A major question concerning the ontogeny of stromal cells is whether endothelial cells that line the bone marrow sinuses, or those present in the stroma of LTBMCs, are derived from a common progenitor cell to fibroblast cells, smooth muscle cells, adipocytes and osteoblasts. Various studies have suggested that a subset of human bone marrow CFU-F colonies cultured in vitro, have endothelial-like characteristics by the presence of collagen type IV and laminin [Lim et al, 1986]. Other studies have identified a subset of murine bone marrow CFU-F colonies in vitro which expressed collagen type IV, laminin and von Willebrand factor VIII [Wang and Wolf; 1990; Perkins and Fleischman, 1990; Pen et al, 1993]. However, in all these studies whole bone marrow mononuclear cells were used, which are contaminated with endothelial cells. Previous studies have shown that human BM cells isolated based on their expression of the STRO-1 antigen can establish functional adherent stromal cell layers under LTBMC conditions that are devoid of both endothelial cells and macrophage [Simmons and Torok-storb, 1991a]. The present study has shown that all, or at least a proportion of stromal progenitor cells do express many cell surface antigens, including PECAM-1, P-Selectin, thrombomodulin, 6-19 and CD34, that display specificity for vascular endothelial cells in vivo. Another Ig superfamily member MUC-18 (CD146) was also found to be constitutively expressed on human BM SPC and their progeny [Filshie et al, 1998]. This molecule has previously been shown to be expressed on metastatic melanoma cells, endothelial cells and smooth muscle cells [Kuzu et al, 1993; Sers et al, 1994]. Mabs that identify the MUC-18 antigen have been shown to effectively isolate differentiated endothelial cells from aspirates of human BM [George et al, 1991; Bardin et al, 1996]. The phenotypic data presented in this study are in accord with similar studies performed in the mouse, where BM stromal precursor cells with osteogenic potential were also shown to express various endothelial cell markers in vivo [Van Vlasselaer et al, 1994]. However, the expression of common antigens between endothelial cells and SPC doesn't necessarily infer a common ontogeny. Further studies are required to identify purified subpopulations of SPC with the capacity to form capillaries

following culture under in vitro conditions, previously shown to promote angiogenesis in human umbilical cord vein endothelial cells [Montesano and Orci, 1985].

To date, the majority of studies have only characterised the progeny of human BM SPC in vitro assayed in the presence of FCS, conditions which may result in the expression of a wide range of differentiation- and proliferation-related genes. Consequentley, the immunophenotype of cultured BM stromal cells may not be a true representation of the phenotype of SPC in vivo. As an example, in this study SPC failed to react with antibodies specific for the adhesion molecule CD44, even though the CD44 mab reacted with the majority (>95%) of the BMMNC. This observation correlates with the lack of expression of CD44 by osteoprogenitors in sections of normal human bone which are thought to be ultimately derived from SPC [Hughes et al, 1994]. Subsequent culture of the SPC isolated from BMMNC, resulted in the progeny of these cells expressing high levels of the CD44 antigen, that has previously been shown to be highly expressed on differentiated bone cells such as osteoblasts and osteocytes in vivo [Hughes et al, 1994]. Therefore the proliferation of BM CFU-F in the presence of serum seems to lead to the expression of a range of differentiation markers associated with osteogenic and adipocytic development in accord with other studies [Castro-Malaspina et al, 1980; Vilamitjana-Amedee et al, 1993; Rickard et al; 1996]. The extensive immunophenotyping of human BM CFU-F presented in this study represents the first real attempt to characterise the phenotype of human BM SPC in vivo.

In the present study, anti-VCAM-1 antibody was found to bind to a minor fraction (7.2% ± 1.2 n=6) of the total BMMNC population. VCAM-1 was found to be universally expressed on SPC and their progeny. This was in contrast to other markers expressed by CFU-F such as THY-1, CD10, CD13, and thrombomodulin, which are also known to react with either haemopoietic cells and or platelets [Baum et al, 1992; Conway and Nowakowski, 1993; Ship and Look, 1993]. VCAM-1 is a transmembrane glycoprotein with a molecular weight of between 95 and 110 kDa present on the membranes of stromal cells and

endothelial cells [Osborn et al, 1989; Simmons et al, 1992]. It binds the integrin receptor $\alpha_4\beta_1$ (VLA-4) present on haemopoietic stem cells, and is involved in the recruitment of lymphocytes and monocytes expressing $\alpha_4\beta_1$ to sites of infection and inflammation [Elices et al, 1990; Simmons et al, 1992].

Significantly, VCAM-1 effectively subset the total STRO-1$^+$ population obtained following MACS, reacting preferentially with the STRO-1^{bright} cell fraction. The CFU-F population was subsequently found to reside exclusively in the STRO-1^{bright}/VCAM-1$^+$ fraction of adult BM. The absolute frequency of CFU-F in bone marrow was determined by limiting dilution experiments using Poisson distribution statistics. Previously studies have demonstrated that using this statistical analysis, stromal precursors with the potential to form mineralized bone nodules in vitro, occured at a frequency of 1 per 1000 murine BM cells plated, based on the phenotype 5-FU-resistant, lineage marker negative [Van Vlasselaer, 1994]. These osteoprogenitors represented approximately 20% of the total CFU-F population in normal murine bone marrow [Falla et al, 1993; Van Vlasselaer, 1994]. Similar analyses of fetal human BMMNC demonstrated a decrease in the frequency of stromal cell progeniotrs of 1 per 1,000 to 1 per 100,000 cells plated, during foetal development at 14 weeks and 24 weeks gestation, respectively, based on the immunophenotype CD34$^+$/CD38$^-$/HLA-DR$^-$ [Waller et al, 1995a]. Furthermore, additional subsetting of foetal BM using the haemopoietic marker CD50, demonstrated no significant difference in the incidence of clonogenic stromal cells sorted on the basis of the phenotype CD34$^+$/CD38$^-$/HLA-DR$^-$/CD50$^-$ [Waller et al, 1995b]. However, no clonogenic growth of stromal progenitors was observed when single cells of adult BM samples were sorted based on the CD34$^+$/CD38$^-$/HLA-DR$^-$ phenotype [Waller et al, 1995a]. This may be due to the inefficiency of a predominately negative selection criteria used to isolate fetal BM SPC and may also reflect the use of the CD34 antigen which demonstrates low level expression on adult BM SPC [Simmons and Torok-Storb, 1991b].

In the present study, the incidence of clonogenic cells (clusters ≥10<50 cells + colonies ≥50) from adult human BM was determined to be 1 per 2 STRO-1^{bright}/VCAM-1^{+} cells plated in SDM containing PDGF and EGF. It must also be stated that a proportion of the wells which were scored as 'negative' contained cell clusters of less than 10 cells. Therefore, by further refining the CFU-F assay, it may be possible to stimulate the growth of these cells in order to increase the overall purity of the SPC population based on the composite STRO-1/VCAM-1 phenotype. The combined MACS/FACS purification technique effectively achieved a 6 x 10^3 fold enrichment of the CFU-F population when compared to unfractionated BMMNC with an approximate incidence of 1 CFU-F colony per 1.2 x 10^4 BMMNC plated. To purify the CFU-F population to homogeneity, the additional two-colour FACS purification step following MACS isolation provided the means for isolating highly enriched populations of CFU-F required to study the biology of SPC in the absence of accessary cells. Thus, dual-color FACS produced an effecient method to discriminate between the STRO-1^{bright} subset from the total STRO-1^{+} BMMNC fraction. This is the first report to describe the purification of adult human BM SPC to virtual homogeneity.

The cells contained within the STRO-1^{bright}/VCAM-1^{+} BM fraction were found to be a homogeneous population of large cells with extensive cytoplasmic processes existing in vivo in a non-cycling state. Other studies have found that CFU-F in vivo are almost entirely non-cycling as shown by ^{3}H thymidine labelling in rodents and by means of the in vitro thymidine suicide technique in adult human BM [Castro-Malaspina et al, 1980; Castro-Malaspina et al, 1981]. This data coinsides with the observations that primitive muli-potential stem cells, identified in the other cell systems such as HSC are by definition quiescent cells [Andrews et al, 1986; Szilvassy et al, 1989; Li and Johnson, 1992]. Given the reported developmental potential of cultured BM CFU-F in vitro and in vivo the question arises as to whether these cells are truly representative of an early uncommitted phenotype with multi-potential or whether all or a proportion of the CFU-F are already committed towards a particular stromal cell lineage.

Analysis of the immunophenotypic characteristics and gene expression pattern of purified adult BM SPC in the present study has revealed that many of the genes expressed by CFU-F in vivo demonstrate a broad stromal tissue distribution related to reticular cells, osteoblasts, adipocytes, chondrocytes and vascular endothelial cells. It is very common to find in the literature that many markers for example osteonectin, osteopontin, and alkaline phosphatase in the bone cell lineage are described as being specific to bone cells when in fact these markers have a wider tissue distribution. Therefore, it is not suprising to find that SPC identified by STRO-1 share common markers with differentiated stromal cell types. Importantly, specific markers of committment such as CBFA-1, collagen type II, PPARγ2, [reviewd in Rodan GA and Harada, 1997] to bone, cartilage and fat respectively were not expressed by the STRO-1^{bright}/VCAM-1^{+} population in vivo. In addition, cytospin preparations of STRO-1^{bright}/VCAM-1^{+} sorted BMMNC failed to show any reactivity to the smooth muscle marker α-smooth muscle actin or with the endothelial marker, FVIII. Overall, the data suggests that SPC residing in the bone marrow exist in an uncommitted state, and may have the potential under different conditions to develop into a few or all of the stromal elements recognised in the bone marrow microenvironment.

CHAPTER FIVE: THE DEVELOPMENTAL POTENTIAL OF BM STROMAL PRECURSOR CELLS

5.0 INTRODUCTION

The stromal connective tissue of the bone marrow (BM) has the unique capacity to support and regulate the development of haemopoietic stem cells throughout normal life. The BM stroma is a complex tissue comprised of a heterogeneous population of stromal cell types including; reticular cells, adipocytes, smooth muscle cells, endothelial cells, pericytes, osteoblasts and chondroblasts [Wiess, 1976; Lichtman, 1981; Bently, 1982; Tavassoli and Friedenstein, 1983; Dexter et al, 1984; Allen et al, 1990]. Single cell suspensions derived from aspirates of BM have the ability to transfer the BM microenvironment in vitro, forming an adherent multi-stromal layer able to support the development of haemopoietic stem cells under defined culture conditions [Dexter et al, 1977]. However, the origin of the various cellular elements of the stroma both during ontogeny and in the post-natal organism remains to be determined. It is still not known if some or all of the different stromal cell lineages represent self-maintaining populations within the BM or whether they are derived from the proliferation and differentiation of a population of multi-potential stromal precursor cells.

To date, our knowledge of stromal cell ontogeny has been based largely on the work of Friedenstein and colleagues who were the first to identify putative BM SPC by their ability to generate adherent clonogenic clusters of fibroblastic-like cells (CFU-F) in liquid culture [Friedenstein, 1970]. A proportion of BM CFU-F clones were shown to have the capacity to develop a functional stromal organ and bone tissue when transplanted beneath the renal capsule in syngeneic hosts [Friedenstein et al, 1980]. However, the majority of CFU-F clones only produced a soft fibrous connective tissue with or without the presence of bone. Based on these studies, Owen and Friedenstein (1988) have proposed the existence of stromal stem cells possessing both the ability to self-renewal while maintaining the potential for cellular differentiation into the fibrous-osteogenic tissues of

the skeleton and the stromal tissue of the BM microenvironment. Similar studies have demonstrated the multi-potential of cultured stromal cells by their capacity to develop into fibrous tissue, fat, bone and cartilage when transplanted into ectopic sites in vivo [Ashton et al, 1980; Friedenstein et al, 1982; Bab et al, 1984; 1986; Mardon et al, 1987; Ohgushi et al, 1989; Goshima et al, 1991; Bennett et al, 1991; Umezawa et al, 1992; Ohgushi et al, 1993; Benayahu et al, 1994; Cassiede et al, 1996; Krebsbach et al, 1997; Mizuno et al, 1997]. While the evidence supporting the existence of a population of stromal precursor cells is mostly circumstantial, this theory has nevertheless provided a useful model to investigate the developmental potential of marrow stromal and skeletal tissues.

In accord with animal studies, several reports have described the presence of cells displaying an osteogenic phenotype in normal human BM stromal cultures by ectopic transplantation into animals using diffusion chambers [Ashton et al, 1985; Davies, 1987; Bab et al, 1988; Gundle et al, 1995]. However, the formation of bone and cartilage was only detected in those diffusion chambers containing stromal cells derived from the bone marrow of young children but not from adult marrow [Bab et al, 1988], suggesting that age-related differences may influence the outcome of these xenogeneic transplant experiments. Moreover, one study has shown that in vivo bone formation can occur in diffusion chambers inoculated with bone marrow stromal cells from a young adult, but only after stimulating the cell cultures with glucocorticoids for several weeks [Gundle et al, 1995]. An alternative, transplantation model has consistently demonstrated the formation of functional bone tissue by cultured adult human bone marrow cells [Haynesworth et al, 1992a; Krebsbach et al, 1997; Kuznetsov et al, 1997]. This open transplantation model involves the loading of cultured stromal cell suspensions into hydroxyapatite coated porous ceramic cubes, and then implanted subcutaneously into immunodeficient mice. These studies imply that other factors other than age, such as culture conditions, the choice of host animal and the type of transplantation vehicle used may be more important variables in assessing the developmental potential of BM SPC in vivo [Haynesworth et al, 1992a; Krebsbach et al, 1997; Kuznetsov et al, 1997].

Several studies have utilised various culture techniques to examine the osteogenic potential of rodent bone marrow stromal cells in vitro following culture in the presence of additives such as ascorbate, dexamethasone, and β-glycerolphoshate [Luria et al, 1987; Maniatopoulos et al, 1988; Schoeters et al, 1988; Leboy et al, 1991]. In similar studies, osteogenic differentiation of human stromal cells was assessed by examining the acquisition of osteoblast-like characteristics including the synthesis of collagen type 1, alkaline phosphatase expression, osteocalcin production and by the capacity of the cells to form a mineralise bone matrix in vitro under similar culture conditions [Long et al, 1990; Long et al, 1990; Haynesworth et al, 1992b; Vilamitjana-Amedee et al, 1993; Cheng et al, 1994; Beresford et al, 1994]. Collectively, these studies demonstrate the presence of cells with osteogenic potential in human bone marrow cultures. However, given the heterogeneity of the stromal cell population in whole bone marrow, the question arises as to the origin of these cells. That is, do the osteoblast-like cells which develop in these culture systems arise from pre-existing cells already committed to the osteogenic lineage, or alternatively, can the osteogenic phenotype be induced in a more primitive multipotential stromal cell precursor population.

To avoid the culture of whole bone marrow which could contain mature functional bone cells, human bone marrow CFU-F were purified by their immunoreactivity with the STRO-1 mab as described in Chapter 4. Bone marrow mononuclear cells sorted on the basis of STRO-1 expression were previously shown to be capable of establishing an adherent stromal layer under long-term culture conditions, consisting of a number of phenotypically distinct stromal cell types including fibroblasts, smooth muscle cells and adipocytes [Simmons and Torok-Storb, 1991a]. Analysis of the phenotypic characteristics of adult BM SPC in the present study (Chapter 4) has revealed that many of the genes expressed by CFU-F in vivo demonstrate a broad stromal tissue distribution related to bone, fat, cartilage and vascular endothelium. However, specific markers of commitment such as CBFA-1, collagen type II and PPARγ2 [reviewed in Rodan and Harada, 1997] to bone,

cartilage and fat respectively were not found to be expressed by the STRO-$_1$bright/VCAM-1$^+$ population in vivo. These data suggest that SPC have the potential to develop into a range of stromal cell types under specific conditions and therefore suggests that this precursor population may be uncommitted. Following the purification of BM SPC, the developmental potential of individual CFU-F clones was assessed under osteogenic conditions both in vitro and in vivo.

5.1 Osteogenic Induction of STRO-1$^+$ BM CFU-F In Vitro

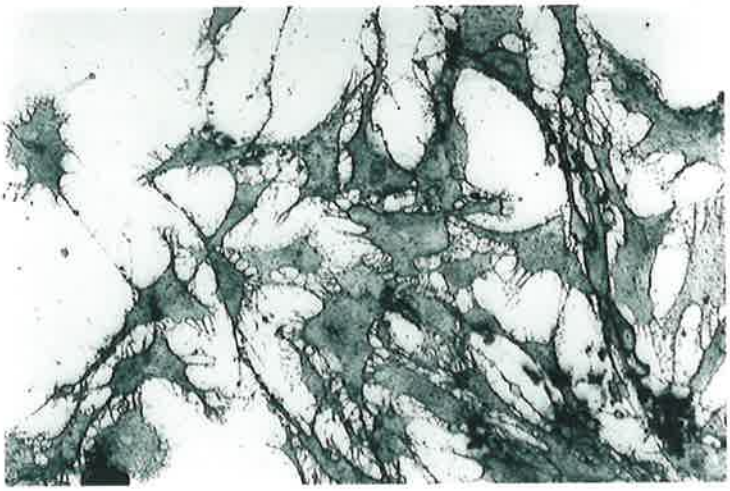
Primary human stromal cultures derived from STRO-1$^+$ BM CFU-F using FACS have the potential to develop an osteoblast-like phenotype under defined culture conditions [Gronthos et al, 1994]. Following two weeks in the presence of the supplements ascorbate-2-phosphate (ASC-2P), dexamethasone (DEX), and inorganic phosphate (PO_{4i}) CFU-F colonies were analysed for their expression of alkaline phosphatase (AP), a well documented marker of osteoblast differentiation. All of the CFU-F colonies were found to react with the mab B4-78, which identifies the bone and liver specific form of the AP enzyme [Lawson et al, 1985] (Figure 37). Moreover, within individual colonies, greater than 95% of the cells were consistently found to react with the B4-78 mab. Alkaline phosphatase activity was also confirmed by histochemical analysis in replicate cultures (Figure 38).

After four to six weeks in osteogenic growth conditions the adherent layers of bone marrow stromal cultures, displayed large areas of mineralized material which were positive for von Kossa staining (Figure 39). Ultrastructural examination of the cultures by transmission electron microscopy, demonstrated that the mineral was associated with collagen fibrils in the extracellular matrix of the adherent layers (Figure 40A and B). EDX was employed to evaluate the nature of the mineral in these cultures. The calcium to phosphorus (Ca/P) ratio in the mineralized cultures was 1.76 ± 0.10 (n=3 different marrow aspirates) (Figure 40C) which was consistent with the Ca/P ratio of hydroxyapatite ($Ca_5(PO_4)_3OH$) in vivo (1.67) [Koutsoukos and Nancollas, 1981]. X-ray diffraction studies

Figure 37. Alkaline phosphatase expression in culture expanded BM CFU-F. Representative photomicrographs depicting the immunoreactivity of primary cultures of CFU-F derived from STRO-1^+ BMMNC isolated by FACS, with the mab, B4-78 (antialkaline phosphatase), and detected by immunoperoxidase staining. The CFU-F were cultured for two weeks in the presence of either FCS alone (A) or under osteo-induction conditions in the presence of ASC-2P, DEX and PO_{4i} (B and C). Following the immunostaining, the cultures were counter stained with haematoxylin. Cultures treated with FCS alone demonstrated a heterogeneous staining pattern of alkaline phoshatase expression, where only a proportion of cells (25%) in each colony were found to express alkaline phosphatase (arrow) (4X) (A). In the cultures treated with ASC-2P, DEX and PO_{4i}, greater than 90% of the cells in each colony were found to express alkaline phosphatase (40X) (B). In addition, the cells cultured under osteo-induction conditions demonstrated a more irregular morphology to the control cultures. No staining occured when the same cells were incubated in the presence of the negative control mab, 3D3 (40X) (C).

A

B



C

Figure 38. Alkaline phosphatase activity in culture expanded BM CFU-F. Representative photomicrographs demonstrating histochemical analysis for alkaline phosphatase activity in primary cultures of CFU-F derived from FACS isolated STRO-1$^+$ BMMNC, grown under osteogenic conditions for 2 weeks in the presence of FCS supplemented with ASC-2P, DEX and PO_{4i} (A and B). The cultures were counter stained with haematoxylin. A high proportion of cells (>95%) in CFU-F colonies were found to be positive for alkaline phosphatase activity when cultured under osteo-induction conditions, as shown in panel A (10X) and panel B (4X).

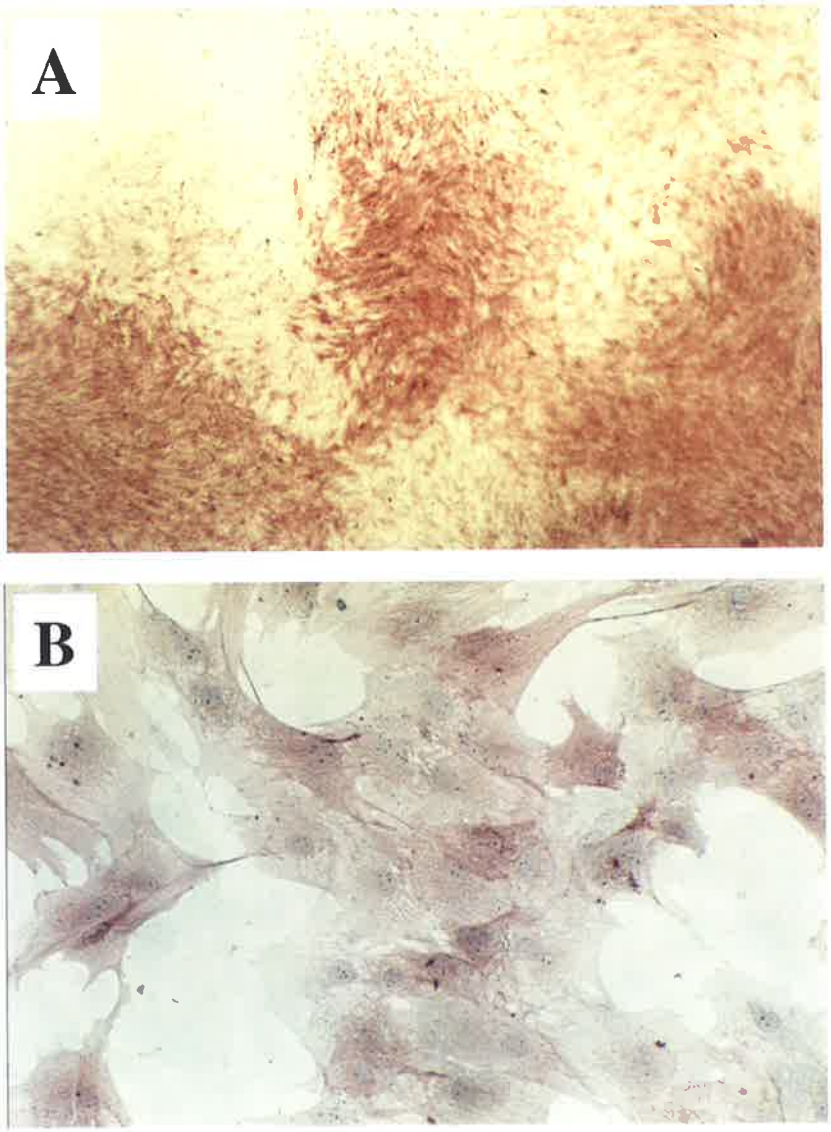

A
B

Figure 39. In vitro formation of a mineralized matrix by cultured BM CFU-F. Representative photomicrographs of histochemical analysis for von Kossa staining of resin embedded transverse sections of the adherent layers from primary cultures of CFU-F. The cultures were derived from STRO-1$^+$ BMMNC isolated by FACS, and grown for six weeks in the presence of: FCS alone (40X) (A); FCS supplemented with ASC-2P, DEX and PO_{4i} (40X) (B). The sections were counter-stained with haematoxylin and eosin. Areas of von Kossa positive mineral deposits (arrow) were observed throughout the adherent layers of the cultures treated with ACS-2P, DEX and PO_{4i} (B). No von Kossa positive mineral could be detected in the adherent layers of the control cultures treated with FCS alone (A).

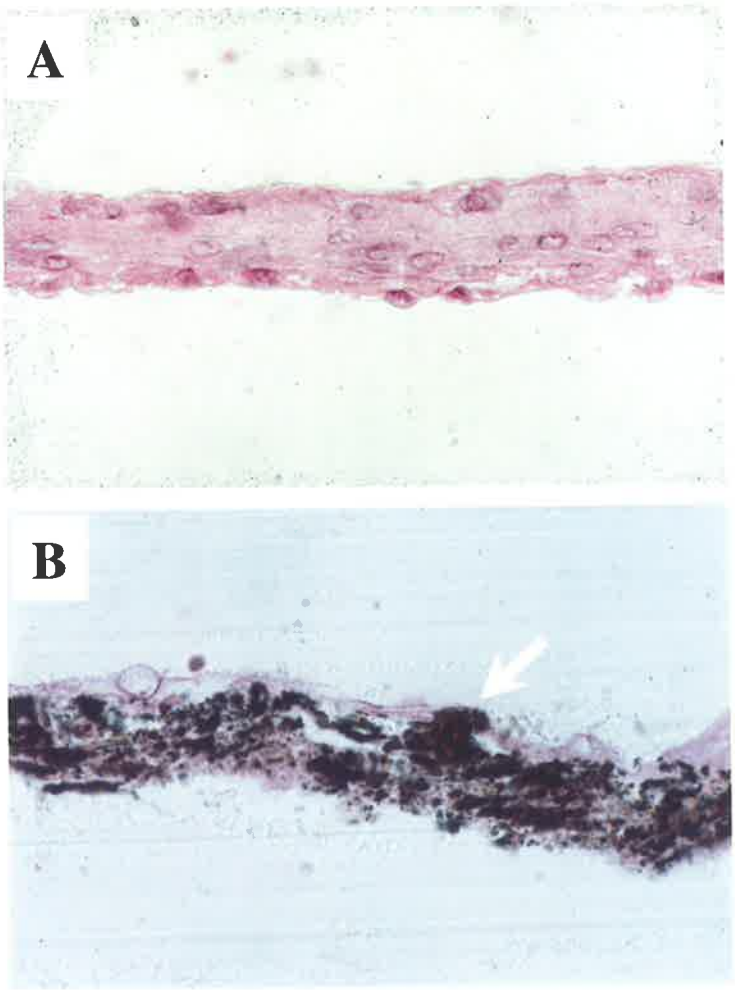
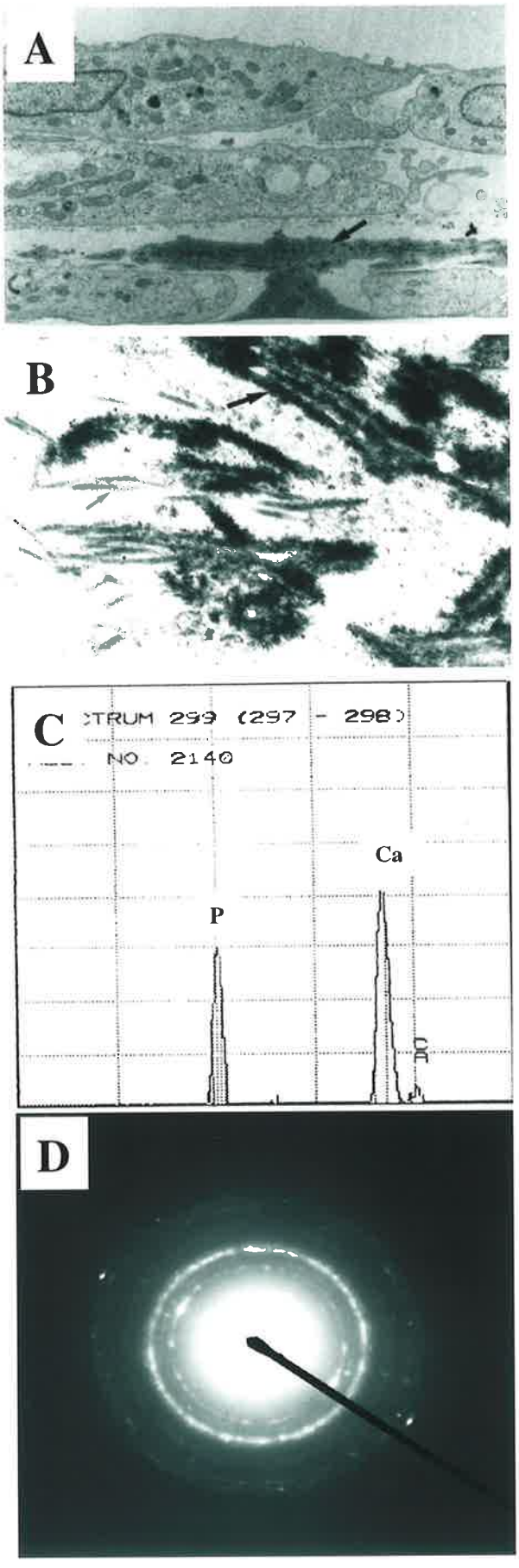

A
B

Figure 40. Analysis of mineralized deposits in BM CFU-F cultures. Transmission electron microscopic examination of the adherent layers of primary CFU-F cultures derived from FACS isolated STRO-1$^+$ BMMNC, grown for six weeks in the presence of ASC-2P, DEX, and PO_{4i}. Panel A: a electron photomicrograph of the formation of a mineral-like material (hydroxyapatite crystals) (arrow) in the extracellular spaces of the adherent layers (3000 X). Examination of the extracellular matrix demonstrated the presence of deposits of hydroxyapatite-like crystals (large arrow) in association with a network of collagen fibrils (small arrow) (25000X) (B). Panel C: representative EDX analysis of similar mineral deposits, demonstrating major peaks for calcium (Ca) and phosphorous (P), with a mean Ca/P ratio of 1.71 ± 0.1 (n=3 different marrow samples). Panel D: X-ray diffraction analysis where the crystaline structure of the mineral deposits, in the same cultures, was found to be identical to that of the hydroxyapatite standard.


A
B
C
NO. 2140
P
Ca
D

also showed that the crystal structure of the mineral deposits in the induced cultures (ASC-2P, DEX and PO_{4i}) was identical to the crystal diffraction patterns obtained from the hydroxyapatite standard (Figure 40D).

The biosynthesis of osteocalcin in the cultures was stimulated by the addition of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) for 48 hours. Samples were then taken from the medium of all the cultures, and analysed for the presence of osteocalcin by RIA. Varying levels of osteocalcin (mean 10.67 ng ± 4.54 SEM/10^6 cells/ 48 hr 1,25-$(OH)_2D_3$, n=3 different marrow samples) were detected in the mineralized bone marrow cultures grown under osteogenic conditions, but not in control cultures grown in the presence of FCS alone (mean 0.42 ng ± 0.19 SEM/10^6 cells/ 48 hours 1,25-$(OH)_2D_3$, n=3). In accord with this data, northern blot analysis of RNA extracted from the mineralized bone marrow stromal cell cultures demonstrated the presence of osteocalcin mRNA, only following stimulation of the cells for 48 hours with 1,25-$(OH)_2D_3$ (Figure 41). No osteocalcin mRNA could be detected in the control cultures.

Following on from this work, we assessed the osteogenic potential of individual CFU-F clones. Ninety CFU-F clones were derived from single STRO-1^{bright}/VCAM-1^+ sorted cells from three individual normal BM donors using flow cytometric single cell deposition as described in the methods. At day 4 of culture single clonogenic clusters were identified visually using an inverted light microscope and then subcultured into 24-well plates in the presence of 10% serum, ASC-2P, DEX and PO_{4i}. The osteogenic potential of each clone was assessed as described above. All colonies were found to express alkaline phosphatase as demonstrated in the bulk CFU-F cultures (data not shown). A von Kossa positive mineralised matrix developed in all of the ninety clones at the end of the sixth week of osteo-induction (Figure 42A and B). Furthermore, a proportion (mean 38% ±15 SEM, n=3 different BM samples) of the clones also gave rise to clusters of lipid containing oil red-O positive adipocytes (Figure 42C and D) demonstrating the bi-potential of at least a proportion of STRO-1^{bright}/VCAM-1^+ SPC. In addition, BM stromal cultures treated

Figure 41. Detection of osteocalcin mRNA by northern blot. An autoradiograph of a nylon membrane representing a formaldehyde-agarose gel, analysed by northern blot analysis, for the detection of osteocalcin mRNA from total RNA isolated from: primary CFU-F cultures derived from FACS isolated STRO-1^+ BMMNC, grown for six weeks in the presence of either FCS supplemented with ASC-2P, DEX and PO_{4i} (lane 1) or FCS alone (lane 2), and stimulated with $1,25(OH)_2$ D_3 for 48 hours; primary human bone cells derived from explants of trabecular bone (positive control), grown for six weeks in the presence of ASC-2P, with $1,25(OH)_2$ D_3 induction for 48 hours (lane 3) or without $1,25(OH)_2$ D_3 induction (lane 4). Descrete bands, characteristic of osteocalcin mRNA (arrow) with a molecular weight of 0.6 kb, were observed in lanes 1 and 3, after hybridization with a specific P^{32} labelled oligo-nucleotide probe (A). The integrity of the RNA (28S and 18S) analysed was confirmed by ethidium bromide staining (B).

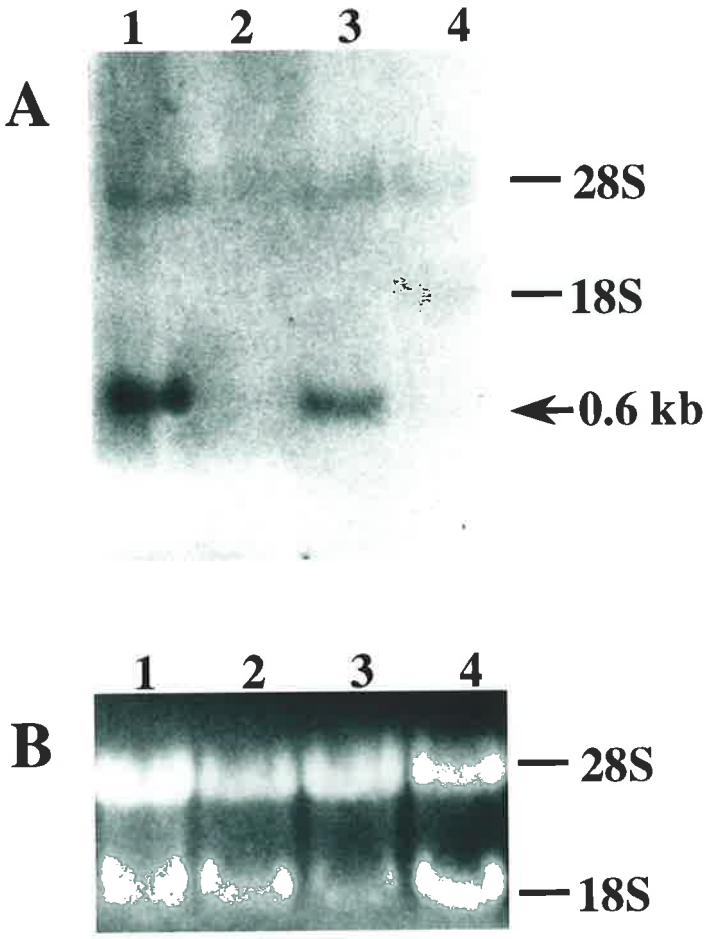
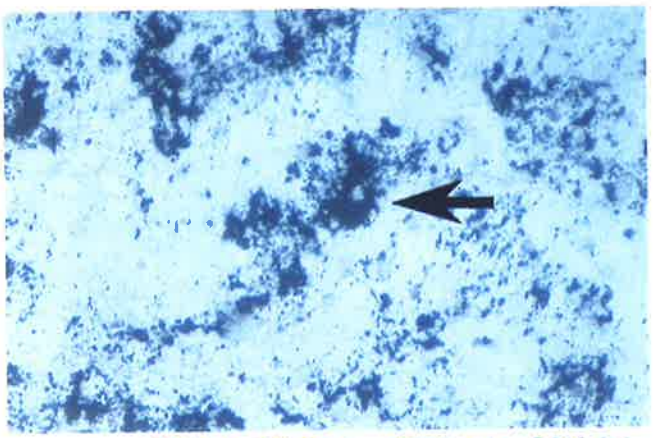
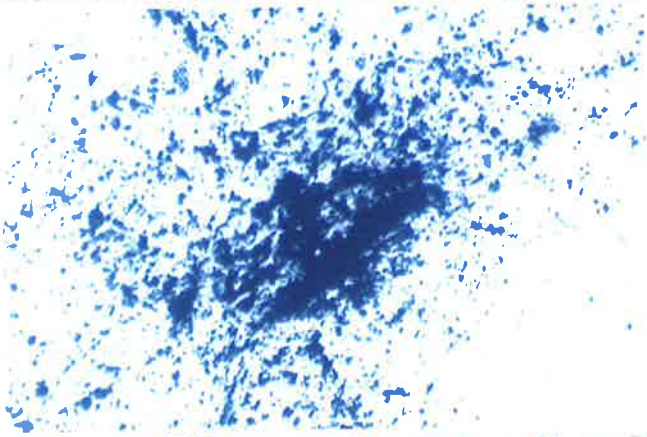

1
2
3
4
A
28S
18S
0.6 kb
1
2
3
4
B
28S
18S

Figure 42. In vitro osteogenic and adipocytic potential of BM CFU-F clones. Representative photomicrographs of in situ histochemical analysis for von Kossa staining and Oil red O staining of CFU-F clones. The clones were derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, grown for six weeks in the presence of FCS supplemented with ASC-2P, DEX and PO_{4i}. The cultures were counter-stained with haematoxylin. Panel A, represents areas of von Kossa positive mineral deposits (arrow), which formed in all of the CFU-F clones examined (10X). Panel B, demonstrates a magnified view of the mineral deposits (40X). In a proportion of CFU-F clones (38%) the formation of clusters of Oil red O positive adipocytes (arrow) was observed, as shown in panel C (10X). Panel D represents a magnified view of the adipocytes (40X).

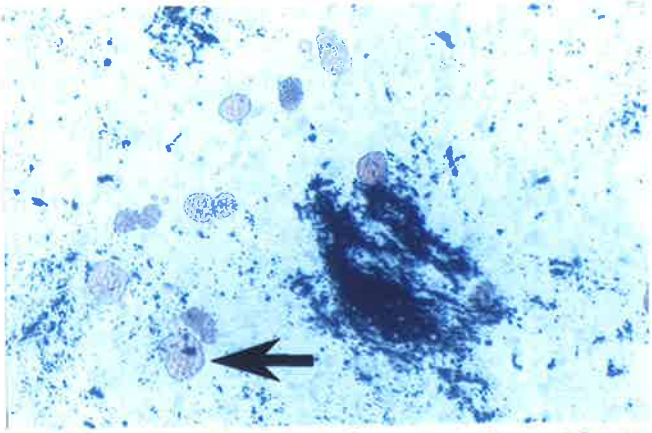
A



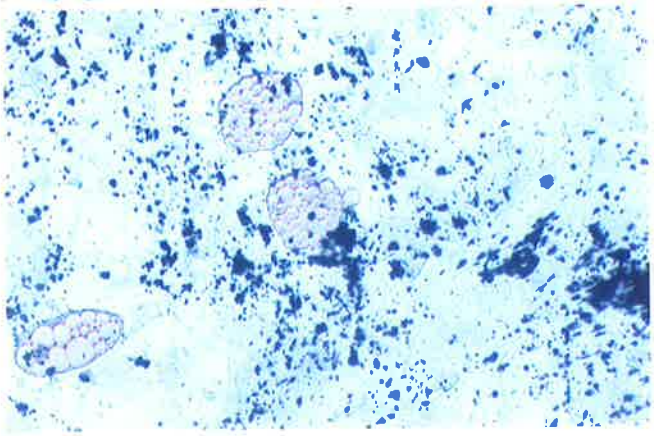
B



C



D



with dexamethasone demonstrated an upregulation in the expression of a variety of bone and fat specific transcripts including CBFA-1 and PPAR-γ2, respectively (Figure 43).

5.2 Developmental Potential of CFU-F In Vivo: Assessment of Different Transplantation Techniques

Given the range of transplantation procedures previously reported, a series of pilot studies were performed in order to determine the appropriate method for assessing the developmental potential of human BM stromal cells in vivo. Primary cultures of CFU-F were established from STRO-1^{bright}/VCAM-1^{+} sorted BMMNC and were allowed to grow to confluence. The cells were subcultured for three passages and then maintained for 8 weeks in the presence of ASC-2P with or without DEX. Following this, single cell suspensions were harvested and then transferred into one of three different transplantation vehicles (polyvinyl sponges, diffusion chambers, and hydroxyapatite ceramic cubes) and then implanted into SCID mice as described in the methods. Alternatively, confluent stromal layers were gently scraped off as an intact 'skin', then transferred beneath the kidney capsule of SCID mice as described in the methods. The early development of a soft fibrous tissue was observed in all of the implants under the different transplantation conditions (Figures 44-47). An increase in the level of vasculature was also observed over the time period for all the open systems used. This was in contrast to the absence of any blood vessels in the diffusion chambers, where there was no direct contact between the human stromal cells and the mouse connective tissue. Bone formation was identified in only the hydroxyapatite cubes (Figure 47). Ectopic bone formation was first detected at week 4 and increased overtime. The effect of dexamethasone stimulation in vitro, was generally found to induce the earlier formation of bone in vivo. No bone formation was detected in control cubes implanted without cells.

Figure 43. BM CFU-F expression of bone-, fat- and cartilage-related transcripts as assessed by RT-PCR in vitro. Total RNA was isolated from cultured CFU-F derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC grown for two weeks in the presence of: ASC-2P and DEX (Lane 1); ASC-2P alone (Lane 2). The various markers examined included: osteonectin (ON); the transcription factor, CBFA1; collagen type I (COLL-1); osteocalcin (OCN); bone sialoprotein (BSP); osteopontin (OP); lipoprotien lipase (LPL); obese gene product (OBGP); the transcription factor PPARγ2; human-adipocyte lipid-binding protein (H-ALBP); aggrecan (AGGN); collagen type II (COLL-2); collagen type X (COLL-X). Transcripts for OCN were examined in cultures treated with 1,25(OH)$_2$D$_3$ (+D$_3$) for 48 h or without 1,25(OH)$_2$D$_3$ (-D$_3$). A number of bone, fat and cartilage related markers were expressed by cultured CFU-F. Other transcripts initially absent in CFU-F cultures (PTH-R, OCN, PPARγ2, and OBGP) were expressed following dexamethasone induction.

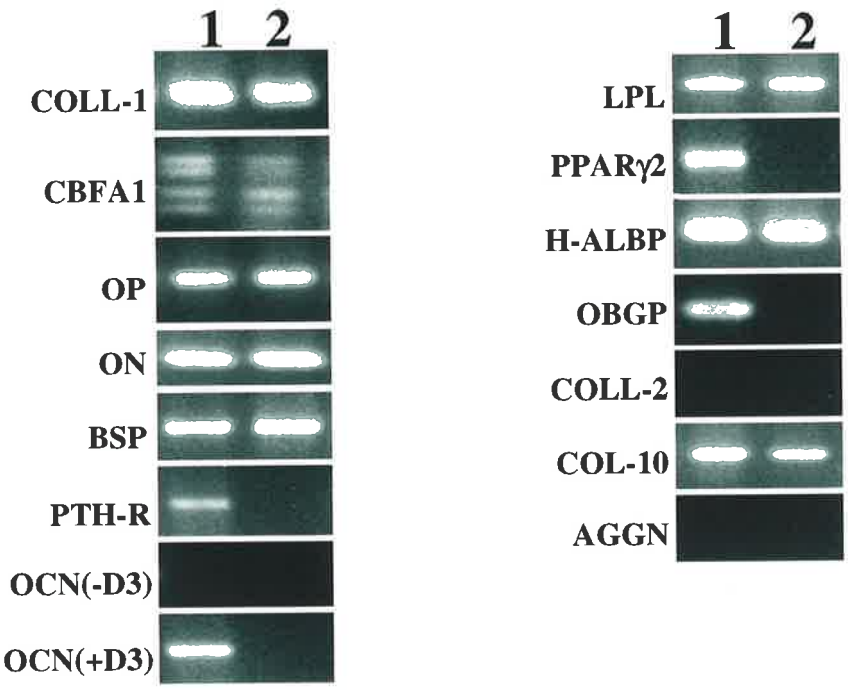

1 2
COLL-1
CBFA1
OP
ON
BSP
PTH-R
OCN(-D3)
OCN(+D3)
1 2
LPL
PPARγ2
H-ALBP
OBGP
COLL-2
COL-10
AGGN

Figure 44. Transplantation of human CFU-F beneath the kidney capsule of mice. CFU-F derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were expanded in vitro and then implanted beneath the kidney capsule of SCID mice for 10 weeks. Representative micrographs of paraffin embedded transverse sections of: a normal SCID mouse kidney (A); a SCID mouse kidney implanted with CFU-F (B and C). The sections were counter stained with haematoxylin and eosin. Panel A: demonstrates the normal histology of the SCID mouse kidney and kidney capsule (arrow) pre-transplantation (10X). Panel B: the development of a soft fibrous tissue beneath the kidney capsule (arrow) harvested 10 weeks post-transplantation (10X). Panel C: demonstrates a magnified view of the same site of transplantation.

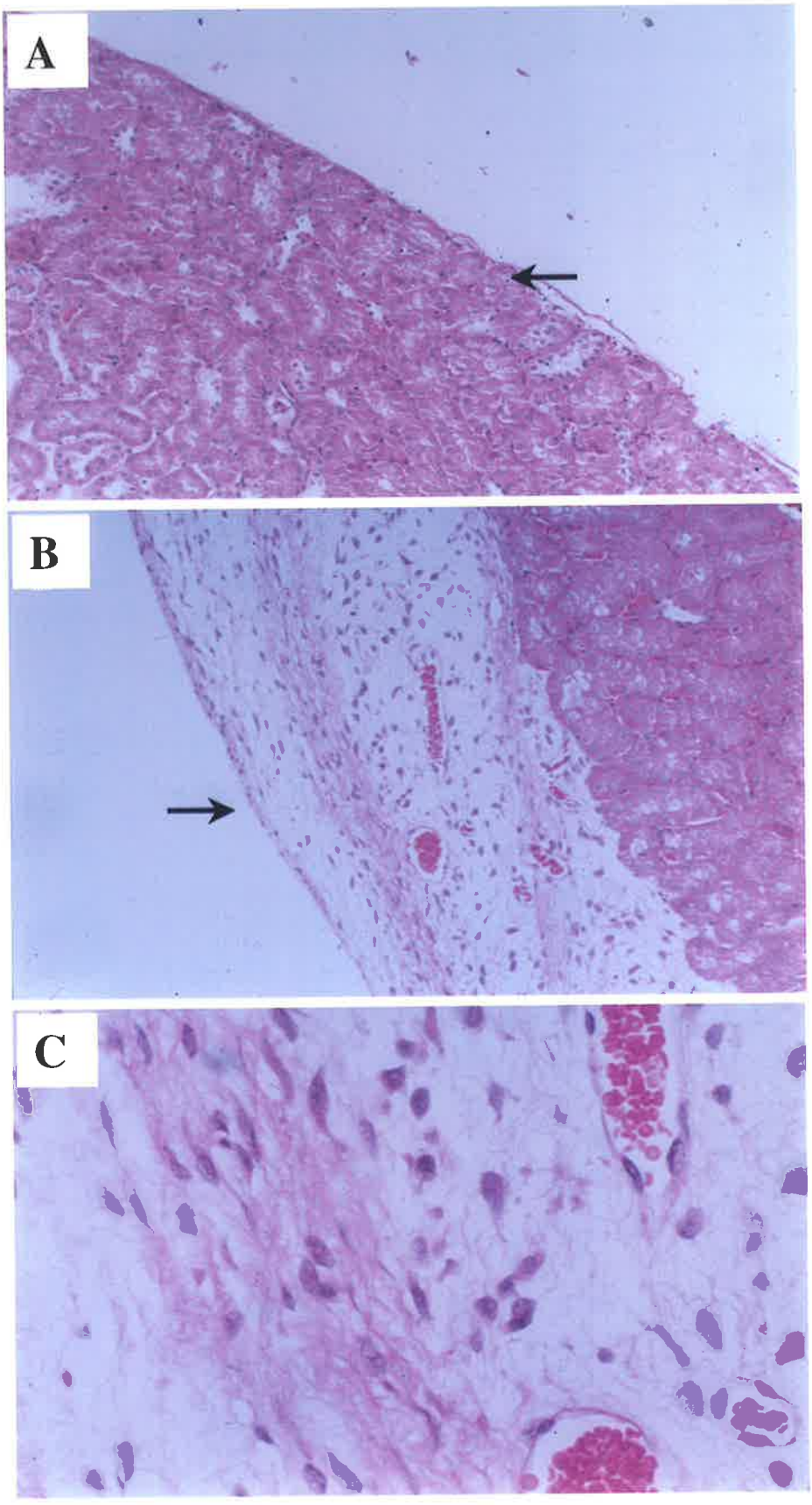

A
B
C

Figure 45. Transplantation of human CFU-F in polyvinyl sponges. CFU-F derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were expanded in vitro and then implanted inside disks of 10 mm polyvinyl sponge, subcutaneously into SCID mice for 10 weeks. Representative micrographs show paraffin embedded cross sections of a disk recovered post-transplant (A and B). The sections were counter stained with haematoxylin and eosin. Panel A: demonstrates the presumed SCID mouse connective tissue (arrow) surrounding the disk and with an ordered histology (10X). Panel B: a magnified view of the same disk where fibroblast-like cells (arrow) are observed covering the internal spongy walls of the disks (40X).

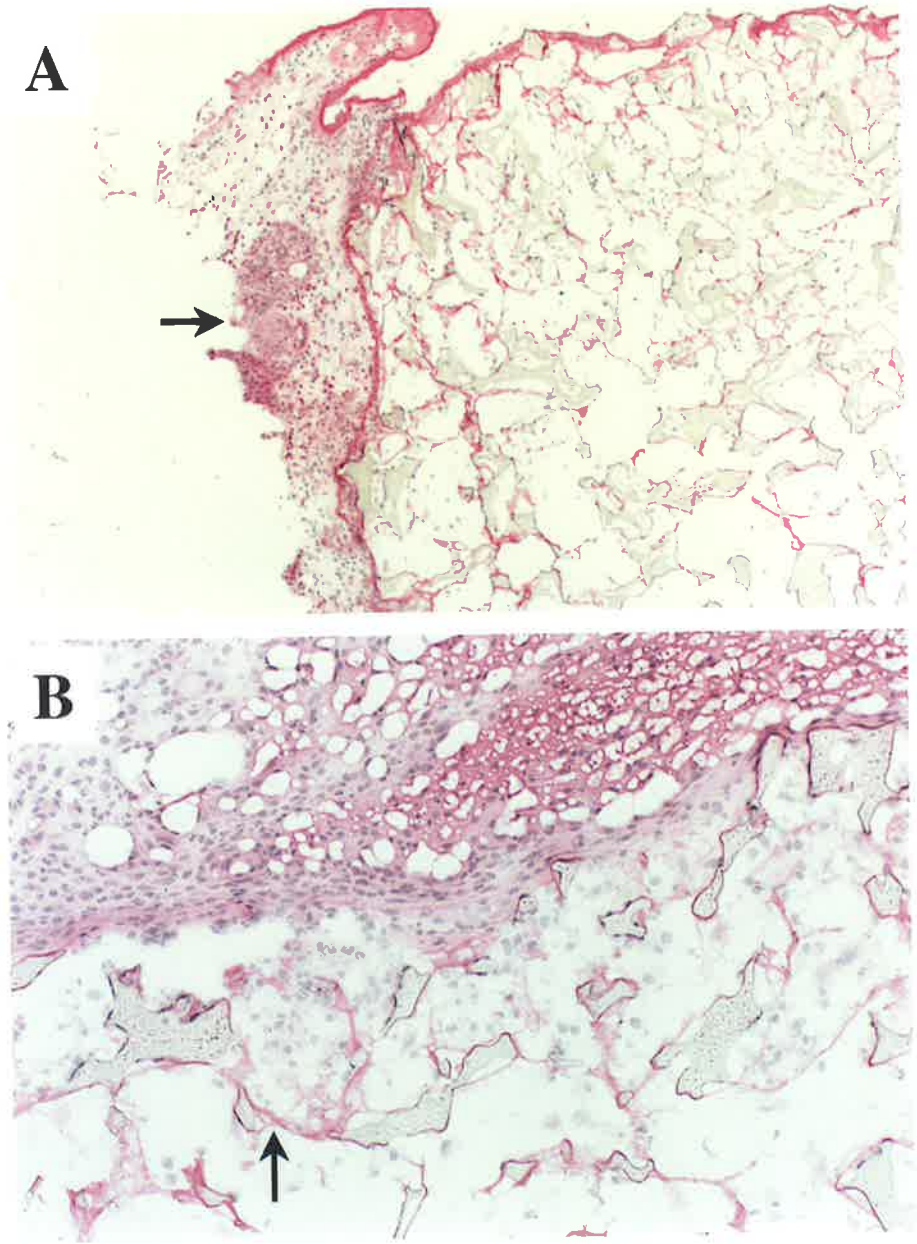

A
B

Figure 46. Transplantation of human CFU-F in diffusion chambers. CFU-F derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were expanded in vitro and then implanted inside diffusion chambers, into the abdominal peritoneal cavity of SCID mice for 10 weeks. Representative photomicrographs show paraffin embedded cross sections of a diffusion chamber recovered post-transplantation (A and B). The sections were counter stained with haematoxylin and eosin. Panel A: demonstrates a layer of mouse connective tissue (small arrow) covering the membrane filter of the diffusion chamber and a mass of human fibrous tissue (large arrow) inside the diffusion chamber (10X). Panel B: depicts a magnified view of the human fibrous tissue in same diffusion chamber (40X).

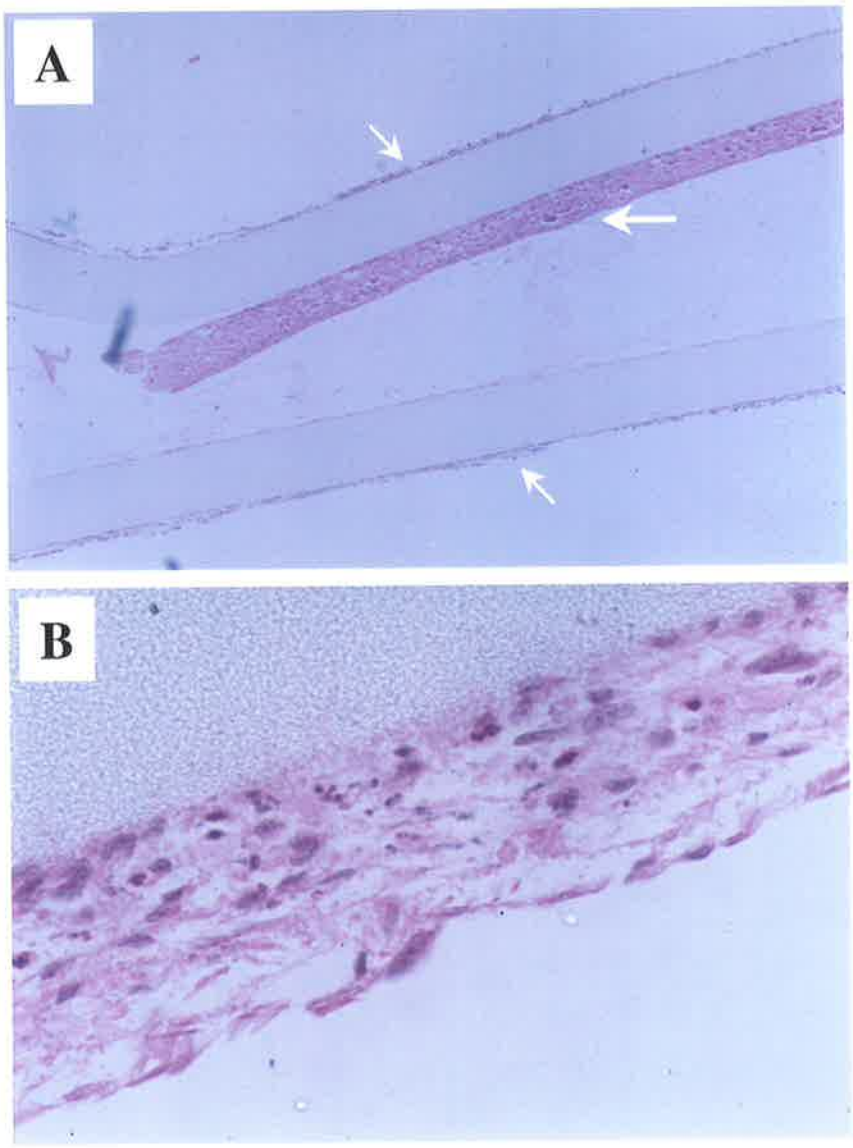
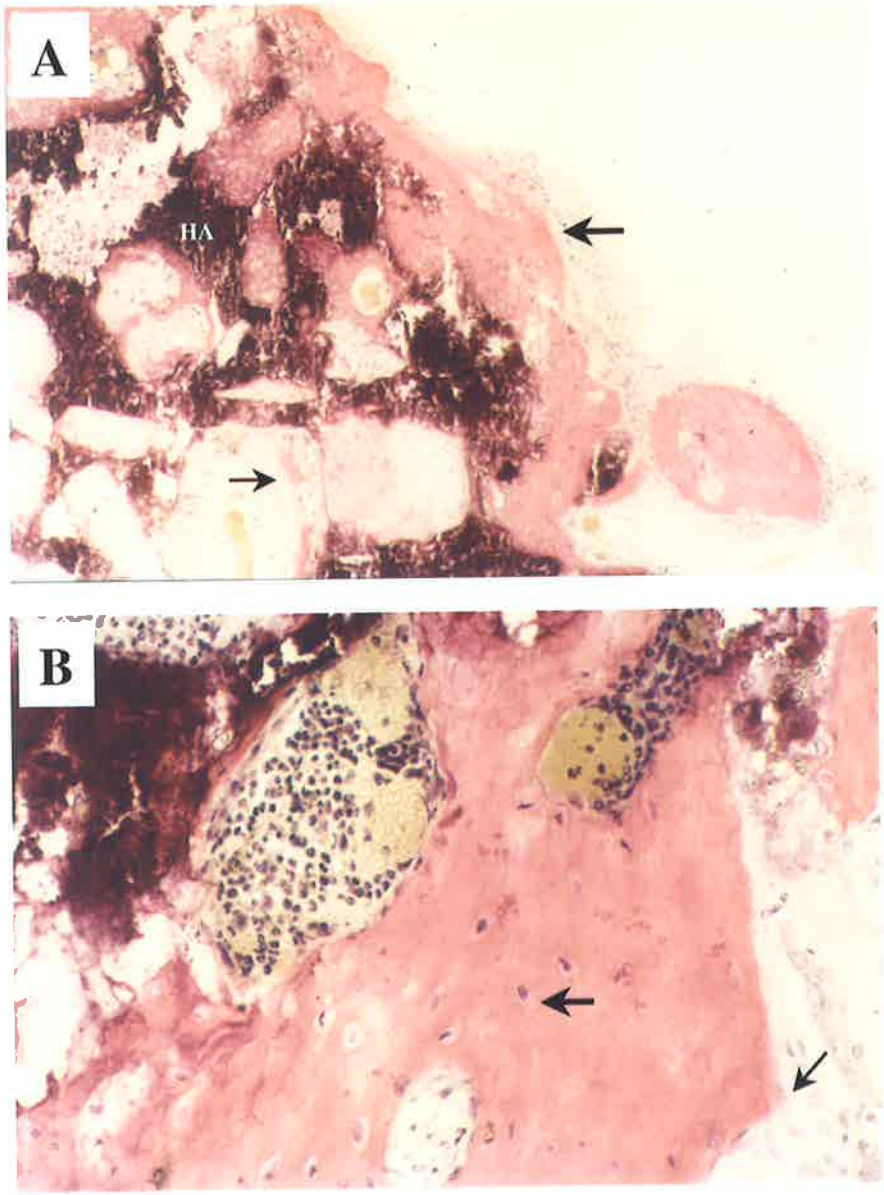

A
B

Figure 47. Transplantation of human CFU-F in hydroxyapatite (HA) ceramic cubes. CFU-F derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were expanded in vitro and then implanted, inside porous HA-ceramic cubes implanted subcutaneously into SCID mice for 10 weeks. Representative micrographs show paraffin embedded cross sections of a HA cube recovered post-transplant (A and B). The sections were counter stained with haematoxylin and eosin. Panel A: demonstrates a layer of connective tissue (large arrow) covering the HA cube surface at the site of new bone formation. Inside the cube, a mass of fibrous tissue (small arrow) is covering the internal walls of the cubes (10X). Panel B: depicts a magnified view of the new bone formation which contains osteocytes (large arrow) trapped with in the bone and osteoblast-like cells (small arrow) lining the bone surface (40X).


A
HA
B

5.3 Identification of Human Stromal Cells Following Transplantation In Vivo

Initial attempts to determine the origin of the cellular material in the implants, involved the use of retroviral vectors to try and mark primary BM stromal cells. Bone marrow stromal cells were found to be readily infected with the retroviral constructs LAPSN or LNPOZ as described in the methods, where greater than 70% of the established clonogenic clusters were shown to express either alkaline phosphatase (human placental-AP, levamisole resistant) (Figure 48) or β-galactosidase (Figure 49) activity respectively, following selection in the presence of G418 containing medium. The ability of the transfected cells to express levamisole sensitive AP expression and to develop a von Kossa positive mineralized matrix in vitro was shown to be comparable to the osteogenic potential of the untransfected control cells cultured in the presence of ASC-2P, DEX and PO_{4i} (data not shown).

The retrovirally marked cells were expanded in culture in the presence of ASC-2P and DEX for several weeks and were then seeded into HA ceramic cubes and implanted into SCID mice as described above. Implants were harvested at various time points, decalcified and then prepared for histological examination as either frozen, wax or resin embedded sections. PL-alkaline phosphatase and β-galactosidase positive cells were only identified in frozen sections (Figure 50). The preparation of the implants as frozen sections caused a loss in the architectural integrity of the implants resulting in poor histology. Therefore no association could be made with the human cells and the formation of new bone. Replicate implants were also sacrificed for enzymatic digestion to liberate the cells from the ceramic cubes which were subsequently cultured in the presence of G418. Cellular material harvested from the implants demonstrated the persistent growth of a G418 resistant cell population in vitro. Histochemical analysis revealed that these cells also expressed the corresponding markers for each of the respective retroviral constructs (Figure 51). However, a proportion of the G418 resistant cells transfected with the LNOPZ vector demonstrated a heterogeneous pattern of expression of the LacZ gene product. Nevertheless, these studies demonstrated that human cells were present in the

Figure 48. LAPSN infection of human BM stromal cells in vitro. Representative photomicrographs demonstrating histochemical analysis for alkaline phosphatase activity in cytospin preparations of cultured stromal cells derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC: untransfected cultures (A); untransfected cultures in the presence of the B/L/K-alkaline phosphatase inhibitor, levamisole (1 μM) (B); cultures transfected with the retroviral vector, LAPSN in the presence of 1μM levamisole (C). The cells were counter stained with haematoxylin. A proportion of cells (20-30%) in the untransfected control cultures (A) were found to be positive for alkaline phosphatase activity (40X). Panel B, demonstrates that alkaline phophatase activity in the untransfected cultures was inhibited in the presence of levamisole (40X). A high expression of alkaline phosphatase activity was observed in all the cells transfected with the retroviral vector, LAPSN following selection in G418 (C), which was not affected by the addition of levamisole (40X).

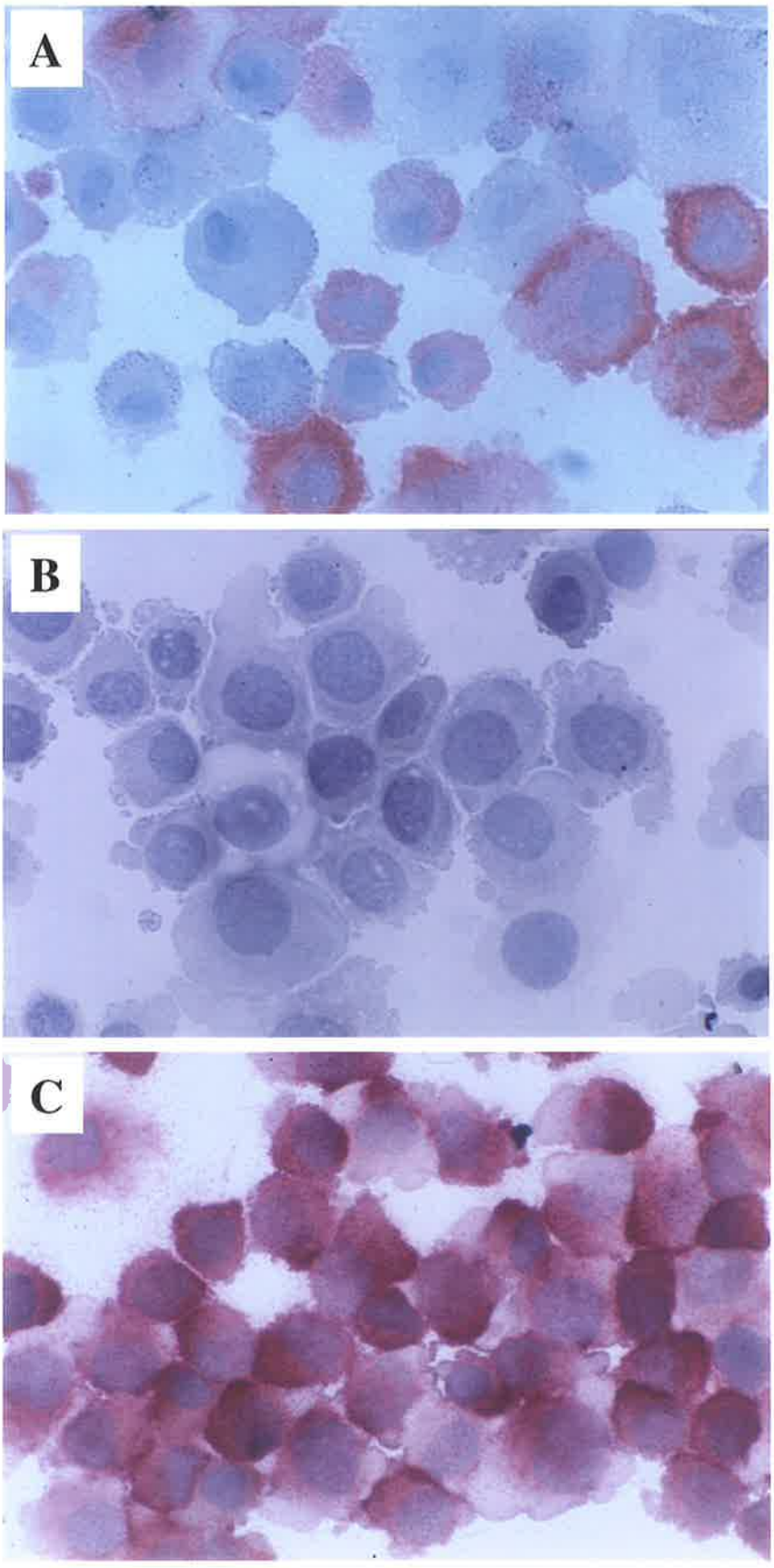
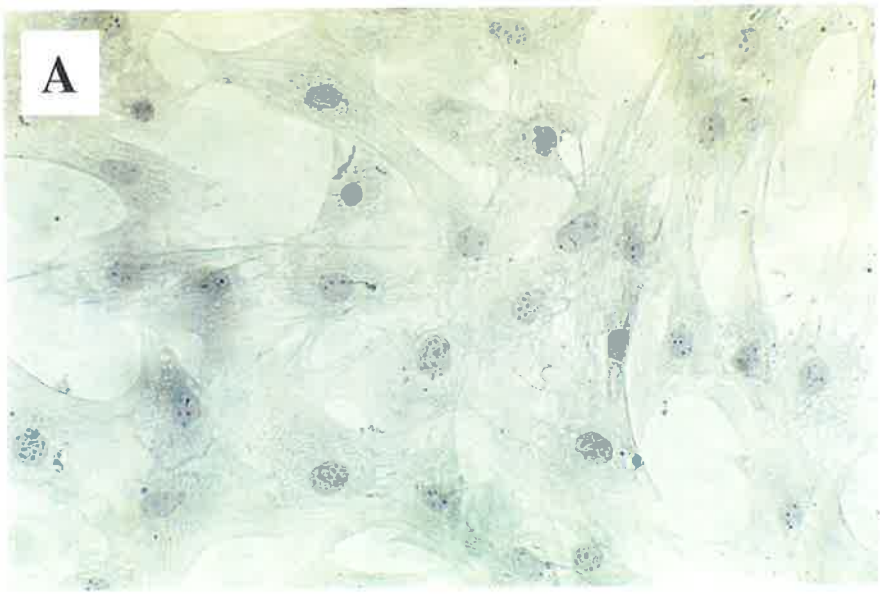

A
B
C

Figure 49. LNPOZ infection of human BM stromal cells in vitro. Representative photomicrographs demonstrating histochemical analysis for β-galactosidase activity in cytospin preparations of cultured stromal cells derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC: untransfected cultures (A); cultures transfected with the retroviral vector, LNPOZ (C). The cultures were counter-stained with haematoxylin. There was no positive expression of β-galactosidase activity in the untransfected control cultures (A) (40X). A high expression of alkaline phosphatase activity was observed in all the cells transfected with the retroviral vector, LNPOZ following selection in G418 (C) (40X).


A

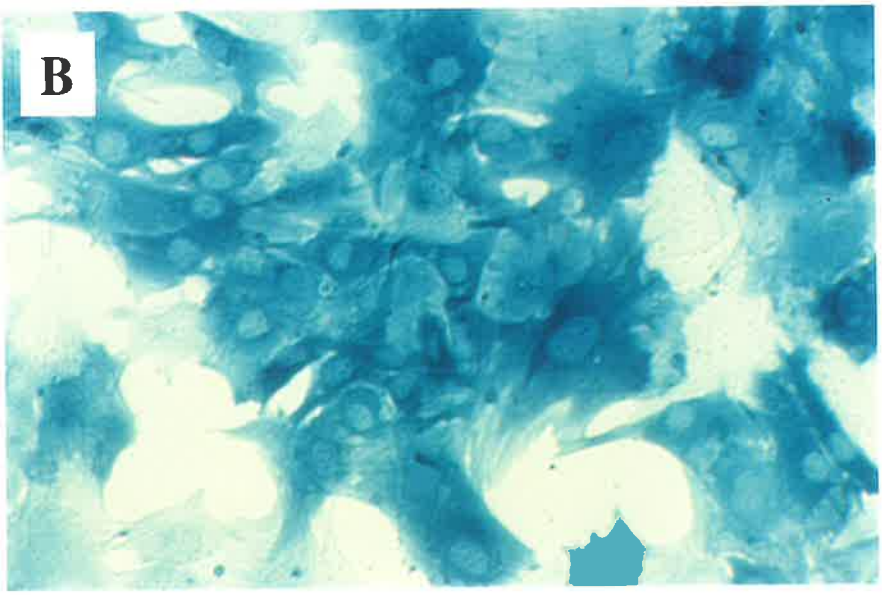

B

Figure 50. Identification of retrovirally marked stromal cells post transplant. Retrovirally marked cultured stromal cells, derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were implanted inside porous hydroxyapatite (HA) ceramic cubes subcutaneously into SCID mice for 10 weeks. The stromal cells were retrovirally marked pre-transplantation with: the retroviral vector, LNPOZ (A); the retroviral vector, LAPSN (B). Representative photomicrographs of histochemical analysis for β-galactosidase activity (arrow, panel A) and alkaline phosphatase activity in the presence of 1μM levamisole (arrow, panel B), in frozen sections HA cubes harvested following transplantation (20X). The sections were counter-stained with haematoxylin. The results demonstrate that retrovirally marked human stromal cells were found lining the inner walls of the HA cubes following 10 weeks transplantion in SCID mice.

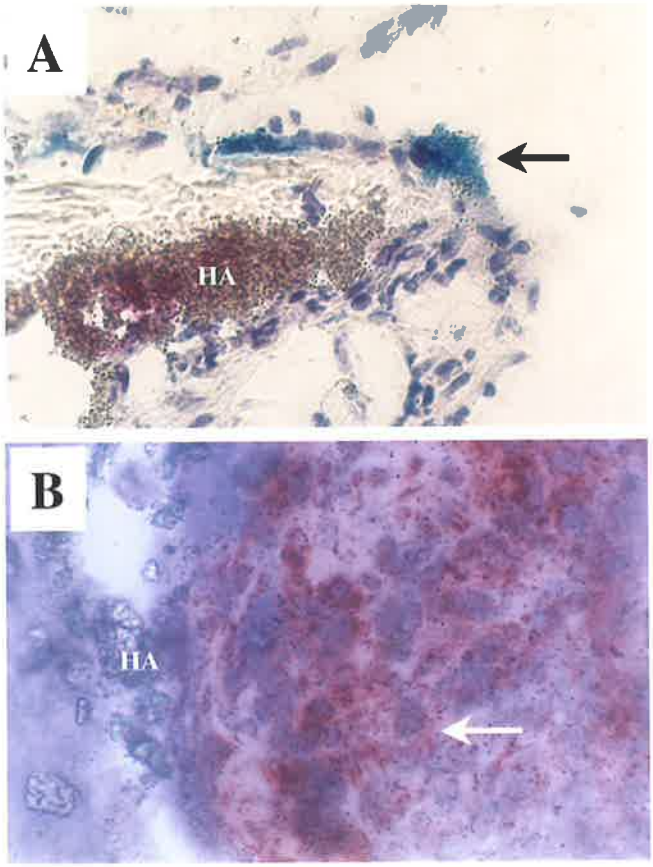
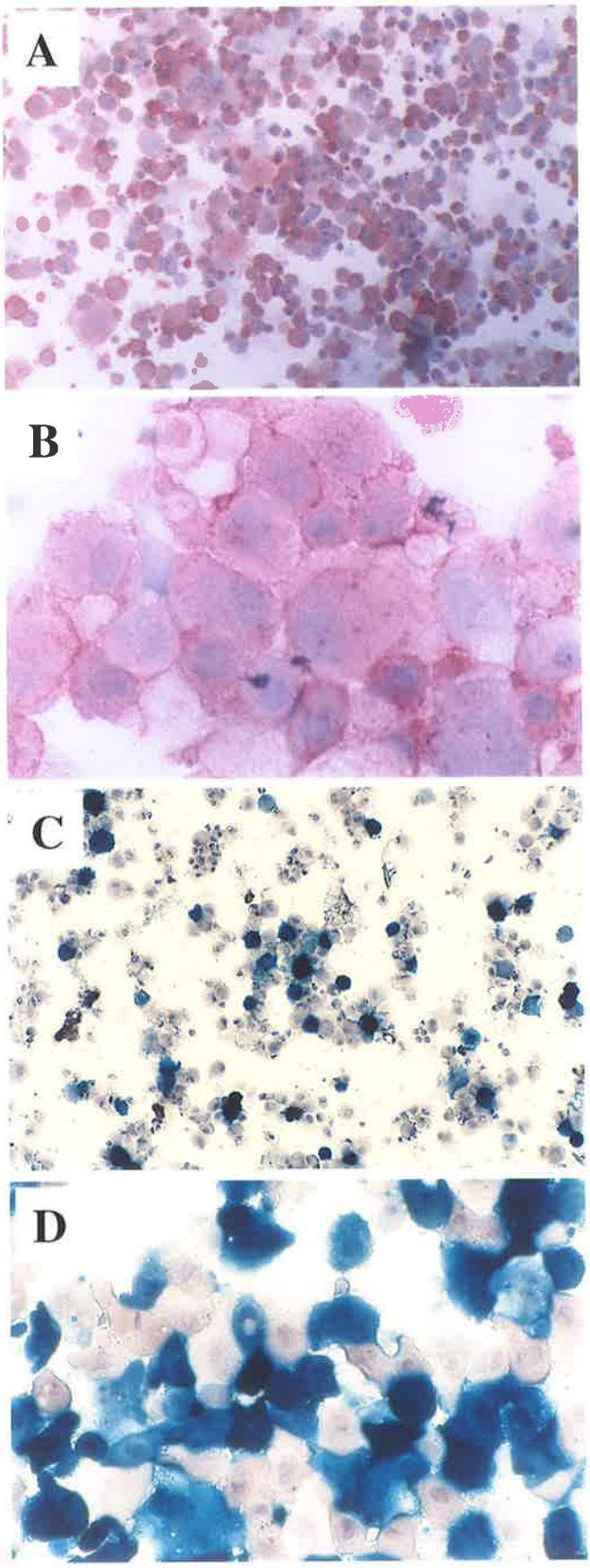

A
HA
B
HA

Figure 51. Recovery and culture of retrovirally marked stromal cells post-transplant. Retrovirally marked cultured stromal cells, derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were implanted inside porous hydroxyapatite (HA) ceramic cubes subcutaneously into SCID mice for 10 weeks. The HA cubes were recovered post-transplantation and the cells within released by treatment with 3 mg/ml collegenase solution for 2 h at 37^{o}C. Liberated cells were then grown in standard culture conditions in the presence of 600 μg/ml G418 for one week. Representative photomicrographs of histochemical analysis for alkaline phosphatase activity in the presence of 1μM levamisole (A and B) and β-galactosidase activity (C and D). Cytospin preparations of cells were prepared following selection in G148 containing medium. The cells were counter stained with haematoxylin. Panel A, shows that all the cells transfected with the retroviral vector, LAPSN exhibited alakline phosphatase activity (10X). Panel B, depicts a magnified view of the same cells (40X). Panel C, demonstrates that only a proportion of cells (40-60%) infected with the retroviral vector LNPOZ expressed positivity for β-galactosidase activity (10X). Panel D, depicts a magnified view of the same cells (40X).


A
B
C
D

implants up to three months post transplantation using two independent markers. No alkaline phosphatase positive or β-galactosidase positive cells could be detected at any time points in HA cubes implanted without cells.

Because of the difficulties associated with identifying human cells in the SCID mice based on their expression of retroviral markers an alternative, less ambiguous method of detection was developed. The origin of the new bone formation within the recovered implants were assessed by in situ hybridisation using a DNA probe specific to the unique human DNA repetitive alu sequence. Control experiments using human cells transplanted in diffusion chambers and sections of normal mouse kidney demonstrated the specificity of the alu probe to human tissue (Figure 52). Ten week old implants of HA ceramic cubes were prepared as paraffin embedded sections and analysed by in situ hybridisation as described in the methods. The fibrous tissue, bone lining cells and osteocytes within the newly formed bone were all found to positive for the alu sequence confirming their human origin (Figure 53). Conversely, the fat and muscle layers surrounding the ceramic cubes did not express the alu sequence and were therefore presumed to have originated from the host mouse (Figure 54). Similarly, the endothelium lining the blood vessels were also found to be negative for the alu sequence implying they were derived from the mouse vasculature. Control sections recovered from the HA cubes implanted without cells demonstrated no reactivity with the alu probe. Having determined that the human stromal cells implanted into the SCID mice had the potential to develop new bone we further investigated the osteogenic potential of individual CFU-F clones in vivo.

5.4 The Developmental Potential of BM CFU-F clones In Vivo

Bone marrow CFU-F clones were established from STRO-1^{bright}/VCAM-1^{+} sorted cells from two individual BM donors using the single cell deposition unit as described in the methods. At day 4 of culture, single clonogenic clusters were identified and expanded by subculture. Some of the cells from the first passage were taken from each clone and grown under osteogenic growth conditions to assess the ability of the clones to develop a

Figure 52. Demonstration of the specificity of the human specific alu DNA probe. In situ hybridization was performed on paraffin embedded transverse sections of: expanded human CFU-F, derived from STRO-1^{bright}/VCAM-1^{+} BMMNC, implanted inside diffusion chambers, into the abdominal peritoneal cavity of SCID mice for 10 weeks (A, B, C and D); normal SCID mouse kidney (E and F). Representative photomicrographs are shown where panel A is counter stained with haematoxylin and eosin while pannels B, C, D, E and F used for in situ hybridization were counter stained with methyl green. Panel A: demonstrates a layer of mouse connective tissue (M) covering the membrane filter(F) of the diffusion chamber and a mass of human fibrous tissue (H) inside the diffusion chamber (10X). Panel B: depicts serial sections of the same diffusion chamber demonstrating positive reactivity with the alu probe in the human tissue only (10X). Panel C: shows a magnified view of the mouse tissue unreactive with the alu probe (40X). Panel D: shows a magnified view of the human tissue with positive reactivity of the alu probe (arrow) in the nucleus of cells (40X). No reactivity was found inside normal SCID mouse kidney (E) or in the outer areas near the kidney capsule (arrow) (20X).

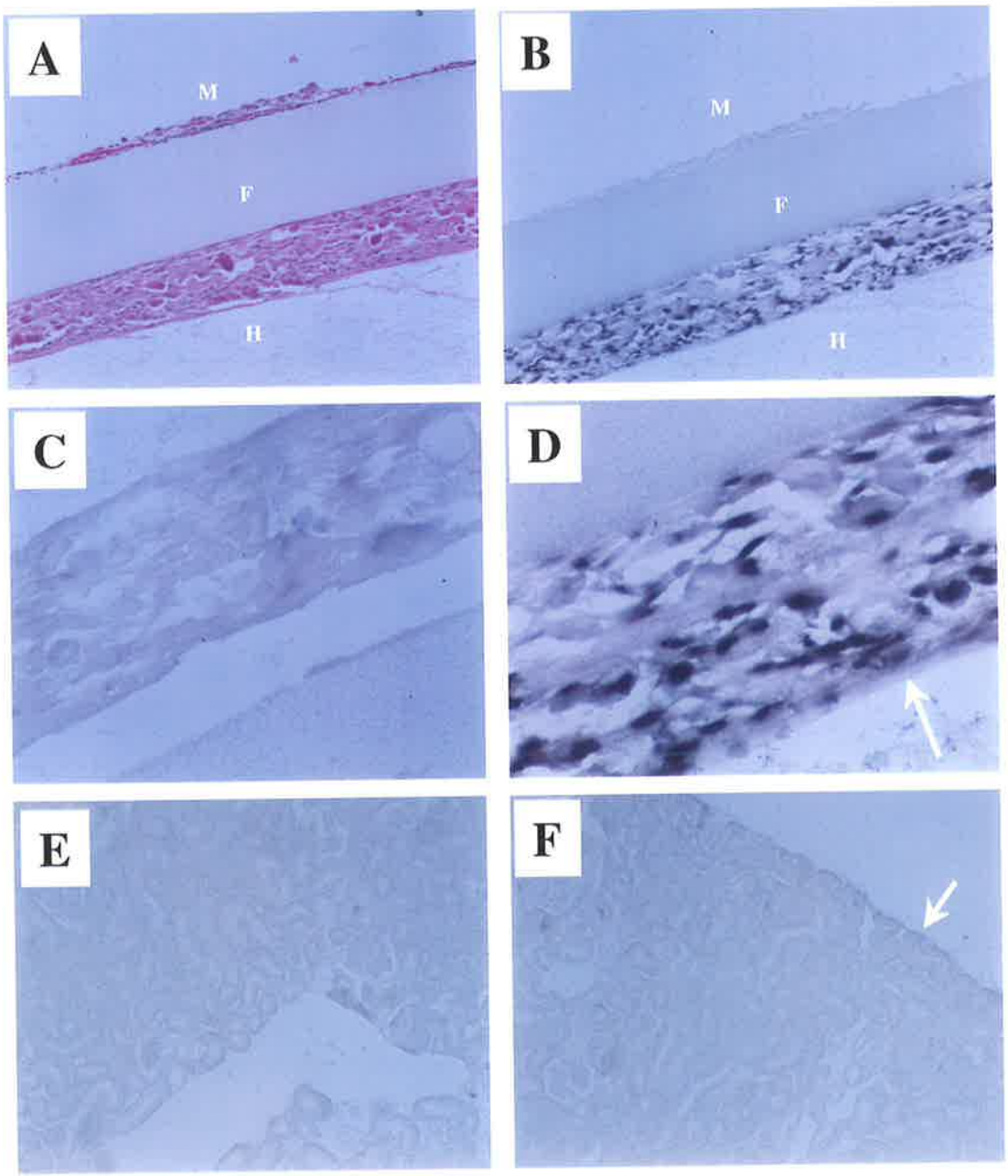

A
M
F
H
B
M
F
H
C
D
E
F

Figure 53. In situ hybridization for the human specific alu DNA sequence in implants of hydroxyapatite (HA) ceramic cubes: new bone formation. Human CFU-F clones, derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were expanded in vitro and then implanted, inside porous HA-ceramic cubes implanted subcutaneously into SCID mice for 10 weeks. Representative micrographs are shown of paraffin embedded cross sections of HA cubes recovered post-transplant and analysed by in situ hybridization for reactivity with the alu probe. Panel A: the formation of new bone on the outer rim of the HA cube (large arrow) and the old HA surface (small arrow) (10X). sections were counter-stained with haematoxylin and eosin. Panel B: depicts a magnified view of the new bone formation which contains osteocytes (arrow) trapped within the new bone (40X). Panel C: in situ hybridization analysis on serial sections of the same HA cube demonstrating positive reactivity to the firous tissue surrounding an area of new bone (arrow) (10X). Panel D: a magnified view of the new bone formation which contains osteocytes (arrow) trapped within the new bone demonstrating positive reactivity with the alu probe (40X). Panels E and F, represent magnified views of new bone formation from two different CFU-F clones, demonstrating that osteocytes within the bone exhibit positive reactivity with the alu probe (40X).

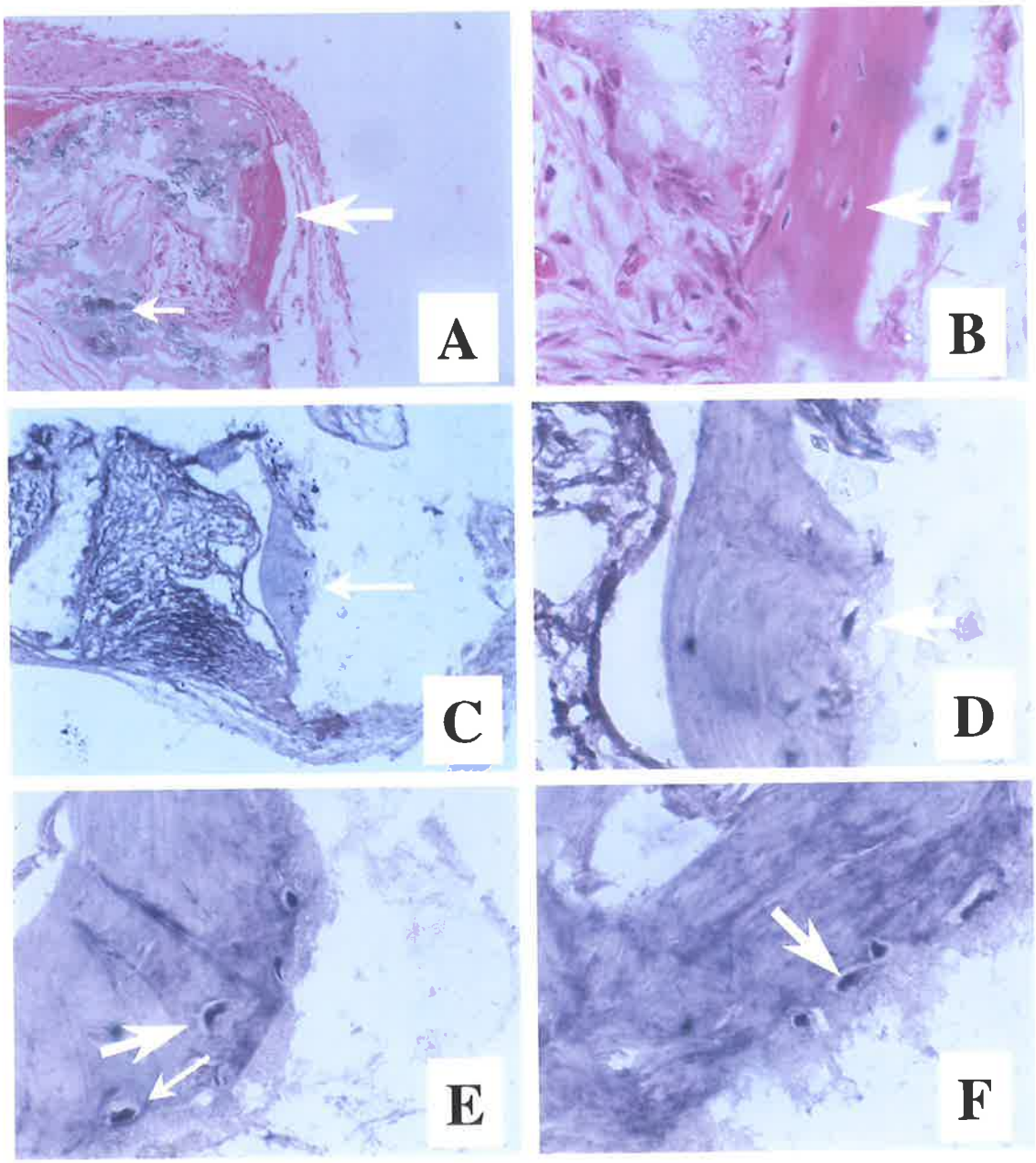
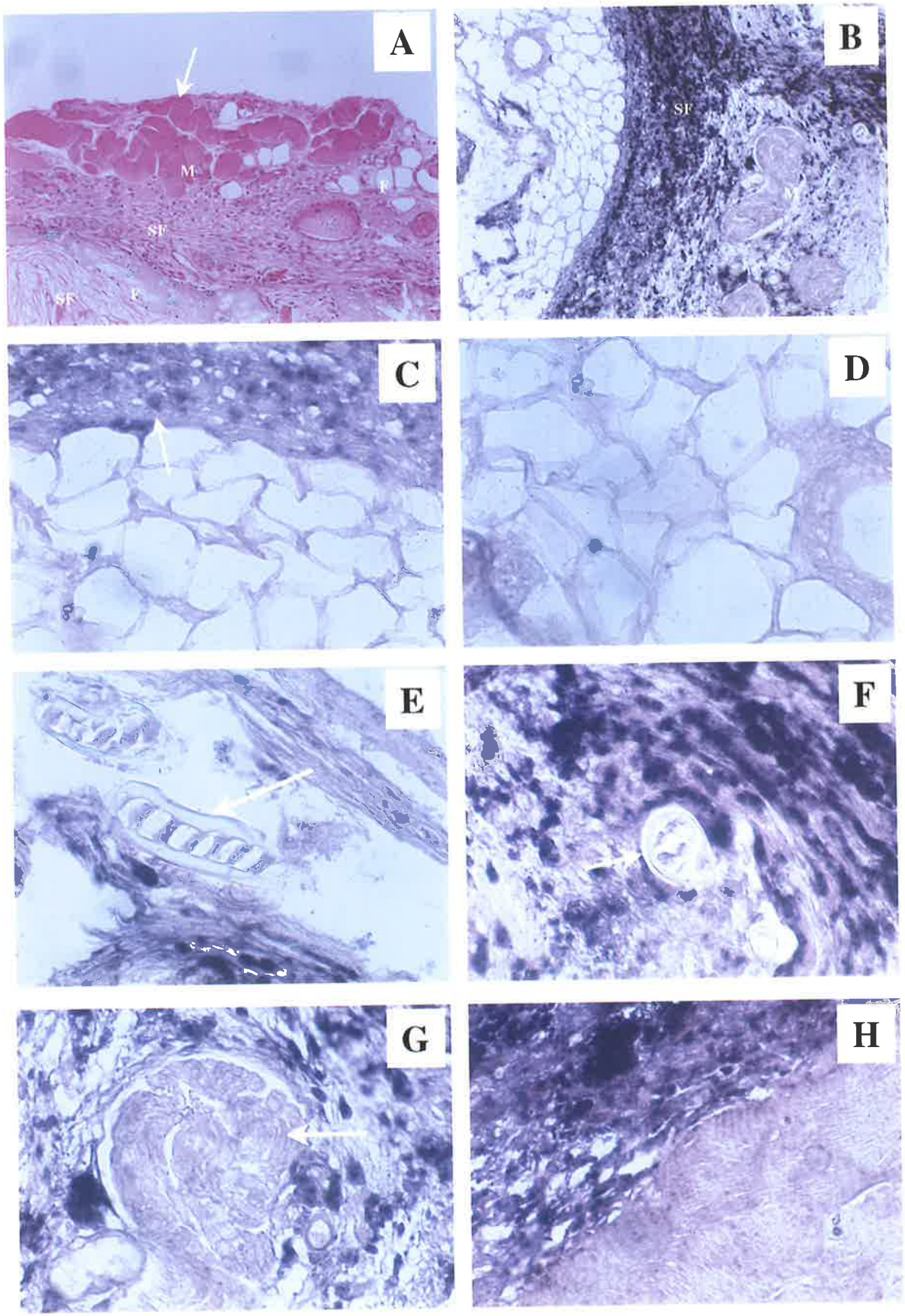

A
B
C
D
E
F

Figure 54. The alu sequence was not expressed in fat, muscle and vascular tissue in implants of hydroxyapatite (HA) ceramic cubes. Human CFU-F clones derived from MACS/FACS isolated STRO-1^{bright}/VCAM-1^{+} BMMNC, were expanded in vitro and then implanted inside porous HA-ceramic cubes implanted subcutaneously into the backs of SCID mice for 10 weeks. Representative micrographs are shown of paraffin embedded cross sections of HA cubes recovered post-transplant and analysed by in situ hybridization for reactivity with the human specific alu probe. Panel A: shows the histology of the soft fibrous tissue (SF) on the outer surface of the HA cube (large arrow) with associate muscle (M) and fat (F) tissues (10X). These sections were counter stained with haematoxylin and eosin. Panel B: depicts the different stromal tissues surrounding the HA cubes analysed by in situ hybridization for reactivity with the alu probe (20X). Panel C: a magnified view of the fat cells inside the HA cube lacking reactivity with the alu probe in comparison to the surrounding fibrous tissue (arrow) which demonstrated positive reactivity (40X). Panel D: a magnified view of fat tissue outside the HA cube failing to react with the alu probe (40X). Panels E and F, represent magnified views of endothelial cells of blood vessels (arrows) showing no reactivity to the alu probe, surrounded by alu positive soft-fibrous tissue (40X). Panels G and H, depict areas of muscle (arrow) showing no reactivity with the alu probe surrounded by alu positive soft fibrous tissue (40X).


A
M
F
SF
SF
F
B
SF
M
C
D
E
F
G
H

mineralised bone-matrix in vitro. The remaining cells were expanded and maintained for several weeks in the presence of ASC-2P and DEX and then seeded into HA ceramic cubes. The ceramic cubes were then implanted subcutaneously on the dorsal surface of SCID mice for a period of 8 weeks as previously described. Following recovery of the implants the cubes were fixed in 10% buffered formalin, decalcified and then embedded in paraffin as described in the methods. Cross-sections of the cubes were prepared and counter stained with haematoxylin and eosin. A total of 46 individual clones from two different marrow samples were transplanted into SCID mice in HA cubes. In all of the implants an extensive network of blood vessels and fibrous tissue was observed. Bone formation was found in 42% (n=26) and 55% (n=20) of the clones isolated from two different BM aspirates A and B, respectively. The developmental potential of each CFU-F clone both in vitro and in vivo is summarised in Table 10. New bone formation detected in the implants was also found to correlate with the in vitro developmental potential of individual clones in respect to the formation of a von Kossa positive mineralised matrix. However, the capacity of CFU-F clones to also form adipocytic clusters in vitro had no bearing on the development of new bone formation in vivo. In addition, there was no cartilage formation observed in sections of different implants and at different time points, as assessed by immunohistochemical analysis using a mab specific for collagen type II (data not shown).

5.5 DISCUSSION

In the present study, osteoprogenitors were identified in the STRO-1$^+$ fraction of human adult bone marrow. While there are no definitive markers of discrete developmental stages of osteogenic lineage, the cells produced under the culture conditions described in this study exhibit characteristics of osteoblasts, as demonstrated by their expression of various bone cell-related markers, and by the production of a mineralized (hydroxyapatite) bone-matrix. The generation of osteoblast-like cells with these phenotypic and functional characteristics was dependent upon culture of the sorted STRO-1$^+$ precursor cells in the presence of ASC-2P (vitamin C), the glucocorticoid DEX and a source of inorganic

Table 10. Developmental potential of BM CFU-F clones. CFU-F clones were generated from single cells expressing the immunophenotype STRO-1^{bright}/VCAM-1^{+} using the combined MACS/FACS isolation procedure for two different BM samples (A and B). Selected CFU-F clones were assessed for their ability to differentiate into osteoblasts and adipocytes in vitro and in vivo as described in the methods.

CFU-F	New Bone Formation In Vivo[1]		Developmental Potential In Vitro	
Clone	Inside cube	Outside Cube	Bone[2]	Fat[3]
2A	-	+	+	-
4A	-	-	+	-
5A	+	+	+	-
7A	-	-	+	-
8A	-	+	+	-
9A	-	-	NA	NA
10A	-	-	+	+
11A	-	+	+	+
13A	+	+	+	+
15A	+	-	+	-
17A	-	-	NA	NA
18A	-	-	+	-
19A	-	-	+	-
20A	-	-	+	-
21A	-	-	+	+
22A	-	+	+	+
24A	+	-	+	+
25A	-	-	+	-
28A	-	-	NA	NA
29A	+	-	+	-

Continued....

Table 10. Developmental potential of BM CFU-F clones in vivo and in vitro, continued.

CFU-F	New Bone Formation In Vivo[1]		Developmental Potential In Vitro	
Clone	Inside cube	Outside Cube	Bone[2]	Fat[3]
30A	-	+	+	-
31A	-	+	+	+
32A	-	-	+	-
39A	-	-	+	-
40A	-	-	+	-
42A	-	-	+	-
1B	-	-	+	-
2B	-	-	+	-
3B	-	+	+	-
4B	-	+	+	-
5B	-	+	+	+
6B	-	-	+	-
8B	-	-	+	+
9B	-	-	+	-
11B	-	-	+	+
13B	+	+	+	-
14B	+	+	+	+
15B	+	+	+	-
18B	-	-	+	+

Continued.....

Table 10. Developmental potential of BM CFU-F clones in vivo and in vitro, continued.

CFU-F	New Bone Formation In Vivo[1]		Developmental Potential In Vitro	
Clone	Inside cube	Outside Cube	Bone[2]	Fat[3]
19B	-	-	+	+
20B	-	-	+	+
21B	+	+	+	+
22B	-	+	+	+
24B	+	+	+	-
25B	+	-	+	-
26B	+	+	+	+

(NA) Not able to be analysed.

(1) Histological identification of areas of new bone formation at the centre of the HA cubes (inside) and on the outer surface of the HA cubes (outside).

(2) Von Kossa positive mineralized matrix.

(3) Oil red O positive lipid conatining adipocytes.

(+) Positive identification.

(-) Absence of new bone formation/von Kossa positive mineral/oil red O positive lipid droplets.

phosphate. This data is in accord with that reported by other investigators based on animal model systems [Maniatopoulos et al, 1988; Schoeters et al, 1988].

Studies have previously shown that ascorbate is essential for bone cell development in vivo and the maintenance of human osteoblasts in vitro [Koshihara et al, 1987]. Ascorbate helps facilitate bone cell proliferation and differentiation by increasing the total protein, collagen synthesis, and alkaline phosphatase activity [Graves et al, 1991; Hitomi et al, 1992; Torii et al, 1994]. In the present study, sodium ascorbate, which has a half life of a few hours in culture was replaced by the long acting derivative ASC-2P, which has a half life of approximately 7 days in culture [Hata et al, 1989]. The role of glucocorticoids in regulating osteogenic differentiation in vitro has not been precisely defined. A number of studies have shown that DEX is critical for inducing terminal differentiation of osteogenic cells in rat BM cultures, however the presence of this steroid is not an absolute requirement for in vitro osteogenesis in other animal systems [Howlett et al, 1986; Maniatopoulos et al, 1988; Bellows et al, 1990; Benayahu et al, 1991; Kamalia et al, 1992; Falla et al, 1993]. Therefore the relevance of some animal based systems to human in vitro models of bone cell development remains questionable. In human studies, glucocorticoid stimulation is required for the differentiation of osteoblast-like cells in bone marrow stromal cultures [Cheng et al, 1994].

Glucocorticoids are a family of steroid hormones known to stimulate the expression of mRNA for many genes associated with osteogenic (AP, PTH-R, OP, OCN) [Leboy et al, 1991; Beresford et al, 1994; Locklin et al, 1995; Rickard et al, 1996] and adipocytic (C/EBPα, PPARγ2, LPL, aP2, adipsin) [Gimble et al, 1990; 1996] differentiation, including various proto-oncogenes such as c-fos [Subramanian et al, 1992] involved in osteogenesis [Ohta et al, 1992]. In the present study, primary cultures of human BM CFU-F were observed to express a range of bone cell-related markers (COL-1, OP, ON, BSP) and fat cell-related markers (LPL, H-ALBP), but lacked the expression of other markers of osteogenic (OCN and PTH-R) and adipocytic (Leptin and PPARγ2) differentiation.

However, transcripts for PTH-R, leptin (Obese gene product) and PPARγ2 were detected in the primary human BM CFU-F cultures following culture for several weeks in the presence of ASC-2P and DEX, but not with ASC-2P alone. Similarly, transcripts for OCN were detected in the DEX stimulated cultures following exposure to 1,25-$(OH)_2D_3$. Comparable levels of osteocalcin protein were also found in the supernatant of primary human BM CFU-F cultures under the same conditions. Therefore the growth of human BM CFU-F in the presence of serum seems to promote osteogenic and adypocytic commitment in at least a proportion of cells, but the development of functional mature bone and fat cell phenotypes requires stimulation with DEX.

The DNA-binding protein CBFA1 has been specifically associated with osteogenic tissue in rodents [Ducy et al, 1997; Komori et al, 1997; Rodan and Harada 1997]. In the present study, primary cultures of stromal cells derived from STRO-1^{bright}/VCAM-1^+ BMMNC were found to express CBFA1 by RT-PCR. In contrast, CBFA1 was undetectable in freshly sorted samples of STRO-1^{bright}/VCAM-1^+ BMMNC prior to culture as shown in Chapter 4. However, the specificity of this transcription factor in human osteogenic tissue is still unclear. It is also remains to be determined what proportion of cultured stromal cells actually express CBFA1 given the sensitivity of RT-PCR analysis. There may exist populations of cells in BM stromal cultures at varying stages of differentiation, analogous to cultures of normal human bone cells as discussed in Chapter 7. Furthermore, our primer sets yielded three distinct splice variants in addition to the expected PCR product for CBFA1 by DNA sequence analysis [unpublished observations by Dr. A. C. W. Zannettino]. A recent study has identified two alternative spliced variants at the 3' end of the human CBFA1 gene which have been shown to affect protein function [Geoffroy et al, 1998]. Studies in our laboratory are currently underway to assess any functional variations between the different CBFA1 splice variants observed in primary cultures of human BM stromal cells.

In vitro, the formation of a mineralized bone-matrix by bone cells has previously been shown to be dependent upon supplementation of their growth medium with a source of inorganic phosphate [Bellows et al, 1992]. In this study, inorganic phosphate was used in place of β-glycerophosphate which is a nonphysiological organic phosphate substrate of alkaline phosphatase [Gerstenfeld et al, 1987]. In vitro studies have shown that β-glycerophosphate is rapidly and virtually completely degraded to inorganic phosphate by AP during the initial stages of mineralisation and that the addition of excess levels of inorganic phosphate can replace the effects of β-glycerophosphate [Bellows et al, 1992]. In addition, the presence of β-glycerophoshate in animal studies has been shown to cause ectopic mineralization in cultures of foetal rat parietal cells and skin fibroblasts, which does not occur using inorganic phosphate at concentrations of less than 1.9 mM [Gronowicz et al, 1989; Khouja et al, 1990; Ishikawa and Wuthier, 1992]. In the present study parallel cultures of human foreskin fibroblasts grown in the presence of ASC-2P, DEX and PO_{4i} for a period of six weeks were found to be von Kossa negative (data not shown).

Typically, cultures of purified STRO-1^+ human BM CFU-F developed a von Kossa positive mineral by twenty one days under osteogenic conditions (ASC-2P, PO_{4i}, DEX). TEM analysis of the adherent layers demonstrated the presence of deposits of hydroxyapatite-like crystals in association with the collagen fibrils in the matrix, consistent with the structure and deposition of hydroxyapatite crystals in vivo [Boskey, 1992]. The presence of mineral deposits, which stained positive for the von Kossa reaction, were demonstrated in all CFU-F clones derived from STRO-1^{bright}/VCAM-1^+ BMMNC. This is in contrast to studies of rodent bone marrow which reported that only a proportion of CFU-F (approximately 20% in murine BM) are capable of developing an osteogenic phenotype with the capacity to form bone nodules in vitro Simmons et al, 1991; Falla et al, 1993]. This discrepancy may be attributed to the presence of accessory cells in the relatively crude bone marrow preparations used in the rodent studies which may affect the expression of the osteogenic phenotype of CFU-F in vitro. In addition, the absence of

dexamethasone [Falla et al, 1993] and ascorbate [Simmons et al, 1991] in the culture medium may also lead to an underestimation of the incidence of osteogenic precursors in rodent bone marrow studies.

In the present study, thirty eight percent of the CFU-F clones examined displayed the capacity to differentiate into adipocytic cell clusters, demonstrating the bi-potential of the human BM CFU-F population in vitro. Moreover, individual CFU-F clones were also found to contain a small proportion of fibroblastic-like cells not associated with mineralization or lipid accumulation. These fibroblast cells may represent as yet undefined stromal populations such as reticular cells, smooth muscle cells, bone lining cells, osteocytes and committed stromal progenitors. To establish whether human BM SPC are indeed multi-potential, there is clearly a need for the development of appropriate culture assays and markers to definitively identify all the different mature stromal cell lineages present in the BM microenvironment. Discrepancies in the ability of some lineage specific markers in vivo to specifically identify similar cell types in vitro has been discussed in Chapter 4. For example some smooth muscle actin-specific isoforms only found in the smooth muscle cells in vivo are also expressed by bone marrow fibroblastic-like cells or different BM stromal cell lines under certain culture conditions [Charbord et al, 1985; Peled et al, 1991; Galmiche et al, 1993].

The developmental potential of selected CFU-F clones was further examined in vivo. Initial experiments found that in all of the transplantation strategies used, only the porous hydroxyapatite coated ceramic cubes reproducible supported the development of human osteogenic tissue in SCID mouse. This is in agreement with the findings in previous studies using rodent and human bone marrow stromal cell cultures transferred inside HA cubes and implanted in immunodeficient mice [Haynesworth et al, 1992a; Krebsbach et al, 1997; Kusnetsov et al, 1997]. In this study, pre-treating the HA ceramic cubes with purified fibronectin was critical to maximise the number of cells retained in the cubes after loading prior to transplantation (data not shown). Pre-treatment of HA cubes with

fibronectin and laminin has been reported to increase cell retention and spreading on the ceramic surface of the cubes [Dennis et al, 1992; Dennis and Caplan; 1993]. Fibronectin and laminin coated cubes were found to augment bone formation from cultured rat BM stromal cells at earlier time points in comparison to untreated cubes [Dennis et al, 1992; Dennis and Caplan, 1993].

The present study failed to detect cartilage formation in any of the transplantation models used, in contrast to other studies which demonstrated cartilage formation in diffusion chambers transplanted with rodent bone marrow or stromal cells derived from the marrow of young children. To date, there have been no reports describing the induction of cartilage formation by adult human bone marrow stromal cells in vivo or in vitro. The recent establishment of culture conditions permissive for rabbit bone marrow cells to develop a chondrocytic phenotype in vitro, could further be extended to determine the chondrocytic potential of purified human CFU-F. The chondrocytic inductive factor in those studies was TGFβ and was shown in the present study to be ineffective in stimulating the proliferation of human CFU-F in vitro (Chapter 3). Therefore, many of the factors unable to stimulate the clonogenic growth of CFU-F such as TGFβ, BMP-2, bFGF, IGF-1, VEFGF and leptin (Chapter 3), may be useful in alternative culture assays to promote the differentiation of CFU-F towards specific stromal cell lineages. In the present study, the expression of the hypertrophic chondrocyte marker collagen type X, by purified adult human BM SPC, is somewhat puzzling, given the presumed specificity of this molecule. The physiological role of collagen type X is not known, therefore its significance in bone marrow remains to be determined.

The present work is in accord with previous studies showing that the formation of new bone in implants of HA cubes is attributed to differentiation of human stromal cells into functional bone cells [Kusnetsov et al, 1997] and did not result from the recruitment of osteoprogenitors from the surrounding host (mouse) tissue. Furthermore, in this study other cell types and tissues such as muscle, adipocytes and vascular endothelial cells

showed no hybridization with the alu probe and are therefore presumed to be host in origin. These findings demonstrate that a proportion of BM SPC within the STRO-1^{bright}/VCAM-1^{+} BM subfraction, demonstrate the capacity to develop into multiple stromal cell types including osteoblasts, adipocytes and fibroblast-like cells. Further studies are still required to characterise the full developmental potential of human BM SPC population.

One area of potential benefit that will occur from a greater understanding of the proliferation and differentiation of SPC, is the ability to manipulate and expand stromal cell populations in vitro for subsequent reimplantation in vivo. Fracture non-union, prosthetic loosening and the replacement of large defects in bone are all common and difficult clinical problems. The use of autologous bone marrow stromal cells with osteogenic potential in combination with biocompatible implant materials, would provide a novel solution to the treatment of these problems and reduce the need to use bone autografts and allografts with all their inherent difficulties. The use of animal models has demonstrated the efficacy of utilising ex vivo expanded BM stromal cells to facilitate bone regeneration and tendon repair in vivo [Bruder et al, 1998b; 1998c; Young et al, 1998].

Furthermore, cultured expanded human stromal cells derived from STRO-1^{bright}/VCAM-1^{+} bone marrow demonstrated a high infection rate with different retroviral constructs. Significantly, transfected stromal cells were also shown to retain their capacity to proliferate and to form bone, fat and fibrous tissue both in vitro and in vivo. Retroviral gene expression in the human stromal cells was found to persist for a period of at least three months post-transplantation in SCID mice. Several other investigators have described how marrow stromal cells from a variety of species are readily infected using either amphotropic retroviruses or adenoviruses [Harigaya and Handa, 1985; Rothstein et al, 1985; Singer et al. 1987; Cicutinni et al, 1992; Roecklein and Torok-Storb, 1995]. In addition, some studies have demonstrated the persistence of transplanted transduced cells over several months in vivo [Li et al, 1995; Anklesaria et al, 1996; Onyia et al, 1998].

Recently, Reiw and colleagues (1998) were able to transduce rabbit BM stromal cells in vitro with a recombinant adenoviral vector carrying the human BMP-2 gene, transforming these cells into an osteoprogenitor line with the capacity to produce new bone in vivo, using an autologous rabbit spinal fusion model. Therefore the ability to harvest purified human SPC from aspirates of BM and to expand these cells ex vivo makes them ideal candidates as possible vehicles for gene transfer in order to treat a variety of diseases and genetic disorders.

CHAPTER SIX: INTEGRIN MEDIATED INTERACTIONS BETWEEN OSTEOBLAST-LIKE CELLS AND THE EXTRACELLULAR MATRIX

6.0 INTRODUCTION

Interactions between cells and their extracellular matrix (ECM) are an essential component of virtually all cellular events [von der Mark et al, 1992]. Such a complex array of interactions occurs through an equally diverse spectrum of cellular receptors and ECM protein ligands. One of the best described families of receptors is that of the integrins. These are transmembrane heterodimers consisting of non-covalently associated α- and β-subunits which are responsible for mediating both cell-cell and cell-matrix interactions [Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1991; Hogg, 1991; Hynes, 1992]. Integrins are arranged in subfamilies according to the eight known β subunits. In turn, each β subunit may associate with either one of as many as nine different α subunits [Ruoslahti, 1991; Hogg, 1991; Hynes, 1992]. Conversely, some α subunits, such as α_V, can be associated with several different β subunits, conferring distinct ligand binding properties to each heterodimer. Furthermore, some integrins such as the fibronectin receptor $\alpha_5\beta_1$ and the laminin receptor $\alpha_6\beta_1$ bind only to single ECM components, while others such as $\alpha_V\beta_3$ react with several different ligands including collagen, vitronectin, laminin and fibronectin [Brown and Juliano, 1985; Albelda and Buck, 1990; Elices et al, 1991; Hynes, 1992]. Thus there arises the potential for a large number of different cellular responses to the cell-matrix and cell-cell interactions depending on the integrin receptor expressed by different cell types and the composition of the surrounding extracellular matrix. The interactions of bone cells with their surrounding ECM are essential for the maintenance of skeletal development and homeostasis. These interactions involve direct cell-matrix adhesion and are subject to regulation by growth and differentiation factors [Horton and Davies, 1989]. Signals from the extracellular matrix can be transduced via integrins to the intracellular mechanisms controlling cell growth, behaviour and differentiation, through an intricate biochemical signal transduction pathway linked to the cytoskeleton [Juliano and Haskill, 1993].

Several BM ECM and bone glycoproteins proteins such as, collagen, fibronectin, vitronectin, thrombospondin, bone sialoprotein and osteopontin exhibit the unique tripeptide (arginine-glycine-aspartate) RGD sequence and are therefore likely candidates for integrin-mediated interactions [Hayman et al, 1983, Pierschbacher and Ruoslahti, 1984; Dedhar et al, 1987; Grzesik and Robey, 1994]. Studies have demonstrated that human bone cells and osteoclasts express a variety of integrin molecules [Clover et al, 1992] and adhere to varying extents to collagen types I and IV, thrombospondin, vitronectin, fibronectin, laminin, osteopontin, bone sialoprotein, gelatin and fibrinogen [Saito et al, 1994; Grzesik and Robey, 1994; Helfrich et al, 1992; 1996]. In the presence of GRGDS peptides, adhesion of osteoblasts to bone sialoprotein, osteopontin and vitronectin was almost completely inhibited. However, the binding of osteoblasts to fibronectin, collagen type I and thrombospondin was only slightly reduced [Grzesik and Robey, 1994] indicating that bone cells use a variety of different mechanisms to attach to their extracellular matrix. Similarly, osteoclast attachment to collagen was found to be RGD-dependent through the integrins $\alpha_2\beta_1$ and $\alpha_v\beta_3$ [Helfrich et al, 1996]. The binding of osteoclasts to other RGD-containing molecules such as fibronectin and the bone sialoproteins is also mediated through multiple β_1 receptors and the integrin $\alpha_v\beta_3$ [Helfrich et al, 1992]. Indeed, it has been shown that the tetrapeptide RGDS which blocks adhesion through the RGD sequence on molecules such as fibronectin, inhibits bone formation and resorption in a mineralising organ culture system derived from foetal rat parietal bones [Gronowicz and De Rome, 1994]. In vitro, osteoclast induced bone resorption has also been shown to be significantly reduced in the presence of $\alpha_2\beta_1$ antibodies [Helfrich et al, 1996].

There is increasing evidence that the consequence of integrin binding to the extracellular matrix may be a reorganisation of the three dimensional structure of the matrix microenvironment. For example, the $\alpha_2\beta_1$ integrin is required for contraction of a type I collagen matrix [Schiro et al, 1991] and my therefore play a role in bone remodelling.

Integrins are coupled to the cytoskeleton via vinculin, talin and α-actin and may play a role as intracellular signal transducers of mechanical stresses on the bone matrix through osteocytes. Mechanical twisting of cell membranes via integrin-bound beads has been found to cause cytoskeletal rearrangements in other cell systems [Wang and Ingber, 1994]. Similarly, one study has shown that mechanical-sensing by bone cells may be mediated via matrix-integrin-cytoskeletal coupling [Meazzini et al, 1998].

Given the potential of primitive BM SPC to develop into functional bone cells (Chapter 5), we next examined the mechanisms mediating the adhesion of differentiated bone cells to different ECM components. Several attempts have been made to identify the integrin subunits or dimers that are present on bone cells [Grzesik and Robey, 1994; Saito et al, 1994; Clover et al, 1992; Hughes et al, 1993]. Results from two studies using cultured human trabecular bone cells or cryostat sections of either adult or foetal human bone indicated that α_4, α_5, α_v, β_1 and β_5 integrin subunits are expressed by osteoblasts [Hughes et al, 1993; Grzesik and Robey, 1994]. In another study using cryostat sections of human bone, osteoclastoma tissue or cultured human bone cells a different pattern of integrin expression was observed with α_1, α_3, β_1 being strongly expressed by osteoblasts and weak α_2 and α_v expression [Clover et al, 1992]. A fourth study which concentrated on cultured human bone cells demonstrated high levels of α_1, α_3, α_5 and $\alpha_v\beta_5$, low levels of α_2, α_v and β_1 and little or no expression of α_4 and α_6 subunits [Saito et al, 1994]. In an attempt to qualify discrepancies in the literature we have studied the pattern of integrin expression by cultured osteoblast-like cells derived from explants of normal human bone that express the characteristics of cells belonging to the osteoblastic lineage [Beresford et al, 1984; Ashton et al, 1985; Auf'mkolk et al, 1985; Beresford et al, 1986; Koshihara et al; 1987; Marie et al, 1989]. The significance of receptors expressed in terms of binding to specific matrix proteins was also examined using a panel of function-blocking monoclonal antibodies against a range of integrin receptors.

6.1 Adhesion of Bone Cells to Purified ECM Glycoproteins

The interaction of differentiated human bone cell populations to different ECM proteins was investigated. Studies were designed to determine the ability of bone cells to bind to a range of purified ECM proteins using primary cultures of normal human bone cells (NHBC) derived from explants of trabecular bone as described in the methods. NHBC demonstrated adhesion to collagen types I, IV, V, fibronectin, laminin, and vitronectin (Figure 55) under serum free conditions. Cell attachment was significantly greater to fibronectin when compared to all of the other ECM proteins used ($p \leq 0.05$, Fisher PLSD). NHBC exhibited equivalent binding to collagen types I, IV and vitronectin all of which in turn demonstrated a significantly greater capacity to support the adhesion of NHBC than either laminin or collagen type V ($p \leq 0.05$, Fisher PLSD). Cultured NHBC did not bind to collagen type III under the conditions described.

6.2 Integrin Expression on Cultured Bone Cells

Given the well documented role of various integrin family members as receptors for many ECM proteins, subsequent studies were performed to examine the expression of these adhesion molecules by means of immunofluorescence flow cytometric analysis using a panel of mabs (Table 3) reactive to different $\alpha\beta$ heterodimers of the β_1, β_3 and β_5 integrin families. Single cell suspensions of primary NHBC obtained by trypsin digestion, were shown to constitutively express a broad range of integrin molecules including; $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, β_1, β_3, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Figure 56). The majority of the different integrin molecules were found to be highly expressed (21.1-99.6% mean fluorescence) on the total NHBC population (Table 11). No cell surface expression of $\alpha_4\beta_1$ or $\alpha_6\beta_1$ could be detected on NHBC. Members of the integrin family known to mediate the adhesion of various cell types to collagen ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$), fibronectin ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$) and laminin ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$) were then assessed for their role in the interaction of bone cells to different ECM proteins.

Table 11. Integrin expression on NHBC. Indirect immunofluorescence flowcytometric analysis was performed on single cell suspensions of primary NHBC cultures. The bone cells were reacted with monoclonal antibodies specific to a wide range of integrin molecules (see Table 1). The data represent the mean values ± SEM (n=3 different NHBC cultures) based on the corresponding isotype matched control antibody.

Antigen	Percent Fluorescence	Mean Fluorescence
3D3	0.3 ± 0.03	3.3 ± 0.6
1A6.11	0.8 ± 0.09	6.1 ± 1.2
$\alpha_1\beta_1$	55.7± 13.9	4.9 ± 2.2
$\alpha_2\beta_1$	65.4 ± 10.6	28.0 ± 3.7
$\alpha_3\beta_1$	94.5 ± 1.4	23.1 ± 3.3
$\alpha_4\beta_1$	0.2 ± 0.1	4.6 ± 1.1
$\alpha_5\beta_1$	95.0 ± 2.3	21.3 ± 3.9
$\alpha_6\beta_1$	1.2 ± 0.1	7.3 ± 2.0
β_1	99.6 ± 0.3	36.6 ± 10.4
β_3	21.1 ± 1.1	7.5 ± 3.5
$\alpha_v\beta_3$	64.9 ± 13.6	10.4 ± 4.3
$\alpha_v\beta_5$	54.0 ± 10.3	6.6 ± 4.4

Figure 55. Human bone cell interactions with extracellular matrix proteins. The ability of NHBC isolated from primary cultures to bind to collagen types I, III, IV, V, fibronectin, laminin, and vitronectin was determined. Bone cells were labelled with ^{51}Cr, and plated in 48-well plates pre-coated with purified matrix proteins in serum-deprived conditions as described in the methods. The data represent the mean values (± SE) from three different experiments expressed as a percentage of the input pouplation (10^5 cells).

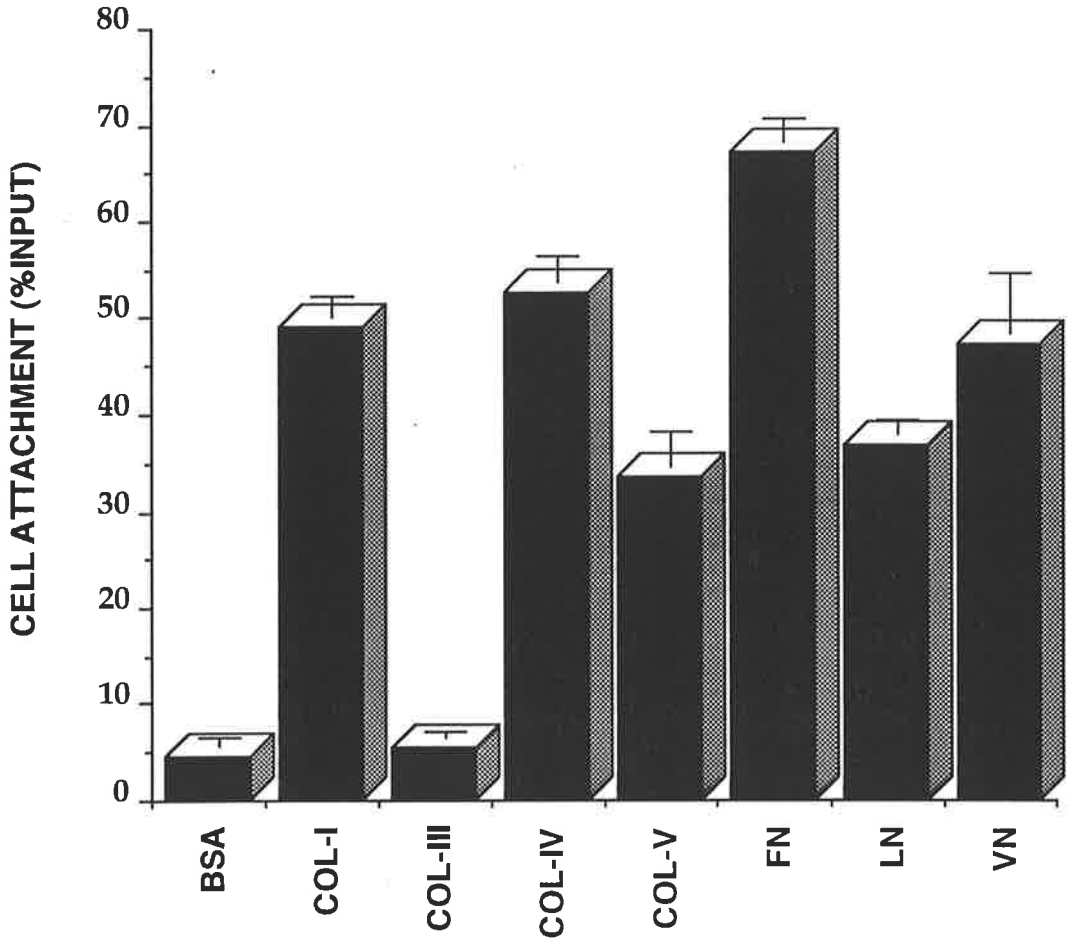
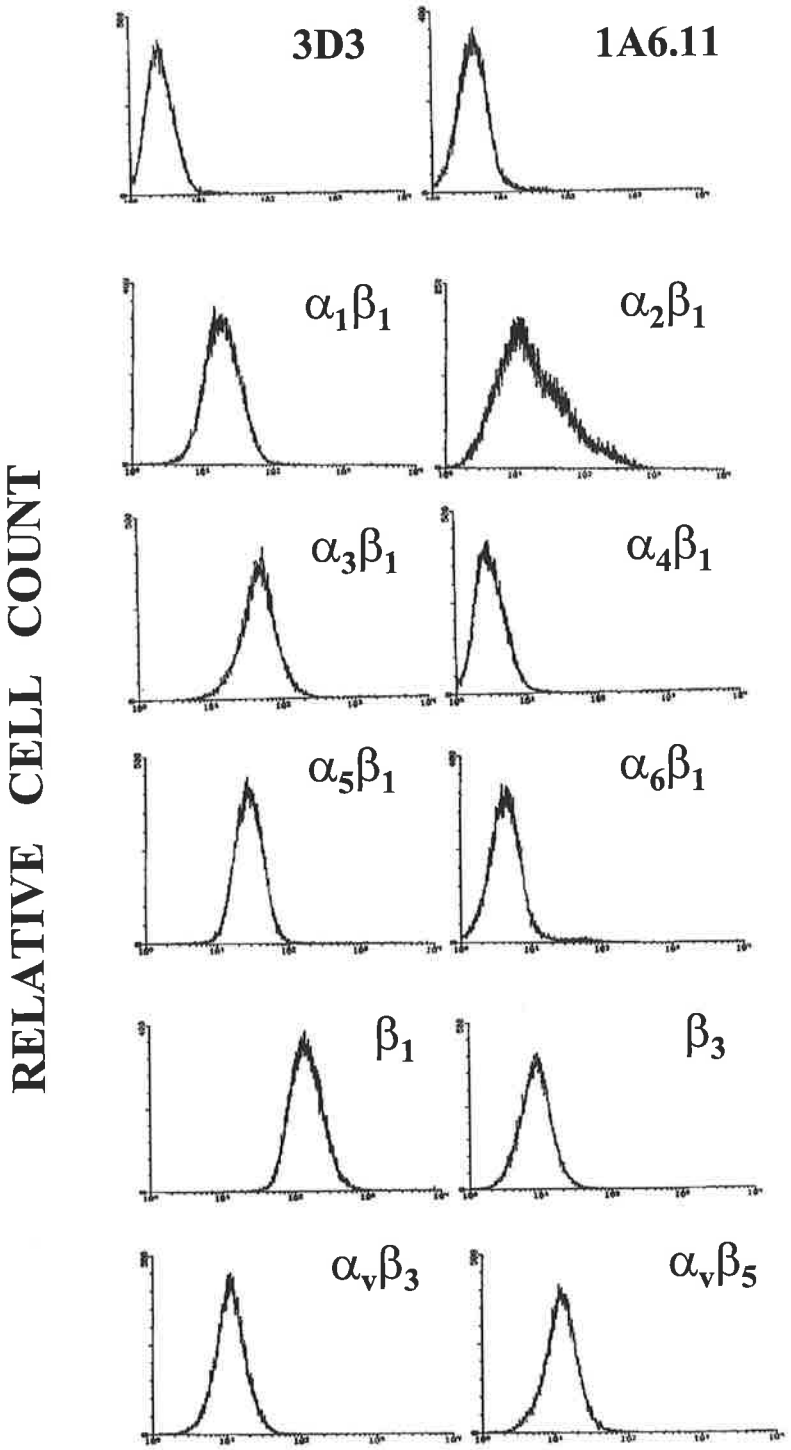

CELL ATTACHMENT (%INPUT)
80
70
60
50
40
30
20
10
0
BSA
COL-I
COL-III
COL-IV
COL-V
FN
LN
VN

Figure 56. Integrin expression ($\alpha_1\beta_1$-$\alpha_6\beta_1$, β_1, β_3, $\alpha_V\beta_3$, and $\alpha_V\beta_5$) on primary normal human bone cells. Representative frequency histograms are shown depicting the level of fluorescence intensity for each integrin molecule in comparison to the negative control antibodies 3D3 ($IgG_{1)}$ and 1A6.11 (IgG_{2b}) as assessed by immunofluorescence flow cytometric analysis. Each histogram is based on 2×10^4 events collected as listmode data. The mean fluorescence levels are shown in Table 11.


3D3
1A6.11
$\alpha_1\beta_1$
$\alpha_2\beta_1$
$\alpha_3\beta_1$
$\alpha_4\beta_1$
$\alpha_5\beta_1$
$\alpha_6\beta_1$
β_1
β_3
$\alpha_v\beta_3$
$\alpha_v\beta_5$
RELATIVE CELL COUNT
FLUORESCENCE INTENSITY FITC

6.3 Bone cell adhesion to Collagen, Laminin and Fibronectin is Mediated by β_1 Integrins

Blocking studies were designed to determine the function of different integrin heterodimers on NHBC. ^{51}Cr-labelled single cell suspensions of NHBC were first incubated with the integrin specific function-blocking mab P4C10 (Table 3) for 1 hour at 4^{o}C as described in the methods. Control wells were plated with ^{51}Cr-NHBC incubated with the negative control antibody, 3D3. The cells were then seeded onto plates coated with either collagen types I, IV, V, laminin, fibronectin, vitronectin or BSA. The binding of NHBC to collagen and laminin was significantly reduced ($p \leq 0.05$, t-test) in the presence of the P4C10 when compared to the control wells containing BSA alone (Figure 57). However, the P4C10 mab did not effect the binding of NHBC to vitronectin and only produced a partial inhibition (approximately 20%) to fibronectin. Furthermore, the addition of both the $\alpha_V\beta_3$ specific function-blocking mab 23C6 and P4C10 mab did not significantly enhance the magnitude of inhibition resulting from the β_1 specific antibody alone indicating that the binding of NHBC to collagen, laminin and fibronectin was mediated in part through members of the β_1 integrin family (Figure 57).

In subsequent experiments the role of different β_1 integrins in the adhesion of NHBC to collagen was determined, using a panel of functional-blocking mabs (P4C10, HB245, AC11, P1B5 and 23C6) specific to the collagen receptors, β_1 $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_V\beta_3$ respectively (Table 11). Adhesion assays were performed as described in the methods following the incubation of the cells with saturating levels of different combinations of the integrin specific mabs. The inclusion of different mabs in the blocking studies was dependent on the expression of the corresponding integrin molecules previously detected on NHBC. There was no inhibition in the attachment of NHBC to collagen when the mabs HB245, AC11 and P1B5 were used individually (data not shown). However, a combination of the three antibodies (HB245, AC11 and P1B5) was partially effective in inhibiting the adhesion of NHBC to collagen types I, IV and V (Figure 58). The further addition of P4C10 to the combination, HB245, AC11, P1B5 resulted in an enhanced inhibition of cell

Figure 57. The mechanisms of integrin mediated adhesion of NHBC to matrix proteins. ^{51}Cr-labelled suspensions of NHBC were incubated with either the β_1 specific antibody P4C10 and/or the $\alpha_V\beta_3$ specific antibody 23C6. The cells were then seeded onto plates coated with either collagen type I, IV, V, laminin, fibronectin, vitronectin or BSA as described in the methods. The results were expressed as a percentage of the radioactivity detected in the control wells containing the negative control antibody 3D3. The level of significance (*: $p \leq 0.05$; t-test) for the percent cell attachment in all conditions was compared with the corresponding BSA control wells (n=3 different bone samples).

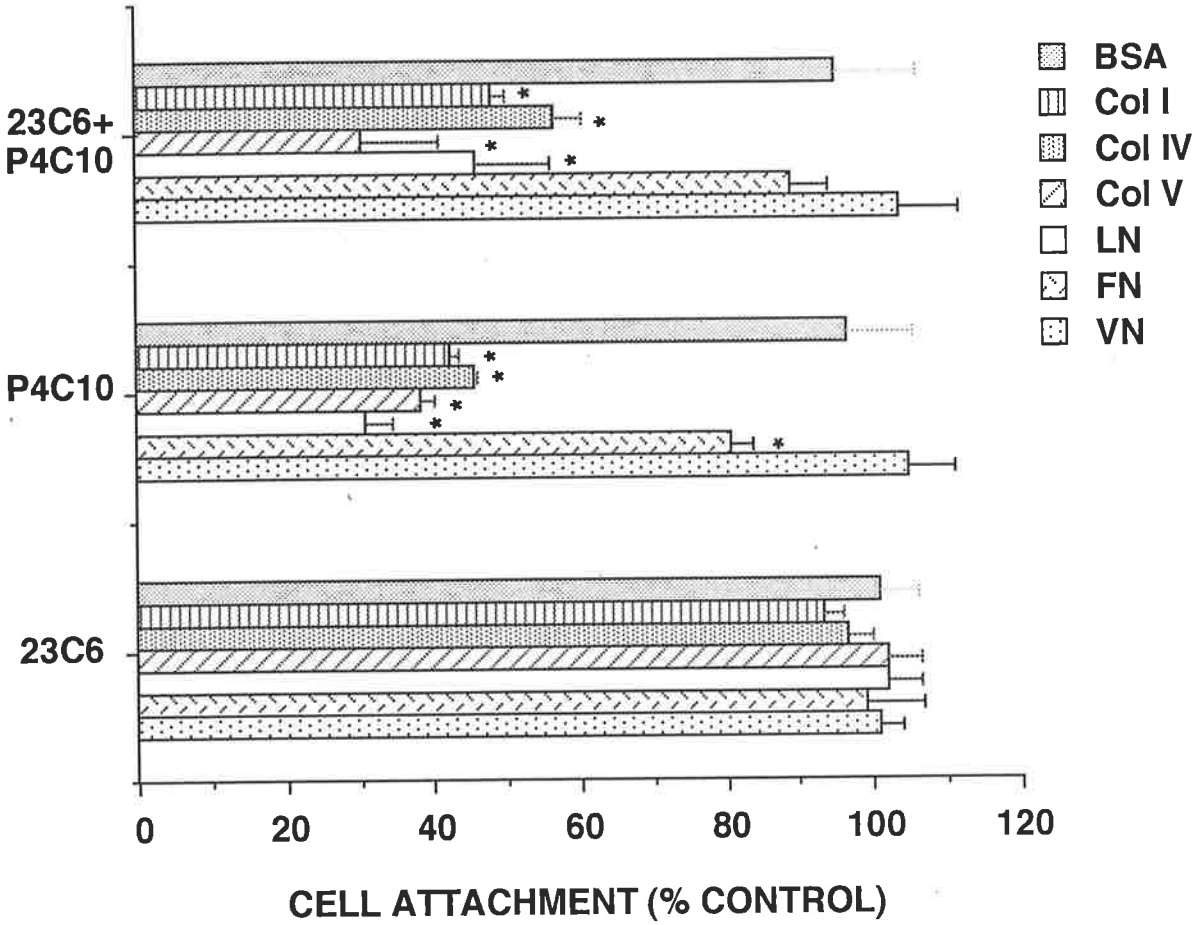
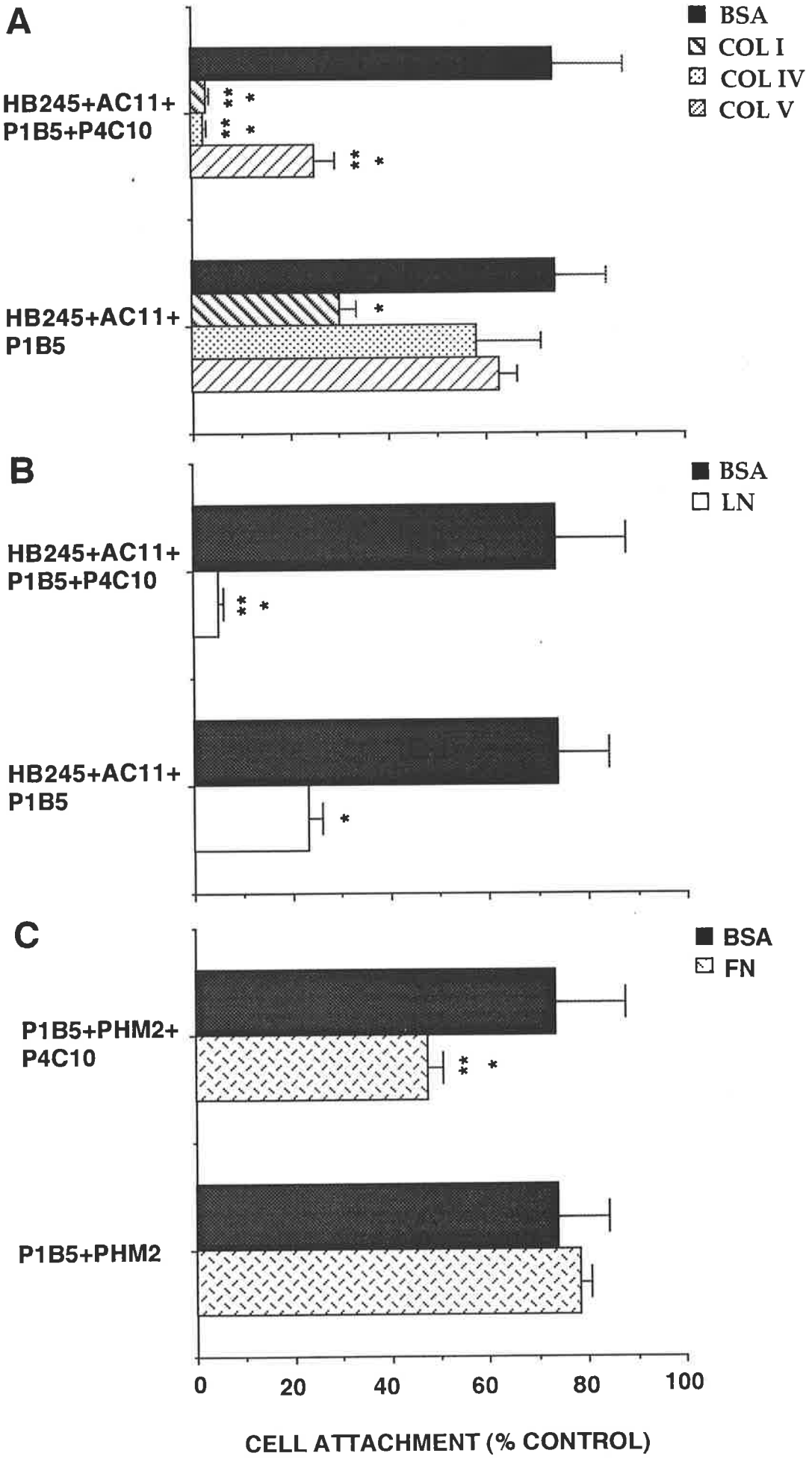

23C6+
P4C10
P4C10
23C6
BSA
Col I
Col IV
Col V
LN
FN
VN
0
20
40
60
80
100
120
CELL ATTACHMENT (% CONTROL)

Figure 58. Integrin β_1 mediated adhesion of NHBC to collagen (A), laminin (B) and fibronectin (C). ^{51}Cr-labelled suspensions of NHBC were incubated with combinations of function-blocking antibodies (HB245, AC11, P1B5, PHM2 and P4C10 specific for $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ $\alpha_5\beta_1$ and β_1 respectively), and were subsequently seeded onto plates coated with either collagen type I, IV, V, laminin, fibronectin, or BSA as described in the methods. The results were expressed as a percentage of the radioactivity detected in the control wells containing the negative control antibody 3D3 (n=3 different bone samples). The level of significance (*: $p \leq 0.05$, t-test) for the percent cell attachment in all conditions was compared with the corresponding BSA control wells. In addition, the level of significance (**: $p \leq 0.05$, t-test) was calculated for the percent cell attachment between the different antibody combinations with or without P4C10.


A
HB245+AC11+
P1B5+P4C10
HB245+AC11+
P1B5
BSA
COL I
COL IV
COL V
B
HB245+AC11+
P1B5+P4C10
HB245+AC11+
P1B5
BSA
LN
C
P1B5+PHM2+
P4C10
P1B5+PHM2
BSA
FN
0
20
40
60
80
100
CELL ATTACHMENT (% CONTROL)

adhesion to each of the three collagen types which was significantly different from the three antibody combination ($p \leq 0.05$, t-test).

A panel of functional mabs (P4C10, HB245, AC11, P1B5, GOH3 and 23C6) specific to the laminin receptors, β_1 $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ respectively, were used to assess the role of β_1 integrins in mediating bone cell adhesion to laminin. There was an inhibition in the binding of NHBC to laminin in the presence of the mabs, HB245, AC11 and P1B5 (Figure 58) which corresponded to that observed with P4C10 alone (Figure 58). As previously observed for the adhesion of NHBC to collagen, a significant decrease in the level of cell attachment to laminin also observed ($p \leq 0.05$, t-test) in the presence of all four antibodies in comparison to the combination of HB245, AC11 and P1B5 (Figure 58).

Finally, blocking studies were conducted to investigate the role of different β_1 integrin molecules in the adhesion of NHBC to fibronectin using the functional mabs; P1B5, PHM2 and P4C10 which identify the fibronectin receptors, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and β_1 respectively. The simultaneous addition of the antibodies P1B5, PHM2 and P4C10 inhibited the binding of NHBC to fibronectin by approximately 50% (Figure 58) in comparison to the 20% inhibition observed in the presence of both P1B5 and PHM2 (Figure 58) or P4C10 alone (Figure 57). Thus the adherence of NHBC to fibronectin appears to be only partly mediated by β_1 integrins.

NHBC appeared to express a non-activated form of the integrin $\alpha_v\beta_3$. The integrin $\alpha_v\beta_3$ is known to mediate binding to a wide range of ECM ligands including collagen, fibronectin, laminin and vitronectin. Blocking studies were performed as described above to determine the function of this integrin heterodimer on NHBC. ^{51}Cr-labelled suspensions of NHBC were first incubated with the $\alpha_v\beta_3$ specific function-blocking antibody 23C6. Control wells were plated with ^{51}Cr-NHBC incubated with the negative control antibody, 3D3. The cells were then seeded onto plates coated with either collagen

types I, IV, V, laminin, fibronectin, vitronectin or BSA. The adhesion of NHBC to each of these ECM proteins was not inhibited by the 23C6 antibody (Figure 57).

6.4 DISCUSSION

The present study has shown that human bone cells are able to bind to different purified proteins present in the extracellular matrix of bone and that these interactions were in part mediated by integrins expressed on the cell surface. Osteoblasts demonstrated a differential binding to collagen types I, IV, V, fibronectin, laminin, and vitronectin and showed preferential adhesion to fibronectin. A previous study has shown that cultured NHBC bind with higher affinity to fibronectin compared to their binding to laminin and collagen type IV [Saito et al, 1994]. Collagen type III is the predominant collagen found in the bone marrow microenvironment but not in the bone-matrix. In contrast to other collagen isotypes examined, collagen type III did not support the adhesion of osteoblasts. We and others have previously shown that cultured NHBC synthesise collagen types I, III, IV and V. The cartilage specific collagen, collagen type II and the basal laminae component, laminin could not be detected in cultures of primary NHBC (data not shown).

To assess the possible mechanisms responsible for the adhesion of bone cells to ECM components we first examined the expression of a wide range of integrin molecules on the cell surface of NHBC. The integrin family of cell adhesion molecules are a series of heterodimeric cell-surface glycoproteins which recognise a range of cell surface and ECM associated ligands including collagen, laminin, fibronectin and vitronectin [Hynes, 1987; 1992]. Integrins have been shown to be involved in osteoblast and osteoclast function during bone deposition and remodelling, respectively [Schiro et al, 1991; Clover et al, 1992; Helfrich et al, 1992; Saito et al, 1994; Grzesik and Robey, 1994; Gronowicz and De Rome, 1994; Helfrich et al, 1996; Meazzini et al, 1998]. However, the exact role of individual integrin molecules in osteoblast function during bone development remains unclear. Our data is in accord with previous studies which show the expression of $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on the surface of osteoblasts both in vivo and in vitro

[Clover et al, 1992; Hughes et al, 1993; Grzesik and Robey, 1994; Saito et al, 1994]. In the present study, integrin expression was found to be uniform throughout the whole bone cell population and did not appear to be restricted to minor subpopulations. Furthermore, NHBC did not express detectable levels of the laminin receptor $\alpha6\beta1$ which is in agreement with previous work [Clover et al, 1992; Saito et al, 1994]. However, there is conflicting data concerning the expression of the integrin $\alpha4\beta1$, on bone cells. In one study [Grzesik and Robey, 1994] high level expression of $\alpha4\beta1$, was detected on osteoblasts in vitro and in vivo as demonstrated by immunohistological staining. This is in contrast to the findings of the present study and that of others [Clover et al, 1992; Hughes et al, 1993; Saito et al, 1994] where osteoblasts were found to express little or no $\alpha4\beta1$ both in vivo and in vitro. These discrepancies may be due to differences in the specific reagents used. Grzesik and Robey (1994) used a rabbit polyclonal antibody against $\alpha4\beta1$ and thus may be more sensitive in detecting low antigen levels in comparison to the mouse monoclonal antibodies used in the other studies. However, further investigations demonstrated that NHBC lacked detectable expression of $\alpha4\beta1$ by flow cytometric analysis using three other monoclonal antibodies (P4C2, 163H, and PS/2) specific to different epitopes on the $\alpha4\beta1$ heterodimer (data not shown).

Similar studies demonstrated that only a proportion (50%) of osteoblast precursors, that is BM CFU-F expressed the integrin $\alpha4\beta1$ using dual-colour FACS analysis (Chapter 4). However, as discussed earlier this differential expression on CFU-F may be due to the presence of contaminating haemopoietic cells and/or platelets bound to CFU-F in vivo. Like NHBC, cultured BM stromal cells derived from culture expanded CFU-F demonstrated no detectable levels of the integrin $\alpha4\beta1$ in vitro (Table 5). The main difference in the integrin expression between BM stromal cells and NHBC was that cultured BM stromal cells and their progenitors were found to constitutively express the laminin receptor $\alpha6\beta1$. The differential expression of the integrin $\alpha6\beta1$ between BM stromal cells and differentiated bone cells may explain the poor ability of NHBC to bind to

laminin and may reflect differences in the matrix requirement of the two cell populations for normal development and function.

Having defined the expression of a variety of integrins on the cell surface of NHBC we then set out to investigate whether these molecules were responsible for mediating the binding of NHBC to a variety of extracellular matrix proteins present in bone. Many integrins react with the unique tripeptide (arginine-glycine-aspartate) RGD sequence found on fibronectin, vitronectin and collagen [Pierschbacher and Ruoslahti, 1984; Dedhar et al, 1987; Hynes, 1987; 1992; Ruoslahti, 1991; Grzesik and Robey, 1994]. Synthetic peptides which recognise the RGD sequence have been shown to inhibit the binding of chick bone cells, rat osteoblasts and human osteosarcoma cells to collagen type I, fibronectin and vitronectin in vitro [Hayman et al, 1985; Dedhar et al, 1987; Puleo and Bizios, 1991; 1992; Gronowicz and De Rome, 1994; Aarden et al, 1996]. One study demonstrated that synthetic RGD specific peptides could completely inhibit the binding of cultured human osteoblasts to vitronectin but only caused a partial inhibition to fibronectin and collagen type I adhesion [Grzesik and Robey, 1994]. Our results showed that the function-blocking antibody 23C6 which reacts with the RGD specific integrin $\alpha_V\beta_3$ did not inhibit the binding of NHBC to either vitronectin, fibronectin, laminin or collagen. This data suggests that human osteoblast-like cells express a non-functional form of $\alpha_V\beta_3$. Work by Horton et al (1991) showed an inhibition of bone resorption and cell spreading by osteoclasts in the presence of the 23C6 antibody or RGD-specific peptides. Furthermore, one study by Chang et al (1995) demonstrated that the binding of human unbilical vein endothelial cells to fibrin and fibrinogen was inhibited by >60% in the presence of either RGD-specific peptides or the $\alpha_V\beta_3$ specific antibody 23C6. The present work is in agreement with recent studies in which the adhesion of chick osteocytes and osteoblasts to vitronectin, fibronectin, laminin and collagen was not affected by the presence of the RGD blocking antibody 23C6 [Aarden et al, 1996]. The stimulation of bone cells by soluble factors such as cytokines may be required for $\alpha_V\beta_3$ activation to occur. This has been observed in other cell systems for example, the adhesion of primitive

haematopoietic progenitor cells to fibronectin can be enhanced by activation of the integrins $\alpha4\beta1$ and $\alpha5\beta1$ following incubation with various cytokines including; interleukin-3, granulocyte-macrophage colony stimulating factor and stem cell factor [Levesque et al, 1995]. Since NHBC adhere to vitronectin via the RGD sequence [Grzesik and Robey, 1994] other RGD specific integrins such as $\alpha v\beta5$ shown in this study to be present on human osteoblast-like cells may contribute to osteoblast binding to vitronectin. Demonstrating a role for $\alpha v\beta5$ as an osteoblast vitronectin receptor would require the development of a functional-blocking antibody to human $\alpha v\beta5$.

Unlike vitronectin, the adhesion of human osteoblasts to collagen and fibronectin were found to be partially inhibited (20-25%) by RGD specific peptides and only in the presence of soluble heparin [Grzesik and Robey, 1994]. Cultured human osteoblasts have been shown to bind to both the cell binding domain (which contains the RGD sequence) and to a lesser extent to the heparin binding domain of fibronectin [Underwood et al, 1992; Dalton et al, 1995]. These observations confirm that bone cells can bind to multiple sites on the fibronectin molecule. The present study showed that NHBC adhesion to fibronectin was inhibited by greater than 50% in the presence of the function-blocking antibodies P1B5, PHM2 and P4C10. This work suggests that NHBC utilise in part the RGD specific $\beta1$ integrins $\alpha3\beta1$ and $\alpha5\beta1$ to mediate fibronectin binding. The observation by Grzesik and Robey (1994) that $\alpha4\beta1$ is expressed on NHBC implicates this integrin as another possible mechanism for the adhesion of osteoblasts to fibronectin. The integrin $\alpha4\beta1$ is known to bind to Glu-Ile-Leu-Asp-Val (EILDV) in the variable region of the fibronectin heparin binding domain, in contrast to the RGD specific integrins $\alpha3\beta1$ and $\alpha5\beta1$ [Guan and Hynes, 1990; Wayner et al, 1990; Elices et al, 1990; Mould and Humphries, 1991; Hynes, 1992; Dalton et al, 1995].

The adhesion of NHBC to collagen and laminin in the present study was found to be completely dependent on $\beta1$ integrins. In particular, inhibition of NHBC attachment to laminin was only achieved by blocking the function of the $\beta1$ integrins $\alpha1\beta1$, $\alpha2\beta1$ and

$\alpha 3\beta 1$. It appears therefore that NHBC express multiple laminin receptors analogous to that observed with fibronectin. The integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are known to recognise the cross region of the laminin molecule while $\alpha 3\beta 1$ recognises a different site on the long arm of laminin [Hynes, 1992]. Similarly, NHBC utilise multiple receptors to bind to collagen; the integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$ which recognise the two different sequences Asp-Gly-Glu-Ala (DGEA) and RGD on the collagen type I molecule respectively [Elices et al, 1990; Staatz et al, 1991]. This redundancy in integrin receptor-ligand interactions and multiple binding sites may have evolved to facilitate a multitude of cellular functions. Continuing studies into the functions of integrins and cell adhesion molecules belonging to other superfamilies may help identify the precise mechanisms for the regulation of osteoclast-osteoblast-matrix interactions involved in bone development and remodelling.

Understanding bone cell-matrix interactions may also lead to the development of appropriate strategies for the successful grafting of biomaterials seeded with autologous bone cells. Evidence exists that the type of biomaterial used or the coating of biomaterials with different ECM proteins has significant effects on the ability of the implanted BM stromal cells to differentiate into functional bone cells in vivo [Dennis and Caplan, 1993; Krebsbach et al, 1997]. In accord with these observations, Sinha and Taun (1996) have shown that NHBC exhibit a differential integrin expression pattern when cultured onto plastic coated with or without collagen, fibronectin or laminin. In addition, differences in the expression of various integrin subunits occurred when bone cells were seeded onto different orthopaedic alloys. Therefore the coating of implant materials with appropriate combinations of purified ECM proteins may enhance the adhesion, proliferation and differentiation of stromal cells in order to facilitate their use in the regeneration and repair of osteogenic defects in vivo.

CHAPTER SEVEN: IDENTIFICATION OF DISCRETE DEVELOPMENTAL STAGES IN OSTEOGENIC DIFFERENTIATION

7.0 INTRODUCTION

Bone is the main constituent of the human adult skeleton and provides a supportive and structural function, due to its ability to with-stand mechanical stress. The hardness of bone makes it a difficult tissue to process, particularly in regard to the isolation of viable cells representative of different stages of osteogenic development. Therefore, early studies on bone cell development have based their findings largely on histological examination of fixed sections of decalcified bone tissue [reviewed in Marks and Hermey, 1996]. These studies have provided an insight into the morphological features and basic functions of the different types of cells associated with the growth and remodelling of osteogenic tissue. To date, histological techniques have been unable to characterise the factors that control these processes including the differentiation of osteogenic cells.

The development of cell lines derived from osteogenic tissues and tumours has significantly enhanced the understanding of bone cell biology. Various culture techniques have been developed to investigate the properties of the cellular constituents of normal rodent bone tissue, through the use of systematic enzyme digestion of bone [Peck et al, 1964; Wong and Cohn, 1974] or by cell migration from the outgrowth of bone explants [Ecarot-Charrier et al, 1983; Lomri et al 1988]. Similar culture systems have been established for investigating the biological properties of normal human bone cells in vitro (NHBC) [Mills et al, 1979; Beresford et al, 1984; Wergedal and Baylink, 1984; Ashton et al, 1985; Robey and Termine; 1985; Auf'mkolk et al, 1985]. Human osteoblast-like cells can be readily cultured from explants of trabecular bone and reproducibly express many of the characteristics of cells belonging to the osteoblastic lineage, including the ability to form a mineralized bone-matrix (hydroxyapatite), the presence of high levels of alkaline phosphatase (AP) activity, the synthesis of osteocalcin (OCN) and the expression of parathyroid hormone receptors (PTH-R) [Beresford et al, 1984; Ashton et al, 1985;

Auf'mkolk et al, 1985; Beresford et al, 1986; Koshihara et al; 1987; Marie et al, 1989]. Studies using cultured bone cells derived from rat and chick bone have identified different cell types within these cultures characteristic of osteoblast-like cells, osteocyte-like cells and fibroblastic cells [Nijweide et al, 1988, Aubin et al, 1993]. Due to the lack of developmental and lineage-specific markers it is unclear in these in vitro culture systems what proportion of the cells are representative of osteoprogenitor cells or indeed other stages of bone cell development such as pre-osteoblasts, osteoblasts and osteocytes, or even other stromal cell lineages.

Several studies have examined the expression of alkaline phosphatase (AP) as a marker of osteogenic differentiation in cultures comprised of heterogeneous cell populations. Flow cytometric analysis has been previously used to quantitate the number of AP expressing stromal cells (following fixation) derived from rat bone marrow [Kamalia et al, 1992a]. Studies by Aubin and colleagues [Turksen and Aubin, 1991; Herbertson and Aubin, 1997] have utilised antibodies reactive to AP in order to isolate living cells with osteogenic potential, from cultures of adult rat bone marrow and foetal rat calvaria. The majority of cells with the ability to form mineralized bone nodules in vitro were found to reside in the AP positive cell fraction, while a minor population of AP negative cells displayed osteogenic potential only when stimulated with dexamethasone [Turksen and Aubin, 1991; Herbertson and Aubin, 1997]. Collectively, these studies imply that there exists a bone cell hierarchy of cellular differentiation where the acquisition of AP expression is associated with osteoblastic differentiation.

In human studies, AP activity was previously found to be differentially expressed in clones of osteoblast-like cells derived from explants of trabecular bone [Manduca et al, 1993]. To better characterise the NHBC culture system, various cellular components that comprise this heterogeneous population of bone cells were identified and isolated, based on the expression of two independent cell surface markers. Firstly, the bone/liver/kidney isoform of AP, was chosen as a general marker of differentiated osteoblasts. Although AP is not

specific to the osteogenic cell lineage, it is an essential feature of functional osteoblasts. In contrast to AP, the STRO-1 antigen was chosen as an alternative marker, because of its unique ability to identify early bone marrow stromal precursor cells with osteogenic potential (Chapter 4 and 5). Therefore the STRO-1 antigen was used as a marker representative of a pre-osteoprogenitor-like phenotype. In previous studies, primary confluent cultures of BM stromal cells initiated with STRO-1^+ BMMNC, demonstrated a loss in the expression of the antigen identified by STRO-1 (Table 5). This decrease of expression in vitro suggests that the STRO-1 mab identifies a differentiation antigen which is eventually lost as the stromal progenitors proliferate and mature. Similarly, alkaline phosphatase activity was found to be differentially expressed in BM stromal cell cultures, where retention of AP activity may reflect a cells commitment to the osteogenic cell lineage (Herbertson and Aubin, 1997).

Based on these previous observations with BM stromal cells, a series of experiments were therefore performed to examine the ability of mabs to AP and STRO-1 to fractionate different NHBC subpopulations in vitro. Dual-colour fluorescence activated cell sorting (FACS) was employed in order to subdivide the NHBC population according to the cell surface expression of STRO-1 and AP. Phenotypically distinct subpopulations were identified and each subjected to an analysis of their osteogenic commitment based both on their expression of bone-associated matrix proteins and the osteogenic transcription factor CBFA1 and by the ability of each subpopulation to form a mineralized bone matrix in vitro.

7.1 Cell Surface Expression of STRO-1 and Alkaline Phosphatase on Primary NHBC

Dual-colour fluorescence activated cell sorting was employed to determine the pattern of expression of the stromal precursor cell marker STRO-1 and the osteoblastic marker alkaline phosphatase (AP) on enzymatically treated single cell suspensions of five-six week old primary NHBC cultured in standard growth conditions in the presence of ASC-

2P. The NHBC population demonstrated a heterogeneous but highly reproducible pattern of expression for both the STRO-1 and AP antigens (Figure 59). The majority of the NHBC (mean 62.2% ±2.7SEM, n=12 different independent bone samples) demonstrated no detectable levels of STRO-1 or AP at the cell surface (STRO-1$^-$/AP$^-$). A high proportion of the remaining NHBC population (mean 30.8% ±2.5 SEM, n=12) expressed the osteoblastic marker AP exclusively (STRO-1$^-$/AP$^+$), while only a minor population (mean 3.6% ±0.6 SEM, n=12) of bone cells expressed the STRO-1 antigen alone (STRO-1$^+$/AP$^-$). In addition, a small proportion of cells (mean 3.4% ±0.7 SEM, n=12) were also found to co-express the AP and STRO-1 antigens (STRO-1$^+$/AP$^+$). Following FACS analysis of the different STRO-1/AP subgroups each cell population was re-sorted and analysed to ensure a purity of >99% (Figure 60) for all subsequent experiments.

7.2 In Vitro Mineralization Potential of The Different STRO-1/AP Subpopulations

The ability of the different STRO-1/AP sorted subpopulations to form a mineralized bone matrix was determined by culturing the cells in osteogenic conditions in the presence of ASC-2P, DEX and PO_{4i} as previously described (Chapter 5). A mineralized matrix formed in all the STRO-1/AP NHBC subsets and was identified as hydroxyapatite by X-ray diffraction and EDX analysis (Figure 61).

To assess the level of mineralisation, calcium concentrations were determined after solubilizing the mineralized cultures under acidic conditions at weekly intervals over a period of seven weeks. A time dependent increase in the level of hydroxyapatite formed in all the NHBC STRO-1/AP subsets (Figure 62) was observed under osteogenic conditions. There was a significant increase (Fisher PLSD test; $p \leq 0.05$) in the level of mineralization observed for the STRO-1$^-$/AP$^+$ subpopulation between 14 days and 35 days in comparison to the STRO-1$^+$/AP$^-$ and STRO-1$^-$/AP$^-$ NHBC subsets. Similarly, the ability of the STRO-1$^-$/AP$^+$ subset to form a mineralized bone-matrix was observed to be significantly ($p \leq 0.05$) higher between days 14 and 28 but not at day 35 when compared to the STRO-1$^+$/AP$^+$ NHBC subpopulation. A significant increase ($p \leq 0.05$) in the level of

Figure 59. Cell surface expression of the STRO-1 and alkaline phosphatase antigens on NHBC. Dual-color flow cytometric analysis depicting the cell surface expression of the STRO-1 and alkaline phosphatase antigens (B4-78 positive) on single cell suspensions derived from primary cultures of NHBC. The dot plot histogram represents 5×10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% fluorescence obtained with the isotype-matched control antibodies 3D3 (PE) and 1A6.12 (FITC) respectively.

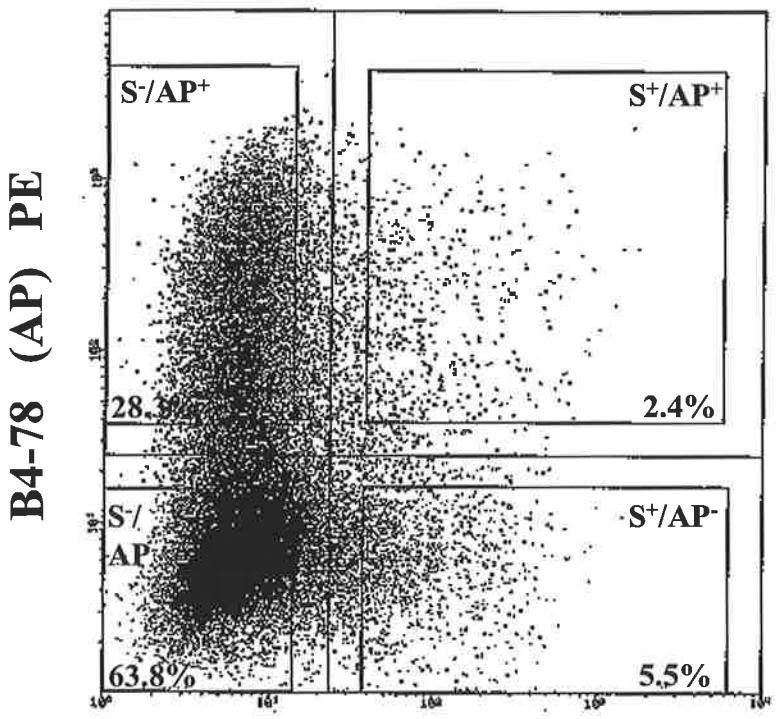

S-/AP+
S+/AP+
2.4%
S-/
AP-
S+/AP-
63.8%
5.5%
B4-78 (AP) PE
STRO-1 FITC

Figure 60. Purification of different STRO-1/AP NHBC populations. Double sorting experiments were conducted on each of the STRO-1/AP NHBC subsets sorted initially from primary cultures to ensure a purity of 99% for each phenotype. (A) STRO-1^-/AP^+; (B) STRO-1^+/AP^+; (C) STRO-1^-/AP^-; STRO-1^+/AP^-. The dot plot histogram represents 2 x 10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% fluorescence obtained with the isotype-matched control antibodies 3D3 (PE) and 1A6.12 (FITC) respectively.

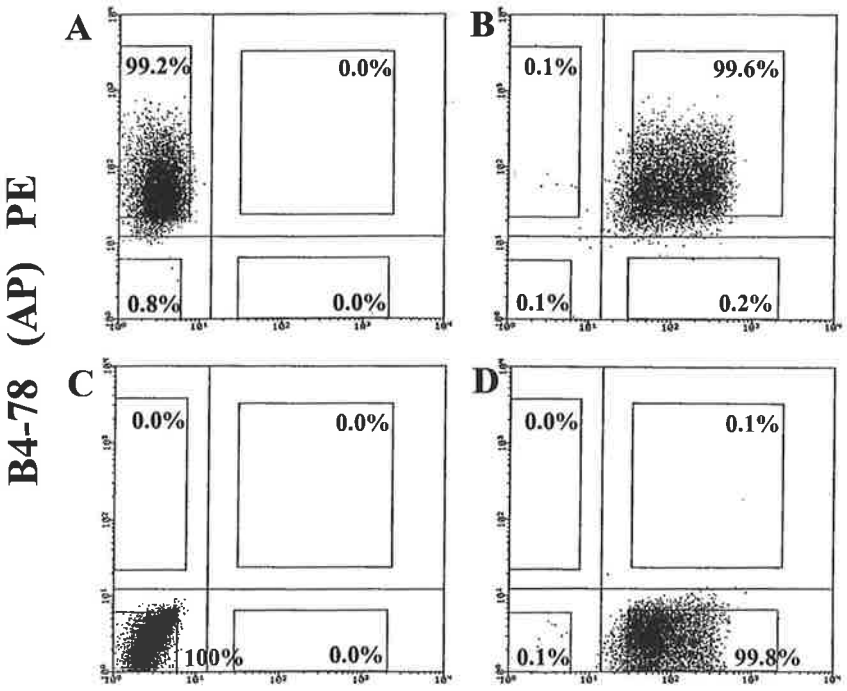
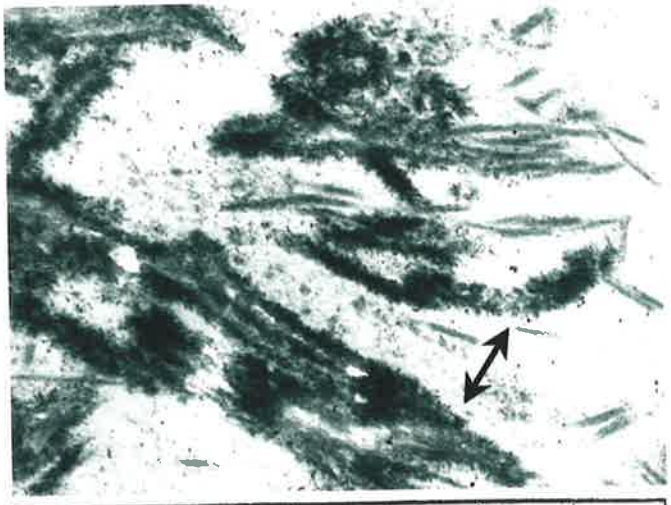
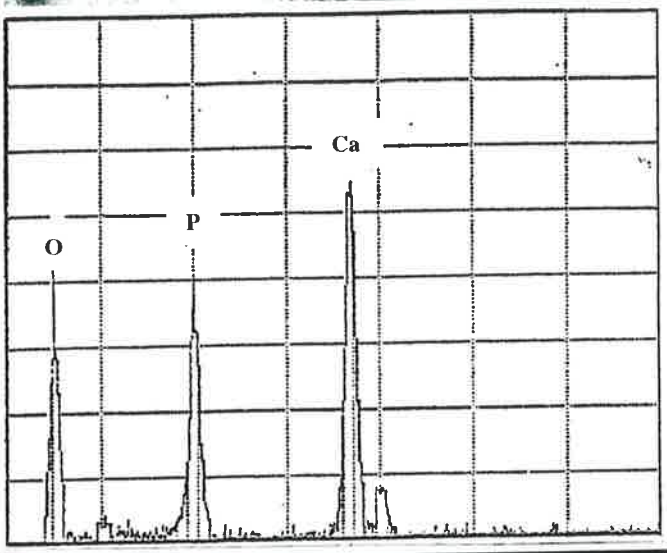

A
99.2%
0.0%
0.8%
0.0%
B
0.1%
99.6%
0.1%
0.2%
C
0.0%
0.0%
100%
0.0%
D
0.0%
0.1%
0.1%
99.8%
B4-78 (AP) PE
STRO-1 FITC

Figure 61. Identification of the mineral formed in NHBC cultures. A representative transmission electronmicrograph dipicting the adherent layer of NHBC cultured for five weeks in the presence of ASC-2P, DEX, and PO_{4i}. The formation of mineral-like material occured throughout the adherent cell layer. Examination of the extracellular matrix demonstrated the presence of deposits of hydroxyapatite-like crystals (double arrow) in association with a network of collagen fibrils (20,000X) (A). EDX analysis of similar mineral deposits demonstrated major peaks for calcium (Ca) and phosphorous (P) with a mean Ca/P ratio consistent with hydroxyapatite (B). The X-ray diffraction pattern of the mineral deposits, in the same cultures was found to be identical to that of the hydroxyapatite standard (C).

A



B



C

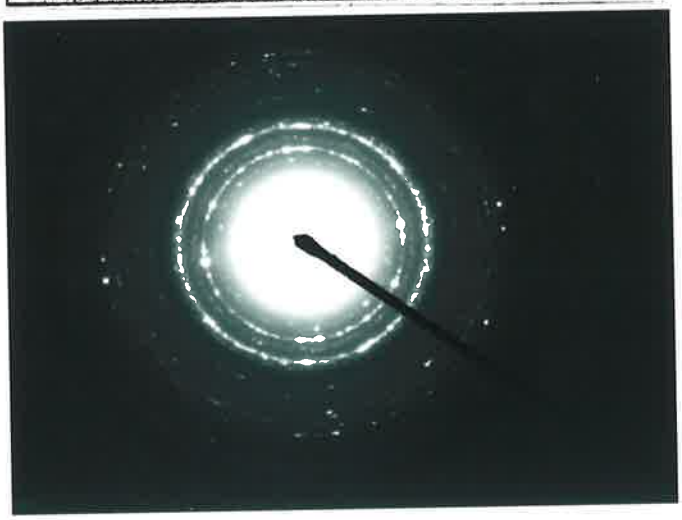
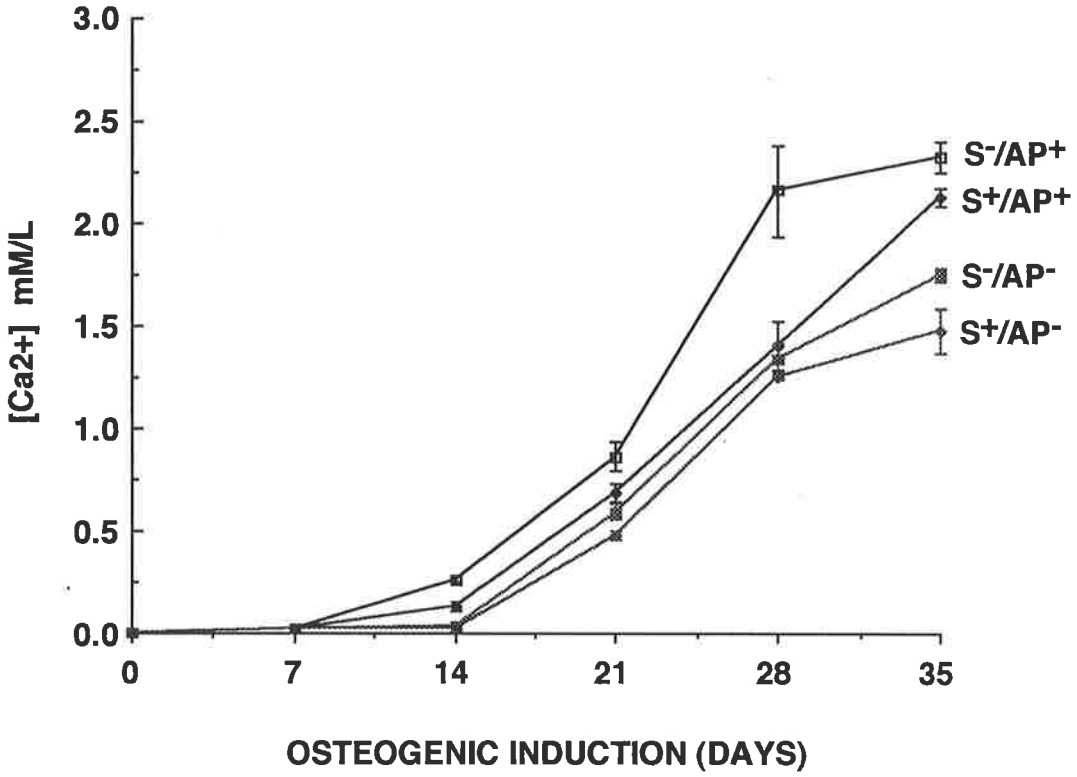


Figure 62. Mineralization rates of the different STRO-1/AP NHBC subpopulations. Calcium levels were determined from the adherent cell layers of triplicate cultures under osteoinductive conditions (ASC-2P, DEX and PO_{4i}). Cultures were sacrificed at weekly intervals for 35 days, representing each of the various normal human bone cells (NHBC) subgroups selected by double FACS analysis based on the expression of STRO-1 (S) and alkaline phosphatase (AP). The release of calcium from the matrix was achieved by treating the cultures under acidic conditions with 0.6N HCL. Samples were then reacted with o-cresol-phthalein-complexon and the colormetric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium for each STRO-1/AP NHBC subset over the time interval (n=3 different bone samples).


3.0
2.5
2.0
1.5
1.0
0.5
0.0
[Ca2+] mM/L
0
7
14
21
28
35
OSTEOGENIC INDUCTION (DAYS)
S-/AP+
S+/AP+
S-/AP-
S+/AP-

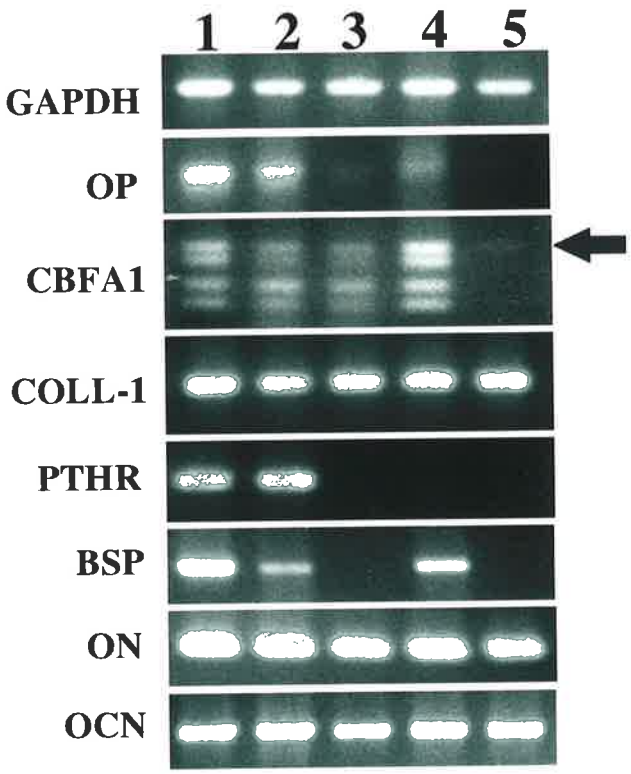
mineralization was also seen for the STRO-1$^+$/AP$^+$ subset at day 14 to day 35 in comparison to the STRO-1$^+$/AP$^-$ subset and at day 35 when compared to the STRO-1$^-$/AP$^-$ subset. However, there was no significant difference in the level of mineralization between the STRO-1$^-$/AP$^-$ and STRO-1$^+$/AP$^-$ subpopulations at all the time points observed, with the exception of day 35 where a higher level of mineralization was detected for the STRO-1$^-$/AP$^-$ subgroup ($p \leq 0.05$) compared to that observed by the STRO-1$^+$/AP$^-$ NHBC subpopulation. Based on these data, the ability of each STRO-1/AP NHBC subpopulation to form a mineralized bone-matrix at the end of the osteogenic induction period can be ranked as follows: STRO-1$^-$/AP$^+$ > STRO-1$^+$/AP$^+$ > STRO-1$^-$/AP$^-$ > STRO-1$^+$/AP$^-$.

7.3 Differential Expression of Bone Related Markers Between the Different STRO-1/ALP Subpopulations

The expression of various genes (CBFA-1, osteocalcin, osteopontin, osteonectin, collagen type I, parathyroid hormone receptor) associated with osteogenic development was determined for all the different NHBC STRO-1/AP subsets. Reverse transcription (RT)-PCR amplification was employed due to the small numbers of cells (2×10^4) available following double sorting where the total cell number for each subgroup was based on the cell yield of the rarest phenotype. The integrity of each sorted subpopulation was confirmed by reproducible and consistent amplification of the "house-keeping" gene GAPDH.

The STRO-1$^-$/AP$^+$ subset expressed all of the osteogenic markers examined and was equivalent to that of the primary NHBC bulk population (Figure 63). Similarly, the STRO-1$^-$/AP$^-$ subgroup was indistinguishable from the former populations, with the exception that this subpopulation lacked the expression of the parathyroid hormone receptor (PTH-R). In contrast, the STRO-1$^+$/AP$^+$ and STRO-1$^+$/AP$^-$ subpopulations consistently lacked expression of osteopontin, bone sialoprotein and PTH-R transcripts. When primers to the bone restricted transcription factor, CBFA1, were used, this resulted in the consistent

Figure 63. Gene expression pattern in the different STRO-1/AP NHBC subpopulations. Representative experiments of the expression of bone-related transcripts as assessed by RT-PCR using total RNA isolated from the various STRO-1/AP sorted NHBC subpopulations: (1) Bulk NHBC; (2) STRO-1^-/AP^+; (3) STRO-1^+/AP^+; (4) STRO-1^-/AP^-; (5) STRO-1^+/AP^-. The various bone-related markers examined included: osteonectin (ON); the transcription factor, CBFA1; collagen type I (COLL-1); osteocalcin (OCN); bone sialoprotein (BSP); osteopontin (OP). Expression of GAPDH was assessed as an indicator of mRNA integrity. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. The STRO-1/AP subpopulations demonstrated a differential expression of the bone-related markers, where only the STRO-1^-/AP^+ subgroup appeared to express all of the transcripts identified in the bulk NHBC population. The arrow indicates the position of a faint upper band for CBFA1 in the STRO-1^+/AP^- subgroup.


1
2
3
4
5
GAPDH
OP
CBFA1
COLL-1
PTHR
BSP
ON
OCN

amplification of four products including the expected 635 bp fragment. DNA sequence analysis of these products indicated that the smaller amplified products represented spliced variants of the full length transcript (as described in Chapter 4). Although all the sorted groups of NHBC expressed CBFA1 transcripts, only the full-length transcript was observed in the STRO-1$^+$/AP$^-$ subgroup although at levels consistently lower that those exhibited by the other subpopulation.

Three-colour FACS analysis of primary NHBC based on their expression of STRO-1/AP and the cell cycling specific antigen Ki-67 [Gerdes et al, 1983; 1894] revealed that the majority of the cells (>80%) in each of the four STRO-1/AP subsets were actively dividing by their expression of the Ki-67 antigen (Figure 64). Therefore the lack of expression of certain bone-related markers in some of the NHBC subgroups was not correlated to the lack of cycling cells in these populations. Studies were also performed to examine any differences in the rate of proliferation between the different STRO-1/AP subgroups following re-culture with equal numbers of cells in each group. The STRO-1$^+$/AP$^-$ population demonstrated a higher proliferation rate (Figure 65) several fold greater compared to that of the other STRO-1/AP subpopulations.

7.4 Differential STRO-1 and ALP expression following re-culture

To assess the level of maintenance of the different STRO-1/AP phenotypes in vitro, primary cultures of NHBC were firstly double sorted into the different STRO-1/AP subpopulations then re-cultured for a period of two weeks. Following re-culture there was no integrity of the characteristic STRO-1/AP expression representative of any of the four subgroups which displayed different patterns of STRO-1/AP expression (Figure 66). The NHBC cells which originally expressed the STRO-1$^+$/AP$^-$ phenotype demonstrated the same STRO-1/AP pattern of expression as observed in the initial primary NHBC cultures (figure 66D). This was in contrast with the other three STRO-1/AP subgroups which demonstrated alternate STRO-1/AP distribution patterns. The majority of the NHBC (mean 73.0% ± 0.1SEM, n=3 different bone samples) sorted on the basis of their STRO-

Figure 64. Cycling status of the different STRO-1/AP NHBC subpopulations. Three-colour immunofluorescence flow cytometric analysis of cultured normal human bone cells (NHBC), derived from explants of trabecular bone, immunoreacted with the mabs STRO-1 coupled to PE and B4-78 (anti-alkaline phosphatase) coupled to TC then fixed in 70% ethanol (-20^{o}C) for 15 min on ice and subsequently immunoreacted with the mab Ki-67 coupled to FITC. The data is presented as fluorescence intensity (log scale) with the expression of STRO-1 and B4-78 (y-axis) versus Ki-67 expression (x-axis). The dot plot histograms represent 2 x 10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% mean fluorescence obtained with the isotype-matched negative control antibodies, 3D3 (TC), 1A6.12 (PE) and IgG_1-FITC. The results demonstrated that the majority (>80%) of cultured NHBC were actively dividing cells which included the majority of the STRO-1^+, STRO-1^-, AP^+ and AP^- cell populations. A minor proportion of cells (<20%) expressing the immunophenotypes, STRO-$1^-/AP^+$ and STRO-$1^-/AP^-$ represented quiescent cell populations (Ki-67 negative).

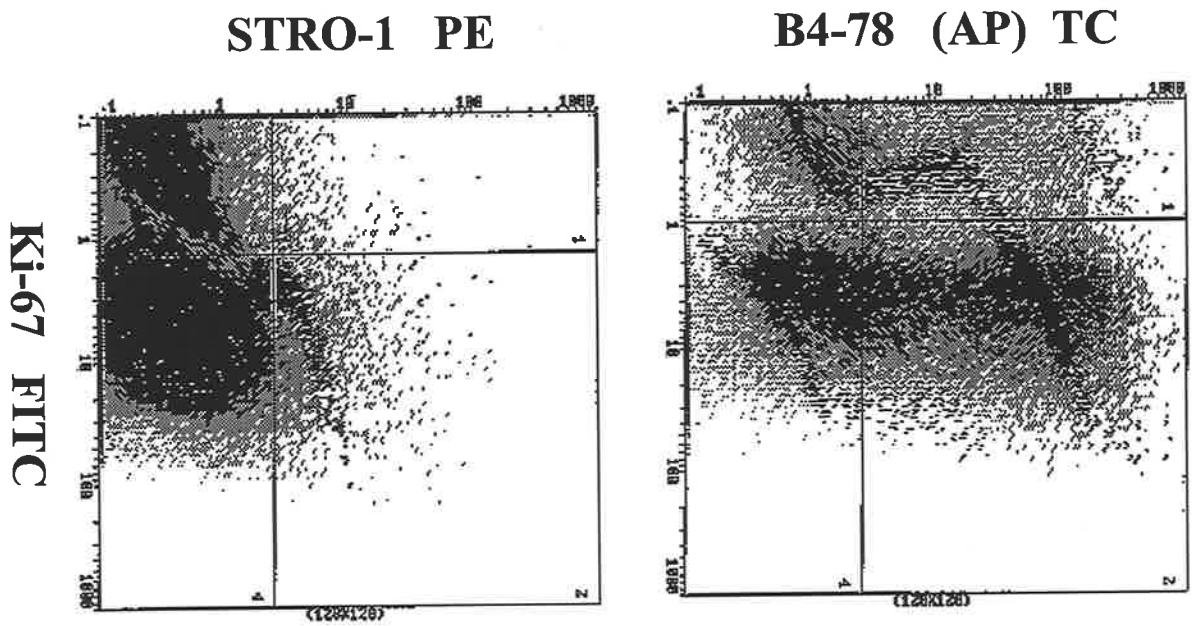

STRO-1 PE
B4-78 (AP) TC
Ki-67 FITC

Figure 65. Proliferation of the different STRO-1/AP NHBC subpoulations. Aliquots of 5 x 10^3 cells per double sorted subfraction (STRO-1^-/AP^+, STRO-1^+/AP^+, STRO-1^-/AP^-, STRO-1^+/AP^-) were seeded into 24-well plates. Following re-culture for two weeks the cells were harvested by collagenase/dispase digest and counted. The data represent the mean number of cells ± SEM (quadruplicate cultures). A significant difference (*: $p \leq 0.05$, t-test) in the number of cells was observed between the STRO-1^-/AP^+, STRO-1^+/AP^+, STRO-1^-/AP^- groups and the STRO-1^+/AP^- subfraction. A significant difference (**: $p \leq 0.05$, t-test) in the number of cells counted was also observed between the STRO-1^-/AP^+ and STRO-1^-/AP^- subfractions.

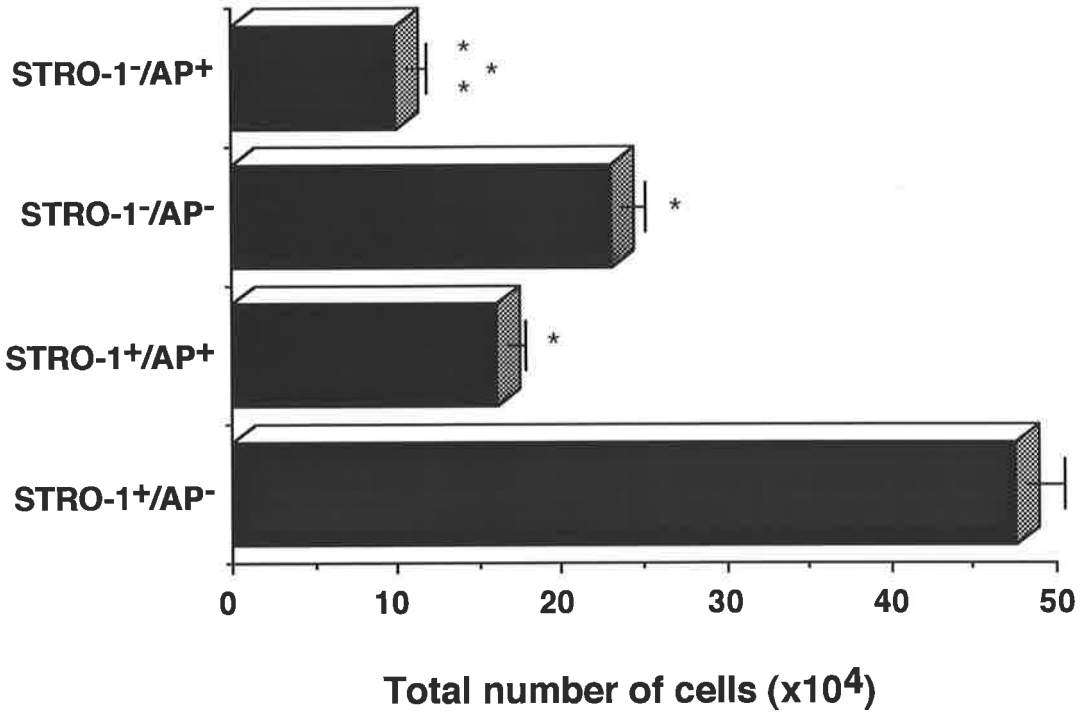
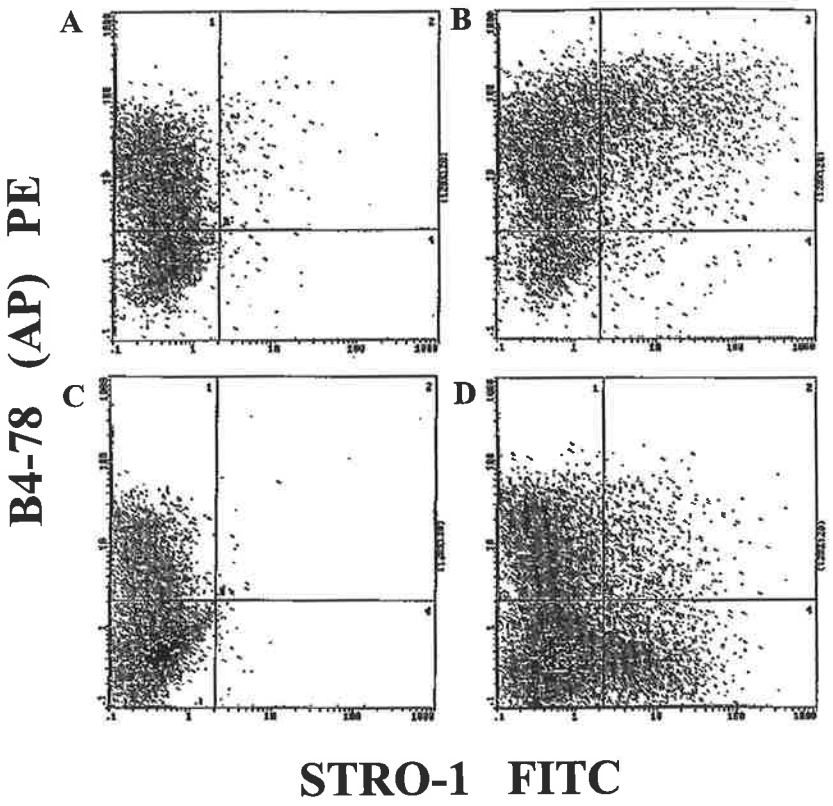

STRO-1-/AP+
STRO-1-/AP-
STRO-1+/AP+
STRO-1+/AP-
0
10
20
30
40
50
Total number of cells (x10^4)

Figure 66. Flow cytometric analysis of the different STRO-1/AP NHBC subpoulations following re-culture. Double sorted NHBC subfractions (A) STRO-1^-/AP^+; (B) STRO-1^+/AP^+; (C) STRO-1^-/AP^-; (D) STRO-1^+/AP^- were re-cultured for two weeks then reanalysed by FACS. The dot plot histogram represents 2 x 10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of <1.0% fluorescence obtained with the isotype-matched control antibodies 3D3 (PE) and 1A6.12 (FITC) respectively. The sorting of the different STRO-1/AP subgroups and the analysis of their respective immunophenotypes following re-culture, demonstrated that only the STRO-1^+/AP^- subpopulation was capable of reconstituting all of the original STRO-1/AP phenotypes from the initial cultures.


A
B
C
D
B4-78 (AP) PE
STRO-1 FITC

$1^+/AP^+$ expression were found to have lost their cell surface expression for STRO-1 but retained their expression of AP after re-culture (Figure 64B). Similarly, the majority (mean 76.5% ± 7.2 SEM, n=3) of the NHBC sorted initially on the basis of the STRO-$1^-/AP^+$ phenoypte retained their expression of AP after two weeks in culture (Figure 66A). A proportion (mean 29.7% ± 5.7SEM, n=3) of those NHBC which initially lacked any cell surface expression of STRO-1 or AP (STRO-$1^-/AP^-$) were now found to express the AP antigen following re-culture (Figure 66C).

7.5 DISCUSSION

The development of osteoblasts from early precursor cells occurs through a series of cellular transitional stages which can be identified based on morphological, biochemical and molecular criteria [reviewed in Aubin and Liu, 1996]. Current evidence suggests that COL-1, the major constituent of the extracellular matrix in bone is expressed in all of the developmental stages within the bone cell lineage and is also synthesised by stromal cells of other non-osteogenic tissues. Therefore, COL-1 on its own is a relatively poor marker of osteogenic commitment. Other markers, such as AP, ON, OP and PTH/PTHrp-R are also thought to be expressed in early pre-osteoblasts and functionally active mature osteoblasts, but are absent or only weakly present in differentiated osteocytes. In contrast, the non-collagenous proteins OCN and BSP appear to be more restricted in their expression to the mature osteoblasts and osteocytes.

In the present study we have utilised primary cultures of NHBC as an in vitro model of osteogenesis. Under appropriate culture conditions, NHBC exhibit characteristics attributed to functionally active osteoblasts including the ability to form a mineralized bone-matrix composed of hydroxyapatite in vitro [Koshihara et al 1987; Beresford et al, 1993; Gundle et al, 1995] and express all the above bone-related markers [Beresford et al, 1984; Ashton et al, 1985; Auf'mkolk et al, 1985; Beresford et al, 1986; Koshihara et al; 1987; Marie et al, 1989; Yao et al, 1994; Boss et al, 1996]. Despite the utility of cultured bone cells as an in vitro model of human bone cell development such culture systems are

inherently complex and contain a range of bone and other cell types at various stages of differentiation.

The present study was therefore performed in order to develop a means of identifying and isolating NHBC at various stages of development, based on the cell surface expression of the STRO-1 and alkaline phosphatase antigens. Other cell surface markers such as VCAM-1, Thy-1, CD10 and CD13 used to isolate CFU-F from freshly isolated aspirates of BM (Chapter 4) were not practical for isolating different cell subpopulations in vitro, since these markers were universally expressed on cultured BM stromal cells (Table 5) and on cultured NHBC (data not shown). Dual-colour FACS analysis was employed to identify and isolate four distinct subsets within the NHBC population based on the cell surface expression of the stromal precursor cell marker STRO-1 and the osteoblastic marker AP. Phenotypically distinct subpopulations were identified. It was hypothesised that NHBC expressing the AP antigen represented a more differentiated bone cell phenotype compared to those cells expressing the STRO-1 antigen alone. To test this hypothesis, the different STRO-1/AP subsets were firstly placed under osteogenic conditions to assess the ability of each phenotype to form a mineralized bone-matrix, a function which is characteristically attributed to mature osteoblasts.

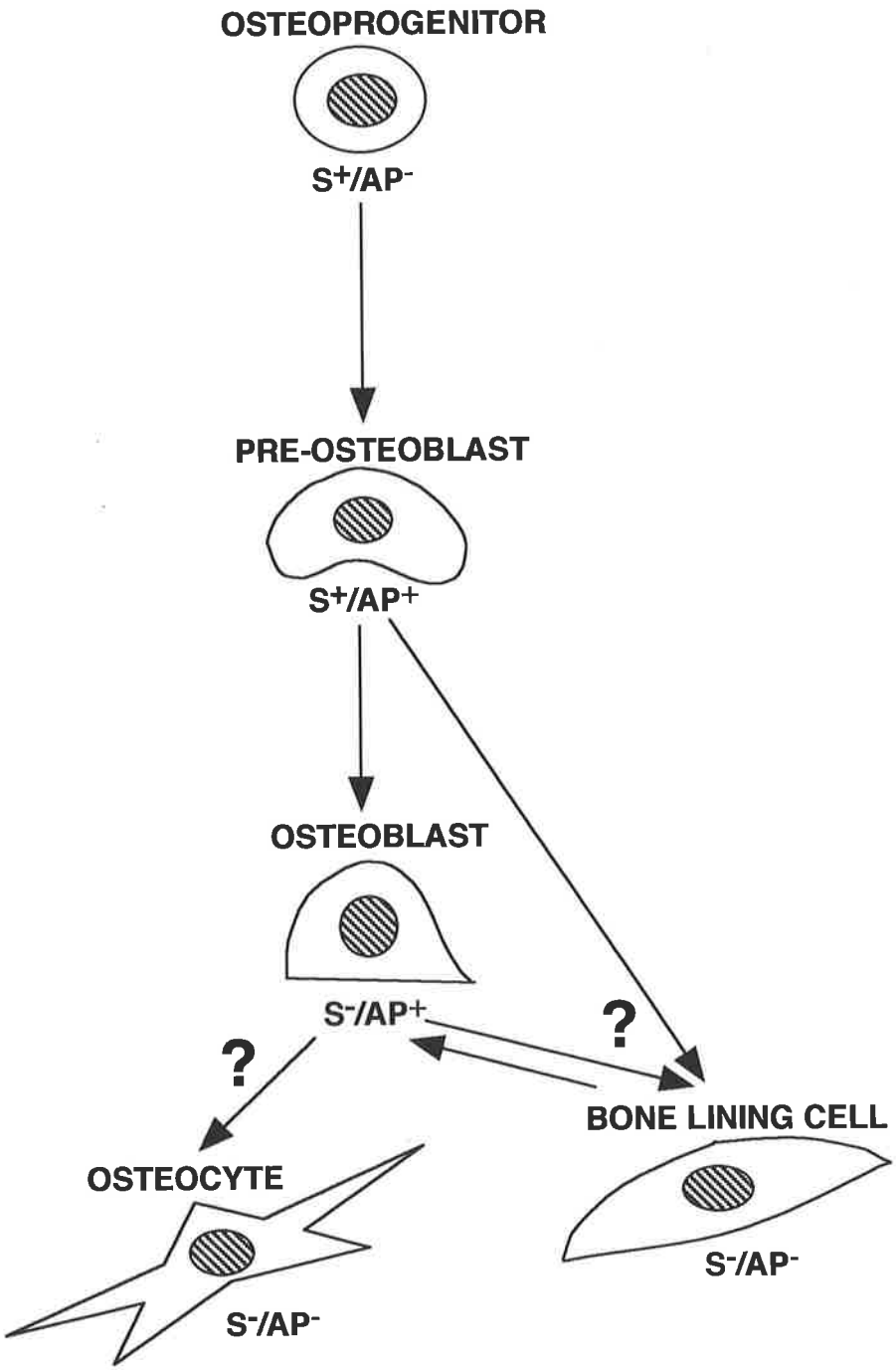
Compared to the other NHBC subpopulations, cells expressing the STRO-1 antigen alone demonstrated both a delayed onset of mineral formation and a reduced capacity for the generation of a mineralised bone-matrix over time. In addition, this subpopulation lacked expression of markers characteristic of more mature bone cells such as BSP and OP and of the four subpopulations examined is the only one which upon sorting and re-culture has the capacity to generate all the four STRO-1/AP fractions de novo. Collectively, this data is consistent with the hypothesis that the STRO-1^{+}/AP^{-} subpopulation in NHBC cultures represents an early osteoprogenitor population. In contrast, the STRO-1^{-}/AP^{+} fraction exhibits both rapid mineralisation kinetics in vitro and expression of all the bone markers examined and thus appears representative of a population of fully differentiated

osteoblasts. Intermediate between the two both phenotypically and functionally is the STRO-1$^+$/AP$^+$ subset representative of a pre-osteoblastic-like cell population. A clear parent-progeny relationship between these three populations is established by the re-sorting experiments which, together with the phenotypic and functional data, constitutes the basis for the hierarchical scheme presented in Figure 67.

In proposing this scheme several points are of note. Firstly, although widely regarded as a specific biosynthetic product of mature osteoblasts, OCN expression was nevertheless detected in all the STRO-1/AP subpopulations without prior stimulation with $1,25(OH)_2D_3$. The reasons for this are unclear but may for example reflect lack of fidelity of expression under the in vitro culture conditions utilised or the presence of contaminating OCN$^+$ cells in each of the sorted fractions. It should be noted that OCN expression in these studies was demonstrated by RT-PCR only and it will therefore be important in future studies to examine OCN expression at the protein level in each of the STRO-1/AP subpopulations. An alternative possibility is that OCN expression may not be tightly restricted to mature osteoblasts as is currently believed and my be expressed at earlier developmental stages. In support of this notion, OCN has been previously detected in pre-osteoblasts in vivo [Mark et al, 1988b].

Secondly, the physiological counterpart of the NHBC population which lacks both STRO-1 and AP remains to be determined. However, given the reduced capacity of this population to mineralise in vitro it is proposed that this cell fraction may be representative of more differentiated bone cells such as osteocytes and/or bone lining cells. In support of this view, osteocytes and bone lining cells have been shown to exhibit little or no AP activity and lack the expression of the PTH-R in vivo [Bianco et al, 1993a; Bianco et al, 1993b; Lee et al, 1993] consistent with the pattern of gene expression exhibited by the STRO-1$^-$/AP$^-$ subpopulation in vitro. In addition, following re-culture of the STRO-1$^-$/AP$^-$ selected subgroup, a minor proportion of the cells were found to develop a STRO-1$^-$/AP$^+$

Figure 67. Proposed hierarchy of bone cell development based on the cell surface expression of the STRO-1 (S) and alkaline phosphatase (AP) antigens in primary culture of normal human bone cells.


OSTEOPROGENITOR
S+/AP-
PRE-OSTEOBLAST
S+/AP+
OSTEOBLAST
S-/AP+
?
?
BONE LINING CELL
S-/AP-
OSTEOCYTE
S-/AP-

phenotype. This may imply a plasticity during bone cell development where for example bone lining cells may be capable of differentiating into osteoblasts and visa versa.

The discovery of the DNA-binding protein CBFA1 has provided a marker of early osteogenic commitment [Ducy et al, 1997; Komori et al, 1997; Rodan and Harada, 1997]. Although an important marker of early osteogenesis, CBFA1 can't be used to identify, isolate and manipulate living osteogenic cells within a heterogeneous stromal cell population. In the present study, all STRO-1/AP NHBC subsets were found to express CBFA1 confirming that primary NHBC cultures are representative of committed osteogenic cells. In addition, our primer sets yielded three distinct splice variants in addition to the expected PCR product for CBFA1 in each of the STRO-1/AP subsets with the exception of the STRO-1^+/AP^- subpopulation. The low levels of CBFA1 expression observed in the STRO-1^+/AP^- fraction may be due to the presence of only a minor population of $CBFA1^+$ cells within this subset. The availability of antibodies reactive to the CBFA1 gene product may resolve this issue. Recent reports have identified two alternative spliced variants at the 3' end of the human CBFA1 gene which have been shown to affect protein function [Geoffroy et al, 1998]. It will be of interest to determine whether the expression of the putative splice variants described in this study are associated with the switch from a pre-osteoblastic phenotype to a functional osteoblast and a mature osteocyte.

Full characterisation of the STRO-1/AP NHBC subpopulations will depend on the further development of monoclonal antibodies with restricted reactivity to specific developmental stages during osteogenesis. Various osteocyte specific mabs, OB7.3 [Nijwiede and Mulder, 1986], OB37.11 [Nijweide et al, 1988] and SB-5 [Bruder and Caplan, 1990], and osteoblast specific mabs SB-2 and SB-3 [Bruder and Caplan, 1990] reactive to avian bone cells, have previously been developed which recognise as yet unidentified antigens. Unfortunately, these reagents do not cross-react with mammalian bone cells in vivo or in vitro and therefore can not be used to dissect our model of bone cell development. In

humans the mab OB/M [Walsh et al, 1994] reacts with a cytoplasmic antigen in osteocytes and may be useful in determining whether the STRO-1$^-$/AP$^-$ NHBC subpopulation represent an osteocyte-like phenotype. To further complicate matters other markers such as the hyaluronate receptor CD44 which is reported to be restricted to osteocytes in vivo [Hughes et al, 1994] appears to be constitutively expressed by all cells in primary cultures of NHBC (data not shown). Similarly, other mabs known to react with human stromal precursors/osteoprogenitors (SH-2, SH-3 and SH-4) [Haynesworth et al, 1992], (SB-10) [Bruder et al, 1997] and osteoblasts (SB-20 and SB-21) [Bruder et al, 1997] in vivo demonstrate ubiquitous expression in stromal cultures derived from human bone and bone marrow. This typifies the limitations of any in vitro culture system where antigen expression may vary from that observed in the cellular counterparts in vivo as a result (at least in part) of the altered or inappropriate regulatory signals provided by the in vitro culture environment. The continuing development of novel lineage and stage specific markers will allow us to use this system to further subset the NHBC population and more accurately identify all the different bone cell types within. Furthermore, because of the use of RT-PCR analysis to measure the expression of the bone-related markers, there is still the possibility that other non-osteogenic stromal elements are also present in some or all of the STRO-1/AP subgroups. Reliable lineage specific markers would also allow the assessment of what proportion of other stromal cell types are contained within the different STRO-1/AP subpopulations.

This data demonstrates that the STRO-1 antigen in addition to being expressed on multi-potential BM SPC is also expressed on cells which exhibit characteristics of osteoprogenitor cells and pre-osteoblasts. Furthermore the significance of AP as a marker of osteoblasts is complicated by the observation that BM SPC express this marker in vivo which is subsequently down regulated in vitro (Table 5). Primitive stromal precursor cells in marrow and near bone surfaces share similar phenotypic traits with osteoprogenitor cells including the expression of AP [Bianco and Riminucci,1998]. Alkaline phosphatase expression may be transiently expressed in cultured bone cell populations in the absence

of appropriate stimulants such as dexamethasone. Nevertheless, the differential expression of the STRO-1 and AP antigens in cultures of NHBC in the present study represents the first real attempt to define the different developmental stages present within human bone cell cultures and provides a means of investigating the factors involved in the development and function of osteogenic cells in vitro. An improved understanding of the factors which regulate the proliferation and differentiation of osteogenic cells is an essential prerequisite for the effective management of many bone diseases such as the various forms of osteoporosis. There is increasing evidence that decreased bone mass in osteoporosis does not result purely from an increase in bone resorption [Ballard and Purdie, 1996]. Recent studies have indicated that decreased bone formation due to a reduction in the number of osteoblasts is the predominant mechanism for continued loss of cancellous bone in both type I (postmenopausal) and type II (age-related) osteoporosis [Parfitt, 1990; Cohen-Solal et al, 1991; Ballard and Purdie, 1996]. The ability of these markers to subset the NHBC population may allow the molecular characterisation of discrete developmental stages of the osteogenic cell lineage which could then be tested in situ in steady state and diseased bone tissue.

CONCLUSIONS AND FUTURE CONSIDERATIONS

This thesis presents a series of novel observations which represent major advances in the study of human stromal and osteogenic precursor cells.

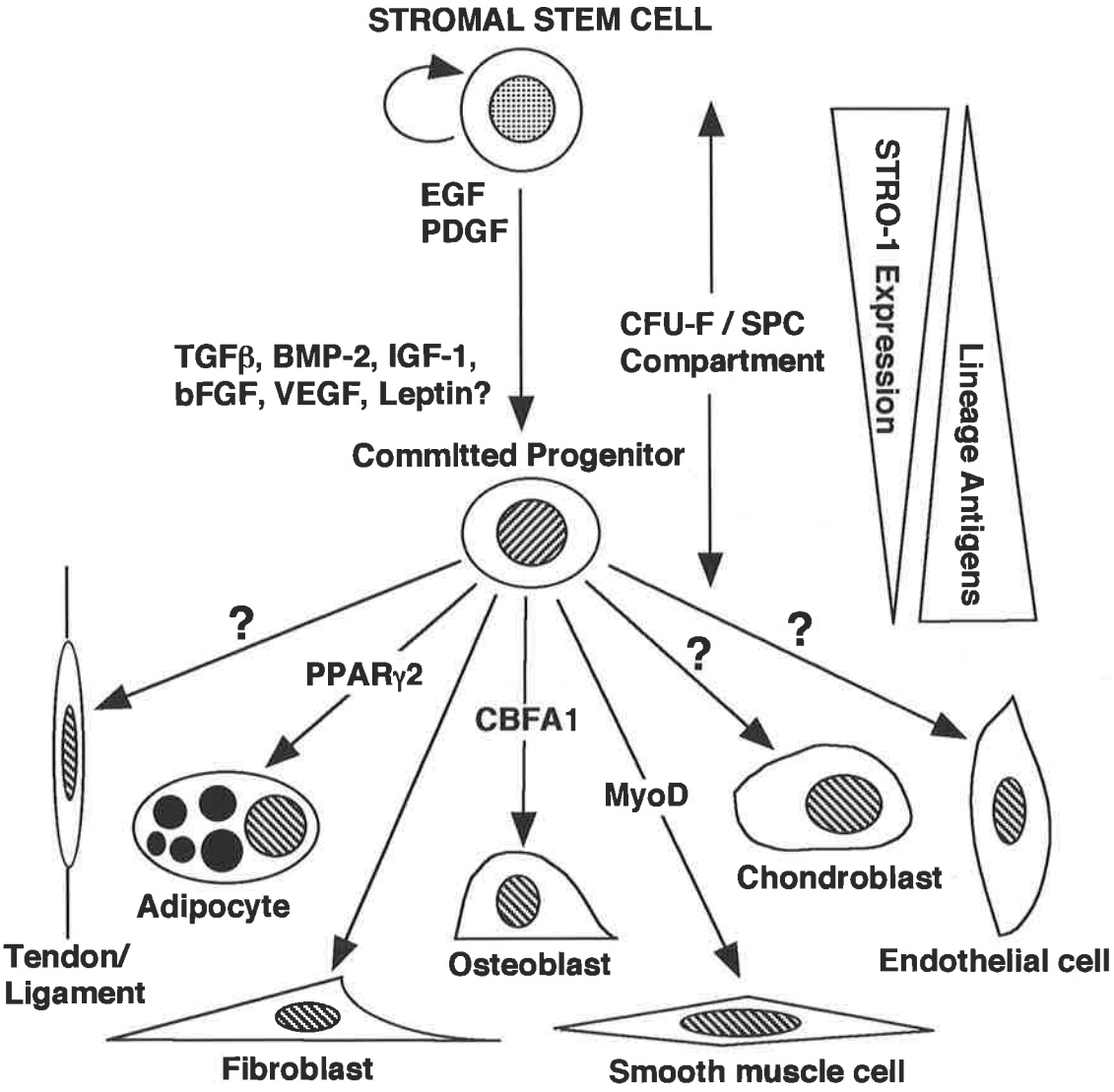
In the present thesis, a modification of the serum based CFU-F clonogenic in vitro culture assay was developed to examine the growth requirements of bone marrow stromal precursor cells (BM SPC) in vitro. This led to the development of a simple and reproducible serum-deprived clonogenic assay for SPC which has allowed for the first time, a stringent examination of the effects of a wide range of cytokines on the growth of SPC in vitro. These studies demonstrated the very specific stimulatory effects of EGF and PDGF on the proliferation of SPC which also demonstrated significantly higher cloning frequencies when compared to serum-replete cultures. This work represent an essential prerequisite for future studies of the role of cytokines in the regulation of stromal cell proliferation, differentiation and development. Further investigations of other cytokines and different combinations of cytokines, may allow identification and improve our understanding of the pathogenesis and possible treatment of conditions of altered stromal cell growth.

The STRO-1 mab, has proven to be a unique and valuable reagent for the identification and isolation of adult human BM SPC. A detailed immunophenotype was compiled demonstrating that SPC appear to express many antigens common to both bone marrow stromal cells, osteogenic and endothelial cells, but lacked expression of haemopoietic specific antigens. The SPC population was subsequently found to be exclusively in the STRO-1^{bright}/VCAM-1^{+} fraction of BMMNC. The ability to purify adult human BM SPC virtually to homogeneity is a significant achievement and has allowed for the first time, a detailed analysis of the cellular properties and functional characteristics of this precursor cell population to be conducted. Collectively, these data demonstrate that purified SPC are a rare and primitive progenitor cell population of non-cycling cells in aspirates of BM.

Clonal studies have shown that a single SPC can give rise to a fibroblast-like colonies where a proportion of clones demonstrate the capacity to develop into multiple stromal cell types including fibroblast cells, osteoblasts and adipocytes in vitro and in vivo. The absence of key regulatory genes such as PPARγ2 and CBFA1 specific for the differentiation of adipocytes and osteoblasts, respectively suggests that BM SPC are uncommitted. These stem cell-like characteristics are in agreement with the observed properties of putative BM SPC identified in animal studies [Friedenstein 1987; Bennett 1991], and support the concept of a BM stromal hierarchy of cellular differentiation in humans (Figure 68). Improvements in existing assay systems and the development of novel bioassays may eventually reveal the full developmental potential of human SPC. Further subsetting of the STRO-1^{bright}/VCAM-1^{+} BM fraction using three- and four-colour FACS analysis may eventually provide a means to discriminate between subpopulations contained within the SPC pool which exhibit different developmental potentials . The purification of SPC with different potential may be used to generate multipotent and bipotent cell lines which could greatly facilitate the design of experimental approaches to study the molecular mechanisms regulating the commitment of early marrow stromal precursors into different stromal cell types.

Expression of the STRO-1 antigen by stromal cells gradually decreases with time in culture. It is hypothesised, therefore that STRO-1 is a specific marker of SPC and that the differentiation of SPC into particular stromal cell types, such as the generation of bone cells, is characterised by sequential changes in the expression of cell surface antigens which represent stage and/or lineage restricted markers of stromal cell development. This is demonstrated in studies where NHBC cultures were found to contain a minor subpopulation of STRO-1^{+}/AP^{-} bone cells which lacked the expression of the bone-related markers AP, BSP, PTH-R. These cells were also found to display functional properties characteristic of a osteoprogenitor cells in comparison to other more mature bone cell phenotypes such as STRO-1^{-}/AP^{-} (osteocyte-like) and STRO-1^{-}/AP^{+} (osteoblast-like). In addition, a fourth intermediate subpopulation (STRO-1^{+}/AP^{+}) was

Figure 68. Revised hypothetical hierarchy of stromal stem cell differentiation (A modification of Figure 6). The stromal precursor cell (SPC) compartment is characterised by high STRO-1 expression, and includes all cells with the ability to form clonogenic clusters (CFU-F) in vitro. The proliferation of SPC is attributed to the growth factors PDGF and EGF. The STRO-1 antigen is proposed to be a developmental marker which is gradually lost as the BM SPC proliferate and differentiate. Differentiation of SPC involves the accquisition of lineage-restricted markers and may be driven by other cytokines such as BMP-2, bFGF, IGF-1, VEGF and Leptin.


STROMAL STEM CELL
EGF
PDGF
TGFβ, BMP-2, IGF-1,
bFGF, VEGF, Leptin?
CFU-F / SPC
Compartment
STRO-1 Expression
Lineage Antigens
Committed Progenitor
?
PPARγ2
CBFA1
MyoD
?
?
Tendon/
Ligament
Adipocyte
Osteoblast
Chondroblast
Endothelial cell
Fibroblast
Smooth muscle cell

found to represents a phenotype characteristic of pre osteoblast-like cells. Given the heterogeneity observed within cultures of NHBC, the differential expression of STRO-1 and AP can be used as a model for further studies of osteogenic development and function. Furthermore, the identification of the cDNA encoding STRO-1 would facilitate studies into the role of this antigen in stromal cell and osteogenic development. Efforts are currently underway, in collaboration with Dr. A. Zannettino, to develop a cDNA expression library [Zannettino et al, 1996] derived from mRNA isolated from MG63 cells, which exhibiting high levels of STRO-1, in order to identify and fully characterise the 60 kDa STRO-1 antigen.

This work also creates a basis to explore a range of future potential clinical benefits. It is likely that purified SPC expanded under serum-deprived conditions, as described in this study, may allow for the expansion of primitive haemopoietic stem cells in vitro, which can then be used to reconstitute the bone marrow following ablative therapy for a variety of malignancies. In particular, the MACS procedure offers a reliable and efficient means to isolate large numbers of partially purified BM SPC required for large clinical scale isolations. Stromal precursor cells could then be cultured and expanded in vitro, using the serum-deprived culture conditions described in the present study in the presence of different differentiation factors. One potential benefit that may develop from a greater understanding of the regulation of stromal stem cell proliferation and differentiation is the ability to manipulate and expand stromal cell precursors in vitro for subsequent re-implantation in vivo. This approach may help to improve the grafting of HSC following ablative therapy by helping to regenerate the BM microenviroment. Furthermore, stromal cell defects such as those seen in many bone diseases and after inductive cancer therapies offer an ideal situation for the treatment of these diseases using autologous stromal cells in combination with biocompatible implant materials and or with stromal cells ectopically expressing a therapeutic gene. The current study also offers insights into bone cell-matrix interactions mediated by molecules belonging to the β_1 integrin family of cell adhesion molecules. Continued studies into the adhesive requirements of bone and other

stromal cells, may eventually contribute to the development of suitable and improved transplantation strategies of biocompatible implant materials and may help to promote the successful infusion and grafting of stromal cells.

In conclusion, this thesis establishes a foundation for future studies investigating the molecular mechanisms regulating the commitment of primitive human SPC to particular developmental pathways and the progression of osteoprogenitors to develop into functional osteoblasts.

BIBLIOGRAPHY

Aarden EM, Nuweide PJ, Van der plas A, Albas MJ, Mackie EJ, Horton MA, Helfrich MH (1996). Adhesive properties of isolated chick osteocytes in vitro. Bone 18:305-313.

Abboud CN, Duerst RE, Frantz CN, Ryan DH, Liesveld JL, Brennan JK (1986). Lysis of human fibroblast colony-forming cells and endothelial cells by the monoclonal antibody (6-19) and complement. Blood 68: 1196-1200.

Abboud SL (1993). A bone marrow stromal cell line is a source and target for platelet-derived growth factor. Blood 81:2547-2553.

Abbou-Samra AB, Jueppner H, Force T, Freeman MWM, Kong X-F, Schipani E, Urena P, Richards J, Bonventre JV, Potts JT, Jr, Kronenburg HM, Segre GV (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of of both cAMP and inositol triphosphates and increases intracellular free calcium. Proc. Natl. Acad. Sci. (USA) 89: 2732-2736.

Adam MA, Miller AD, Osborne WRA (1991). Internal initiation of translation in retroviral vectors carrying picornavirus 5' nontranslated regions. J. Virology 65: 4985-4990.

Albelda SM and Buck CA (1990). Integrins and other cell adhesion molecules. FASEB J. 4: 2868-2880.

Albelda SM, Muller WA, Buck CA, Newman PJ (1991). Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J Cell Biol. 114: 1059-1068.

Alexander WS (1998). Cytokines in hematopoiesis. Int. Rev. Immunol. 16: 651-82.

Allen TD (1981). Haemopoietic microenvironment in vitro: Ultrastructural aspects. In: Inporter R, Whelan J (eds) "Microenvironments in haemopoietic and lymphoid differentiation." CIBA Foundation Symposium 84. London. Pitman Medical, p 38.

Allen, TD, Dexter, TM, Simmons, PJ (1990). Marrow Biology and Stem Cells. In: Dexter TM, Garland JM, Testa NG (eds): Colony Stimulating Factors: Molecular and Cellular Biology. Marcel Dekker. New York. p. 1-38.

Andrews, RG, Singer, JW, Bernstein, ID (1986). Monoclonal antibody 12.8 recognises a 115 kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 67: 842-845.

Anklesaria P, Kase K, Glowacki J, Holland CA, Sakakeeny MA, Wright JA, FitzGerald TJ, Lee C-Y, Greenberger JS (1987). Engraftment of a clonal bone marrow stromal cell line in vivo stimulates hematopoietic recovery from total body irradiation. Proc. Natl. Acad. Sci. (USA) 84: 7681-7685.

Aplin AE, Howe A, Alahari SK, Juliano RL (1998). Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. Pharmacol. Rev. 50: 197-263.

Appelbaum FR, Fefer A, Cheever MA, Sanders JE, Singer JW, Adamson JW, Mickelson EM, Hansen JA, Greenberg PD, Thomas ED (1980). Treatment of aplastic anamia by bone marrow transplantation in identical twins. Blood 55: 1033-1039.

Arao M, Yamaguchi T, Sugimoto T, Fukase M, Chihara K (1994). Involvement of protein kinase C in the stimulation of sodium-dependent phosphate transport by parathyroid hormone in osteoblast-like cells. Eur. J. Endocrinol. 131: 646-651.

Arkin S, Naprstek B, Guarini L, Ferrone S, Lipton JM (1991). Expression of intercellular adhesion molecule-1 (CD54) on hematopoietic progenitors. Blood 77: 948-953.

Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990). CD44 is the principal cell surface receptor for hyaluronate. Cell 61: 1303-1313.

Ashton B, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen ME (1980). Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin. Orthop. 151: 294-307.

Ashton B, Cave F, Williamson M, Sykes B, Couch M, Poser J (1985). Characterization of cells with high alkaline phosphatase activity derived from human bone and marrow: Preliminary asessment of their osteogenicity. Bone 6: 313-319.

Aubin JE, Turksen K, Heersche JNM (1993). Osteoblastic cell lineage. In: Noda M (ed), Cellular and molecular biology of bone. Academic Press, San Diego. p. 1-45.

Aubin JE and Liu F (1996). The osteoblast lineage. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 51-67.

Auf'mkolk B, Hauschka PV, Schwartz ER (1985). Characterization of human bone cells in culture. Calcif. Tissue Int. 37: 228-235.

Aye MT, Hashemi S, Leclair B, Zeibdawi A, Trudel E, Halpenny M, Fuller V, Cheng G (1992). Expression of stem cell factor and c-kit mRNA in cultured endothelial cells,

monocytes and cloned human bone marrow stromal cells (CFU-RF). Exp. Hematol. 20: 523-527.

Bab I, Ashton BA, Syftestad G, Owen M (1984). Assessment of an in vivo diffusion chamber method as a quantitative assay for osteogenesis; Calcif. Tissue Int. 36: 77-82.

Bab I, Gazit D, Massarawa A, Sela J (1985). Removal of tibial marrow induces increased formation of bone and cartilage in rat mandibular condyle. Calcif. Tissue Int. 37: 551-555.

Bab I, Ashton BA, Gazit D, Marx G, Williamson MC, Owen ME (1986). Kinetics and differentiation of marrow stromal cells in diffusion chambers in vivo. J. Cell Sci. 84: 139-151.

Bab I, Passi-Even L, Sekeles E, Ashton B, Peylan-Ramu N, Ziv I, Ulmansky M (1988). Osteogenesis in in vivo diffusion chamder cultures of human marrow cells. Bone Mineral 4: 373-386.

Bagby GC Jr. (1994). Hematopoiesis. In: Stamatoyannopoulos G. (ed), Molecular basis of blood diseases. W.B. Saunders, Philadelphia, second edition. p. 71-103.

Baldwin CT, Reginato AM, Smith C, Jimenez SA, Prockop DJ (1989). Structure of cDNA clones coding for human type II procollagen. Biochem. J. 262: 521-528.

Ballard PA and Purdie DW (1996). The natural history of osteoporosis. Br. J. Hosp. Med. 55: 503-507.

Bardin N, Frances V, Lesaule G, Horschowski N, George F, (1996). Identification of the S-Endo 1 endothelial-associated antigen. Biochem. Biophys. Res. Commun. 218: 210-216.

Barling PM, Bennett JH, Triffitt JT, Owen ME (1989). The adenylate cyclase response to parathyroid hormone in cultured rabbit marrow fibroblastic cells. Bone Mineral 7: 23-30.

Bathija A, Davis S, Trubowitz S (1979). Bone marrow adipose tissue: Response to starvation. Am. J. Hematol. 6: 191-198.

Baum CM, Weissman L, Tsukamoto AS, Buckle AM, Peault B (1992). Isolation of a candidate human haemopoietic stem-cell population. Proc. Natl. Acad. Sci. (USA). 89: 2804-2808.

Bazil V, Baudys M, Hilgert I, Stefanová I, Low MG, Zbrozek J, Horejsí V (1989). Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. Mol. Immunol. 26: 657-662.

Beck K, Hunter I, Engel J (1990). Structure and function of laminin: anatomy of a multidomain glycoprotein. FASEB J. 4: 148-160.

Bellahcene A, Merville MP, Castronovo V (1994). Expression of bone sialoprotein, a bone matrix protein, in human breast cancer. Cancer Res. 54: 2823-2826.

Bellows CG, Aubin JE, Heersche JNM, Antosz M (1986). Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif. Tissue Int. 38: 143-154.

Bellows CG, Aubin JE, Heersche JNM (1987). Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in virto. Endocrinology 121: 1985-1992.

Bellows CG and Aubin JE (1989). Determination of the number of osteoprogenitors present in isolated fetal rat calvaria cells in vitro. Dev. Biol. 133: 8-13.

Bellows CG, Heersche JNM, Aubin JE, (1990). Determination of the capacity for proliferation and differentiation of osteoprogenitor cells in the presence and absence of dexamethasone. Dev. Biol. 140: 132-138.

Bellows CG, Aubin JE, Heersche JNM (1991). Initiation and progression of mineralization of bone nodules formed in vitro: the role of alkaline phosphatase and organic phosphate. Bone and Mineral 14: 27-40.

Bellows CG, Heersche JNM, Aubin JE (1992). Inorganic phosphate added exogenously or released from β-glycerolphosphate initiates mineralization of osteoid nodules in vitro. Bone and Mineral 17: 15-29.

Benayahu D, Fried A, Zipori D, Wientroub S (1991). Subpopulations of marrow stromal cells share a variety of osteoblastic markers. Calcif. Tissue Int. 49: 202-207.

Benayahu D, Gurevitch O, Zipori D, Wientroub S (1994). Bone formation by marrow osteogenic cells (MBA-15) is not accompanied by osteoclastogenesis and generation of hematopoietic supportive microenvironment. J. Bone Miner. Res. 9: 1107-1114.

Benayahu D, Shamay A, Wientroub S (1997). Osteocalcin (BGP), gene expression, and protein production by marrow stromal adipocytes. Biochem. Biophys. Res. Commun. 231: 442-446.

Bennett JH, Joyner CJ, Triffitt JT, Owen ME (1991). Adipocyte cells cultured from marrow have osteogenic potential. J. Cell Sci. 99: 131-139.

Bentley SA, Alabaster O, Foidart JM (1981). Collagen hetergeneity in normal human bone marrow. British Journal of Haematology 48: 287- 291.

Bentley SA (1982a). Bone marrow connective tissue and the haemopoietic microenvironment. British Journal of Haematology 50: 1-6.

Bentley SA, Knutsen T, Whang-Peng J (1982b). The origin of the hematopoietic microenvironment in continuous bone marrow culture. Experimental Hematology 10: 367-372.

Berenson RJ, Andrews RG, Bensinger WI, Kalamasz DF, Knitter G, Buckner CD, Bernstein ID (1988). Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons. Journal of Clinical Investigation 81: 951-955.

Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF, Still BJ, Spitzer G, Buckner CD, Bernstein ID, Thomas ED (1991). Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma. Blood 77: 1717-1722.

Beresford HN, Gallagher JA, Poser JW, Russell RGG (1984). Production of osteocalcin by human bone cells in vitro. Effects of $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, parathyroid hormone and glucocorticoids. Metab. Bone Dis. Relat. Res. 5: 229-234.

Beresford HN, Gallagher JA, Russell RGG (1986). $1,25(OH)_2D_3$ and human bone-derived cells in vitro: Effects on alkaline phosphatase, type 1 collagen synthesis and proliferation. Endocrinology 119: 1776-1781.

Beresford JN, Graves SE, Smoothy CA (1993). Formation of mineralized nodules by bone derived cells in vitro: a model of bone formation? Am. J. Med. Genet. 45: 163-178.

Beresford JN, Joyner CJ, Devlin C, Triffitt JT (1994). Osteogenic differentiation in human marrow stromal cell cultures: Effects of dexamethasone and 1,25-dihydroxyvitamin D_3. Arch. Oral Biol. 39: 941-947.

Bessis M (1973). Livng Blood Cells and Their Ultrastructure. Springer-Verlag, New York. p. 477-517.

Bhargava U, Bar-Lev M, Bellows CG, Aubin JE (1988). Ultrastructure analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells. Bone 9: 155-163.

Bianco P, Constantini M, Dearden LC, Bonucci E (1988). Alkaline phosphatase positive precursors of adipocytes in the human bone marrow. Brit. J. Haemat. 68-401-403.

Bianco P and Bonucci E (1991). Endosteal surfaces in hyperparathyroidism: an enzyme cytochemical study on low temperature-processed, glycol methacrylate embedded bone biopsies. Virchow's Arch. Pathol. Anat. Histopath. 419: 425-431.

Bianco P, Fisher LW, Young MF, Termine JD, Robey PG (1991). Expression of the bone sialoprotein (BSP) in developing human tissues. Calcif. Tissue Int. 49: 421-426.

Bianco P and Boyde A (1993). Confocal images of marrow stromal (Westen-Bainton) cells. Histochemistry 100:93-99.

Bianco P, Riminucci M, Bonucci E, Termine JD, Gehron Robey P (1993a). Bone sialoprotein (BSP) secretion and osteoblast differentiation: Relationship to bromodeoxyuridine incorporation, alkaline phosphatase, and matrix deposition. J Histochem Cytochem 41:183-191

Bianco P, Riminucci M, Silvestrini G, Bonucci E, Termine JD, Fisher LW, Gehron Robey P (1993b). Localization of bone sialoprotein (BSP) to Golgi and post-Golgi secretory structures in osteoblasts and to discrete sites in early bone matrix. J. Histochem. Cytochem. 41: 193-203.

Bianco P and Riminucci M (1998). The bone marrow stroma in vivo: ontogeny, structure, cellular composition and changes in disease. In: Beresford JN and Owen ME(eds): Marrow stromal cell culture. Handbooks in practical animal cell biology, Cambridge University Press, UK. Chapter 2, p. 10-25.

Bilbe G, Roberts E, Birch M, Evans DB (1996). PCR phenotyping of cytokines, growth factors and their receptors and bone matrix proteins in human osteoblast-like cell lines. Bone 19: 437-445.

Bonaventure J, Kadhom N, Cohen-Solal L, Bourguignon NGJ, Lasselin C, Freisinger P (1994). Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Experimental Cell Research 212: 97-104.

Boni-Schnetzler M and Pilch PF (1987). Mechanism of epidermal growth factor receptor autophosphorylation and high affinity binding. Proc. Natl. Acad. Sci. (USA) 84: 7832-7836.

Boskey AL (1992). Mineral-matrix interactions in bone and cartilage. Clin. Ortho. Rel. Res. 281: 244-273.

Boskey AL, Maresca M, Ulrich W, Doty SB, Butler WT, Prince CW (1993). Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite formation and growth in a gelatin-gel. Bone Miner. 22: 147-159.

Boss MP, van der Meer JM, Feyen JHM, Hermann-Erlee MPM (1996). Expression of the parathyroid hormone receptor and correlation with other osteoblastic parameters in fetal rat osteoblasts. Calcif. Tissue Int. 58: 95-100.

Bowen-Pope DF, Dicorleto PE, Ross R (1983). Interaction between the receptors for platelet derived growth factor and epidermal growth factor. J. Cell Biol. 96: 679-683.

Brant J, Srour EF, Besien KV, Briddell RA, Hoffman R (1990). Cytokine-dependent long-term culture of highly enriched precursurs of hematopoietic progenitor cells from human bone marrow. J. Clin. Invest. 86: 932-941.

Brizzi MF, Avanzi GC, Pegoraro L (1991). Hematopoietic growth factor receptors. Int. J. Cell Cloning 9: 274-300.

Brockband KGM, de Jong JP, Piersma AH, Voerman JSA (1986). Haemopoiesis on purified bone marrow derived reticular fibroblasts in vitro. Exp. Hematol. 14: 386-394.

Brown PJ and Juliano RL (1985). Selective inhibition of fibronectin-mediated adhesion by monoclonal antibodies to a cell surface glycoprotein. Science 228:1448-1451.

Broxmeyer HE, Sherry B, Lu L, Cooper S, Oh K-O, Tekamp-Olson P, Kwon BS, Cerami A (1990). Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells. Blood 76: 1110-1116.

Bruder SP and Caplan AI (1990). Terminal differentiation of osteogenic cells in the embryonic chick tibia is revealed by a monoclonal antibody against osteocytes. Bone 11: 189-198.

Bruder SP, Fink DJ, Caplan AI (1994). Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J. Cell. Biochem. 56: 283-294.

Bruder SP, Horowitz MC, Mosca JD, Haynesworth (1997). Monoclonal antibodies reactive with human osteogenic cell surface antigens. 21: 225-235.

Bruder SP, Ricalton NS, Boynton RE, Connolly TJ, Jaiswal N, Zaia J, Barry FP (1998a). Mesenchymal stem cell surface antigen SB-10 corresponds to activated leukocyte cell adhesion molecule and is involved in osteogenic differentiation. J. Bone Miner. Res. 13: 655-663.

Bruder SP, Kraus KH, Goldberg VM, Kadiyala S (1998b). The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J. Bone Joint Surg. (AM.) 80: 985-996.

Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S (1998c). Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J. Orthop. Res. 16: 155-162.

Brunner G, Nguyen H, Gabrilove J, Rifkin DB, Wilson EL (1993). Basic fibroblast growth factor expression in human bone marrow and peripheral blood cells. Blood 81: 631-638.

Butler WT (1989). The nature and significance of osteopontin. Connect. Tissue Res. 23: 123-126.

Caligaris-Cappio F, Berguli L, Gregoretti MG (1991). Role of bone marrow stromal cells in the growth of human multiple myeloma. Blood 77: 2688-2693.

Canalis E (1993). Insulin-like growth factors and the local regulation of bone formation. Bone 14: 273-276.

Caplan AI (1988). Bone development. In: Evered D and HarnettS, (eds). Cell and molecular biology of vertabrate hard tissues. John Wiley & Sons, Chichester. Ciba Foundation Symposium 136: 3-21.

Caplan AI and Dennis JE (1996). Mesenchymal stem cells: Progenitors, progeny, and pathways. J. Bone Miner. Metab. 14: 193-201.

Carlos TM, Harlan JM (1994). Leukocyte-endothelial adhesion molecules. Blood 84: 2068-2101.

Carpenter G and Zendegui JG (1986). Epidermal growth factor, its receptor and related proteins. Exp. Cell Res. 164: 1-10.

Cassiede P, Dennis JE, Ma F, Caplan AI (1996). Osteochondrogenic potential of marrow mesenchymal progenitor cells exposed to TGF-β1 or PDGF-BB as assayed in vivo and in vitro. J. Bone Miner. Res. 11: 1264-1273.

Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MAS (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood 56: 289-301.

Castro-Malaspina H, Rabellino EM, Yen A, Nachman RL, Moore MAS (1981). Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts. Bood 57: 781-787.

Cattoretti G, Schiro R, Orazi A, Soligo D, Colombo MP (1993). Bone marrow stroma in humans: Anti-nerve growth factor receptor antibodies selectively stain reticular cells in vivo and in vitro. Blood 81: 1726-1738.

Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA, Wozney JM (1986). Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. EMBO J. 5: 1885-1890.

Chang M-C, Wang B-R, Huang T-F (1995). Characterization of endothelial cell differential attachment to fibrin and fibrinogen and its inhibition by Arg-Gly-Asp-containing peptides. Thrombosis and Haemostasis 74: 764-769.

Charbord P, Gown AM, Keating A, Singer JW (1985). CGA-7 and HHF, two monoclonal antibodies that recognise muscle actin and react with adherent cells in human long-term bone marrow cultures. Blood 66: 1138-1142.

Chen Y and Rabinovitch PS (1989). Platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor 1 regulate specific cell-cycle parameters of human diploid fibroblasts in serum-free culture. J. Cell Physiol. 140: 59-67.

Chen J, Shapiro HS, Wrana JL, Reimers S, Heersche JNM, Sodek J (1991a). Localization of bone sialoprotein (BSP) expression to sites of mineralized tissue formation in fetal rat tissues by in situ hybridization. Matrix 11: 133-143.

Chen Z, Van Bockstaele DR, Buyssens N, Hendrics D, Meester ID, Vanhoof G, Scharpe SL, Peetermans ME, Berneman ZN (1991b). Stromal populations and fibrosis in human long-term bone marrow cultures. Leukemia 5: 772-781.

Cheng S-L, Yang JW, Rifas L, Zhang S-F, Avioli LV (1994). Differentiation of human bone marrow osteogenic stromal cells in vitro. Induction of the osteoblast phenotype by dexamethasone. Endocrinology 134: 277-286.

Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G (1998). A common precursor for hematopoietic and endothelial cells. Development 125:725-32.

Chomczynski P and Sacchi N (1987). Single-step method of RNA isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform extraction. Anal. Biochem. 162: 156-159.

Chu ML, de Wet W, Bernard M, Ramirez F (1985). Fine structural analysis of the Human pro alpha 1(I) collagen gene. Promoter structure, AluI repeats, and polymorphic transcripts. J. Biol.Chem. 260: 2315-2320.

Clark SC and Kamen R (1987). The hematopoietic colony stimulating factors. Science 236: 1229-1237.

Clemmons DR, Underwood LE, Van Wyk JJ (1981). Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. J. Clin .Invest. 67: 10-19.

Clemmons DR and Shaw DS (1983). Variables controlling somatomedin production by cultured human fibroblasts. J. Cell Physiol. 115: 137-142.

Clezardin P, Malaval L, Ehrensperger A-S, Delmas PD, Dechavanne M, McGregor JL (1988). Complex formation of human thrombospondin with osteonectin. Eur. J. Biochem. 175: 275-280.

Clover J, Dodds RA, Gowen M (1992). Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. J. Cell Sci .103: 267-271.

Cohen-Solal ME, Shih MS, Lundy MW, Parfitt AM (1991). A new method for measuring cancellous erosion depth: application to cellular mechanisms of bone loss in postmenopausal osteoporosis. J. Bone Miner. Res. 6: 1331-1237.

Cole SR, Ashman LK, Ey PL (1987). Biotinylation: An alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. Mol. Immunol. 24: 699-705.

Conforti G, Dominguez-Jimenez C, Zanetti A, Gimbrone M., Cremona O, Marchisio PC, Dejana E (1992). Human endothelial cells express integrin receptors on the luminal aspect of their membrane. Blood 80: 437-446.

Constantinides PG, Jones PA, Gevers W (1977). Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. Nature 267: 364-366.

Conway EM and Nowakowski B (1993). Biologically active thrombomodulin is synthesized by adherent synovial fluid cells and is elevated in synovial fluid of patients with rheumatoid arthritis. Blood 81: 726-733.

Coulombel L, Vuillet MH, Tchernia G (1988). Lineage and stage specific adhesion of human hematopoietic progenitor cells to extracellular matrices from marrow fibroblasts. Blood 71: 329-334.

Craig AM, Bowden GT, Chambers AF, Spearman MA, Greenberg AH, Wright JA, McLeod M, Denhardt DT (1990). Secreted phosphoprotein mRNA is induced during multistage carcinogenesis in mouse skin and correlates with the metastatic potential of murine fibroblasts. Int. J. Cancer 46: 133-137.

Crosby AH, Edwards SJ, Murray JC, Dixon MJ (1995). Genomic organization of the human osteopontin gene: exclusion of the locus from a causative role in the pathogenesis of dentinogenesis imperfecta type II. Genomics 27: 155-160.

Cross M and Dexter TM (1991). Growth factors in development, transformation and tumorigenesis. Cell 64: 271-280.

Dalton BA, McFarland CD, Underwood PA, Steele JG (1995). Role of the heparin binding domain of fibronectin in attachment and spreading of human bone-derived cells. J. Cell Science 108: 2083-2092.

D'Amore (1990). Culture and study of pericytes. In: Cell culture techniques in heart and vessel research, Piper HM (ed). Sprigger-Verlag, Berlin. p.299-314.

Davies JE (1987). Human bone marrow cells synthesise collagen in diffusion chambers implanted into the normal rat. Cell Biol. Int. Rep. 11: 125-130.

Davis RL, Weintraub H, Lasser AB (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51: 987-1000.

Davis PY, Frazier CR, Shapiro JR, Fedarko NS (1997). Age-related changes in effects of insulin-like growth factor I on human osteoblast-like cells. Biochem. J. 324: 753-760.

Dedhar S, Ruoslahti E, Pierschbacher MD (1987). A cell surface receptor complex for collagen type I recognises the Arg-Gly-Asp sequence. J. Cell Biol. 104: 585-593.

Dennis JE, Haynesworth SE, Young RG, Caplan AI (1992). Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously:

Effect of fibronectin and laminin on cell retension and rate of osteogenic expression. Cell Transplantation 1: 23-32.

Dennis JE and Caplan AI (1993). Porous ceramic vehicles for rat-marrow-derived osteogenic cell delivery: effects of pre-treatment with fibronectin or laminin. J. Oral Implant. 19: 106-115.

Depollack C, Arnaud E, Renier D, Marie PJ (1997). Age-related changes in bone formation osteoblastic cell proliferation, and differentiation during postnatal osteogenesis in human calvaria. J. Cell Biochem. 64 128-139.

Deryugina EI and Muller-Sieburg CE (1993). Stromal cells in long-term cultures: Keys to the elucidation of hematopoietic development? Crit. Rev. Immunol. 13: 115-150.

Dexter TM (1987). Growth factors involved in haemopoiesis. J. Cell. Sci. 88: 1-6.

Dexter TM, Allen TD, Lajtha LG (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. Journal of Cellular Physiology 91: 335-344.

Dexter TM, Spooncer E, Simmons P, Allen TD (1984). Long term bone marrow culture: an overview of techniques and experience. In: Long term Bone Marrow Culture, DG Wright, JS Greenberger, (eds). Kroc Foundation Series Vol 18. Alan R Liss, Inc., New York. p. 57-96.

DiCorleto PE and Bowen-Pope DF (1983). Cultured endothelial cells produce a platelet-growth factor-like protein. Proc. Natl. Acad. Sci. (USA) 80: 1919-1923.

Dobnig H and Turner RT (1995). Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. Endocrinology 136: 3632-3638.

Doege KJ, Sasaki M, Kimura T, Yamada Y (1991). Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms. J. Biol. Chem. 266:894-902.

Dommergues M, Aubeny E, Dumez Y, Durandy A, Coulombel L (1992). Hematopoiesis in the human yolk sac: quantitation of erythroid and granulopoietic progenitors between 3.5 and 8 weeks of development. Bone Marrow Transplantation 9 Suppl (1): 23-27.

Dorheim M-A, Sullivan M, Dandapani V, Wu X, Hudson J, Segarini PR, Rosen DM, Aulthouse AL, Gimble JM (1993). Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. J. Cell. Physiol. 154: 317-328.

Dorshkind K (1990). Regulation of hemopoiesis by bone marrow cells and their products. Ann. Rev. Immunol. 8: 111-137.

Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullirich A, Schlessinger J, Waterfield MD (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequence. Nature 307: 521-527.

D'Souza S, Ginsberg M, Plow E (1991). Arginyl-glycl-aspartic acid (RGD): a cell adhesion motif. Trends Biochem. Sci. 16: 246-249.

Ducy P and Karsenty G (1996a). Skeletal gla proteins: gene structure, regulation of expression, and function. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 183-195.

Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G (1996b). Increased bone formation in osteocalcin-deficient mice. Nature 382:448-452.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997). Osf2/Cbfa1: A transcription activator of osteoblast differentiation. Cell 89: 747-754.

Ebell W, Castro-Malaspina H, Moore MAS, O'Reilly RJ (1985). Depletion of stromal cell elements in human marrow grafts separated by soybean agglutinin. Blood 65: 1105-1111.

Ecarot-Charrier B, Glorieux FH, Van der Rest M, Pereira G (1983). Osteoblasts isolated from mouse calvaria initiate matrix mineralization. J. Cell Biol. 96: 639-643.

Ecarot-Charrier B, Shepard N, Charette G, Grynpas N, Glorieux FH (1988). Mineralization in osteoblast cultures: A light and electron microscopy study. Bone 9: 147-154.

El-Badri NS, Wang B-Y, Cherry, Good RA (1998). Osteoblasts promote engraftment of allogeneic hemopoietic stem cells. Experimental Hematology 26: 110-116.

Elias JA, Tang W, Horowitz MC (1995). Cytokine and hormone stimulation of human osteosarcoma IL-11 production. Endocrinology 136: 489-498.

Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyl S, Hemler M, Lobb R (1990). VCAM-1 on activated endothelium interacts with the leukocyte antigen VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell 60: 577-584.

Elices MJ, Urry LA, Helmer ME (1991). Receptor functions for the integrin VLA-3: Fibronectin, collagen and laminin binding are differentially influenced by Arg-Gly-Asp peptide and by divalent cations. J Cell Biol 112: 169-181.

Epichina SY and Latzinik NV (1976). Proliferative activity of bone marrow stromal clonogenic cells. Bull. Exp. Biol. Med. 81: 55-58.

Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R (1995). Toward a molecular understanding of skeletal development. Cell 80: 371-378.

Ershler WB, Ross J, Finlay JL, Shahidi NT (1980). Bone marrow microenvironment defect in congenital hypoplastic anemia; The New England Journal of Medicine 302: 1321-1326.

Eyre DR (1996). Biochemical basis of collagen metabolites as bone turnover markers. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 1143-153.

Falla N, Van Vlasselaer P, Bierkens J, Borremans B, Schoeters G, Van Gorp U (1993). Characterization of a 5-flurouracil-enriched osteoprogenitor population of murine bone marrow. Blood 82: 3580-3591.

Favaloro E., Moraitis N, Bradstock K, Koutts J (1990). Co-expression of haemopoietic antigens on vascular endothelial cells: a detailed phenotypic analysis. Br. J. Haematol.74: 385-394.

Fedarko NS, Vetter U, Weistein S, Robey PG (1992). Age-related changes in the hyaluronan, proteoglycan, collagen and osteonectin synthesis by human bone cells. J. Cell physiol. 151: 215-227.

Fialkow PJ, Jacobson PJ, Papayannopoulou T (1977). Chronic myeloid leukemia: Clonal origin in a stem cell common to granulocytes, erythrocytes, platelets and monocytes/macrophage. Am. J. Med. 63: 125-130.

Filshie RJ, Zannettino AC, Makrynikola V, Gronthos S, Henniker AJ, Bendall LJ, Gottlieb DJ, Simmons PJ, Bradstock KF (1998). MUC18, a member of the immunoglobulin superfamily, is expressed on bone marrow fibroblasts and a subset of hematological malignancies. Leukemia 12:414-421.

Findlay DM, Fisher LW, McQuillan CI, Termine JD, Young MF (1988). Isolation of the osteonectin gene: evidence that a variable region of the osteonectin molecule is encoded within one exon. Biochemistry 27: 1483-1489.

Fisher LW, Whitson SW, Avioli LV, Termine JD (1983). Matrix sialoprotein of developing bone. J. Biol. Chem. 258: 12723-12727.

Fisher LW, McBride OW, Termine JD, Young MF (1990). Human bone sialoprotein. Deduced protein sequence and chromosomal localization. J. Biol. Chem. 265: 2347-2351.

Fleet JC and Hock JM (1994) Identification of osteocalcin mRNA in nonosteoid tissue of rats and humans by reverse transcription-polymerase chain reaction. J. Bone Miner. Res. 9: 1565-1573.

Flier JS (1997). Leptin expression and action: New experimental paradigms. Proc. Natl. Acad. Sci. (USA) 94: 4242-4245.

Fowler JH, Wu AM, Till JE, McCulloch EA, Siminovitch L (1967). The cellular composition of haemopoietic spleen colonies. Journal of Cellular Physiology 69: 65-71.

Fried W, Chamberlin W, Kedo A, Barone J (1976). Effects of radiation on hemopoietic stroma. Experimental Hematology 4: 310-314.

Fried W, Kedo A, Barone J (1977). Effects of cyclophosphamide and of bulsulfan on spleen colony-forming units and on haemopoietic stroma. Cancer Research 37: 1205-1209.

Friedenstein AJ, Chailakhyan RK, Lalykina KS (1970). The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Tissue Kinetics 3: 393-402.

Friedenstein AJ (1976). Precursor cells of mechanocytes. International Review of Cytology 47: 327-355.

Friedenstein AJ, Ivanov-Smolenski AA, Chailakhjan RK, Gorskeja UF, Kuralesova AI, Latzinik NV, Gerasimov UF (1978). Origin of bone marrow stromal mechanocytes in radiochimeras and heterotropic transplants; Exp. Hematol. 6: 440-444.

Friedenstein AJ (1980). Stromal mechanisms of bone marrow: cloning in vitro and retransplantation in vivo. In: Thienfelder S, (ed) Immunology of Bone Marrow Transplantation. Springer-Verlag Berlin. p. 19-29.

Friedenstein AJ, Latzinik NW, Grosheva AG, Gorskaya UF (1982). Marrow microenvironment transfer by heterotopic transplantation of freshly isolated and cultured cells in porus sponges. Exp. Hematol. 10: 217-227.

Friedenstein AJ, Latzinik NV, Gorskaya YF, Luria EA, Moskvina IL (1992). Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone and Mineral 18: 199-213.

Frohman MA, Dush MK, Martin GR (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. (USA) 85: 8998-9002.

Fromigue O, Marie PJ, Lomri A (1998). Bone morphogenic protein-2 and transforming factor-beta2 interact to modulate human bone marrow stromal cell proliferation and differentiation. J. Cell Biochem. 68: 411-426.

Gallagher JT (1989). The extended family of proteoglycans: social residents of the pericellular zone. Curr. Opin. Cell. Biol. 1:1201-1218.

Gallagher JA, Gundle R, Beresford JN (1996). Isolation and culture of bone-forming cells (osteoblasts) from human bone. In: Jones GE (ed), Methods in Molecular Medicine: Human Cell Culture Protocols. Humana Press, Totowa, NJ. p. 233-262.

Galmiche MC, Koteliansky VE, Briere J, Herve P, Charbord P (1993). Stromal cells from long-term marrow cultures are mesenchymal cells that differentiate following a smooth muscle differentiation pathway. Blood 82: 66-76.

Gartner S and Kaplan H (1980). Long-term culture of human bone marrow cells. Proc. Natl. Acad. Sci. (USA) 77: 4756-4759.

Gazit D, Karmish M, Holzman L, Bab I (1990). Regenerating marrow induces systemic increase in oseto- and chondrogenesis. Endocrinology 126: 2607-2613.

Geoffroy V, Corral DA, Zhou L, Lee B, Karsenty G (1998). Genomic organization, expression of the human CBFA1 gene, and evidence for an alternative splicing event affecting protein function. Mammalian Genome 9:54-57.

George F, Poncelet P, Laurent JC, Massot O, Arnoux D, Lequeux N, Ambrosi P, Chicheportiche C, Sampol J (1991). Cytofluorometric detection of human endothelial cells in whole blood using S-Endo 1 monoclonal antibody. J. Immunol. Methods 139: 65-75.

Gerasimov YV and Chailakhyam RK (1979). Effects of curettage of the medullary cavity on bone marrow precursor cells. Bull. Exp. Biol. Med. 86: 362-365.

Gerdes J, Schwab U, Lemke H, Stein H (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer 31: 13-20.

Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H (1984). Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J. Immunol. 133: 1710-1715.

Gerstenfeld LC and Shapiro FD (1996). Expression of bone-specific genes by hypertrophic chondrocytes: implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. J. Cell Biochem. 62: 1-9.

Gerstenfeld LC, Chipman SD, Glowaki J, lian JB, (1987). Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Develop. Biol. 122: 49-60.

Gerstenfeld LC, Chipman SD, Kelly CM, Hodjens KJ, Lee D, Landis W (1988). Collagen expression ultra-structure assembly and mineralization in cultures of chicken embryo osteoblasts. J. Cell Biol. 106: 979-989.

Gerstenfeld LC, Zurakowski D, Schaffer JL, Nichols DP, Toma CD, Broess M, Bruder SP, Caplan AI (1996). Variable hormone responsiveness of osteoblast populations isolated at different stages of embryogenesis and its relationship to the osteogenic lineage. Endocrinology 137: 3957-3968.

Gimble JM, Dorheim M-A, Cheng Q, Medina K, Wang C-S, Jones R, Koren E, Pietrangeli C, Kincade PW (1990). Adipogenesis in a murine bone marrow stromal cell line capable of supporting B lineage lymphocyte growth and proliferation: biochemical and molecular characterization. Eur. J. Immunol. 20: 379-387.

Gimble JM, Robinson CE, Wu X, Kelly KA (1996). The function of adipocytes in the bone marrow stroma: An update. Bone 19:421-428.

Glenn K, Bowen-Pope DF, Ross R (1982). Platelet derived growth factor III. Identification of a platelet derived growth factor receptor by affinity labeling. J. Biochem. 257: 5172-5176.

Goldblum SE, Ding X, Funk SE, Sage EH (1994). SPARC (secreted protein acidic and rich in cysteine) regulates endothelial cell shape and barrier function. Proc. Acad Sci. 91: 3448-3452.

Golde DW, Hocking WG, Quan SG, Sparkes RS, Gale RP (1980). Origin of bone marrow fibroblasts. Br. J. Haematol. 44: 183-187.

Gordon MY, Riley GP, Watt SM, Greaves MF (1987). Comparmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 326: 403-405.

Goshima J, Goldberg V, Caplan A (1991). The osteogenic potential of culture expanded rat marrow mesenchymal cells assayed in vivo in calcium phosphate ceramic blocks. Clin. Orthop. 262: 298-311.

Graves S, Smoothy CA, Beresford JN, Francis MJO (1991). L-ascorbic acid 2-phosphate promotes proliferation, differentiation and matrix production of human bone-derived cells. J. Bone Joint Surg. [Br] 73-B: 126 (absract).

Greenberger JS (1978). Sensitivity of corticosteroid-dependent insulin-resistant lipogenesis in marrow preadipocytes of obese-diabetic (db/db) mice. Nature 275: 752-754.

Greenberger JS (1979). Corticosteroid-dependent differentiation of human marrow preadipocytes in vitro. In Vitro 15: 823-828.

Grigoriadis AE, Heersche JNM, Aubin JE (1988). Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: Effect of Dexamethasone. J. Cell Biol. 106: 2139-2151.

Gronowicz G, Woodiel FN, McCarthy M-B, Raisz LG (1989). In vitro mineralization of fetal rat parietal bones in defined serum free medium: effect of beta-glycerol phosphate. J. Bone Min. Res. 4: 313-324.

Gronthos S, Ohta S, Graves SE, Simmons PJ (1994). The STRO-1$^+$ fraction of adult human bone marrow contains the osteogenic precursors. BLOOD 84: 4164-4173.

Gronthos S, Simmons PJ (1995). The growth factor requirements of STRO-1$^+$ human bone marrow stromal precursors under serum-deprived conditions. BLOOD 85: 929-940.

Gronthos S, Simmons PJ (1996). The Biology and Application of Human Bone Marrow Stromal Cell Precursors (Review). Journal of Hematotherapy 5: 15-23.

Gronthos S, Stewart K, Graves SE, Hay S, Simmons PJ (1997). Integrin Expression and Function on Human Osteoblast-like Cells. Journal of Bone and Mineral Research 12: 1189-1197.

Gronthos S, Graves SE, Simmons PJ (1998): Isolation, Purification And In Vitro Manipulation Of Human Bone Marrow Stromal Precursor Cells. In: Owen M and Beresford JN (eds), Practical Animal Cell Biology, Cambridge University Press UK, Chapter 3, pp. 26-42.

Gronowicz G and De Rome ME (1994). A synthetic peptide containing Arg-Gly-Asp inhibits bone formation and resorption in a mineralizing organ culture system of fetal rat parietal bones. J. Bone Miner. Res. 9: 193-201.

Grzesik WJ and Gehron Robey P (1994). Bone matrix RGD glycoproteins: Immunolocalization and interaction with human primary osteoblastic bone cells in vitro. J. Bone Miner. Res. 9: 487-496.

Guan J-L and Hynes RO (1990). Lymphoid cells recognize an alternately spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. Cell 60: 53-61.

Gundle R, Joyner CJ, Triffit JT (1995). Human bone tissue formation in diffusion chamber culture in vivo by bone-derived and marrow stromal fibroblastic cells. Bone 16: 597-601.

Hall BK (1978). Developmental and cellular skeletal biology. Academic Press, New York.

Hall AK and DicksonIR (1985). The effects of parathyroid hormone on osteoblast-like cells from embryonic chick calcaria. Acta. Endocrinol. 108: 217-233.

Hanada K, Dennis JE, Caplan AI (1997). Stimulatory effects of basic fibroblast growth factor and bone morphogenic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. J. Bone Miner. Res. 12: 1606-1614.

Harada S-I, Matsumoto T, Ogata E (1990). Role of ascorbic acid on the action of insulin-like growth factor in osteoblast like MC3T3-E1 cells. Bone and Mineral 10: S292.

Harada S, Matsumoto T, Ogata E (1991). Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells. J. Bone Min. Res. 6: 903-908.

Harris H (1990). The human alkaline phosphatases: what we know and what we don't know. Clin. Chim. Acta 186: 133-150.

Hart CE, Forstorm JW, Kelly JD, Seifert RA, Smith RA, Ross R, Murray MJ, Bowen-Pope DF (1988). Two classes of PDGF receptor recognise different isoforms of PDGF. Science 240: 1529-1531.

Hata R-I and Senoo H (1989). L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three dimensional tissue-like substance by skin fibroblasts. J. Cell Phisiol. 138: 8-16.

Hattersley G, Kerby JA, Chambers TJ (1991). Identification of osteoclast precursors in multilineage hemopoietic colonies. Endocrinology 128: 259-262.

Hauner H, Rohrig K, Petruscke T (1995). Effects of epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) on human adipocyte development and function. Eur. J. Clin. Invest. 25: 90-96.

Hauschka PV, Lian JB, Gallop PM (1975). Direct identification of the calcium-binding amino acid γ-carboxglutamate, in mineralized tissue. Proc. Natl. Acad. Sci. (USA) 72: 3925- 3929.

Haylock DN, To, LB, Dowse, TL, Juttner CA, Simmons, PJ (1992). Ex vivo expansion and maturation of peripheral blood $CD34^+$ cells into the myeloid lineage. Blood 80: 1405-1412.

Hayman EG, Pierschbacher MD, Ohgren Y, Ruoslahti E (1983). Serum spreading factor (vitronectin) is present at the cell surface and in tissues. Proc. Natl. Acad. Sci. (USA) 80: 4003-4007.

Hayman EG, Pierschbacher MD, Ruoslahti E (1985). Detachment of cells from culture substrate by soluble fibronectin peptides. J. Cell Biol. 100: 1948-1954.

Haynesworth SE, Goshima J, Goldberg VM, Caplan AI (1992a). Characterization of cells with oseogenic potential from human marrow. Bone 13: 81-88.

Haynesworth SE, Baber MA, Caplan AI (1992b). Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 13: 69-80.

Heldin CH, Backstrom G, Ostman A, Hammacher A, Ronnstrand L, Rubin K, Nister M, Westermark B (1988). Binding of dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J. 7: 1387-1393.

Helfrich MH, Nesbitt SA, Dorey EL, Horton MA (1992). Rat osteoclasts adhere to a wide range of RGD (Arg-Gly-Asp) peptide containing proteins, including the bone sialoproteins and fibronectin via a β_3 integrin. J Bone Miner. Res. 7: 335-343.

Helfrich MH, Nesbitt SA, Lakkakorpi PT, Barnes MJ, Bodary SC, Shankar G, Mason WT, Mendrick DL, Vaananen HK, Horton MA (1996). β_1 integrins and osteoclast function: involvement in collagen recognition and bone resorption. Bone 19: 317-328.

Herbertson A and Aubin JE (1997). Cell sorting enriches osteogenic populations in rat bone marrow stromal cell cultures. Bone 21: 491-500.

Hirata J, Kaneko S, Nishimura J, Motomura S, Ibayashi H (1985). Effects of platelet-derived growth factor and bone marrow-conditioned medium on the proliferation of human bone marrow derived fibroblastoid colony-forming cells. Acta. Haemat. 74: 189-194.

Hitomi K, Torii Y, Tsukagoshi N (1992). Increase in the activity of alkaline phosphatase by L-ascorbate 2-phosphate in a human osteoblast cell line, HuO-3N1. J. Nutr. Sci .Vitaminol. Tokyo 38: 535-544.

Hogg N (1991). An integrin overview. Chem. Immunol. 50: 1-12.

Holyoake TL and Alcorn MJ (1994). $CD34^+$ positive haemopoietic cells: Biological and clinical applications. Blood Reviews 8: 113-124.

Hooper WC (1991). The role of transforming growth factor beta in hematopoiesis. A review. Leuk. Res. 15: 179-184.

Horton MA and Davies J (1989) Perspectives: Adhesion receptors in bone. J. Bone Miner. Res. 4: 803-808.

Horton MA, Taylor ML, Arnett TR, Helfrich MH (1991). Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. Exp. Cell Res. 195: 368-375.

Howlett C, Cave J, Williamson M, Farmer J, Ali S, Bab I, Owen ME (1986). Mineralization in in vitro cultures of rabbit marrow stromal cells. Basic Sci. Pathol. 213: 251-263.

Huang S and Terstappen LWMM (1992). Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells. Nature 360: 745-749.

Hughes DE, Salter DM, Dedhar S, Simpson R (1993). Integrin expression in human bone. J. Bone Miner. Res. 8: 527-533.

Hughes DE, Salter DM, Simpson R (1994). CD44 expression in human bone: a novel marker of osteocyte differentiation. J. Bone Miner. Res. 9: 39-44.

Humphries MJ, Newham P (1998). The structure of cell-adhesion molecules. Trends Cell Biol. 8:78-83.

Hunt P, Robertson D, Weiss D, Rennick D, Lee F, Witte ON (1987). A single bone marrow derived stromal cell type supports the in vitro growth of early lymphoid and myeloid cells. Cell 48: 997-1007.

Hunter GK, Heersche JN, Aubin JE (1983). Isolation of three species of proteoglycan synthesized by cloned bone cells. Biochemistry 22: 831-837.

Hunter GK, Kyle CL, Goldberg HA (1994). Modulation of crystal formation by bone phosphoproteins: Structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J. 300: 723-728.

Hynes RO (1985). Molecular biology of fibronectin. Ann. Rev. Cell Biol. 1:67-90.

Hynes RO (1987) Integrins: a family of cell surface receptors. Cell 48: 549-554.

Hynes RO (1990). Fibronectins. Springer-Verlag, New York.

Hynes RO (1992). Integrins: Versatility, modulation and signalling in cell adhesion. Cell 69: 11-25.

Irlin Y and Peled A (1992). Thy-1 antigen-mediated adhesion of mouse lymphoid cells to stromal cells of haemopoietic origin. Immunol. Lett. 33: 233-238.

Ishida Y and Heersche JNM (1997). Progesterone stimulates proliferation and differentiation of osteoprogenitor cells in bone cell populations derived from adult female but not from adult male rats. Bone 20: 17-25.

Ishikawa Y, Wuthier RE (1992). Development of an in vitro mineralization model with growth plate chondrocytes that does not require β-glycerophosphate. Bone and Mineral 17: 152-156.

Isogai Y, Akatsu T, Ishizuya T, Yamaguchi A, Hori M, Takahashi N, Suda T (1996). Parathyroid hormone regulates osteoblast differentiation positively or negatively depending on the differentiation stages. J. Bone Miner. Res. 11: 1384-1393.

Issekutz TB (1991). Inhibition of in vivo lymphocyte migration to inflamation and homing to lymphoid tissues by the TA-2 monoclonal antibody: A likely role for VLA-4 in vivo. The Journal of Immunology 147: 4178-4184.

Itoh K, Tezuka H, Sakoda H, Konno M, Nagata K, Uchiyama T, Uchino H, Mori KJ (1989). Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. Exp. Hematol. 17: 145-153.

Iyer J, Duerst RE, Looney JN, Humphries RK, Abboud CN, Frantz CN (1990). An 80,000-kd glycoprotein cell surface antigen found only on nonhematopoietic cells in human bone marrow. Exp. Hematol. 18: 384-389.

Jacobsen K, Kravitz J, Kincade PW, Osmond DG (1996). Adhesion receptors on bone marrow stromal cells: in vivo expression of vascular cell adhesion molecule-1 by reticular cells and sinusoidal endothelium in normal and gamma-irradiated mice. Blood 87: 73-82.

Jackson RL, Busch SJ, Cardin AD (1991). Glycosaminoglycans: molecular properties, protein interactions and role in physiological processes. Physiol. Rev. 71: 481-539.

Jaiswal N, Haynesworth SE, Capaln AI, Bruder SP (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J. Cell Biochem. 64: 295-312.

James IE, Dodds RA, Olivera DL, Nuttall ME, Gowen M (1996). Human osteoclastoma-derived stromal cells: correlation of the ability to form mineralized nodules in vitro with formation of bone in vivo. J. Bone Miner. Res. 11: 1453-1460.

Johansen JS, Williamson MK, Rice JS, Price PA (1992). Identification of proteins secreted by human osteoblastic cells in culture. J. Bone Miner. Res. 7: 501-512.

Johnson A and Dorshkind K (1986). Stromal cells in myeloid and lymphoid long-term bone marrow cultures can support multiple hemopoietic lineages and modulate their production of hemopoietic growth factors. Blood 68: 1348-1354.

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998). In vitro chondrogenesis of bonemarrow-derived mesenchymal progenitor cells. Exp. Cell Res. 238: 265-272.

Joyner CJ, Bennett A, Triffitt JT (1997). Identification and enrichment of human osteoprogenitor cells by using differentiation stage-specific monoclonal antibodies. Bone 21: 1-6.

Juliano RL and Haskill S (1993). Signal transduction from the extracellular matrix. J. Cell Biol. 120: 577-585.

Junqueira LC, Carneiro J, Kelly RO (1989). Basic Histology. A Lange Medical Book Sixth Edition, Prentice-Hall International Inc. p137.

Kamalia N, McCulloch AG, Tenenbaum HC, Limeback H (1992a). Direct flow cytometric quantification of alkaline phophatase activity in rat marrow stromal cells. J. Histochem. Cytochem. 40: 1059-1065.

Kamalia N, McCulloch CAG, Tenebaum HC, Limeback H (1992b). Dexamethasone recruitment of self-renewing osteoprogenitor cell in chick bone marrow stromal cultures. Blood 79: 320-326.

Kaneko S, Motomura S, Ibayashi H (1982). Differentiation of human bone marrow derived fibroblastoid colony forming cells (CFU-F) and their roles in haemopoiesis in vitro. Br. J. Haematol. 51: 217-225.

Kasperk C, Wergedal JE, Strong D, Farley J, Wangerin K, Gropp H, Ziegler R, Baylink DJ (1995). Human bone cell phenotypes differ depending on their skeletal site of origin. J. Clin. Endocrinol. Metab. 80: 2511-2517.

Kassem M, Risteli L, Mosekilde L, Melsen F, Eriksen EF (1991). Formation of osteoblast-like cells from human mononuclear bone marrow cultures. APMIS 99:269-74.

Kasugai S, Todescan R Jr, Nagata T, Yao K-L, Butler WT, Sodek J (1991). Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells in vitro: inductive effects of dexamethasone on the osteoblastic phenotype. J Cell Physiol. 147: 111-120.

Keating A, Singer JW, Killen PD, Striker GE, Salo AC, Sanders J, Donnall Thomas D, Thorning D, Failkoow PJ (1982). Donor origin of the in vitro haematopoietic microenvironment after marrow transplantation in man. Nature 298: 280-283.

Keiliss-Borok IV, Latzinik NV, Epichina SY, Friedenstein AJ (1971). Dynamics of the formation of fibroblast colonies in monolayer cultures of bone marrow according to ^{3}H thymidine incorporation experiments. Cytologia 13: 1402-1411

Kelly K, Cochran BH, Stiles CD, Leder P (1983). Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35: 603-610.

Kerby JA, Hattersley G, Collins DA, Chambers TJ (1992). Derivation of osteoclasts from hematopoietic colony-forming cells in culture. J. Bone Miner. Res. 7: 353-362.

Khouja HI, Bevington A, Kemp GJ, Russell RGG (1990). Calcium orthophosphate deposits in vitro do not imply osteoblast-mediated mineralization: mineralization by beta-glycerolphosphate in the absence of osteoblasts. Bone 11: 385-391.

Kiefer MC, Stephans JC, Crawford K, Okino K, Barr PJ (1990). Ligand-affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA 87: 6985-6989.

Kim CW, Goldberger OA, Gallo RL, Bernfield M (1994). Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns. Mol. Biol. Cell 5:797-805.

Kim RH, Li JJ, Ogata Y, Yamauchi M, Freedman LP, Sodek J (1996). Identification of a vitamin D3-response element that overlaps a unique inverted TATA box in the rat bone sialoprotein gene. Biochem. J. 318: 219-226.

Kimura A, Katoh O, Kuramoto A (1988). Effects of platelet derived growth factor, epidermal growth factor and transforming growth factor-β on the growth of human marrow fibroblasts. British J. Haematol. 69: 9-12.

Kincade PW, Lee G, Pietrangeli CE, Hayashi SI, Gimble JM (1989). Cells and molecules that regulate B-lymphopoiesis in bone marrow. Ann. Rev. Immunol. 7: 111-143.

King AC and Cuatrecassas P (1982). Resolution of high and low affinity epidermal growth factor receptors. Inhibition of high affinity component by low temperature, cycloheximide and phorbol ester. J. Biol. Chem. 257: 3053-3060.

Knospe WH, Blom J, Crosby WH (1966). Regeneration of locally irradiated bone marrow. Blood 28: 398-415.

Knospe WH, Gregory SA, Husseini SG et al (1972). Origin and recovery of colony-forming units in locally curetted bon marrow in mice. Blood 39: 331-340.

Knospe WH, Husseini SG, Zipori D, Fried W (1993). Hematopoiesis on cellular ester membranes. XIII. A combination of cloned stromal cells is needed to establish a hematopoietic microenvironment supportive of trilineal hematopoiesis. Exp. Hematol. 21: 257-262.

Kodama H, Hagiwara H, Sudo H (1986). MC3T3-G2 PA6 preadipocytes support in vitro proliferation of haemopoietic stem cells through a mechanism different to that of interleukin-3. J. Cell Physiol. 129: 11-26.

Koenigsmann M, Griffin JD, DiCarlo J, Cannistra SA (1992). Myeloid and erythroid progenitor cells from normal bone marrow adhere to collagen type I. Blood 79: 657-665.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Degucchi K, Shimizu Y, Bronson RT, Gao Y-H, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997). Targeted disruption of CBFA1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89: 755-764.

Koshihara Y, Kawamura M, Oda H, Higaki S (1987). In vitro calcification of human osteoblastic cell line derived from periosteum. Biochem. Biophys. Res. Comm. 145: 651-657.

Koutsoukos PG and Nancollas GH (1981). Crystal growth of calcium phosphates-epitaxial considerations. J. Crystal Growth 53: 10-15.

Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RVB, Rowe DW, Robey PG (1997). Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation 63: 1059-1069.

Kruijer W, Cooper JA, Hunter T, Verma IM (1984). Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. Nature 312: 711-716.

Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, Robey PG (1997). Single-colony derived strains of human stromal fibroblasts form bone after transplantation in vivo. J. Bone Miner. Res. 12: 1335-1347.

Kuzu I, Bicknell R, Fletcher CDM, Gatter KC (1993). Expression of adhesion molecules on the endothelium of normal tissue vessels and vascular tumors. Lab. Invest. 69: 322-328.

Lajeunesse D, Kiebzak GM, Frondoza C, Sacktor B (1991). Regulation of osteocalcin secretion by human primary bone cells and the human osteocarcoma cell line MG-63. Bone and Mineral 14: 237-250.

Lansdorp PM and Dragowska W (1992). Long-term erythropoiesis from constant numbers of $CD34^+$ cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. J. Exp. Med. 175: 1501-1509.

Latsinik NV, Fridenshtein A, Gorskaia I, Moskvina IL, Iui KC (1990). The dependence of the formation of stromal CFU-f colonies on the stimulating action of hematopoietic cells. Biull. Eksp. Biol. Med. 110:519-22.

Lawson GM, Katzmann JA, Kimlinger TK, O'Brien JF (1985). Isolation and preliminry characterization of a monoclonal antibody that interacts preferentially with the liver isoenzyme of human alkaline phosphatase. Clin. Chem. 31: 381-385.

Leboy PS, Beresford JN, Devlin C, Owen ME (1991). Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J. Cell Physiol. 146: 370-378.

Lee K, Deeds JD, Bond AT, Juppner H, Abou-Samara A-B, Segre GV (1993). In situ localization of PTH/PTHrPreceptor mRNA in the bone of fetal and young rats. Bone 14: 341-345.

Lee K, Deeds JD, Chiba S,. Un-no M, Bond AT, Segre GV (1994). Parathyroid hormone induces sequential c-fos expression in bone cells in vivo: In situ localization of its receptor and c-fos messenger ribonucleic acids. Endocrinology 134: 441-450.

Levesque J-P, Leavesley DI, Niutta S, Vadas M, Simmons PJ (1995). Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. J. Exp. Med. 181: 1805-1815.

Lewis JP and Trobaugh FE (1964). Haematopoietic stem cells. Nature 204: 589-590.

Li CL and Johnson GR (1992). Rhodamine 123 reveals heterogeneity within the lin^-, $sca\text{-}1^+$ hemopoetic cells. J. Exp. Med. 175: 1443-1447.

Li KJ, Dilber MS, Abedi MR, Bjorkstrand B, Smith CIE, Garoff H, Gahrton G, Xanthopoulos KG (1995). Retroviral-mediated gene transfer into human bone marrow stromal cells: studies of efficiency and in vivo survival in SCID mice. Eur. J. Haematol. 55: 302-306.

Lichtman MA (1981). The ultrastructure of the hemopoietic environment of the marrow: A Review. Experimental Hematology 9: 391-410.

Lim B, Izaguirre CA, Aye MT, Huebsch L, Drouin J, Richardson C, Minden MD, Messner HA (1986). Characterization of reticulofibroblastoid colonies (CFU-RF) derived from bone marrow and long-term marrow culture monolayers. J. Cell Physiol. 127: 45-54.

Liu F, Malaval L, Aubin JE (1997). The mature osteoblast phenotype is characterized by extensive plasticity. Exp. Cell Res. 232: 97-105.

Locklin RM, Williamson MC, Beresford JN, Triffit JT, Owen ME (1995). In vitro effects of growth factors and dexamethasone on rat marrow stromal cells. Clin. Orthop. 313: 27-35.

Lomri A, Marie PJ Tran PV, Hott M (1988). Characterization of endosteal osteoblastic cells isolated from mouse caudal vertebrae. Bone 9: 165-175.

Long MW, Williams JL, Mann KG (1990). Expression of human bone-related proteins in the hematopoietic microenvironment. J. Clin. Invest. 86: 1387-1395.

Long MW (1992). Blood cell cytoadhesion molecules. Exp. Hematol. 20: 288-301.

Long MW, Briddell R, Walter AW, Bruno E, Hoffman R (1992). Human hematopoietic stem cell adherence to cytokines and matrix molecules. J. Clin. Invest. 90: 251-255.

Lopez-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, Massague J (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-β receptor system. Cell 67: 785-795.

Lowenthal JW, Castle BE, Christiansen J, Schreurs J, Rennick D, Arai N, Hoy P, Takebe Y, Howard M (1988). Expression of high affinity receptors for murine interleukin-4 (BSF-1) on hemopoietic and nonhemopoietic cells. J. Immunol. 140: 456-464.

Luria EA, Owen ME, Friedenstein AJ, Morris JF, Kuznetsow SA (1987). Bone formation in organ cultures of bone marrow. Cell Tissue Res. 248: 449-454.

MacDonald D, Adams JA, McCarthy D, Barret AJ (1990). Interleukin-2 inhibits Growth of Fibroblasts Derived from Human Bone Marrow. Acta. Haemat. 83: 26-30.

MacDougald OA and Lane MD (1995). Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem. 64: 345-373.

MacManus PM and Weiss L (1984). Bulsulfan-Induced Chronic Bone Marrow Failure: Changes in Cortical Bone, Marrow Stromal Cells, and Adherent Cell Colonies. Blood 64: 1036-1041.

MacNeil RL, Sheng N, Strayhorn C, Fisher LW, Somerman MJ (1994). Bone sialoprotein is localized to the root surface during cementogenesis. J. Bone Miner. Res. 9: 1597-1606.

Macrum JA, Reilly CF, Rosenberg RD (1987). In: Wight TN and Mecham RP, (eds) Biology of proteoglycans. Academic Press, San Diego, CA. p.301-343.

Manduca P, Sanguineti C, Pistone M, Boccignone E, Sanguineti F, Santolini F, Federici A (1993). Differential expression of alkaline phosphatase in clone of human osteoblast-like cells. J. Bone Miner. Res. 8: 291-300.

Maniatis A, Tavassoli M, Crosby WH (1971). Origin of osteogenic precursor cells in extramedullary marrow implants. Blood 38: 569-575.

Maniatopoulos C, Sodek J, Melcher AH (1988). Bone formation in vitro by stromal cells obtained from the bone marrow of young rats. Cell Tissue Res. 254: 317-330.

Mardon HJ, Bee J, von der Mark K, Owen ME (1987). Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. Cell Tissue Res. 250: 157-165.

Marie PJ, Lomri A, Sabbagh A, Basle M (1989). Culture and behavior of osteoblastic cells isolated from normal trabeculer bone surfaces. In Vitro Cell Dev Biol 25: 373-379.

Mark MP, Prince CW, Oosawa T, Gay S, Bronkers ALJJ, Butler WT (1987). Immunohistochemical demonstration of a 44 kDa phosphoprotein in developing rat bones. J. Histochem. Cytochem. 35: 707-716.

Mark MP, Prince CW, Gay S, Austin RL, Butler WT (1988a). 44 kDa bone phosphoprotein antigenicity at ectopic sites in newborn rats: Kidney and nervous tissue. Cell Tissue Res. 251: 23-30.

Mark MP, Butler WT, Prince CW, Finkelman RD, Ruch J-V (1988b). Developmental expression of a 44 kDa bone phosphoprotein (osteopontin) and bone γ-carboxyglutamic acid (gla)-containing protein (osteocalcin) in calcifying tissue of rats. Differentiation 37: 123-136.

Marks SC and Hermey DC (1996). The structure and development of bone. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 3-14.

Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R (1997). Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology 138: 4456-4462.

Martinet Y, Bitterman PB, Mornex JF, Grotendorst GR, Crystal RG (1986). Activated human monocytes express the c-sis proto-oncogene and release a mediator showing PDGF-like activity. Nature 319: 158-160.

Massague J and Pandiella A (1993). Membrane-anchored growth factors. Ann. Rev. Biochem. 62: 515-541.

Masuzaki H, Ogawa Y, Isse N, Satoh N, Okazaki T, Shigemoto M, Mori K, Tamura N, Hosoda K, Yoshimasa Y, Jingami H, Kawada T, Nakao K (1995). Human Obese gene expression. Adipocyte-specific expression and regional differences in adipose tissue. DIABETES 44: 855-858.

Mathieu E, Schoeters G, Vander Plaetse F, Merregaert J (1992). Establishment of an osteogenic cell line derived from adult mouse bone marrow stroma by use of a recombinant retrovirus. Calcif. Tissue Int. 50: 362-371.

Matsui T, Heidaran M, Miki T, Popescu N, La Rochelle W, Kraus M, Pierce J, Aaronsson S (1989). Isolation of a novel receptor cDNA establishes the existance of two PDGF receptor genes. Science 243: 800-804.

Matsumoto T, Igarashi C, Takeuchi Y, Harada S, Kikuchi T, Yamato H, Ogata E (1991). Stimulation by 1,25-dihydroxyvitamin D_3 of in vitro mineralization induced by osteoblast-like MC3T3-E1 cells. Bone 12: 27-32.

McCulloch CAG, Strugurescu M, Hughes F, Melcher AH, Aubin JE (1991). Osteogenic progenitor cells in rat bone marrow stromal poulations exhibit self-renewal in culture. Blood 77: 1906-1911.

McIntyre AP and Bjornson BH (1986). Human bone marrow cell colonies: response to hydrocortisone and dependence on platelet-derived growth factor. Exp. Hematol. 14: 833-839.

McNiece IK, Langley KE, Zsebo KM (1991). Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages. Exp. Hematol. 19: 226-231.

Meazzini MC, Toma CD, Schaffer JL, Gray ML, Gerstenfeld LC (1998). Osteoblast cytoskeletal modulation in response to mechanical strain in vitro. J. Orthop. Res. 16: 170-80.

Metcalf D (1993). Hematopoietic regulators: redundancy or subtlety?. Blood. 82: 3515-3523.

Migliaccio G, Migliaccio AR, Petti S, Mavillio F, Russo G, Lazzaro D, Testa U, Marinucci M, Peschle C (1986). Human embryonic hemopoiesis. J. Clini. Invest. 78: 51-60.

Migliaccio G., Migliaccio A.R., Adamson J.W (1988). In vitro differentiation of human granulocyte/macrophage and erythroid progenitors: comparative analysis of the influence of recombinant human erythropoietin, G-CSF, GM-CSF, and IL-3 in serum-supplemented and serum-deprived cultures. Blood 72: 248-256.

Millan JL (1988). Oncodevelopment expression and structure of alkaline phosphatase genes. Anticancer Res. 8: 995-1004.

Miller AD and Buttimore C (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell Biol. 6: 2895-2902.

Miller AD, Miller DG, Garcia JV, Lynch CM (1993). Use of retroviral vectors for gene transfer and expression. Methods in Enzymology 217: 581-599.

Mills BG, Singer FR, Weiner LP, Holst PA (1979). Long-term culture of cells from bone affected by Paget's disease. Calcif. Tissue Int. 29: 79-87.

Mintz KP, Fisher LW, Grzesik WJ, Hascall VC, Midura RJ (1994). Chlorate-induced inhibition of tyrosine sulfation on bone sialoprotein synthesized by a rat osteoblast-like cell line (UMR 106-01 BSP). J. Biol. Chem. 269: 4845-4852.

Miyajima A, Kitamura T, Harade N, Yokota T, Arai K (1992). Cytokine receptors and signal transduction. Ann. Rev. Immunol. 10: 295-331.

Mizuno M, Shindo M, Kobayashi D, Tsuruga E, Amemiya A, Kuboki Y (1997). Osteogenesis by bone marrow stromal cells maintained on type I collagen matrix gells in vivo. Bone 20: 101-107.

Montesano R and Orci L (1985). Tumour promoting phorbal esters induce angiogenesis in vitro. Cell 42: 469-477.

Moore MAS and Metcalf D (1970). Ontology of haempoietic system: yolk sac origin of in vivo and invitro colony forming cells in the developing mouse embryo. British Journal of Haematology 18: 279-296.

Moore MAS (1991). Clinical implications of the positive and negative hematopoietic stem cell regulators. Blood 78: 1-19.

Morris SA and Bilezikian JP (1996). Signal transduction in bone physiology: messenger systems for parathyroid hormone. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 1203-1215.

Morrison SJ, Uchida N, Weissman IL (1995). The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. 11: 35-71.

Moss DW (1992). Perspectives in alkaline phosphatase research. Clin. Chem. 28: 2486-2492.

Mould AP and Humphries MJ (1991). Identification of a novel recognition sequence for the integrin $\alpha 4\beta 1$ in the COOH-terminal heparin-binding domain of fibronectin. EMBO J. 10: 4089-4095.

Muench MO, Firpo MT, Moore MAS (1993). Bone marrow transplantation with interleukin-1 plus kit-ligand ex vivo expanded bone marrow accelerates reconstitution in mice without the loss of stem cell lineage and proliferative potential. Blood 81: 3463-3473.

Nakahara H, Goldberg VM, Caplan AI (1991). Culture-expanded human periosteal-derived cells exhibit osteochondral potential in porous calcium phosphate ceramics in vivo. Clin. Orthop. Rel. Res. 276: 291-298.

Nefussi J, Boy-Lefevre M, Boulekbache H, Forest N (1985). Mineralization in vitro of matrix formed by osteoblasts isolated by collagenase digestion. Differentiation 29: 160-168.

Nijweide PJ and Mulder RJP (1986). Identification of osteocytes in osteoblast like cultures using a monoclonal antibody specifically directed against osteocytes. Histochemistry 84: 343-350.

Nijweide PJ, Van der Plas A, Olthof AA (1988). Osteoblastic differentiation. In: Everd D and Harnett S (eds) Cell and molecular biology of vertabrate hard tissues. John Wiley & Sons, Chichester. Ciba Found. Symp. 136: 66-77.

Nijweide PJ, Burger EH, Klein Nulend J, Van der Plas A (1996). The osteocyte. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, pp 115-126.

Nilsson J, Thyberg J, Heldin CH, Westermark B, Wasteson A (1983). Surface binding and internalization of platelet-derived growth factor in human fibroblasts. Pro. Natl. Acad. Sci. (USA) 80: 5592-5596.

Nilsson J, Sjolund M, Palmberg L, Thyberg J, Heldin CH (1985). Arterial smooth muscle cells in primary culture produce a platelet derived growth factor-like protein. Proc. Natl. Acad. Sci . (USA) 82: 4418-4422.

Nomura S, Wills A, Edwards DR, Heath JK, Hogan BLM (1988). Developmental expression of 2ar (osteopontin) and SPARC (osteonectin) RNA as revealed by insitu hybridization. J. Cell Biol. 106: 441-450.

Ogawa E, Maruyama M, Kagoshima H, Inuzuka M, Lu J, Satake M, Shigesada K, Ito Y (1993). PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. Proc. Natl. Acad. Sci. (USA) 90: 6859-6863.

Oguri K, Okayama E, Caterson B, Okayama M (1987). Isolation, characterization, and localization of glycosaminoglycans in rabbit bone marrow. Blood 170: 501-510.

Ohgushi H, Goldberg VM, Caplan AI (1989). Heterotopic osteogenisis in porous ceramics induced by marrow cells. J. Orthop. Res. 7: 568-578.

Ohgushi H, Dohi Y, Tamai S, Tabata S (1993). Osteogenic differentiation of marrow stromal stem cells in porous hydroxyapatite ceramics. J Biomed. Mater. Res. 27: 1401-1407.

Ohta S, Hiraki Y, Shigeno C, Suzuki F, Kasai R, Toshihiko I, Kohno H, Lee K, Kikuchi H, Konishi J, Bentz H, Rosen DM, Yamamuro T (1992). Bone morphogenic proteins (BMP-2 and BMP-3) induce the late phase expression of the proto-oncogene c-fos in murine osteoblastic MC3T3-E1 cells. FEBS LETTERS 314: 356-360.

Oldberg A, Franzen A, Heinegard D, Pierschbacher M, Ruoslahti E (1988). Identification of bone sialoprotein receptor in osteosarcoma cells. J. Biol. Chem. 263: 19433-19436.

Oliver LJ, Rifkin DB, Gadrilove J, Hannocks MJ, Wilson EL (1990). Long-term culture of human stromal cells in the presence of basic fibroblast growth factor. Growth Factors 3: 231-236.

Olsson I, Gullberg U, Lantz M, Richter J (1992). The receptors for regulatory molecules of hematopoiesis. Eur. J. Haematol. 48: 1-9.

Olwin BB and Rapraeger A (1992). Regression of myogenic differentiation by aFGF, bFGF and K-FGF is dependent on cellular heparan sulphate. J. Cell Biol. 118: 631-639.

Onyia JE, Clapp DW, Long H, Hock JM (1998). Osteoprogenitor cells as targets for ex vivo gene transfer. 13: 20-30.

Osborn L, Hession C, Tizard R, Vassallo C, Luhowsky S, Chi-Rosso G, Lobb R (1989). Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59: 1203-1211.

Oster G (1989). Cell motility and tissue morphogenesis. In: Stein WD and Bronner F, (eds) Cell shape: Determinants, regulation and regulatory role. Academic Press, San Diego. p. 33-61.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GWH, Beddington RSP, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765-771.

Owen ME (1985). Lineage of osteogenic cells and their relationship to the stromal system. Bone and Mineral Research 3: 1-25.

Owen ME, Cave J, Joyner CJ (1987). Clonal analysis in vitro of osteogenic differentiation of marrow CFU-F. J. Cell Sci. 87: 731-738.

Owen ME and Friedenstein AJ (1988). Stromal stem cells: marrow derived osteogenic precursors. CIBA Foundation Symposium 136: 42-65.

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming LW, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990). Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with

osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell Physiol. 143: 420-430.

Ozari A, Cattoretti G, Schiro R, Siena S, Bregni M, DiNicola M, Gianni M.A (1992). rhIL-3 and rhGM-CSF administered in vivo after high dose cyclophosphamide cancer chemotherapy: the effect on the hemtopoiesis and microenvironment in human bone marrow Blood 79: 2610-2619.

Parfitt AM (1990). In: Christiansen C et al (eds) Osteoporosis. Third International Symposium on Osteoporosis. Osteopress ApS, Copenhagen p. 429.

Patt HM and Maloney MA (1975). Bone marrow regeneration afterlocal injury: A review. Exp. Haematol. 3: 135-148.

Peck WA, Binge SJ, Fedack SA (1964). Bone cells: biochemical and biological studies after enzymatic isolation. Science 146: 1476-1477.

Peled A, Zipori D, Abramsky O, Ovadia H, Shezen E (1991). Expression of α-smooth muscle actin in murine bone marrow stromal cells. Blood 78: 304-309.

Pen PE, Jiang D-Z, Fei R-G, Sitnicka E, Wolf NS (1993). Dissecting the hematopoietic microenvironment. IX. Further characterization of murine bone marrow stromal cells. Blood 81: 1205-1213.

Perkins S and Fleischman RA (1990). Stromal cell progeny of murine bone marrow fibroblast colony-forming units are clonal endothelial-like cells that express collagen IV and laminin. Blood 75: 620-625.

Pfeilschifter J, Diel I, Pilz U, Brunotte K, Naumann A, Ziegler R (1993). Mitogenic responsiveness of human bone-cells in vitro to hormones and growth-factors decreases with age. J. Bone Miner. Res. 8: 707-717.

Pickl WF, Majdic O, Fischer GF, Petzelbauer P, Fae I, Waclavicek M, Stockl J, Scheinecker C, Vidicki T, Aschauer H, Johnson JP, Knapp W (1997). MUC18/MCAM (CD146), an activation antigen of human T lymphocytes. J. Immunol. 158: 2107-2115.

Pierschbacher MD and Ruoslahti E (1984). The cell attachment activityof fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309: 30-33.

Prchal JT, Throckmorton DW, Carroll AJ, Fusion EW, Gams RA, Prchal JF (1978). A common progenitor for human myeloid and lympoid cells. Nature 274: 590-591.

Preissner KT (1991). Structure and biological role of vitronectin. Ann. Rev. Cell Biol. 7: 275-310.

Price PA, Otsuka AS, Poser JW, Kristaponis J, Raman J (1976). Characterization of the γ-carboxyglutamic acid containing proetin from bone. Proc. Natl. Acad. Sci. (USA) 73: 1447-1451.

Price PA and Baukol SA (1980). 1,25-dihydroxyvitamin D_3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. J. Biol. Chem. 24: 11660-11663.

Pri-Chen S, Pitaru S, Lokiec F, Savion N (1998). Basic fibroblast growth factor enhances the growth and expression of the osteogenic phenotype of dexamethasone-treated human bone marrow derived bone-like cells in culture. Bone 23: 111-117.

Puleo DA and Bizios R (1991). RGDS tetrapeptide binds to osteoblasts and inhibits fibronectin-mediated adhesion. Bone 12: 271-276.

Puleo DA and Bizios R (1992). Mechanisms of fibronectin-mediated attachment of osteoblasts to substrates in vitro. Bone and Mineral 18: 215-226.

Quarto R, Thomas D, Liang CT (1995). Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calcif. Tiss. Int. 56: 123-129.

Raynal C, Delmas PD, Chenu C (1996). Bone sialoprotein stimulates in vitro bone resorption. Endocrinology 137: 2347-2354.

Rawlinson SCF, Mosley JR, Suswillo RFL, Pitsillides AA, Lanyon LE (1995). Calvarial and limb bone cells in organ and monolayer culture do not show the same early responses to dynamic mechanical strain. J. Bone Miner. Res. 10: 1225-1232.

Reich KM, Gay CV, Frangos JA (1990). Fluid shear stress as a mediator of osteoblast cyclic adenosine monophosphate production. J. Cell Physiol. 143: 100-104.

Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I (1994). Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev. Biol. 161: 218-228.

Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL (1996). Isolation and characterization of osetoblast precursor cells from human bone marrow. J. Bone Miner. Res. 11: 312-324.

Riew KD, Wright NM, Cheng S, Avioli LV, Lou J (1998). Induction of bone formation using a recombinant adenviral vector carrying the human BMP-2 gene in a rabbit spinal fusion model. Calcif. Tissue Int. 63: 357-360.

Rios M and Williams DA (1990). Systematic analysis of the ability of stromal cell lines derived from different murine adult tissues to support maintenance of hematopoietic sten cells in vitro. J. Cell Physiol. 145: 434-443.

Risau W and Flamme I (1995). Vasculogenesis. Ann. Rev. Cell Dev. Biol. 11: 73-91.

Roberts R, Gallagher JT, Spooncer E, Allen TD, Bloomfield F, Dexter TM (1988). Heparan sulphate bound growth factors: a mechanism of stromal mediated haemopoiesis. Nature 332: 376-378.

Robertson M (1979). Bone-marrow transplantation: Man and mouse. Nature 280: 720-721.

Robey PG and Termine JD (1985). Human bone cells in vitro. Calcif. Tissue Int. 37: 453-460.

Robey PG, Bianco P, Termine JD (1992). The cell biology and molecular biochemistry of bone formation. In: Favus MJ, Coe FL (eds). Disorders of Mineral Metabolism. Raven Press, New York, p. 241-263.

Robey PG and Boskey AL (1995). The biochemistry of bone. In: Marcus R, Feldman D, Bilizekian JP and Kelsey J (eds). Osteoporosis. Academic Press, New York, p. 95-183.

Robey PG (1996). Bone Matrix Proteoglycans and Glycoproteins. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 155-165.

Rodan GA and Harada S-I (1997). The missing bone. Cell 89: 677-680.

Roghan M and Moscatelli D (1992). Basic fibroblast growth factor is internalized through both receptor-mediated and heparan sulphate-mediated mechanisms. J. Biol. Chem. 267: 22156-22162.

Romberg RW, Werness PG, Riggs BL, Mann KG (1986). Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry 25: 1176-1180.

Rosenfeld ME, Bowen-Pope DF, Ross R (1984). Platelet derived growth factor: morphologic and biochemical studies of binding, internalization and degradation. J. Cell Physiol. 121: 263-274.

Rosenfeld ME, Keating A, Bowen-Pope DF, Singer JW, Ross R (1985). Responsiveness of the in vitro hematopoietic microenvironment to platelet derived growth factor. Leukemia Research 9: 427-.

Ross R (1989). Platelet derived growth factor. The Lancet 1: 1179-1187.

Ross M, Francis GL, Szabo L, Wallace JC, Ballard FJ (1989). Insulin-like growth factor (IGF)-binding proteins inhibit the biological activities of IGF-1 and IGF-II but not des(1-3) IGF-1. Biochem. J. 258: 267-272.

Ross FP, Chappel J, Alvarez JI, Snader D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresh DA (1993). Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin $\alpha_V\beta_3$ potentiate bone resorption. J. Biol. Chem. 268: 9901-9907.

Rossert J and de Crombrugghe B (1996). Type I collagen: structure, synthesis, and regulation. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 127-142.

Rubinstein M, Dinarello CA, Oppenheim JJ, Hertzog P (1998). Recent advances in cytokines, cytokine receptors and signal transduction. Cytokine Growth Factor Rev. 9: 175-81.

Ruoslahti E and Pierschbacher MD (1987). New perspectives in cell adhesion: RGD and integrins. Science 238: 491-497.

Ruoslahti E (1988). Structure and biology of proteoglycans. Ann. Rev. Cell Biol. 4: 229-255.

Ruoslahti E (1989). Proteoglycans in cell regulation. 264: 13369-13372.

Ruoslahti E (1991). Integrins. J. Clin Invest. 87: 1-5.

Ruoslahti E and Yamaguchi Y (1991). Proteoglycans as modulators of growth factor activities. Cell 64: 867-869.

Saeland S, Duvert V, Caux C, Pandrau D, Favre C, Valle A, Durand I, Charboard P, de Vries J, Banchereau J (1992). Distribution of surface membrane molecules on bone marrow and cord blood $CD34^+$ hematopoietic cells. Exp. Hematol. 20: 24-33.

Saito T, Albelda SM, Brighton CT (1994). Identification of integrin receptors on cultured human bone cells. J. Orthop. Res. 12: 384-394.

Sambrook J, Fritsch EF, Maniatis T (1989). In Molecular cloning: a laboratory manual., 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Santra M, Mann DM, Mercer EW, Skorski T, Calabretta B, Lozzo RV (1997). Ectopic expression of decorin protein core causes a generalized growth suppression in neoplastic cells of various histogenetic origin and requires endogenous p21, an inhibitor of cyclin-dependent kinases. J. Clin. Invest. 100: 149-157.

Satomura K, Derubeis AR, Fedarko NS, Ibaraki-O'Connor K, Kuznetsov SA, Rowe DW, Young MF, Gehron Robey P (1998). Receptor tyrosine kinase expression in human bone marrow stromal cells. J. Cell Physiol. 177: 426-438.

Saxne T, Zunino L, Heinegard D (1995). Increased release of bone sialoprotein into synovial fluid reflects tissue destruction inrheumatoid arthritis. Arthritis Rheum. 38: 82-90.

Schiro JA, Chan BMC, Roswit WT, Kassner PD, Pentland AP, Hemler ME, Eisen AZ, Kupper TS (1991). Integrin $\alpha 2\beta 1$ (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. Cell 67: 403-410.

Schmid C, Schlapfer I, Peter M, Boni-Schnetzler M, Schwander J (1994). Growth hormone and parathyroid hormone stimulate IGFBP-3 in rat osteoblasts. Am. J. Physiol. 267: E226-E233.

Schoeters GER, de Saint-Georges L, Van Den Heuvel R, Leppens H, Vanderborght O (1988). Mineralization of adult mouse bone marrow cells in vitro Cell. Tissue Kinet. 21: 1-12.

Schoeters GER, Leppens H, Van Gorp U, Van Den Heuvel R (1992). Haemopietic long-term bone marrow cultures from adult mice show osteogenic capacity in vitro on 3-dimensional collagen sponges. Cell Prolif. 25: 587-603.

Schor AM, Canfield AE, Sutton AB, Arciniegas E, Allen TD (1995). Pericyte differentiation. Clin. Orthop. Rel. Res. 313:81-91.

Schor AM and Canfield AE (1998). Osteogenic potential of vascular pericytes. In: Beresford JN and Owen ME(eds): Marrow stromal cell culture. Handbooks in practical animal cell biology, Cambridge University Press, UK. Chapter 8, p. 128-148.

Seibel MJ, Woitge HW, Percherstofer M, Karmatschek M, Horn E, Ludwig H, Ambruster FP, Ziegler R (1996). Serum immunoreactive bone sialoprotein as a new marker of bone turnover in metabolic and malignant bone disease. J. Clin. Endocrinol. Metab. 81: 3289-3294.

Senger DR, Peruzzi CA, Gracey CF, Papadopoulos A, Tenen DG (1989). Secreted phosphoproteins associated with neoplastic transformation, close homology with plasma proteins cleaved during blood coagulation. Cancer Res. 48: 5770-5774.

Ser C, Riethmuller G, Johnson JP (1994). MUC18, a melanoma-progression molecule, and its potential role in tumor vascularization and hematogenous spread. Cancer Res. 54: 5689-5694.

Shapiro JR, Primorac D, Rowe DW (1996). Osteogenesis imperfecta: current concepts. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, p. 889-902.

Shepro D and Mrel NML (1993). Pericyte physiology. FASEB J. 7 1013-1018.

Shi S, Kirk M, Kahn AJ (1996). The role of type I collagen in the regulation of the osteoblast phenotype. J. Bone Miner. Res. 11: 1139-1145.

Siczkowski M, Clarke D, Gordon MY (1992). Binding of primitive progenitor cells to marrow stromal cells involves heparan sulfate. Blood 80: 912-919.

Shigeno Y and Ashton BA (1995). Human bone cell proliferation in vitro decreases with human donor age. J. Bone Joint Surg. 77B: 139-142.

Ship MA and Look AT (1993): Hematopoietic differentiation antigens that are membrane-associated enzymes: cutting is the key! Blood 82: 1052-1070.

Siminovitch L, McCulloch EA, Till JE (1963). The distribution of colony-forming cells among spleen colonies. Journal of Cellular and Comparative Physiology 62: 327-336.

Simon LS, Slovik SM, Neer RM, Krane SM (1988). Changes in serum levels of type I and III procollagen extension peptides during infusion of human parathyroid hormone fragment (1-34). J Bone Miner. Res. 3: 241-246.

Simmons DJ, Seitz P, Kidder L, Klein GL, Waeltz M, Gundberg CM, Tabuchi C, Yang C, Zhang RW (1991). Partial characterization of rat stromal cells. Calcif. Tissue Int. 48: 326-334.

Simmons PJ, Kent GN, Jika RL, Scott DM, Fallon M, Cohn DV (1982). Formation of bone by isolated cultured osteoblasts in millipore diffusion chambers. Calcif. Tissue Int. 34: 291-294

Simmons PJ, Przepiorka D, Donnall Thomas E, Torok-Storb B (1987). Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature 328: 429-432.

Simmons PJ and Gronthos S (1991). Isolation and characterization of human bone marrow stromal cell precursors. International Journal of Cell Cloning 9: 408 [abstract].

Simmons PJ and Torok-Storb B (1991a). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 78: 55-62.

Simmons PJ and Torok-Storb B (1991b). CD34 expression by stromal precursors in normal human adult bone marrow. Blood 78: 2848-2853.

Simmons PJ, Masinovsky B, Longenecker BM, Berenson R, Torok-Storb B, Gallatin WM (1992). Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. Blood 80: 388-395.

Simmons PJ, Zannettino A, Gronthos S, Leavesley D (1994a). Potential adhesion mechanisms for localisation of haemopoietic progenitors to bone marrow stroma. Leukemia and Lymphoma 12: 353-363.

Simmons PJ, Gronthos S, Zannettino A, Ohta S, Graves SE (1994b). Isolation, characterization and functional activity of human marrow stromal progenitors in hemopoiesis. Prog. Clin. Biol. Res.389: 271-280.

Sinha RK and Tuan RS (1996). Regulation of human osteoblast integrin expression by orthopedic implant material. Bone 18: 451-457.

Soligo D, Schiro R, Luksch R, Manara G, Quirici N (1990). Expression of integrins in human bone marrow. Br. J. Haematol. 76: 323-332.

Spangrude GJ, Heimfeld S, Weissman, IL (1988). Purification and characterization of mouse haemopoietic stem cells. Science. 241: 58-62.

Spooncer E, Gallagher JT, Krizsa F, Dexter TM (1983). Regulation of haemopoiesis in long-term bone marrow cultures. IV. Glycosaminoglycan synthesis and the stimulation of haemopoiesis by beta-D-xylosides. J. Cell Biol. 96: 510-514.

Staatz WD, Fok KF, Zutter MM, Adams SP, Rodriguez BA, Santoro SA (1991). Identification of a tetrapeptide recognition sequence for the $\alpha 2\beta 1$ integrin in collagen. J. Biol. Chem. 266: 7363-7367.

Strobel ES, Gay RE, Greenberg PL (1986). Characterization of the in vitro stromal microenvironment of human bone marrow. Int. J. Cell. Clon. 4: 341-356.

Subramanian M, Colvard D, Keeting PE, Rasmussen K, Riggs BL, Spelsberg TC, (1992). Glucocorticord regulation of alkaline phosphatase, osteocalcin, and proto-oncogenes in normal human osteoblast like cells. J. Cell Biochem. 50: 411-424.

Suda T and Dexter TM (1981). Effects of hydrocortisone on long-term human marrow cells. Br. J. Haematol. 48: 661-664.

Suda T, Udagawa N, Takahashi N (1996). Cells of bone: osteoclast generation. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, pp 87-102.

Suda T, Nakamura I, Jimi Eijiro, Takahashi N (1997). Regulation of osteocalst function. J. Bone Miner. Res. 12: 869-879.

Sudo N, Kodama H-A, Amagai Y, Yamamoto S, Kasai S (1993). In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J. Cell Biol. 96: 191-198.

Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM (1989). Characterization and partial purification of human marrow cells capable of initiating long-term hemopoiesis in vitro. Blood 74: 1563-1570.

Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proc. Nat. Acad. Sci. (USA) 87: 3584-3588.

Suzuki J, Fujita J, Taniguchi S, Sugimoto K, Mori KJ (1992). Characterization of murine hemopoietic-supportive (MS-1 and MS-5) and non-supportive (MS-K) cell lines. Leukaemia 6: 452-458.

Swaroop A, Hogan BL, Francke U (1988). Molecular analysis of the cDNA for human SPARC/ Osteocalcin/ BM-40: Sequence, expression, and localization of the gene to chromosome 5q31-q33. Genomics 2: 37-47.

Szabo L, Mottershead DG, Ballard FJ, Wallace JC (1988). The bovine insulin-like growth factor (IGF) binding protein purified from condition medium requires the N-terminal tripeptide in IGF-1 for binding. Biochem. Biophys. Res. Commun.151: 207-214.

Szilvassy SJ, Lansdorp PM, Humphries RK, Eaves AC, Eaves CJ (1989). Isolation in a single step of a highly enriched hemopoietic stem cell population with competitive long-term repopulating ability. Blood 74: 930-939.

Taichman RS, Emerson SG (1994). Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. J. Exp. Med. 179: 1677-1682.

Taichman RS, Emerson SG (1998). The role of osteoblasts in the hematopoietic microenvironment. STEM CELLS 16: 7-15.

Tamayo E, Charbord P, Li J, Herve P (1994). A quantative assay that evaluates the capacity of human stromal cells to support granulomonopoiesis in situ. Stem Cells Dayt. 12: 304-315.

Tanaka Y, Morimoto I, Nakano Y, Okada Y, Hirota S, Nomura S, Nakamura T, Eto S (1995). Osteoblasts are regulated by the cellular adhesion through ICAM-1 and VCAM-1. J. Bone Miner. Res. 10: 1462-1469.

Tavassoli M and Crosby WH (1968). Transplantation of marrow to extramedullary sites. Science 161: 54-56.

Tavassoli M, Maniatis A, Crosby WH (1974). Induction of sustained haemopoiesis in fatty marrow. Blood 43: 33-38.

Tavassoli M, Sacks PV, Crosby WH (1975). The effect of in vitro irradiation on the subsequent growth of ectopic splenic autoimplants. Proc. Soc. Exp. Biol. Med. 148: 780-783.

Tavassoli M (1976). Ultrastructure development of bone marrow adipose cell. Acta Anat. 94: 65-77.

Tavassoli M, Houchin DN, Jacobs P (1978). Fatty acid composition of adipose cells in red and yellow marrow. Scand. J. Haematol. 18: 47-53.

Tavassoli M and Khademi R (1980). The origin of hemopoietic cells in ectopic implants of spleen and marrow. Experientia 36: 1126-1127.

Tavassoli M and Friedenstein AJ (1983). Hemopoietic stromal microenvironment. Ann. J. Hematol. 15: 195-203.

Tavassoli M and Hardy CL (1990). Molecular basis of homing of intravenously transplanted stem cells to the marrow. Blood 76: 1059-1070.

Taylor SM and Jones PA (1979). Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17: 771-779.

Taylor SM and Jones PA (1982). Changes in phenotypic expression in embryonic and adult cells treated with 5-azacytidine. J. Cell Physiol. 111: 187-194.

Teixido J, Hemler ME, Greenberger JS, Anklesaria P (1992). Role of Beta-1 and Beta-2 intergrins in the adhesion of human CD34 stem cells to bone marrow stroma. J. Clin. Invest. 90: 358-367.

Termine JD, Kleinman HK, Whitson SW, Conn KM, Mcgarvey ML, Martin GR (1981). Osteonectin. a bone-specific protein linking mineral to collagen. Cell 26: 99-105.

Thiede MA, Smock SL, Petersoen DN, Gasser WA, Thompson DD, Nishimoto SK (1994). Presence of messenger ribonucleic acid encoding osteocalcin, a marker of bone turnover, in bone marrow megakaryocytes and peripheral blood platelets. Endocrinology 135: 929-937.

Thomas L, Byers HR, Vink J, Stamenkovic I (1992). CD44H regulates tumor cell migration on hyaluronate-coated substrate. J. Cell Biol. 118: 971-977.

Thomson BM, Bennett J, Dean V, Triffitt JT, Meikle MC, Loveridge N (1993). Preliminary characterizartionof porcine bone marrow stromal cells: Skeletogenic potential, colony-forming activity and response to dexamethasone, transforming growth factor β and basic fibroblast growth factor. J Bone Miner. Res. 8: 1173-1183.

Till JE and McCulloch EA (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Reasearch 14: 213-222.

Timpl R (1989). Structure and biological activity of basement membrane proteins. Eur. J. Biochem. 180: 487-502.

Tingstrom A, Reuterdahl C, Lindahl P, Heldin CH, Rubin K (1992). Expression of paletelet-derived growth factor-β receptors on human fibroblasts. J. Immunol. 148: 546-554.

Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, Sakiyama S (1987). Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer. Res. 47: 5616-5619.

Tokunaga K, Ogose A, Endo N, Nomura S, Takahashi HE (1996). Human osteosarcoma (OST) induces mouse reactive bone formation in xenograft system. Bone 19: 447-454.

Torii Y, Hitomi K, Tsukagoshi N (1994). L-ascorbic acid 2-phosphate promotes osteoblastic differentiation of MC3T3-E1 mediated by accumulation of type I collagen. J. Nutr. Sci. Vitaminol (Tokyo) 40:229-238.

Traianedes K, Martin TJ, Findlay DM (1996). Regulation of osteopontin expression by type I collagen in preosteoblastic UMR201 cells. Connective Tissue Research 34: 63-74.

Tsai S, Patel V, Beaumont E, Lodish HF, Nathan DG, Sieff CA (1987). Differential binding of erythroid and myeloid progenitors to fibroblasts and fibronectin. Blood 69: 1587-1594.

Tsuji T, Hughes FJ, McCullochCAG, Melcher AN (1990). Effects of donor age on osteogenic cells of rat bone marrow in vitro. Mech. Aging Pevel. 61: 121-132.

Tursken K and Aubin JE (1991). Positive and negative immunoselection for enrichment of two classes of osteoprogenitor cells. J. Cell Biol. 14: 373-384.

Uchida N and Weissmann IL (1992). Searching for hematopoietic stem cells: evidence that Thy-1.1^{lo}Lin^{-}Sca-1^{+} cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J. Exp. Med. 175: 175-184.

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yanden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH (1984). Human EGF-receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309: 418-429.

Umezawa A, Maruyama T, Segawa K, Shadduck RK, Waheed A, Hata JI (1992). Multiple marrow stromal cell line is able to induce hematopoiesis in vivo. J. Cell Physiol. 151: 197-205.

Underwood PA, Dalton BA, Steele JG, Bennett FA, Strike P (1992). Anti-fibronectin antibodies that modify heparin binding and cell adhesion: evidence for a new cell binding site in the heparin binding region. J. Cell Science 102: 833-845.

Vaananen K (1996). Osteoclast function: biology and mechanisms. In: Bilezikian JP, Raisz LG, Rodan GA (eds) Principles of Bone Biology. Academic Press, San Diego, pp 103-113.

Van Den Heuvel RL, Versele SRM, Schoetters GER, Vanderborght OLJ (1987). Stromal stem cells (CFU-F) in yolk sac, liver, spleen and bone marrow of pre- and postnatal mice. Brit. J. Haematol. 66: 15-20.

Van Den Heuvel RL, Schoeters G, Leppens H, Vanderborght O (1991). Stromal cells in long-term cultures of liver, spleen and bone marrow at different developmental ages have different capacities to maintain GM-CFC proliferation. Exp. Hematol. 19: 115-121.

Van der Plas A and Nijweide PJ (1992). Isolation and purification of osteocytes. J. Bone Min. Res. 7: 389-396.

Van der Plas A, Aarden EM, Feyen JHM, de Boer AH, Wiltink A, Alblas MJ, de Ley L, Nijweide PJ (1994). Characteristics and properties of osteocytes in culture. J. Bone Miner. Res. 9:1697-1704.

Van Vlasselaer P, Falla N, Snoeck H, Mathieu E (1994). Characterization and Purification of Osteogenic Cells from Murine Bone Marrow by Two-Color Cell Sorting Using Anti-Sca-1 Monoclonal Antibody and Wheat Germ Agglutinin. Blood 84: 753-763.

Vidal-Puig AJ, Considine RV, Jimenez-Linan M, Werman A, Pories WJ, Caro

JF, Flier JS (1997). Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. J. Clin. Invest. 99: 2416-2422.

Vilamitjana-Amedee J, Bareille R, Rouais F, Caplan AI, Harmand M-F (1993). Human bone marrow stromal cells express an osteoblastic phenotype in culture. In Vitro Dev. Biol. 29A: 699-707.

Villarreal XC, Mann KG, Long GL (1989). Structure of human osteonectin based upon analysis of cDNA and genomic sequences. Biochemistry 28: 6483-6491.

Villarreal XC, Grant BW, Long GL (1991). Demonstration of osteonectin mRNA in megakaryocytes: the use of the polymerase chain reaction. Blood 78: 1216-1222.

von der Mark K, von der Mark H, Goodman S (1992). Cellular responses to extracellular matrix. Kidney Int. 41: 632-640.

Waddington RJ, Embery G, Last KS (1989). Glycosaminoglycans of human alveolar bone. Arch. Oral Biol. 34: 587-589.

Waddington RJ, Embery G (1991). Structural characterization of human alveolar bone proteoglycans. Arch. Oral Biol. 36: 859-866.

Waller EK, Huang S, Terstappen L (1995a). Changes in the growth properties of $CD34^{+}$, $CD38^{-}$ bone marrow progenitors during fetal development. Blood 86: 710-718.

Waller EK, Olweus J, Lund-Johansen F, Huang S, Nguyen M, Guo G-R, Terstappen L (1995b). The "Common Stem Cell" Hypothesis Reevaluated: Human Fetal Bone Marrow

Contains Seperate Populations of Hematopoietic and Stromal Progenitors. Blood 85: 2422-2435.

Walsh S, Dodds RA, James IE, Bradbeer JN, Gowen M (1994). Monoclonal antibodies with selective reactivity against osteoblasts and osteocytes in human bone. J. Bone Miner. Res. 9: 1687-1696.

Walthall BJ and Ham RG (1981). Multiplication of human diploid fibroblasts in a synthetic medium supplemented with EGF, insulin, and dexamethasone. Exp. Cell Res. 134: 303-311.

Wang JC, Lang HD, Liao P, Wong A (1992). Recombinant alpha interferon inhibits colony formation of bone marrow fibroblast progenitor cells (CFU-F). Am. J. Hematol. 40: 81-85.

Wang N and Ingber DE (1994). Control of cytoskeletal mechanisms by extracellular matrix, cell shape and mechanical tension. Biophys. J. 66: 2181-2189.

Wang Q-R.and Wolf NS (1990). Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp. Hematol. 18: 355-359.

Wang Q-R, Yan Z-J, Wolf NS (1990). Dissecting the hematopoietic microenvironment. VI. The effects of several growth factors on the in vitro growth of murine bone marrow CFU-F. Exp. Hematol. 18: 341-347.

Watanabe Y (1966). An electron microscopic study on the reticuloendothelial system in bone marrow. Tohoku J. Exp. Med. 89: 167-176.

Watt SM, Karhi K, Gatter K, Furley AJW, Katz FE, Healy LE, Atlass LJ, Bradley NJ, Sutherland DR, Levinsky R, Greaves MF (1987). Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with the human hemopoietic progenitor cells. Leukemia 1: 417-426.

Wayner EA, Garcia-Pardo A, Humphries MJ, McDonald JA, Carter WG (1990). Identification and characterisation of the T-lymphocyte receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J. Cell Biol. 109: 1321-1330.

Weinreb M, Shinar D, Rodan G (1990). Different patterns of alkaline phophatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. J. Bone Miner. Res. 8: 831-842.

Weiss L (1961). An electron microscopic study of vascular sinuses of the bone marrow of the rabbit. Bull. Johns Hopkins Hosp. 108: 171-199.

Weiss L (1965). The structure of bone marrow. Functional interrelationships of vascular and hemopoietic compartments in experimental haemolytic anaemia: an electron microscopic study.J. Morph. 117: 467-537.

Weiss L (1970). Transmural cellular passage in vascular sinuses of rat bone marrow. Blood 36: 189-207.

Weiss L and Chen LT (1975). The organisation of hemopoietic cords and vascular sinuses in bone marrow. Blood Cells 1: 617-638.

Weiss L (1976). The hematopoietic microenvironment of the bone marrow: An ultrastructure study of the stroma in rats. Anatomical Record 186: 161-184.

Weiss L and Sakai H (1984). The hematopoietic stroma. Am. J. Anat. 170: 447-463.

Wergedal JE and Baylink DJ (1984). Characterization of cells isolated and cultured from human bone. Proc. Soc. Exp. Biol. Med. 176: 60-69.

Westen H and Bainton DF (1979). Association of alkaline phosphatase-positive reticulum cells in bone marrow with granulocytic precursors. J. Exp. Med. 150: 919- 937.

Westermark B and Heldin CH (1985). Similar action of Platelet derived growth factor and epidermal growth factor in the prereplicative phase of human fibroblasts suggests a common intracellular pathway. J. Cell Physiol. 124: 43-48.

Whitlock CA and Witte ON (1982). Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci. (USA) 79: 3608-3612.

Whyte MP (1994). Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr. Rev. 15: 439-461.

Wight TN, Kinsella MG, Keating A, Singer JW (1986). Proteoglycans in human long-term bone marrow culture: Biochemical and ultrastructural analysis. Blood 67: 1333-1343.

Wion KL, Kirchgessner TG, Lusis AJ, Schotz MC, Lawn RM (1987). Human lipoprotein lipase complementary DNA sequence. Science 235: 1638-1641.

Witte PL, Frantsve LM, Hergott M, Rahbe SM (1993). Cytokine production and heterogeneity of primary stromal cells that support B lymphopoiesis. Eur. J. Immunol. 23: 1809-.

Wolf NS, Kone A, Priestly GV, Bartelmez SH (1993). In vivo and in vitro characterisation of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Exp. Hematol. 21: 614-622.

Wong GL and Cohn DV (1974). Separation of parathyroid hormone and calcitonin sensitive cells from non-responsive bone cells. Nature 252: 713-715.

Wrann M, Fox CF, Ross R (1980). Modulation of epidermal growth factor receptors on 3T3 cells by platelet derived growth factor. Science 210: 1363-1365.

Yamaguchi Y, Mann DM, Ruoslahti E (1990). Negative regulation of TGF-β by the proteoglycan decorin. Nature 346: 281-284.

Yamaguchi A and Kahn AJ (1991). Clonal osteogenic cell lines express myogenic and adipocytic development potential. Calcif. Tissue Int. 49: 221-225.

Yamamoto T, Escarot B, Glorieux FH (1991). In vivo osteogenic activity of isolated human bone cells. J. Bone Miner. Res. 6: 45-51.

Yao K-L, Todescan R JR, Sodek J, (1994). Temporal changes in matrix protein synthesis and mRNA expression during mineralized tissue formation by adult rat bone marrow cells in culture. J. Bone Miner. Res. 9: 231-240.

Yoffey JM and Yaffe P (1980). The phagocytic central reticular cell of the erythroblastic island in rat bone marrow. J. Recticuloendothel. Soc. 28: 37-47.

Yoon K, Buenaga R, Rodan G (1987). Tissue specificity and developmental expression of rat osteopontin. Biochem. Biophys. Res. Commun. 148: 1129-1136.

Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ (1998). Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J. Orthop. Res. 16: 406-413.

Yurchenco PD and O'Rear JJ (1994). Basal lamina assembly. Curr. Opin. Cell Biol. 6: 674-681.

Zannattino ACW, Rayner JR, Ashman LK, Gonda TJ, Simmons PJ (1996). A powerful new technique for isolating genes encoding cell surface antigens using retroviral expression cloning. J. Immunol. 156: 611-620.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994). Positional cloning of the mouse obese gene and its human homologue. Nature 372. 425-432.

Zipori D, Toledo J, von der Mark K (1985). Phenotypic heterogeneity among stromal cell lines from mouse bone marrow disclosed in their extracellular matrix composition and interactions with normal and leukemica cells. Blood 66: 447-455.

Zuckerman KS and Wicha MS (1983). Extracellular matrix production by the adherent cells of long-term murine bone marrow cultures. Blood 61: 540-547.

Zuckerman KS, Rhodes RK, Goodrum DD, Patel VR, Sparks B, Wells J, Wicha MS, Mayo LA (1985). Inhibition of collagen deposition in the extracellular matrix prevents the establishment of a stroma supportive of hematopoiesis in long-term murine bone marrow cultures. J. Clin. Invest. 75: 970-975.

Zumstein P and Stiles CD (1987). Molecular cloning of gene sequences that are regulated by insulin-like growth factor I. J. Biol. Chem. 262: 11252-11260.