Quantitation of the Growth and Differentiation of Normal and Malignant Bone Marrow derived Stromal Cells

A thesis submitted for the degree of Doctor of Philosophy to the University of Wales

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Dedication

To family and friends who have supported me throughout

Acknowledgements

Abstract

Bone marrow derived stromal cells (BMdSC) can be easily isolated from the mononuclear cell fraction within the bone marrow by plastic adherence following inoculation into tissue culture flasks. They include a heterogeneous population of cells from osteocytes and adipocytes to endothelial cells, smooth muscle cells and stromal fibroblasts, and include a wide spectrum of cells from the proliferative stem-like and progenitor cells (colony forming unit-fibroblasts: CFU-F) to the terminally differentiated cells (no proliferative capacity). It is the progenitor cells within this population that are able to proliferate *in vitro* and differentiate into a wide range of mesenchymal tissues including bone (osteogenesis), fat (adipogenesis), tendon (tendrogenesis), cartilage (chondrogenesis) and stromal fibroblasts.

BMdSCs also function to provide a microenvironment for haemopoiesis through cytokine production and cell to cell or cell to extracellular matrix interactions. These help regulate proliferation, differentiation and cell fate of both the stromal and haemopoietic cells. The close interactions between the two environments suggest that abnormalities within one will affect the other. It is currently unknown if the leukaemic haemopoietic cells create abnormalities within the stromal layer, or whether abnormalities within the stromal layer contribute to the disease.

This study aimed to quantitate markers of proliferation (cell counts, cell doubling and CFU-F colony formation), osteogenesis (calcium deposition, alkaline phosphatase (ALP), collagen I, osteopontin and osteocalcin gene expression) and adipogenesis (multidroplet cell cluster formation, aP2, LPL and PPAR-γ gene expression) in BMdSCs derived from normal and pathological bone marrows (affected by haemopoietic diseases), to determine the presence of any abnormalities within the BMdSC layer. The antigenic expression profiles of these cells were also compared.

Normal and pathological passaged BMdSCs were successfully grown to confluence over 3 passages and induced to differentiate down the osteogenic and adipogenic lineage with the formation of mineral deposit and multidroplet cell clusters respectively. Juvenile Myelomonocytic leukaemia (JMML) BMdSCs had a significantly increased cell doubling (n=7, n=53; p=0.000001) and MDS BMdSCs had significantly increased number of small colonies (n=5, n=54; p=0.016) in comparison to normal. Lymphoma BMdSCs had an increased CD105, CD49b and CD45 antigenic expression; myeloma BMdSCs had an increased CD105 antigenic expression, and the lymphoproliferative BMdSCs had a significantly reduced CD90, CD105 and CD49b expression in comparison to normal. With osteogenic induction, AML and lymphoma (mineralised) cultures showed a reduced osteocalcin gene expression in comparison to normal. With adipogenic induction, AML and lymphoma cultures showed reduced multidroplet cell cluster formation with comparable gene expression to normal.

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Abbreviations

ALCAM:	Activated leukocyte cell adhesion molecule
ALP:	Alkaline phosphatase
AML:	Acute myeloid leukaemia
ALL:	Acute lymphoblastic leukaemia
α-MEM:	Alpha-modified minimal essential medium eagle
AMP:	2-amino-2-methylpropanol
aP2:	Adipocyte-specific lipid binding protein
APC:	Allophycocyanin
B-ALL:	B-cell ALL
bcr/abl fusion gene:	formed during (9;22) translocation
BMDC:	Bone marrow derived cells (spectrum of hemangioblast, haemopoietic and Mesenchymal cells)
BMdSC:	Bone marrow derived stromal cell
BMT:	Bone marrow transplant
CaCl$_2$:	Calcium chloride
CAM:	Cell adhesion molecule
cAMP:	Cyclic AMP
C/EBP:	CCAAT/enhancer binding proteins
CFU-F:	Colony forming unit-fibroblast
CFU-GM:	Colony forming unit-granulocyte monocyte
CML:	Chronic myeloid leukaemia
CLL:	Chronic lymphoid leukaemia
CSF:	Colony stimulating factors
CT:	Cycle threshold
Cx:	Connexin
DMEM:	Dulbecco's modified minimal essential medium eagle
DMSO:	Dimethylsulphoxide
ECM:	Extracellular matrix
ER:	Endoplasmic reticulum
ES:	Embryonic stem (cell)
FAB:	French, American, British classification of leukaemias
FCS:	Foetal calf serum
FGF:	Fibroblast growth factor

FITC:	Fluorescein isothiocyanate
FLT3:	fms-like tyrosine kinase
FSC:	Forward scatter (indication of size of cell)
G-CSF:	Granulocyte-colony stimulating factor
GM-CSF:	Graunolocyte, macrophage- colony stimulating factor
GFP:	Green fluorescent protein
G-protein:	GTP-binding protein
HC:	Hydrocortisone
HCAM:	Homing associated cell adhesion molecule
HD:	Hodgkin's disease
HS:	Horse serum
HSC:	Haemopoietic stem cell
IBMX:	3-Isobutyl-1-methylxanthine
ICAM:	Intercellular cell adhesion molecule
IL:	Interleukin
ITD:	Internal tandem duplication
IVF:	*In vitro* fertilisation
JMML:	Juvenile myelomonocytic leukaemia
LPL:	Lipoprotein lipase
LTC-IC:	Long term culture initiating cells (stem cells)
MAPC:	Multipotent adult progenitor cell
M-CSF:	Macrophage colony stimulating factor
MDS:	Myelodysplastic Syndrome
MM:	Multiple Myeloma
MNC:	Mononuclear cell
MPD:	Myeloproliferative disorder
MRD:	Minimal residual disease
mRNA:	Messenger RNA
MSC:	Mesenchymal stem cell
NAC:	Non adherent cell
NaOH:	Sodium hydroxide
NBAC:	National Bioethics Advisory Commission
NHL:	Non-Hodgkin's lymphoma
PBS:	Phosphate buffered saline
PE:	Phycoerythrin

PerCP:	Peridinin-chlorophyll-a
Ph:	Philadelphia (chromosome)
PLL:	Prolymphocytic leukaemia
PM:	Plasma membrane
PMT:	Photomultiplier tube
PPAR:	Peroxisome proliferator activated receptor
RS:	Rabbit serum
SCNT:	Somatic cell nuclear transfer
SDS:	Sodium dodecyl sulfate
SE:	Standard error
SF:	Steel factor
SR:	Sarcoplasmic reticulum
SSC:	Side scatter (indication of granularity of a cell)
TBE:	Tris-Borate EDTA
T_m:	melting temperature
TGF:	Transforming growth factor
TNF:	Tumour necrosis factor
TPO:	Thrombopoietin
UC:	Universal container
VCAM:	Vascular cell adhesion molecule
WHO:	World Health Organisation

Chapter 1

General Introduction

1.1. Stem Cells: General Concepts

1.1.1. What is a stem cell?

A stem cell is defined by its ability to produce daughter cells that either remain stem cells by the process of self-renewal, or differentiate out of the stem cell population via a specific lineage to produce more specialised progenitor cells of various lineages (McCulloch 2003). It is still unclear whether this is an equal process, producing either two differentiated daughter cells or two stem cells (symmetric division), or an unequal process, producing one stem cell and one differentiated daughter cell (asymmetric division). In the hierarchical differentiative model, whereby stem cells differentiate into progenitor cells, followed by maturation to more mature differentiated cell-types (figure 1.1), self-renewal is thought of as a horizontal division of the stem cell (no differentiation). This mechanism is usually maintained by the actions of telomerase, which preserves the telomere cap on the end of chromosomes during cell division and prevents the cell from entering replicative senescence (failure of a cell to replicate its DNA and divide into two cells by mitosis) (Raff 2003).

The niche in which stem cells reside is important for their maintenance and regulation (proliferation and / or differentiation), which is usually dictated by the environmental conditions. *In vitro,* cell confluence (involving cell-cell contact), often promotes growth arrest and / or differentiation rather than cell division (Raff 2003). A question, which remains unanswered, is how stem cells remain as stem cells? It is possible that the stem cells are maintained in a quiescent state by specific molecules or proteins that protect them from differentiative signals until the environment dictates their requirement for maintenance or repair (Blau et al. 2001).

1.1.2. Stem cell differentiation

Differentiation is the vertical progression from a more primitive cell type to a more mature cell type via a particular lineage (figure 1.1) (Moore & Quesenberry 2003). Whilst stem cells employ telomerase production for maintenance and self-renewal, progenitor cells are more limited in that they lack telomerase activity, and can only divide a limited number of times before reaching terminal differentiation and / or senescence and apoptosis (programmed cell death) (Raff 2003). The mechanism that determines the number of divisions is unknown, but it is possible that committed progenitor cells divide a set number of times before terminal differentiation (Obinata et al. 1998). Progenitor cells are considered as committed, partially differentiated intermediate cells and are responsible for greatly amplifying a population of cells to efficiently attain a large number of terminally differentiated cell types (Bellantuono 2004).
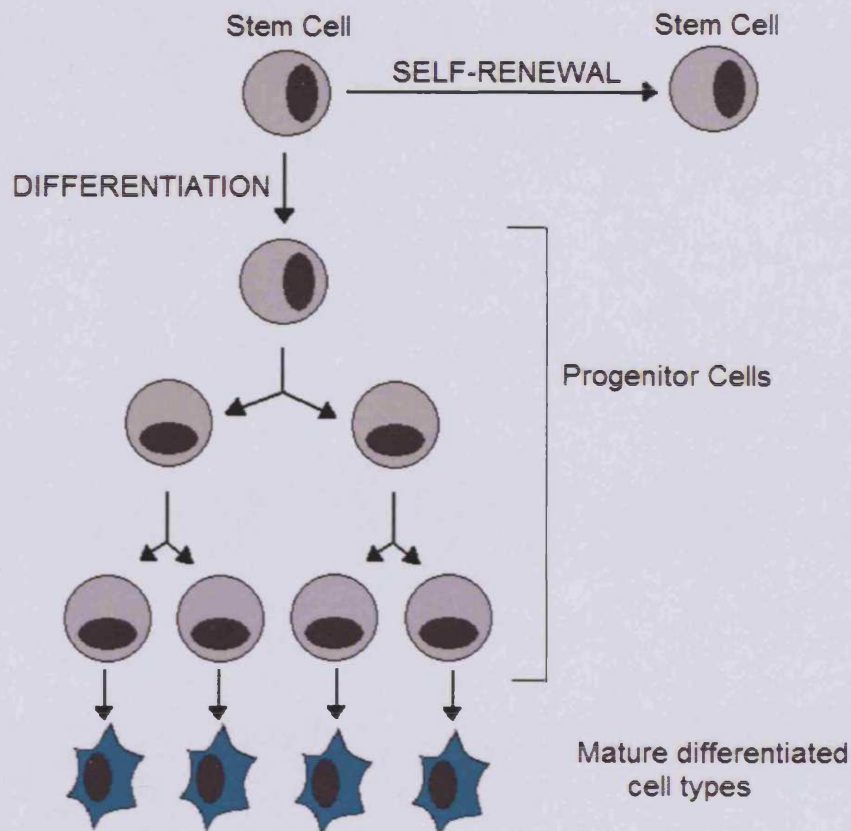
Figure 1.1. The hierarchical differentiative model showing self-renewal and differentiation down a particular lineage to produce mature cell types.

In the hierarchical differentiative system it is impossible to tell where the stem cell population finishes and the progenitor population starts, producing a large spectrum of cells with varying maturity. A cell will gradually become more differentiated and less prolific the further down the vertical progression it gets, which until recently has always been thought to be a one-way system.

The mechanisms responsible for causing a stem cell to differentiate are unclear. Environmental stimuli such as soluble growth factors and cytokines secreted from surrounding cells or circulating in the peripheral bloodstream are likely to be involved, in addition to cell-to-cell contact or cell-to-extracellular matrix interactions (Bellantuono 2004).

1.1.3. **Stem cell potential?**

Stem cells have varying levels of developmental potential in terms of the number of lineages they can differentiate into. The initial zygote (egg) and immediate daughter cells are the only single cells considered to be totipotent because they can produce all embryonic tissues,

including extra-embryonic tissues, required to produce a multicellular organism (Herzog et al. 2003). This totipotency is lost as the blastocyst cells become more committed by germ layer specification to form the ectoderm, mesoderm and endoderm lineages (Deasy et al. 2001). This is then followed by further differentiation to more specific cell types (Camargo et al. 2004). Embryonic stem cells (ES cells) can be derived from the inner cell mass during embryogenesis (1.2.1. 'Embryonic stem cells') (Toyooka et al. 2003) and are considered to be pluripotent because although they can produce most embryonic cell types of the three germ layers (ectoderm, endoderm and mesoderm), they cannot produce all the cells required for mammalian development (Conley et al. 2004; McKay 2000). It is still doubtful as to whether ES cells exist *in vivo*. Adult stem cells are more developmentally restricted and are only considered to be multipotent if capable of producing more than one cell type (Jiang et al. 2002). Recently many investigations have been carried out to show that adult stem cells may be more pluripotent than thought (1.3. 'Adult stem cell plasticity').

1.1.4. Why do we need stem cells?

Stem cells are vital for the survival of the organism. Without them many tissues and organs would stop functioning, ultimately leading to system failure and the demise of the organism (1.6. 'Cellular systems'). Many tissues such as the gut epithelium, epidermis (skin), haemopoietic system, hair and nails are rapidly regenerating and need a constant supply of cells to replace those that have been lost. Other organs including the liver, bone and kidneys renew more slowly with an estimated renewal time of ten years (van Bekkum 2004). Cells are replaced by the continued self-renewal and differentiation of the stem cell population. Mature, functioning blood cells within the haemopoietic system, which have a relatively short life span, need constantly replenishing from the stem cell population to meet the demands of the body. If red cells (erythrocytes), which carry oxygen to the tissues, are being prematurely lost from the peripheral blood stream by intravascular destruction or bleeding, signals must be sent to the stem cell population in the bone marrow to increase their production. This creates flexibility within the haemopoietic stem cell population to vary rates of production and vary the lineages into which cells are differentiated (1.6. 'Cellular systems'). Another important property of stem cells is their regeneration capacity to replace cells lost through apoptosis or necrosis following injury or infection. This allows the organism to repair itself in the maintenance of system function and survival.

1.1.5. **Where can we find stem cells?**

The haemopoietic system within the bone marrow is one of the most characterised stem cell systems. During the Second World War with the atomic bombing of Hiroshima and Nagasaki it was found that high doses of irradiation destroyed the haemopoietic system and killed the victim. Shortly after it was shown in mice that infusion of bone marrow cells from a healthy donor could reconstitute the blood system (previously destroyed by irradiation) and save the recipient (Raff 2003).

Adult stem cells are rare, residing in many tissues such as the epidermis, muscle, pancreas, lung and arguably liver, where they remain quiescent until activated to divide according to the demands of the body (Camargo, Chambers, & Goodell 2004). The epidermis needs constantly regenerating from its stem cell compartment within the basal layer of the epidermis and at the base of hair follicles. The epidermal stem cells give rise to keratinocytes and the follicular stem cells give rise to the hair follicle and epidermis (National Institute of Health 2004). Other stem cell compartments are only active at low levels during steady state conditions and increase in activity following tissue injury. Skeletal muscle cells (satellite cells) proliferate extensively in response to injury with high self-renewal and multipotential differentiative capacity (Deasy, Jankowski, & Huard 2001). Liver stem cells are still surrounded by controversy because although the liver has an enormous capacity to regenerate following injury (in mammals the cell number and total liver mass can be restored within two to three weeks after surgical removal of up to 75%), the regeneration often appears to be due to the mitotic division of pre-existing differentiated liver cells (hepatocytes), rather than by hierarchical lineage differentiation (Grompe 2003; van Bekkum 2004).

Stem cells have also been found within organs that were previously thought to be post mitotic with no intrinsic regenerative capacity such as the central nervous system and heart (Gojo & Umezawa 2003). Self-renewing multipotent stem cells have been located in the developing brain, hippocampus and olfactory bulb of the mammalian central nervous system (McKay 2000), and have been found to give rise to both neurons and non-neuronal cells such as astrocytes and oligodendrocytes (Almeida-Porada et al. 2001). Neurons of the olfactory system are (in mice and sub-human primates) continuously renewed from stem cells within the lateral walls of the ventricles of the brain (van Bekkum 2004). These stem cells, however, have minimal proliferative ability with continuous low-level proliferation. Their regeneration capacity following injury is also limited, which explains the progressive nature of many neurodegenerative disorders (Steindler & Pincus 2002). Neural stem cells can be isolated from

postnatal organisms and grown and differentiated *in vitro* into multiple neural phenotypes (Blau, Brazelton, & Weimann 2001).

Stem cells have been recently identified in the myocardium. A population of self-renewing, clonogenic and multipotent cardiac stem cells (Nadal-Ginard et al. 2003a) have been found to give rise to cardiomyocytes, smooth muscle and endothelial vascular cells (Beltrami et al. 2003). These stem cells differentiate into myocyte progenitors that proliferate further before terminally differentiating and exiting from the cell cycle, acting to continuously replace myocytes lost through apoptosis or necrosis. The heart is therefore not terminally differentiated as previously suggested but can regenerate, to a limited extent, following injury. This limited capability is clearly seen by the stem cells' inability to repair damaged cardiomyocytes following myocardial infarction. Evidence also suggests that the regenerative capacity of the heart decreases in later life or in certain pathological conditions when cell death begins to exceed myocyte renewal, and the myocardium compensates for the loss in mass by myocyte hypertrophy (Nadal-Ginard et al. 2003b).

1.1.6. Stem cell identification?

Cell biologists have persistently looked for a commonality between stem cells of different tissues for the purpose of identification but as yet none have been found (Evsikov & Solter 2003). It may be that tissue or organ stem cell populations are so heterogeneous that one single stem cell marker cannot be found. Their rarity within tissues in addition to the lack of suitable markers for identification makes isolation and purification of stem cells very difficult (Steindler & Pincus 2002). Unspecialised (undifferentiated) ES cells can be identified from the presence of the Oct4 surface epitope, but no similar marker has been found for the direct identification of adult stem cells. Currently cellular biologists indirectly identify stem cells by labelling cells such as haemopoietic cells with GFP or / and transfusing Y-chromosome positive cells into female recipients and tracking them *in vivo* to determine the specialised cell types they produce. The presence of stem cells can also be established by their ability to repopulate a stem cell system such as the haemopoietic system following myeloablative therapy. If the environmental conditions are known, cells can also be differentiated *in vitro* indirectly identifying the presence of more immature, potentially uncommitted cells (National Institute of Health 2004).

In summary, stem cells are immature unspecialised cell types capable of long-term self-renewal and differentiation into a large number of specialised cell-types. They can persist past embryonic development into adult life and are found in many actively proliferating tissues of

the body. They are essential for sustaining tissue homeostasis and organ mass for the life span of the organism and are important in tissue regeneration post injury (Dabeva & Shafritz 2003).

1.2. Stem Cells for *in vitro* Use

1.2.1. Embryonic stem cells

Pluripotent ES cells have been harvested from the totipotent cells of the inner cell mass (50-100 cells) of the early (<5 days old) blastocyst from both mice (Evans & Kaufman 1981) and humans (Thomson et al. 1998).
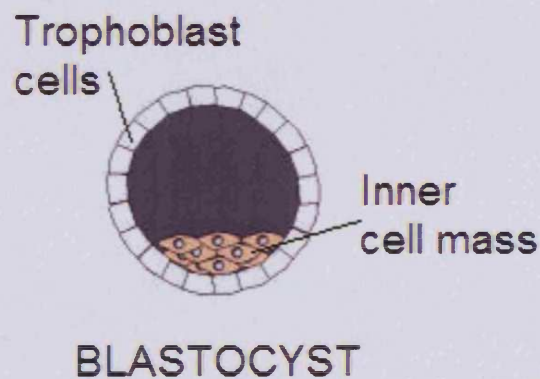
Figure 1.2. Blastocyst with an outer layer of trophoblast cells and an inner cell mass.

A blastocyst is composed of an outer layer of trophoblast cells that give rise to the placenta, the blastocoel (hollow cavity inside the blastocyst) and the inner cell mass, which has the potential to form embryonic tissues (figure 1.2) (Conley et al 2004). The ES cells are relatively short lived within the embryo (Rippon & Bishop 2004) but can proliferate extensively *in vitro* whilst retaining the potential to differentiate into a broad range of cell types (Orkin & Morrison 2002).

1.2.2. Adult stem cells

Multipotent adult stem cells are found in multiple tissues within an organism (1.1.4. 'Why do we need stem cells?'). In comparison to embryonic stem cells they have a much more limited proliferative capacity. Bone marrow derived mesenchymal cells (non-haemopoietic cells of the bone marrow, derived from the mesoderm) do not grow indefinitely *in vitro* and show a tendency to lose their proliferative potential and multipotentiality with extended culture (Banfi et al. 2000). Verfaillie has recently identified a more pluripotent adult stem cell within the bone marrow, called a multipotent adult progenitor cell or MAPC, that appears to be more primitive than bone marrow derived mesenchymal cells and possesses more ES cell-like qualities including telomerase production (Verfaillie et al. 2003). When grown *in vitro* at low density

(400-800 cells/cm^2) (Grompe 2002) mouse and rat MAPCs were found to proliferate for more than 150 population doublings and human MAPCs for up to 80 population doublings, whilst retaining the multipotentiality to differentiate into various tissues (Verfaillie et al 2003).

1.2.3. Comparison between adult and embryonic stem cells

Embryonic and adult stem cells (excluding MAPCs) differ greatly in their proliferative capabilities and in the number of lineages they can differentiate in to (National Institute of Health 2004), however, both show an immense potential for replacing cells lost or damaged by disease. ES cells and MAPCs can be expanded extensively from a single clone with continued multipotentiality to contribute to a wide range of tissues *in vivo* and *in vitro* but the work is very labour intensive and expensive. Although embryos are readily available from *in vitro* fertilisation clinics, they are surrounded by an ethical minefield (1.2.4. 'Ethical concerns about ES cell research'), and it is unlikely that the limited pool of ES cells will be immunologically compatible with all patients (Grompe 2002). The isolation and *in vitro* growth of ES cells is difficult with a low success rate, and it is difficult to differentiate a population of ES cells uniformly and homogeneously into a target tissue (known to spontaneously differentiate in an uncontrolled manner). Also, ES cells are capable of forming tumours or promoting tumour formation *in vivo* (Raff 2003), which currently makes them a poor alternative for therapy. Adult stem cells (e.g. bone marrow mesenchymal stromal cells) are not surrounded by the same ethical and moral dilemmas as ES cells, and are easily isolated from the post-natal bone marrow for growth *in vitro*. They can be differentiated into numerous lineages, but their growth and multipotentiality is limited in culture. For the effective long-term use of autologous immunologically compatible cells for treating common diseases, they would need to be greatly amplified in culture without any genetic transformation, which may cause unknown problems when transfused back into the patient (McLaren 2001). Adult stem cells are not reported to form tumours *in vivo* and are already being transfused into patients (haemopoietic stem cell transplants) without any problems. Adult stem cells may, therefore, offer a great alternative to ES cells for cellular therapy if the problems of supply (increasing cell number and life-span in culture) can be sorted.

1.2.4. Ethical concerns about ES cell research

The ethical issues surrounding stem cell research mainly focuses on the use of ES cells because their derivation involves the destruction of embryonic material, which ultimately has the potential for life if implanted into the uterus of a woman (McLaren 2001). Pro-life groups argue that human embryos deserve respect as a form of human life and have rights as that

human. Others argue that the moral status of an early embryo is equivalent to any somatic cell of the body (Orive et al. 2003). It is therefore important to weigh up embryonic destruction against the potential therapeutic advantages of ES cell research and the long-term benefit to the human population (Childress 2004; McLaren 2001; Orive et al. 2003).

Outside the scientific community there is great diversity of opinion, with no general consensus within religious groups or within the general public. Catholics traditionally believe that embryos have a moral status upon conception and therefore argue against their destruction for research on a parallel with selective abortions (Caulfield 2004). Ethicists against using embryonic material argue that research on ES cell lines is unnecessary because adult stem cells show the multipotentiality needed for cell therapy (Grompe 2002; McWhir et al. 2003). It is argued that both lines of research need to be pursued because adult stem cells do not hold the same potential as ES cells and may not be utilisable for the treatment of all diseases (McLaren 2001).

a) Sources of ES cells

Whilst ES cells hold an enormous potential for medical research, their sources remain controversial. Research embryos can be derived from *in vitro* fertilisation (IVF), selective abortions or from somatic cell nuclear transfer (SCNT) (Childress 2004) (described later). The most acceptable source is from excess eggs produced for reproductive purposes by IVF that are destined for destruction, and which are donated with informed consent from the parents. It is argued that human embryos must not be created for the purposes of scientific research (Rippon & Bishop 2004). The National Bioethics Advisory Commission (NBAC) within America and the Council of Europe's convention on Human Rights and Biomedicine have recognised the importance of drawing a distinction between creating embryos through IVF to have children and creating them for research (Childress 2004; Orive et al 2003).

b) International views about ES cell research

Most developed countries, including Britain, South Korea, China and France, have recognised the distinction between using embryonic research for therapeutic purposes (therapeutic cloning) and for cloning humans (reproductive cloning), which is banned. They therefore allow embryonic research to proceed under tightly regulated guidelines, using surplus embryos from fertility clinics (Anonymous 2004; Palmer 2004).

In Britain the Human Fertilisation and Embryology Act was enacted in 1990 (regulated by the Human Fertilisation and Embryology Authority) to control the creation, fertilisation and disposal of embryos for research and fertility treatment. This allowed embryonic research to proceed within the first 14 days of development whilst banning all research towards cloning. In 2001, the Act was expanded to include therapeutic cloning with existing embryos (Palmer 2004) and in 2004 the world's first stem cell bank was opened in Hertfordshire, based at the National Institute for Biological Standards and Control and funded by the Medical Research Council and the Biotechnology and Biological Sciences Research Council. This bank manages and supplies a wide range of ethically approved and quality controlled stem cell lines for research around the world (Alok Jha & Stephen Minger 2004).

A licence is required to undertake embryonic cell research in Britain, which enables the work to be regulated. There are currently eight institutions in Britain with a licence to generate human ES cells, which is likely to increase in the next few years (Alok Jha & Stephen Minger 2004).

America, Germany and Italy operate more restriction on the use of ES cells for research. In America, President Bush laid down guidelines that federal funding would only be provided for research on ES cell lines harvested from left over embryos derived with private or non-federal funds for reproductive purposes, and with informed consent from the parents, by 9pm on 9 August 2001 (Childress 2004), preventing any further destruction of human embryos. Italy has laws preventing the freezing of embryos and all research on ES cell lines, whilst Germany, along with the majority of Europe, has strict laws banning stem cell nuclear transfer and therapeutic cloning.

c) Somatic cell nuclear transfer (SCNT)

Somatic cell nuclear transfer used in the creation of Dolly the sheep in 1996, replaces the nucleus of an unfertilised oocyte with a somatic cell nucleus. This implanted nucleus is then reprogrammed by the oocyte's cytoplasm to produce a blastocyst from which ES cells can be derived (figure 1.3). Its use for therapeutic cloning remains controversial, and illegal in many countries, although it is permitted in South Korea, China and Singapore, and in Britain from 2004 under strict licensing laws. The university of Newcastle upon Tyne was the first institution in Britain to be licensed to experiment with human cloning for therapeutic purposes by using somatic skin cell nuclei (Henderson 2004).
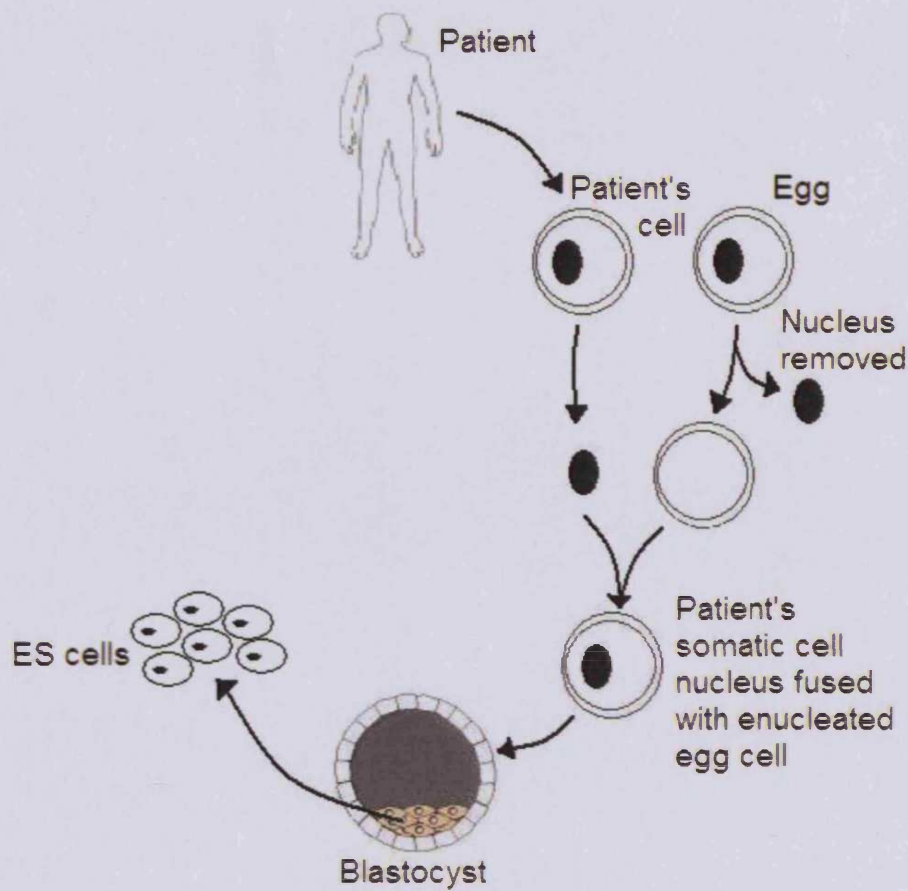
Figure 1.3. Somatic cell nuclear transfer: the creation of a blastocyst from an unfertilised oocyte for the production of an ES cell line that is immunologically compatible to the patient (Palmer 2004)

SCNT does not require the use of fertilised oocytes and can create ES-like cells that are immunologically compatible to the patient (Vogel 2004). However, the technique utilises donated unfertilised eggs that could be used for infertile couples; purposely produces a human embryo for research, and has the potential to develop into a human life if implanted into a uterus. Ethicists worry that as the technique becomes more advanced researchers may use it for cloning humans (reproductive cloning), which is still strictly banned in all countries (Alok Jha & Stephen Minger 2004; Vogel 2004). Human reproductive cloning is still considered unsafe and unethical, considering the death of Dolly in 2003 following abnormalities such as arthritis and lung disease, although this has not been attributed to the cloning experiment (Wilmut 2005).

Stem cell nuclear transfer is expensive in comparison to preventing transplant rejection with immunosuppressive drugs (McLaren 2001), and its efficiency is currently extremely low, requiring a large number of unfertilised oocytes to produce a small number of ES cell lines

(McLaren 2001; Rippon & Bishop 2004). Unless success rates increase, the technique is unlikely to be effective for therapeutic purposes.

Another technique recently described is parthenogenesis by which an oocyte can be chemically stimulated into cell division as though it had been fertilised. These are much easier to grow and have been shown to produce ES cells that differentiate into multiple cell types of all three germ layers. Parthenotes do not have two sets of chromosomes as if from each parent, but contain one doubled set. Artificially created parthenotes in animal studies do not grow to term and generally develop many abnormalities within the womb, and for this reason are not viable for human reproductive cloning (Henahan 2002; Lanza & Rosenthal 2004).

It may be possible in the future to create cells with ES-like qualities (increased lifespan and pluripotentiality) without the use of somatic cell nuclear transfer or the need for embryonic material, by dedifferentiating adult stem cells (Anonymous 2004; Westphal & Cohen 2004). With a better understanding of how the oocyte cytoplasm reprograms a nucleus, it may also be possible to reprogram a somatic nucleus without the use of an embryo (McLaren 2001). These manipulated cells would have the same potential as ES cells for cell therapy and would be ethically acceptable for research.

1.3. **Adult Stem Cell Plasticity**

It was originally thought that adult stem cells were restricted in their differentiative and regenerative potential to their tissue of origin (tissue or organ-specific), for example, epidermal stem cells would be restricted to generate skin cells and satellite cells restricted to repair damaged skeletal muscle (Blau, Brazelton, & Weimann 2001). Recent research, however, has suggested that, given a change in environmental cues, they may be able to differentiate into tissues very different from their tissue of origin. One of the first papers challenging the long held belief that adult mammalian stem cells were lineage restricted was published in 1998 by Ferrari (Ferrari et al. 1998) who demonstrated that bone marrow derived cells could differentiate into skeletal muscle. This led to the belief that adult stem cells were more 'plastic' than first thought (Frisen 2002) in that they may be able to cross lineage barriers and adopt expression profiles (cell surface markers and gene expression) and functional properties of different cells (Herzog, Chai, & Krause 2003). Recent research has even suggested that tissue-specific stem cells may be able to cross embryologically determined boundaries from mesodermal to ectodermal or endodermal lineages, for example, bone marrow derived cells

may not only be able to replace bone marrow and blood tissues but also contribute to epithelial tissues, brain, muscle, heart, endothelium, lung and liver tissues (figure 1.4) (Abedi et al. 2004).
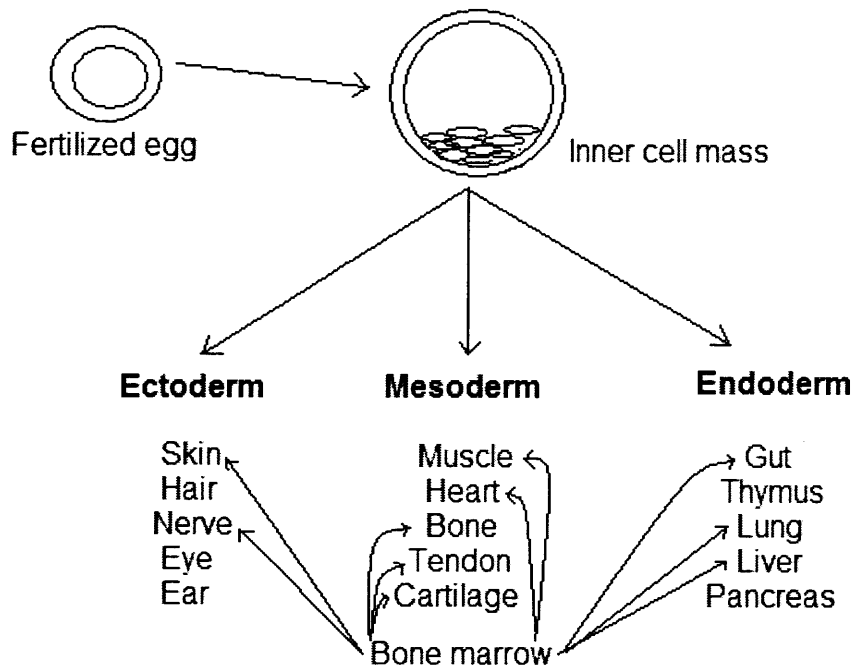
Figure 1.4. Differentiation within and between the three germ layers (Tao & Ma 2003)

1.3.1. Mechanisms for lineage switching

Several pathways have been put forward to explain the concept of stem cell plasticity (figure 1.5) but the mechanisms by which the cells appear to switch lineage are not yet understood (Martin-Rendon & Watt 2003b). One pathway thought to be responsible is **dedifferentiation** whereby a mature or lineage-restricted cell dedifferentiates to a more immature stem or progenitor cell, followed by redifferentiation to another lineage (figure 1.5). This is seen in newts after limb amputation where chondrocytes and skeletal muscle cells dedifferentiate and redifferentiate into specialised mesodermal cell types of the regenerated limb (Raff 2003). A second pathway is **transdetermination** whereby the potential of a progenitor cell programmed to generate certain lineages is redirected to another progenitor cell giving rise to different lineages (figure 1.5). A third pathway is **transdifferentiation** whereby a differentiated cell type can gain the phenotypic characteristics of another differentiated cell type (Frisen 2002) (figure 1.5). For example, smooth muscle cells often take on the phenotype of skeletal myocytes in the oesophagus (Martin-Rendon & Watt 2003a) and terminally differentiated pancreatic epithelial cells can give rise to a hepatic phenotype (Herzog, Chai, & Krause 2003).
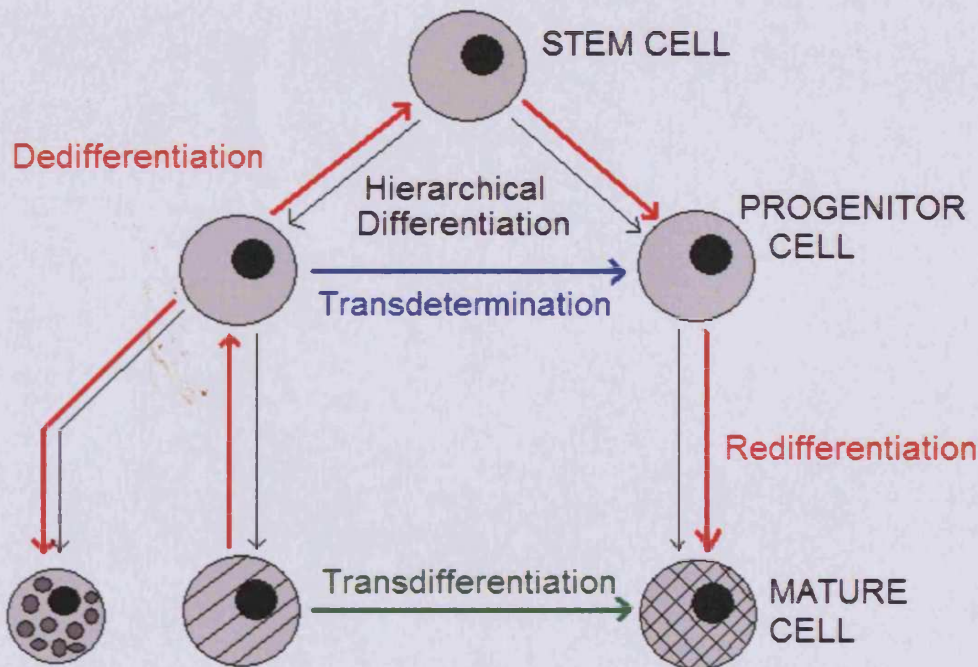
Figure 1.5. Possible mechanisms for switching lineages. Adapted from Moore & Quesenberry (Moore & Quesenberry 2003)

No conclusive evidence has yet been provided for any one of the pathways outlined above (Frisen 2002) and it is unlikely that only one pathway is responsible (Herzog, Chai, & Krause 2003).

1.3.2. Stem cell plasticity questioned

Are cells really switching lineage or does it just appear that way? Recently, much of the work demonstrating stem cell plasticity has been called into question, mainly because of a lack of reproducibility between cellular biologists (Herzog, Chai, & Krause 2003; Raff 2003). Variability exists between researchers because stem cell populations are heterogeneous and techniques differ between laboratories (Mariani 2002). Many experiments demonstrating plasticity have been performed *in vivo* under extreme selective pressure, or following induced (artificial) damage, which is intended to model a particular disease or injury, but may not reflect true *in vivo* physiology (Flake 2004; Frisen 2002). It is also unclear if adult stem cells naturally exhibit this plasticity, or whether they only transdifferentiate when manipulated experimentally. Previous demonstrations of stem cell plasticity are now being questioned, searching into other possible explanations for the apparent change in cell fate.

a) Rare, immature, uncommitted stem cells?

One explanation that may give the appearance of stem cell plasticity is the presence of rare, immature, uncommitted, non-tissue specific stem cells. These may be more immature and less committed than haemopoietic stem cells or epidermal stem cells. In this respect there may be a population of stem cells with varying commitment potentials, which when placed into a particular environment with the correct stimuli can begin to differentiate into cells of a more committed and progenitor-like nature (Horwitz 2003; Tisdale & Dunbar 2002).

b) Circulating stem cell populations?

Stem cell plasticity may be explained by tissue-specific stem cells residing within various unrelated tissues or organs (Kucia et al. 2004; Verfaillie 2002). For example, haemopoietic stem cells (HSCs) have been found in muscle; results initially interpreted to suggest the transdifferentiation of muscle stem cells into HSCs (Camargo, Chambers, & Goodell 2004; Kawada & Ogawa 2001; Orkin & Zon 2002). Adult stem cells have access to all organs of the body via the circulation and are often recruited from this 'stem cell highway' (Blau, Brazelton, & Weimann 2001) in response to homing signals secreted by organs during tissue injury. Once in residency within the tissue, they are presumably influenced by local environmental factors that determine their function. They can either remain within the stem cell pool or generate into a differentiated phenotype. This stem cell migration to various organs of the body may explain why there is such heterogeneity within stem cell populations, highlighting the difficulty in identifying them. The heterogeneous nature of stem cells may also explain the variability between cellular biologists when demonstrating cell plasticity if different populations of cells are being isolated and manipulated.

Quesenberry (Quesenberry et al. 2004) posed a working hypothesis that conversion (arguably transdifferentiation) from the bone marrow may be due to the existence of one, or more than one type of bone marrow stem cell with a broad differentiation potential. The stem cells may then differentiate according to the environmental stimuli. Tissue engraftment may, therefore, be explained by either the presence of a single multipotential stem cell, or multiple stem cells with restricted potential (McWhir et al. 2003).

c) Cell fusion?

Another pathway recently identified that could explain stem cell plasticity is cell fusion whereby two cells of different lineages fuse to produce a hybrid cell with the genetic material

and phenotypic characteristics of both lineages (figure 1.6). This phenomenon has been frequently shown between ES and somatic cells (Verfaillie 2002).

Stem cell or more mature cell of a committed lineage

New committed lineages
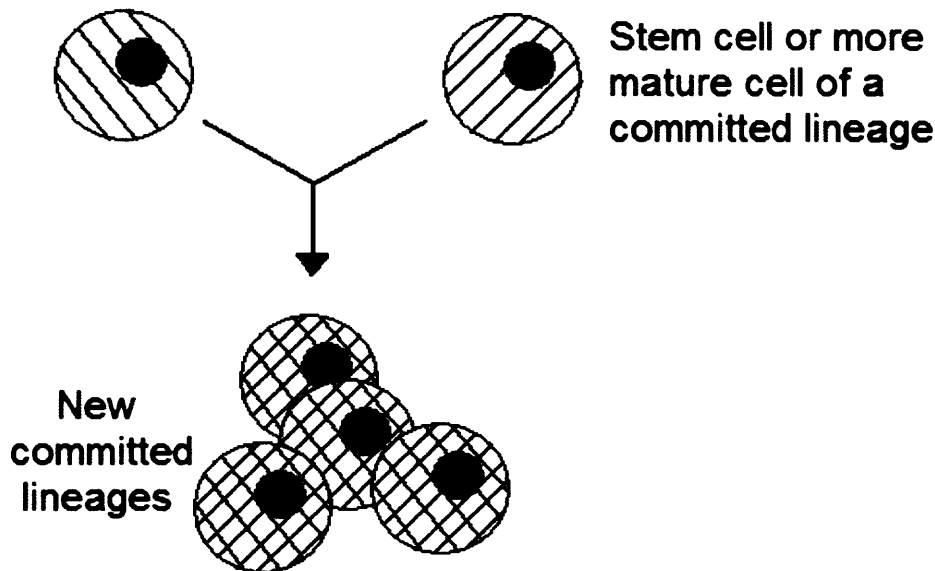
Figure 1.6. Cell Fusion. Modified from Martin-Rendon & Watt (Martin-Rendon & Watt 2003a)

For an adult stem cell to become more pluripotent it needs to take on a more embryonic-like nature expressing different characteristics and a different gene expression profile. Cell fusion between a somatic cell and an ES cell can give the appearance that the adult cell has become reprogrammed to be more pluripotent when in fact it has only taken on certain traits of the ES cell during the fusion event (Mariani 2002). Initial reports in 2002 by Terada (Terada et al. 2002) and Ying (Ying et al. 2002) showed that ES cells could fuse with murine bone marrow cells ($1:10^5$ to $1:10^6$ HSCs) or brain cells ($1:10^4$ to $1:10^5$ brain cells) (Frisen 2002) respectively *in vitro*, producing hybrids that were phenotypically like ES cells with twice the number of chromosomes (Horwitz 2003; Verfaillie 2002; Wurmser & Gage 2002). This gave the appearance that the neural cells or HSCs had become reprogrammed to become more pluripotent, but the cells still possessed surface markers of the somatic cell type. Cell fusion, therefore, shows conversion from one lineage to another, making it imperative that it is excluded when demonstrating cell plasticity in animal models.

Reports have shown that bone marrow derived cells can contribute to the regeneration of liver, brain, heart and muscle (1.4.4. 'Evidence of plasticity in bone marrow derived stem cells') but questions are now being asked as to whether these cells converted from one lineage to another, or whether they adopted the phenotype of the resident cell through spontaneous fusion (Scott

2004)? Alvarez-Dolado (Alvarez-Dolado et al. 2003) showed no evidence of transdifferentiation when bone marrow-derived cells fused spontaneously with neural progenitors *in vitro* to form multinucleated cells. Neither did they show transdifferentiation *in vivo* following the fusion of bone marrow derived cells with liver hepatocytes, neurons and cardiac muscle cells following bone marrow transplantation.

It is argued that cell fusion is not efficient enough to be responsible for all the transdifferentiation events recorded (Horwitz 2003) with rare fusion rates estimated to be $1:10^4$ to $1:10^6$ (Herzog, Chai, & Krause 2003; Tao & Ma 2003). Also, cell fusion is known to be a normal physiologic mechanism in tissue maintenance and function, particularly within the liver (Alison et al. 2004; Flake 2004), albeit at very low frequencies. So it is not surprising that fusion events occur during transdifferentiation experiments.

It may be that the exchange of genetic information during fusion leads to the reprogramming of the hybrid cell, initiating a lineage switch (Flake 2004; Quesenberry et al 2004; Vassilopoulos & Russell 2003).

1.3.3. Nuclear reprogramming

Developmentally restricted somatic cells can now be reprogrammed to become more pluripotent by SCNT, which inserts a partially or fully differentiated somatic cell nucleus into an enucleated oocyte to create a blastocyst (figure 1.3). The somatic nucleus is then reprogrammed by unknown factors within the egg's cytoplasm (Verfaillie 2002) to differentiate into various cell types. In this respect blood or epithelial cells can be reprogrammed to become muscle or nerve cells (figure 1.7) (Gurdon et al. 2003).

With a better understanding of nuclear reprogramming, particularly the unknown factors within the oocyte's cytoplasm, it may be possible to utilise the mechanisms responsible to induce lineage switching in somatic cells without nuclear transplantation (Verfaillie 2002).
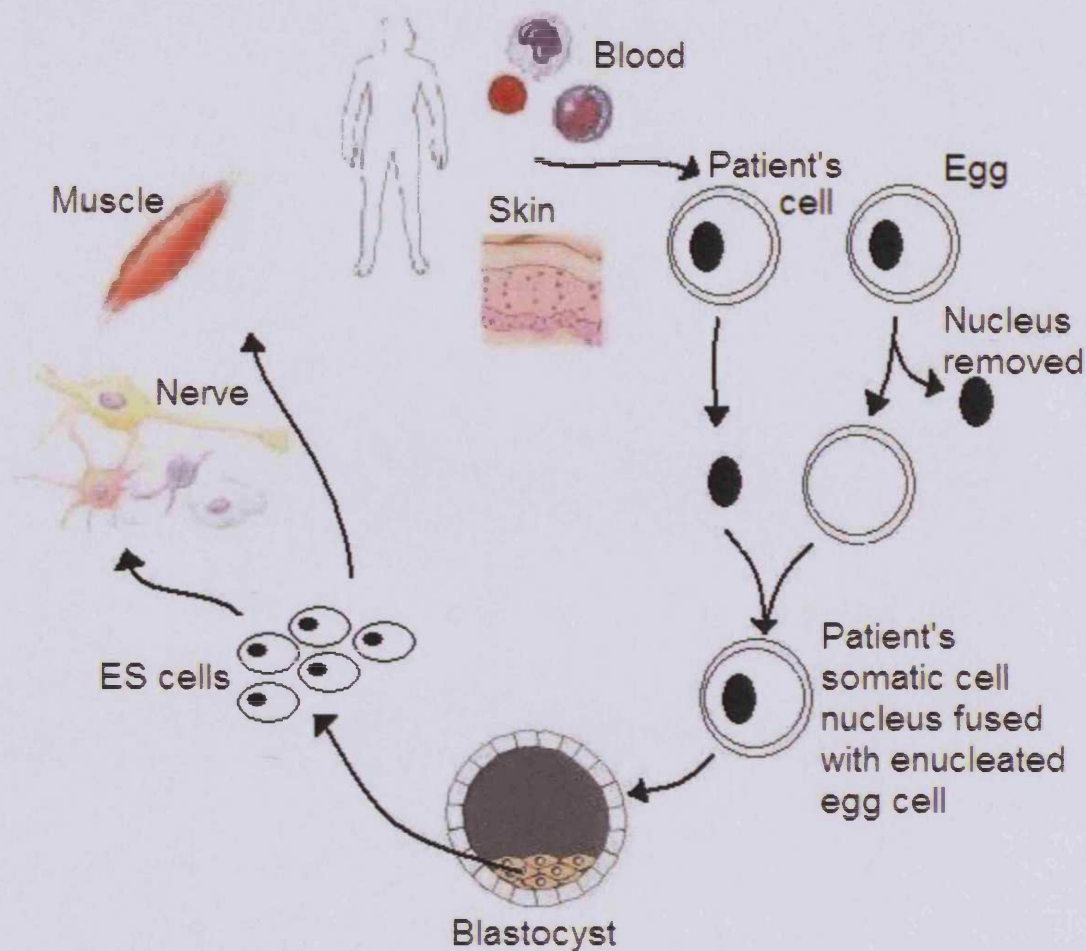
Figure 1.7. Nuclear reprogramming by somatic cell nuclear transfer.

1.3.4. **Difficulty in establishing stem cell plasticity**

Proving stem cell plasticity is extremely difficult, first and foremost because stem cells are very rare and lack that crucial specific marker to identify them. Definitive proof of conversion requires expected and unexpected tissues to be derived from a single somatic stem cell, which presents an enormous problem when even the most purified stem cell population is heterogeneous (Horwitz 2003; Moore & Quesenberry 2003; Quesenberry et al 2004). It is also vital when proving stem cell plasticity to eliminate contamination by unwanted cells that may be residing in unexpected locations *in vivo* (Horwitz 2003). Differentiation must be accurately assessed by, at the very least, morphological characterisation and antigenic expression profiles. A demonstration of the contribution to tissue function is also desirable. The starting-point must be identified at the single cell or homogeneous level, and the end-point cell population must be fully characterised including the structural and functional contribution to the recipient tissue, eliminating cell fusion as a possible cause (Martin-Rendon & Watt 2003a).

Experimental approaches to identify stem cell plasticity usually rely on the detection of the Y-chromosome in female recipients, or markers such as LacZ or GFP (green fluorescent protein), in addition to antigenic expression profiles to prove lineage (Alison et al 2004). Krause (Krause et al. 2001) transplanted a single cell into a recipient organism and demonstrated multi-lineage engraftment by detection of membrane dye-tagged Y-chromosome positive cells. They used an enriched population of male haemopoietic stem cells that had homed to the female recipient's bone marrow, which were recovered and transplanted at the single cell level into lethally irradiated secondary female mice. The technique resulted in long-term survival, demonstrating donor derived cells in the blood, lung, skin, liver and GI tract at eleven months post transplant (Holden & Vogel 2002; Tao & Ma 2003; Tisdale & Dunbar 2002). However, the cells did not appear to be functional and cell fusion was not assessed. Also, other researchers have failed to replicate these results (Rosenthal 2003; Wagers et al. 2002).

The demonstration of plasticity may be more advantageous *in vitro* because it is likely that many unknown, and therefore uncontrolled, events occur *in vivo*. However, recreating *in vivo* environments for differentiation *in vitro* is very difficult (Almeida-Porada, Porada, & Zanjani 2001).

1.4. Bone Marrow

1.4.1. Bone marrow derived stem cells

Bone marrow is found within the various long bones and some flat bones, including ribs and sternum, of vertebrates. There are two main interacting populations of cells within the bone marrow, the haemopoietic or blood forming cells and the non-haemopoietic mesenchymal stromal cells. The mesenchymal stromal matrix provides a heterogeneous support system or microenvironment for the production of all blood cell lineages through the secretion of an extracellular matrix involving various collagens, glycoproteins (fibronectin, laminin and thrombospondin) and glycosaminoglycans (hyaluronic acid) (Hoffbrand, Moss, & Pettit 2001), and over forty different growth factors that interact through specific receptors to regulate the proliferation, differentiation and cell fate of both haemopoietic and stromal cells (figure 1.8) (Prosper & Verfaillie, 2001). Many cytokines produced by the stromal cell layer, such as colony stimulating factors (CSF), thrombopoietin (TPO) and steel factor (SF) act to stimulate haemopoiesis, whilst others, such as tumour necrosis factor-α, (TNF-α), TNF-β or interferon-γ act to inhibit haemopoiesis by inducing apoptosis (Mayani 2001).
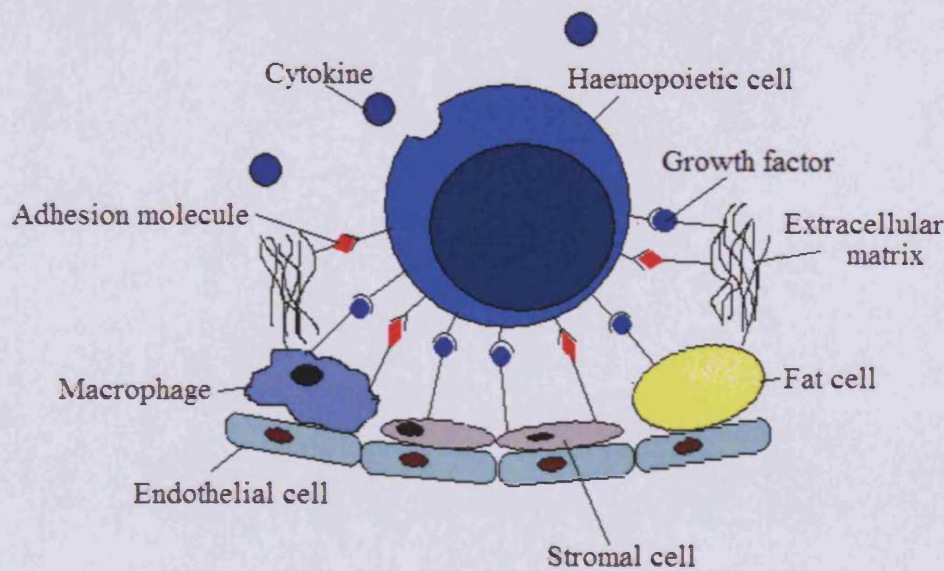
Figure 1.8. A 3D meshwork of stroma, ECM and haemopoietic cells. The stromal matrix forms a niche for the attachment of haemopoietic cells by a series of adhesion molecules and secretes a range of growth factors, detected by growth factor receptors, and cytokines. (Hoffbrand, Moss, & Pettit 2001)

Haemopoiesis takes place within the medullary cavity of the bone marrow, which is subdivided into compartments by venous sinusoids that radiate from the endosteal surface towards the central sinus (Montecino-Rodriguez & Dorshkind 2001). Sinusoids are different to capillaries in that they slow the blood flow down and allow for the transendothelial migration of individual cells from the marrow into the circulation (Bianco & Gehron 2000). Haemopoiesis, in association with the support matrix, occurs in the extravascular space between these marrow sinuses. The blood cells form within the matrix, mainly at the endosteal surfaces, and mature towards the arterioles and sinuses, which are lined with endothelial cells (Lichtman 1984). Eventually, by the interaction of cellular integrins with adhesion molecules and the extracellular matrix, the mature blood cells diapedese through the endothelial layer into the circulation. Integrin signalling plays a large part within the niche to determine the function of the stem cell as to whether it remains quiescent, self-renews or differentiates (Rosendaal & Krenacs 2000). The heterogeneity of the stromal matrix (haemopoietic inductive microenvironment) provides a number of different niches, which may provide varying levels of support to the haemopoietic system, for example, some niches may support the perturbation of the stem cell population, whilst others control the differentiation of progenitor cells (Dexter 1979b). The mesenchymal cells are also responsible for generating a number of mesenchymal tissues including the bone itself.

1.4.2. Bone marrow stem cell differentiation

The two interacting differentiative systems share numerous commonalities, although the more primitive nature of the mesenchymal system is less well understood (figures 1.9 & 1.10). It is thought that both systems follow a hierarchical model beginning with a stem cell (haemopoietic or mesenchymal), before differentiating into numerous progenitor cells with commitment and subsequent maturation. In the case of haemopoiesis, maturation produces a range of functioning blood cells, which release from the bone into the sinusoid (centre of the bone marrow) and into the peripheral circulation. In the mesenchymal system the progenitor cells mature into mesenchymal tissues including bone, fat, tendon, cartilage and mature stromal cells that form the matrix within the bone cavity. Recent work has suggested that the more primitive mesenchymal stromal cells are also able to differentiate into cells outside the mesoderm (1.4.4. 'Evidence of plasticity in bone marrow derived stem cells').

The bone marrow's cytokine and growth factor rich environment provides an ideal niche for the various complex and interacting stem cell systems (Orkin & Zon 2002). The bone marrow may also serve as a reservoir or back up stem cell system for the regeneration of other organs in the body (Gojo & Umezawa 2003). This cytokine rich milieu must be controlled by outside influences to meet the demands of the body. For example, if the organism is depleted in a particular bone-forming lineage, signals (cytokines) will be sent from remote areas of the body to the various sites of haemopoiesis to increase production of that lineage. This allows the various sites of haemopoiesis within the organism to operate in unison.

More recently, another population of bone marrow derived stem cells have been discovered called multipotent adult progenitor cells or MAPCs (1.4.3. 'Multipotent adult progenitor cells'), which are reported to be more primitive with a greater differentiative potential than the mesenchymal cells (Lodie et al. 2002), however, their existence *in vivo* is still in question.

a) Haemopoiesis

Haemopoietic stem cells (HSC), in addition to extensive self-renewal capability, give rise, after a number of cell divisions and differentiation steps, to a large array of haemopoietic cell lines, including erythrocytic, granulocytic, monocytic, megakaryocytic and lymphoid precursor cells (B and T-cell types and natural killer cells) (figure 1.9) (Colvin et al. 2004). They are relatively rare with a frequency of 1 in 10^4 to 10^5 total marrow-nucleated cells (Martin-Rendon & Watt 2003a) but individually are capable of producing approximately 10^6 mature blood cells after 20 cell divisions (Hoffbrand, Moss, & Pettit 2001). HSCs cycle very slowly, so primitive

progenitor cells, comprising a heterogeneous population of cells with overlapping commitment potentials, are more likely to be responsible for this extensive haemopoietic proliferation.

The stem cell compartment can be divided into two overlapping populations, which can be seen as a continuum of primitive to more mature cells. Long-term repopulating cells (HSCs), which proliferate for the life-time of the organism (high levels of telomerase expression) are capable of repopulating a haemopoietic system. Short-term (progenitor) cells do not have telomerase production, proliferating for a short period of time before differentiating into myeloid and / or lymphoid precursors. It is very difficult, if not impossible, to directly identify either a long-term or a short term repopulating cell *in vivo* or *in vitro* (Dept.Health and Human Services 2001).

i. Flexibility within the haemopoietic system

Blood cells are one of the shortest surviving cell types within the body whilst being vital for survival. Mature granulocytes survive for only a few hours and erythrocytes survive for approximately 120 days in the bloodstream. Therefore, the haemopoietic system must be under constant and extensive renewal to meet the demands of the body (Testa & Dexter 1998). The system must also have the flexibility to control differentiation into various lineages during times of stress such as infection or haemorrhage. When the bone marrow is at capacity or unable to function efficiently, the body is able to compensate for the reduction in blood cells by allowing haemopoiesis to take place at extramedullary sites such as the spleen or liver. These organs can be greatly enlarged in certain haemopoietic malignancies where the bone marrow is severely affected by disease and failing to cope with the demands of blood production. HSCs must therefore be able to migrate into the peripheral circulation from the bone marrow in response to particular signals to begin haemopoiesis external to the struggling bone marrow. It is possible to manipulate HSCs into the peripheral circulation for a stem cell harvest using growth factors such as G-CSF (Granulocyte – colony stimulating factor) either alone or in combination with cytotoxic therapy. These treatments can often increase the number of circulating progenitor cells about 100-fold for collection (Hoffbrand, Moss, & Pettit 2001). For haemopoiesis to take place there must also be the formation of a stromal matrix at extramedullary sites, indicating the movement of mesenchymal precursors within the peripheral circulation.

Pluripotent Stem Cell

Multipotent Myeloid Stem Cell   Lymphoid Stem Cell

CFU-GEMM

BFU-E   CFU-Meg   CFU-GM   CFU-Eo   CFU-Ba   Pre-B cell   Pre-T cell

CFU-G   CFU-M

Progenitor Cells

Erythroblast Megakaryoblast Myeloblast Monoblast Eosinophilblast Basophilblast B lymphoblast T lymphoblast

Normoblast Megakaryocyte Neutrophilic Promonocyte Eosinophilic Basophilic
                           myelocyte   myelocyte   myelocyte

Mature and Maturing Cells

Erythrocyte   Platelets   Neutrophil   Monocyte   Eosinophil   Basophil   B-cell   T-cell

Plasma Cell

Macrophage

Figure 1.9. Traditional
hierarchical model of
haemopoiesis, modified from
Ranocyte lenograstim.

Key
BFU-E: Burst forming unit-erythroid
CFU: colony forming unit
GEMM: Granulocyte, erythrocyte, monocyte, macrophage
Meg: Megakaryocyte
GM: Granulocyte, macrophage
Eo: Eosinophil
Ba: Basophil

ii. Characterisation of haemopoietic cells

Undifferentiated HSCs and progenitor cells are characterised by $CD34^+$ (expressed on 0.5-5% bone marrow cells and early progenitors), $CD45^+$, $CD38^-$, c-Kit^+, Lin^- and $Thy1.1^{low}$ expression but even with the most rigorous enrichment techniques HSC populations remain heterogeneous and cannot be directly isolated (Bellantuono 2004). Although they proliferate extensively *in vivo*, their life span *in vitro* is significantly reduced and they either differentiate out of the stem cell population or apoptose. Therefore, a great amount of research has gone into the components and niche required to maintain the stem cell compartment *in vitro* in addition to the cell-cell interactions and cell-matrix interactions required to control their proliferation and differentiation, with the hope that *in vivo* environments can be replicated *in vitro* (Dept.Health and Human Services 2001).

iii. Haemopoietic microenvironment

In the late 1970s it was found that the crucial feature of an *in vitro* haemopoietic long-term cell culture system was the formation of an adherent cell layer (Dexter 1979a). In siliconised flasks (inhibited adherence), or in the absence of, or presence of only a poor adherent cell layer, haemopoietic cultures survived for 1-2 weeks, whereas in the presence of a confluent haemopoietic inductive stromal microenvironment, haemopoietic cultures survived for months. One of the main features Dexter noticed within mouse long-term cultures was the success of granulopoiesis in the presence of giant fat cell aggregates, although a direct link has never been proven (Dexter 1979b). The success of a haemopoietic long-term culture is heavily influenced by the serum batch used and this may be partly due to the availability of corticosteroids (provided by the serum) necessary for lipogenesis. If granulopoiesis relies on fat cell production a poor serum batch would result in a poor haemopoietic culture system. Hydrocortisone supplementation was also found to stimulate bone marrow derived stromal cell growth and adipogenesis in culture (Suda & Dexter 1981). It is interesting to note that human long-term cultures have a shorter survival times than mouse cultures, and that these giant fat cells are not found in human long-term culture systems.

Within haemopoietic cultures, cobblestone areas of granulopoiesis form in association with macrophages and flattened endothelial cells beneath a sheet of blanket cells (large well spread polygonal cells) that aid in the production of an extracellular matrix. The stromal microenvironment produces a series of niches in which granulocyte progenitors (CFU-GM) reside for maintenance and differentiation. Granulocytes then rise up through the extracellular

matrix and eventually release into the culture supernatant (Allen & Dexter 1984; Dexter et al. 1984).

iv. The hierarchical model questioned?

Colvin (Colvin et al 2004) proposed a different model to the traditional hierarchical structure by suggesting that no hierarchy exists within haemopoiesis. There is known to be a overlap between the stem cells and cells that have developed further (early progenitor cells) due to the continuum of the system (Testa & Dexter 1998), but Colvin suggests that within the more primitive compartments there is no hierarchy and that stem / progenitor cells represent different phenotypes of the same cell, which behave differently at different positions within the cell cycle. The phenotype of a primitive cell may change throughout the cell cycle through chromatin remodelling, altering surface phenotype and ultimately determining its response to environmental stimuli. One cell may act as a progenitor at one point in the cell cycle before returning to stem cell capability in another.

b) The bone marrow stromal matrix

Bone marrow derived mesenchymal cells were first identified in rats by Friedenstein (Friedenstein & Petrakova 1966) as fibroblast-like cells and have since been isolated from a number of species including mice, human, dog, baboon, pig, sheep, goat, rabbit and cat (Javazon et al. 2004; Meirelles & Nardi 2003). Mesenchymal precursors are rare in bone marrow, estimated to be 0.3 in 10^4 bone marrow cells (Flores-Figueroa et al. 2005; Wexler et al. 2003). Mesenchymal cells are known to arise from the mesoderm during embryonic development alongside the HSCs, but their origin remains elusive. One theory proposed is that a common stem cell exists for both HSCs and mesodermal precursors, but as yet no proof exists of this (Flores-Figueroa et al 2005). Bone marrow derived mesenchymal cells form the non-haemopoietic element of the bone marrow as a heterogeneous population of stromal or fibroblast-like cells, endothelial cells, adipocytes, osteoblasts, smooth muscle, macrophages and reticular cells (Galotto et al. 1999; Hoffbrand, Moss, & Pettit 2001).

i. Terminology

No standardised terminology exists for the heterogeneous population of bone marrow derived mesenchymal cells. They are referred to in the literature by many names including colony forming unit-fibroblasts (CFU-F), mesenchymal stem cells (MSCs), mesenchymal progenitor cells (MPCs) and mesenchymal stromal cells (Meirelles & Nardi 2003) and retain a broad capacity to differentiate along numerous nonhaemopoietic mesenchymal pathways including

osteogenic, chondrogenic, adipogenic and tendogenic lineages (figure 1.10) in response to the appropriate stimuli (Horwitz 2003). The term MSC, used by many authors, is largely an assumption, as MSCs have never been identified. The term may represent a mixed multilineage population, rather than the multilineage differentiation of a common undifferentiated MSC type into a heterogeneous MPC pool (La Russa et al. 2002). In the absence of direct mesenchymal stem cell identification, their *in vivo* stem cell nomenclature arises from their ability to self-renew; differentiate into multiple cell types and regenerate mesenchymal tissues following injury.

Simplistic model of mesenchymal differentiation
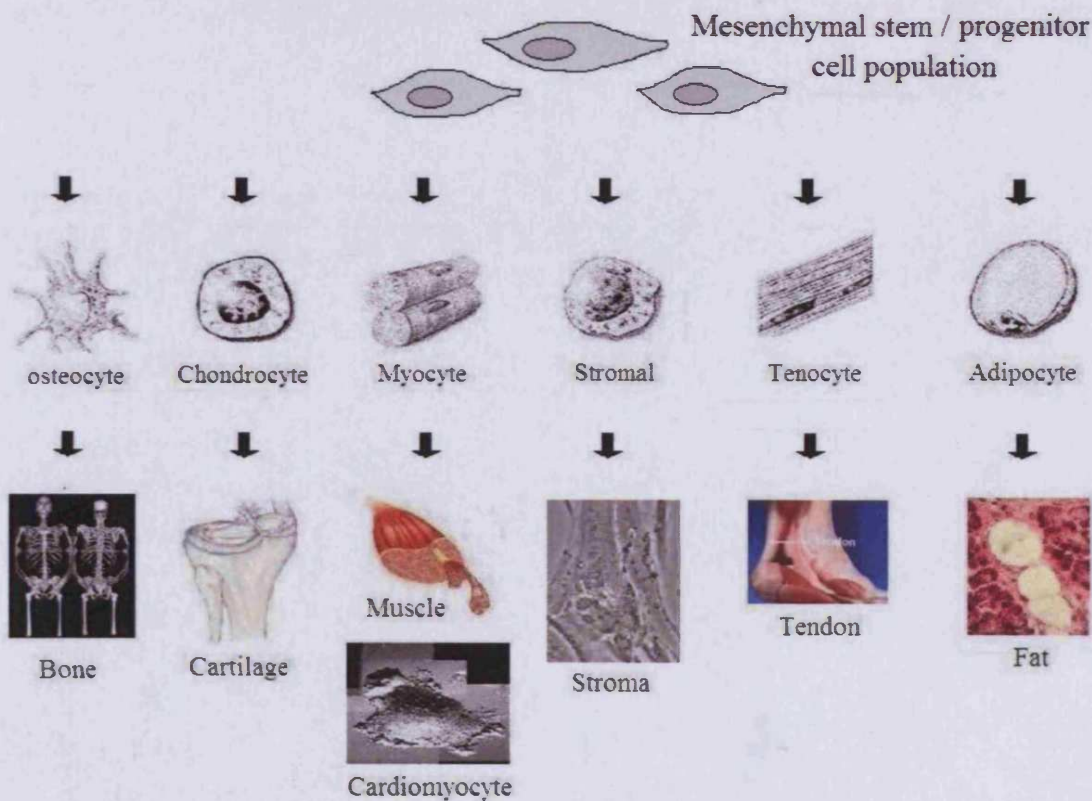
Figure 1.10. Hierarchical mesenchymal lineage model. Adapted from Pittenger (Pittenger et al. 1999).

Because terminology of the heterogeneous mesenchymal fibroblast-like population is so difficult and because the presence of mesenchymal stem cells have never been identified *in vivo* or *in vitro*, this investigation will use the term Bone Marrow derived Stromal Cells (BMdSCs) to include all stem, progenitor and terminally differentiated cell types within the heterogeneous stromal layer. The heterogeneous nature of the BMdSCs (includes all stages of development from terminally differentiated stromal cells to stem cells) makes identification and

characterisation by specific cell markers difficult to establish. They are uniformly negative for the haemopoietic markers CD45, CD11b and CD117 (c-kit) (Herzog, Chai, & Krause 2003). CD34 expression is variable particularly with mouse stromal cells (Javazon, Beggs, & Flake 2004). No specific cell marker or combination of markers has been established to identify the stromal cells but positive expression for the adhesion molecules CD106 (Stro-1, VCAM-1), CD166 (ALCAM), CD44 (HCAM-1), CD49b (VLA-α2) and CD105 (endoglin) has been ascertained.

ii. BMdSC isolation

Currently the main method for isolating BMdSCs from the mononuclear cell fraction of the bone marrow is by plastic adherence, however, macrophages, endothelial cells, lymphocytes and smooth muscle cells, also present within the mononuclear cell fraction, are initially able to adhere to plastic, contaminating early bone marrow preparations (Javazon, Beggs, & Flake 2004). Several attempts have been made to isolate the more primitive BMdSCs from the bone marrow using CD106 expression (Gronthos et al. 1994; Stewart et al. 2003) or by using negative selection to remove contaminating haemopoietic cells (positive for CD45 and CD34) (Short et al. 2003), but the resulting population was still largely heterogeneous. The life span of proliferating BMdSCs *in vitro* is limited (20-30 population doublings, remembering that not all BMdSCs in culture have the capability to divide) indicating that the *in vitro* environment cannot sustain self-renewal. It is unclear where BMdSCs stop proliferating during the hierarchical differentiative model (figure 1.10) and only a small percentage of cells are likely to be stem / progenitor cells within the population. Either the stem cells rapidly differentiate out of the stem cell population during culture or they senesce. This limited proliferative capacity for BMdSCs in culture is currently a disadvantage for their use in cell therapy (McWhir et al. 2003).

1.4.3. Multipotent Adult Progenitor Cells

MAPCs become apparent in adherent BMdSC cultures after 25-35 population doublings when grown at low density and cultured on fibronectin in the presence of little or no foetal bovine serum, and in the presence of epidermal growth factor and platelet-derived growth factor (Verfaillie et al 2003). They have been isolated from the mononuclear cell fraction of both mouse and human bone marrow, and can be grown indefinitely *in vitro* without genetic instability (Horwitz 2003).

Many authors have suggested that MAPCs are created *in vitro* and do not exist *in vivo*, but they have been shown to differentiate into a wide variety of tissues of mesodermal, ectodermal and

endodermal origin when injected into early blastocysts (e.g. brain, lung, myocardium, liver, intestine and kidney) or transplanted into irradiated animal models (e.g. cells of marrow blood and spleen; epithelial cells in liver, lung and intestine) (Grompe 2002; Herzog, Chai, & Krause 2003; Horwitz 2003). MAPCs have also been differentiated *in vitro* at high cell densities into cells with expression profiles and functional characteristics of neurons and hepatocytes (Verfaillie et al 2003).

1.4.4. Evidence of plasticity in bone marrow derived stem cells

The bone marrow appears to be central to the regeneration of tissues, encompassing a number of interacting stem cell systems (mesenchymal, possibly including MAPCs, haemopoietic and hemangioblastic), which are responsible for differentiating into a wide range of cell types of various germ lines. This is clearly seen following bone marrow transplantation (BMT) with the engraftment of bone marrow derived cells into various tissue types (Okamoto et al. 2002). BMT does not indicate the actions of a single cell but rather a heterogeneous population of cells (Bellantuono 2004), so when interpreting plasticity studies following, for example BMT, it is often very difficult to determine the specific cell type responsible for the engraftment and conversion (McWhir et al. 2003). This has led many authors to encompass BMdSCs, including mesenchymal stem / progenitor cells, and HSCs into one heading: 'bone marrow derived cells' (BMDC), during plasticity studies, unless the specific cell type was identified (Martin-Rendon & Watt 2003a).

a) Bone marrow to mesoderm

BMdSCs are known to differentiate into a wide range of mesodermal tissues including bone, fat, cartilage, muscle and tendon. BMdSCs have been clearly shown to differentiate into osteoblasts-like cells *in vitro* in the presence of the appropriate environmental stimuli, with the resultant production of hydroxyapatite mineralised matrix, alkaline phosphatase, collagen I, osteopontin and osteocalcin expression (markers of osteogenic lineage) (Ahdjoudj et al. 2001; Frank et al. 2002; Jaiswal et al. 1997). The formation of new bone following BMT, for example in children with osteogenesis imperfecta (1.5.3.'Experimental successes') also indicates the conversion of BMdSCs into bone *in vivo* (Tao & Ma 2003). Cartilage-like structures can be stimulated to form *in vitro* with the formation of chondrocyte-like lacunae and representative expression profiles including cartilage matrix glycans, proteins and type II collagen. Tendon repair is also possible from the conversion of BMdSCs to tenocytes using scaffolds and cell seeded implants (Tao & Ma 2003).

Muscle fibres have been regenerated at low levels in mouse models with Duchenne's muscular dystrophy following infusion of highly purified male HSCs into female recipients (Martin-Rendon & Watt 2003b), and damage-induced skeletal muscle cells have been shown to regenerate following BMT (Krause 2002). The systemic (Barbash et al. 2003) or direct injection of male GFP transfected BMdSCs into the hearts of female mice following experimentally induced myocardial infarcts can promote tissue regeneration with the production of GFP and Y-chromosome positive cardiac myocyte, smooth muscle and endothelial-like cells, resulting in improved myocardial capillary vasculature and ventricular function (Fukuhara et al. 2003; Herzog, Chai, & Krause 2003; Horwitz 2003; Martin-Rendon & Watt 2003b; Orlic et al. 2001a). Studies by Orlic (Orlic et al 2001a; Orlic et al. 2001b) showed that nine days following injection into infarcted female mouse hearts, approximately 50% of myocytes, endothelial cells and smooth muscle cells were donor-derived (Fraser et al. 2004). The differentiation of BMdSCs has also been shown to form elongated contractile myotubule-like structures *in vitro* with the resultant expression of contractile proteins, adrenergic receptors and cardiac-specific transcription factors following stimulation with 5-azacytidine (Tao & Ma 2003).

b) Bone marrow to ectoderm

Following BMT, a small proportion of donor male BMDCs (identified using Y-chromosome) have been discovered in several regions of the female mouse brain including cortex, hippocampus, thalamus, brain stem and cerebellum. Once *in situ* they appear to respond to local regulation differentiating into multiple cells expressing various neuronal, microglial and astroglial specific markers (Martin-Rendon & Watt 2003a; Martin-Rendon & Watt 2003b). Other reports outlined by Horwitz (Horwitz 2003) have reported similar findings of systemically transfused male mouse or rat BMDCs being found throughout the brain of lethally irradiated female recipients expressing neuronal characteristics. This provides strong evidence that BMDCs can migrate to the brain after BMT and differentiate in response to local environmental cues into many neuronal phenotypes, but the true nature of the specific BMDC responsible has not yet been elucidated. More convincing evidence is provided by the direct injection of BMDCs into the lateral ventricle of the adult rodent brain, which migrate to the different areas of the brain including forebrain and cerebellum before differentiating into cells with various neuronal characteristics (Tao & Ma 2003). This type of investigation led to an improvement in the behaviour of mice with Parkinson's disease as the BMDCs contributed to the regeneration of degenerated nervous tissue (Kim et al. 2002). There are also multiple reports of *in vitro* differentiation of BMdSCs into neuronal-like cells, expressing various neuronal specific markers such as nestin, neural specific enolase, NeuN and Neurofilament

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protein (Black & Woodbury 2001; Sanchez-Ramos et al. 2000; Tisdale & Dunbar 2002; Vogel et al. 2003).

Following sex-mismatched BMT, male Y-chromosome cytokeratin[+] BMDCs have been found in the skin of female recipients, the BMDCs engrafting as epithelial and endothelial cell types during the formation of scar tissue. This engraftment is significantly enhanced following injury (Herzog, Chai, & Krause 2003).

c) Bone marrow to endoderm

Krause (Krause et al 2001) provided the first experimental evidence that a single haemopoietic reconstituting cell could engraft into lung, liver, intestine and skin (Tisdale & Dunbar 2002) (1.3.4. 'Difficulty in establishing stem cell plasticity'). Experimental conversion of bone marrow to liver is still controversial. Many have reported the differentiation of HSCs (CD34^{+}, Lin^{-}) or unfractionated bone marrow to oval cells and hepatocytes in the liver by identifying the Y-chromosome after sex mis-matched transplantations in mice, particularly following the induction of liver damage. However, hepatocytes are well known for fusing with various cells, so it is possible that BMDCs fused with the recipient hepatocytes to take on the phenotypic characteristics of the liver rather than by transdifferentiating (Herzog, Chai, & Krause 2003; Martin-Rendon & Watt 2003a; Martin-Rendon & Watt 2003b).

The lung has an inherent cellular complexity and slow parenchymal cell turnover, which makes plasticity experiments difficult to carry out and interpret, but there are reports of BMDCs having differentiated into lung phenotypes (expressing cytokeratin for epithelial cells and surfactant B for pneumocytes) post transplantation in mice. This could be significantly increased following tissue damage (Fine 2004), but again cell fusion was not assessed.

Following sex mis-matched BMT, BMDCs have been demonstrated to differentiate into kidney mesangial and interstitial cells as well as renal tubular cells following ischemic renal disease in mice. Y-chromosome positive and GFP positive BMDCs have also been found in the pancreas, oesophagus, stomach and bowel of females suggesting a wide engraftment of BMDCs into endodermal tissues (Herzog, Chai, & Krause 2003).

1.5. **Future Uses of Embryonic and Adult Stem Cells**

Both embryonic and adult stem cells have an immense potential for cellular therapy but there are many problems to contend with before they can be used routinely to treat diseases. The cells would need to be amplified in sufficient number without transformation and must not cause tumour formation *in vivo* (1.2.3. 'Comparison between adult and embryonic stem cells'). Immunologically compatible cells would be beneficial for use in cell therapy (more likely with adult stem cells rather than embryonic) but may not be necessary if rejection can be treated efficiently enough (Donovan & Gearhart 2001).

1.5.1. **Stem cells for cellular therapy**

The presence of stem cells, even rapidly renewing stem cells, may not always be enough to regenerate organs following significant injury. Deeper wounds or larger areas of damaged tissue require several cell types to be produced in an orderly fashion to restore tissue structure, at which point mammalian repair usually fails (van Bekkum 2004). With the finding that adult stem cells may be more pluripotent than originally thought and with the versatility of ES cells, the possibilities for cell replacement therapy are immense. Many animal studies have demonstrated the repair of mesenchymal tissues including muscles, tendons, cartilage and bone (Devine 2002), but various stem cells may also be useful for neurological repair in neurodegenerative diseases such as Alzheimer's or Parkinson's disease. Other diseases that may benefit from stem cell therapy include strokes, heart disease, burns, diabetes, multiple sclerosis, spinal cord injuries, osteoarthritis or rheumatoid arthritis among many others (Orive et al 2003). There are still, however, many questions that remain to be answered before stem cells can be used in clinical trials. For example, what are the role(s) of endogenous stem cells in the adult and how are they activated to divide or kept in a quiescent-like state? What are the natural environments of stem cell niches *in vivo* and what are their functions? Can we reproduce these environments *in vitro*? What are the mechanisms responsible for homing, engraftment and differentiation (Barry & Murphy 2004)? Transplantation of mesenchymal cells by systemic infusion is already known to cause long-term engraftment into various tissues around the body (Roufosse et al. 2004), but how are they homed to specific sites for regeneration, and can this be controlled to mobilise endogenous stem cells (Barry & Murphy 2004)? If adult stem cells are plastic in their ability to differentiate into many cell types can we define the molecular mechanisms underlying lineage switching to enhance its use for cellular therapy? If plasticity could be utilised it wouldn't be necessary to use allogeneic cells to replace diseased cells because autologous cells could be isolated and induced to differentiate down very different lineages before transplantation back into the patient. Better characterisation of the stem cells,

currently flawed by the lack of specific cell surface markers, would lead to a better understanding of the cell types involved in certain repair mechanisms. It is also vital to ensure the genetic stability of *in vitro* modified cells before transplantation to prevent oncogenesis *in vivo* (Verfaillie 2002).

1.5.2. Stem cells for scientific research

Stem cells, particularly ES cells, may not only be useful for therapeutic purposes but may also be valuable resources for scientific research. ES cells could be used to analyse how cells proliferate and differentiate into particular lineages in the presence of specific gene and chromosomal abnormalities in comparison to normal cells. Also chromosomal abnormalities underlying many congenital defects, for example, those that cause tumours in children, could be determined, whilst allowing the disease to be studied *in vitro* (Rosenthal 2003). ES cells carrying the defect can be created from patients with the disorder by SCNT, or they can be induced from knock-in or knock-out technology, which induce the defect into cells (Barratt et al. 2004). It may even be possible to correct the gene mutation(s) in ES cells created by SCNT by gene therapy to create healthy ES cells for transplant back into the patient. ES cells could therefore be very important in furthering our understanding of the early development and occurrence of many diseases and birth defects, whilst being able to gain a better understanding of many physiological disease processes (Mariani 2002; Wilmut 2004). ES cells, created from multiple human tissue types, could also be used for developing and testing new therapeutic drugs or for performing toxicity studies (Rosenthal 2003).

1.5.3. Experimental successes

Many successes have been documented in animal studies with possible relevance to a clinical setting, perhaps one of the most prominent being the treatment of neurodegenerative disorders such as Parkinson's disease. This disease affects more than 2% of the population over 65 years of age and is caused by a loss of dopamine-producing neurons leading to tremors, rigidity and hypokinesia (National Institute of Health 2004). The differentiation of BMdSCs into dopamine producing neurons has been well documented to relieve symptoms of neurological disorders (Kim et al 2002; Tao & Ma 2003; Vogel et al 2003; Zhao et al. 2002). ES cells have also been genetically manipulated and differentiated into dopamine neurons *in vitro* for transplantation into mouse models of Parkinson's disease, the mice showing profound improvements in abnormal behaviour (Grompe 2002; Orkin & Morrison 2002; Schuldiner et al. 2001).

BMTs have been successfully used in humans since the Second World War to reconstitute haemopoiesis after myeloablation and to treat a variety of haematological malignancies in humans. Infusion of BMdSCs with HSCs during BMT may also facilitate engraftment and reconstitution of the damaged microenvironment to improve haemopoiesis (Deans & Moseley 2000; Dominici et al. 2001).

Allogeneic BMTs have been used to treat bone disorders such as osteogenesis imperfecta in children. This is a disease in which bone cells (osteoblasts) produce defective type 1 collagens leading to osteopenia, bone fractures, deformities and shortened lifespan. Following BMT, the growth rate and bone mineral content was increased with reduced bone fractures (Tao & Ma 2003).

1.5.4. Bone marrow derived cells in cellular therapy

Bone marrow derived cells, in particular BMdSCs, are proving to be the most promising area for cellular replacement because of their ease of isolation, growth potential *in vitro* without transformation and varied differentiation capability (figure 1.11) (Lodie et al 2002). BMdSCs have been shown to not only differentiate into the mesenchymal lineages such as bone, fat, tendon, cartilage and muscle, but also into ectodermal tissues such as epithelial cells or neurons. BMdSCs may also differentiate into other cells of the ectoderm or endoderm pathways including lung, liver, pancreas or kidney tissues. With many experimental procedures performed *in vivo* it has been impossible to determine whether the cells were mesenchymal or haemopoietic in origin, or whether in fact they were caused by other stem cells residing in the bone marrow, which is the reason for the vagueness within the diagram (figure 1.11: the arrows moving outward from the bone marrow to various organs).
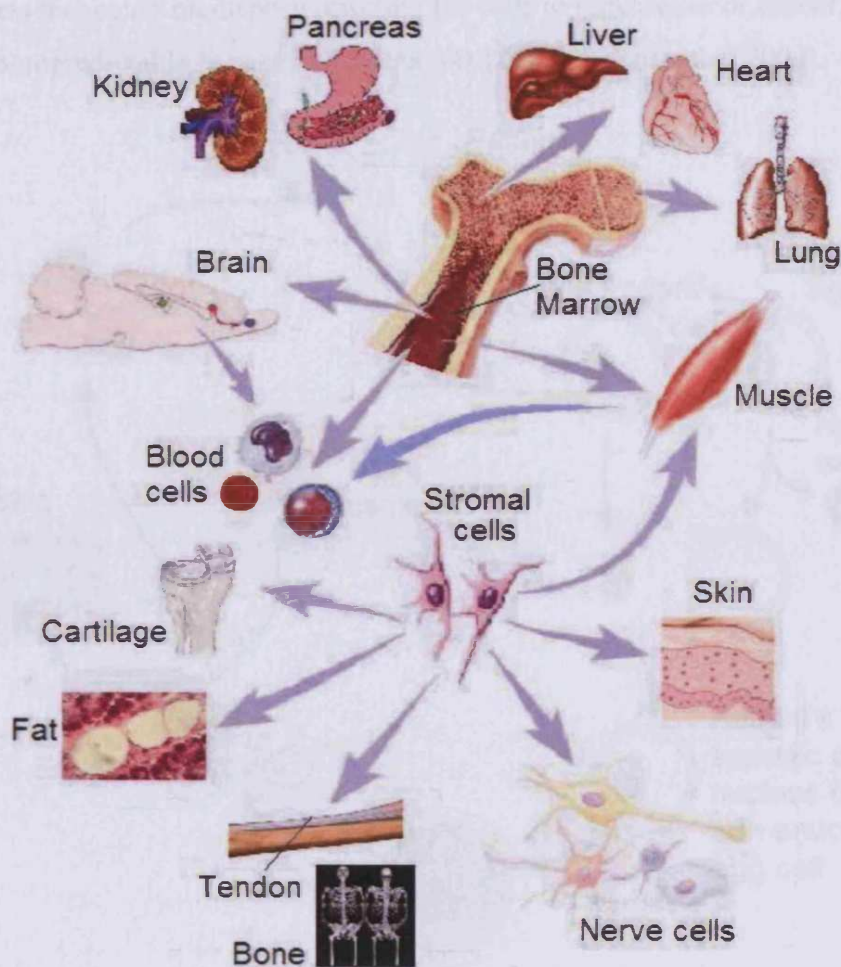
Figure 1.11. Bone marrow derived cells from mesenchymal and haemopoietic lineages have been shown to differentiate into numerous tissues. Modification from Dept. Health and Human Services, 2001

1.5.5. **ES cells in cellular therapy**

A big problem with using ES cells for therapy is the lack of immunological compatibility with the patient. Although autologous adult stem cells are immunologically compatible they have limited potential in comparison to ES cells. Therapeutic cloning by SCNT can create immunologically compatible ES cells for transplantation by injecting the patient's somatic cell nucleus into an enucleated oocyte to form a blastocyst from which the ES cells can be isolated (figure 1.12) (Westphal & Cohen 2004).

The immunologically compatible ES cells are instructed to differentiate *in vitro* before being transplanted into the patient. In effect, the ES cells are customised to each patient in a long drawn out and expensive process, which may make the technique unlikely for large numbers of patients (Rippon & Bishop 2004). It is also still uncertain whether this technique may introduce

gene mutations that could predispose resulting ES cells to senescence or cancer, making the technique too unpredictable for use in humans, yet (Lanza & Rosenthal 2004).
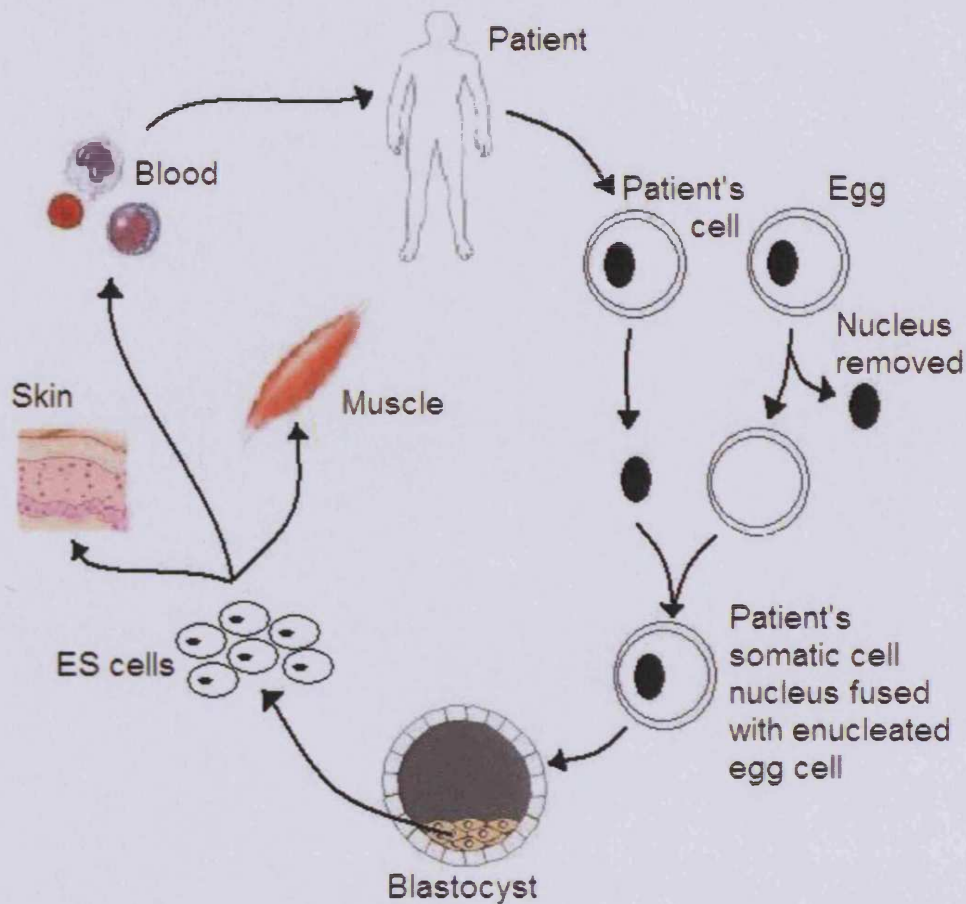
Figure 1.12. A patient's nucleus is inserted into an enucleated oocyte to form a blastocyst from which ES cells can be harvested and differentiated into specific cell types *in vitro*. The cells can then be transplanted into the patient. (Palmer 2004).

Before ES cells can be transplanted into humans it is vital that pure populations of mature differentiated progeny are isolated to prevent tissue function perturbation and teratoma formation (Rippon & Bishop 2004). It could be useful if ES cells could be coaxed into a more stable, yet flexible, progenitor-like cell stage before administration because this would allow local environmental cues to control lineage without the occurrence of spontaneous uncontrolled differentiation. However, the underlying mechanisms that control development in human ES cells are still largely unknown (Heins et al. 2004). Another problem associated with ES cells is that they are grown on murine feeder layers (the absence of which results in the formation of embryoid bodies that randomly and spontaneously differentiate) (Alok Jha & Stephen Minger 2004), which creates a risk of animal pathogen transmission to humans upon transplantation (Rippon & Bishop 2004). It is therefore important to find ways to isolate and grow ES cells without using animal products before they can be used in a clinical setting. ES cells have been

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expanded up to 130 population doublings using extracellular matrices such as laminin in place of feeder layers but conditioned media containing animal products is still required (Conley et al 2004; McWhir, Thomson, & Sottile 2003). ES cell isolation and culture is also very laborious and time consuming with the report that after successful isolation of the inner cell mass and establishment of an ES cell population, the cell line still takes 6 months to create, making large-scale ES cell production difficult for viable ES cellular based therapies (Alok Jha & Stephen Minger 2004).

Despite the many ethical and technical problems associated with therapeutic cloning, many still believe that it holds an enormous potential to provide patients with tissue-matched stem cells, including Ian Wilmut's team who were responsible for cloning Dolly the sheep (Wilmut et al. 1997).

Stem cell research still offers a great potential for human therapeutics although many political, ethical and technical obstacles remain to be determined and corrected before it can be used in humans. It is important that research continues into both embryonic and adult stem cells until many more mechanisms are understood that can be utilised in the laboratory (Orive et al 2003).

1.5.6. Cell fusion in therapy

The problems associated with cell fusion during cellular therapy are still unclear with the possibility of transplanted cells fusing with host cells. Often, hybrid cells that form during normal development fail to proliferate further so it is possible that fusion during tissue repair will create cells with limited developmental potential. It is also possible that these cells may be genetically unstable with the potential to form tumours *in vivo*. No evidence exists to show that fusion of normal non-transformed cells causes genetic instability and we know that cell fusion is a natural event *in vivo*, but the possibility must first be assessed (Vassilopoulos & Russell 2003).

In summary it has not been proven that somatic stem cells are more pluripotent than originally thought, but there is enough evidence suggesting their plasticity to warrant continued investigation, with the intention of eventually using various cell types such as bone marrow to cure or improve many genetic or neurodegenerative diseases (Verfaillie 2002).

1.6. **Cellular Systems**

1.6.1. **Network interactions**

A multicellular organism is composed of many biological systems, which are organised into functional networks of varying complexity. A protein network, dictated by a genetic network, is relatively simple in comparison to the cellular network it creates, and a cellular network is less complex than the tissues or organs they form. The genes dictate the functions of the proteins, which dictate the functions of the cell, which dictate the functions of the tissue, which help dictate the function of the organ, required for the organism to function (figure 1.13).

Increasing network complexity

| GENES | → | PROTEINS | → | CELLS | → | TISSUES | → | ORGANS |

Positive and negative
feedback systems
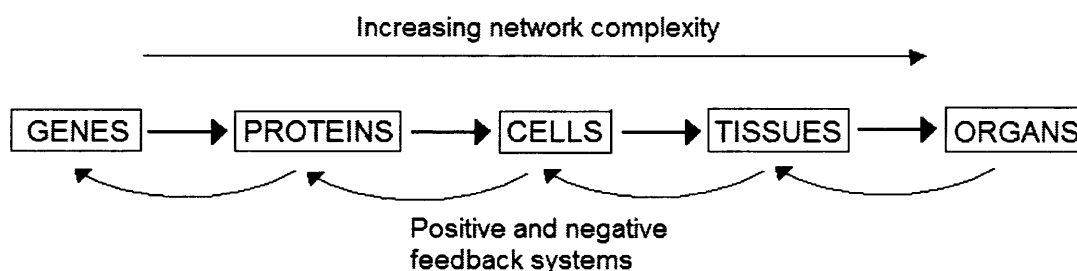
Figure 1.13. Progressive unfolding of information to create different levels of complexity and organisation, based on the activities of a small number of functional modules, organised hierarchically with increasing levels of complexity (Arias & Stewart 2002b).

A hierarchical system is based on the activities of the individual networks, which are organised into levels of increasing complexity (Arias & Stewart 2002a) (figure 1.14). Each component within a network makes a small, but essential contribution to the overall function of the system (Dunkley & Ralston 1992) and the number of networks involved determines the complexity of the system. The bone marrow environment is extremely complex because it involves a number of interacting stem cell networks including the interplay between the haemopoietic and mesenchymal systems.

For the organism to function efficiently each system must be controlled by the regulation of the individual components of the networks. Networks are often self-organising in that they have intrinsic regulation involving positive and negative feedback loops that control function. For example, in normal conditions the differentiation of particular haemopoietic lineages within the bone marrow can be precisely controlled to prevent excessive cellular formation, whilst satisfying demand. External signals (extrinsic regulation) continuously instruct intrinsic regulation to alter accordingly to meet the demands of the body. During haemorrhage, external signals, such as erythropoietin, may alter positive and negative feedback loops to favour

erythrocyte production, or during infection, activation factors may stimulate granulocyte, monocyte lineage production. Therefore, cells within different tissues can signal the need for different levels of cellular production when needed (Testa & Dexter 1998).
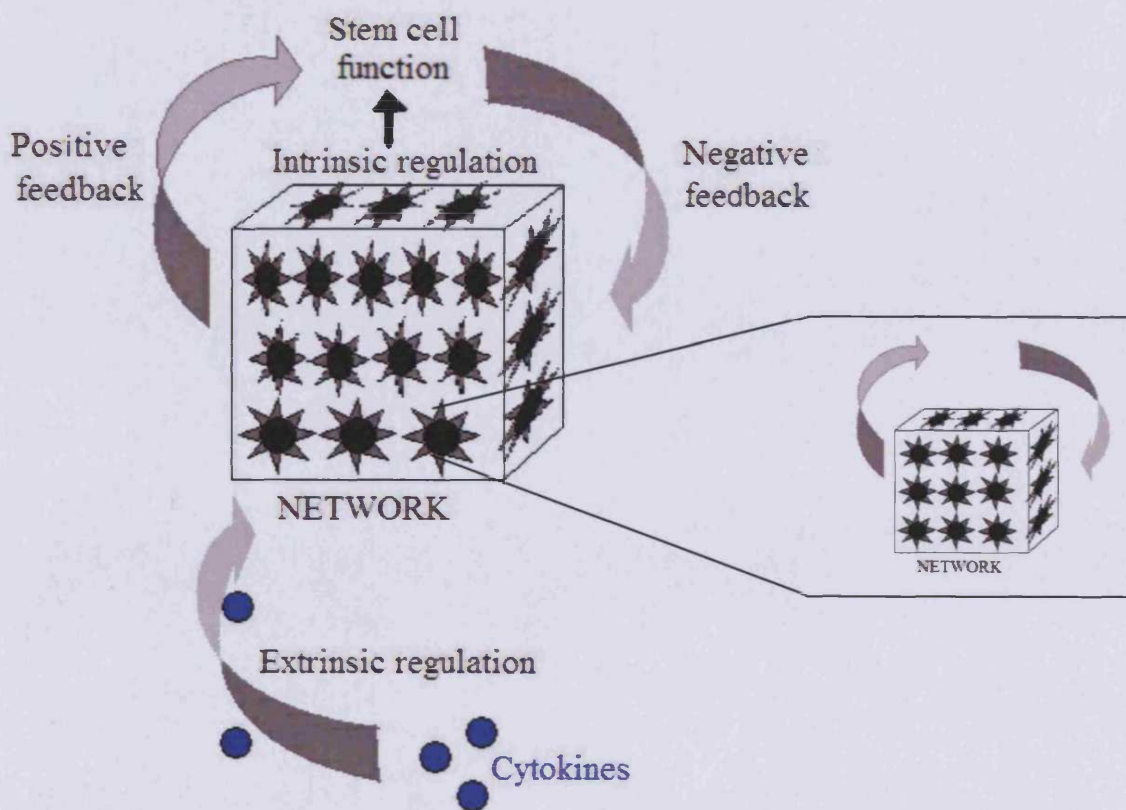
Figure 1.14. A stem cell network involving intrinsic and extrinsic regulation. A series of these networks make up a system. Modified from Flake (Flake 2004).

Some cellular biologists believe that complex systems can only be understood by studying their individual components (Cartesian science), for example, CFU-GM (colony forming unit – granulocyte, macrophage) assays, which investigate the presence of the granulocyte, monocyte precursor cells, are performed *in vitro* out of context of the system. This is unlikely to represent behaviours present within the system because the cells will be affected by *in vitro* influences rather than systemic influences. Others believe that living systems must be studied as a whole and that 'reductionism' or removing parts from the larger whole may alter the function of that component (Systems science) (Flake 2004). Many plasticity studies have been performed *in vivo* in the context of Systems science, however these have often involved artificial injury to promote plasticity. This is likely to alter network function, the ability of a cell to undergo a conversion event being determined by the disruption in network function. However, demonstration of stem cell plasticity *in vitro* in the context of Cartesian science is likely to skew

the behaviour of the assay as to what is expected of it without representing *in vivo* function. True stem cell plasticity, or the natural tissue response to injury or disease, can only be studied accurately on unmanipulated cells using Systems science. Systems science, however, is currently limited, forcing cellular biologists to use Cartesian science and imply an interpretive view with potential clinical relevance (Flake 2004).

1.6.2. Adaptation

For a multicellular organism to survive, systems must be able to adapt, for example, during times of injury or stress. Networks must be highly reactive to external signals to adapt their function as necessary to change system output. More complex systems, with a greater degree of network interaction, have a larger flexibility to alter function in a shorter space of time, and are termed complex adaptive systems (Theise & d'Inverno 2004). It is important to note that there is a great deal of redundancy (non-functioning components) within a complex network. This allows for adaptation to rapidly occur when the equilibrium is disturbed, for example, during bleeding, multiple pathways may be activated to directly or indirectly stimulate erythropoiesis. Having various routes to exert the same effect allows systems to rapidly respond to any given environmental situation, but under steady state situations, not all these routes may be activated.

Haemopoiesis is highly reactive to external influences to enable a rapid change in response to changes in the haemostasis, for example, during haemorrhage. Each lineage within haemopoiesis can be thought of as a reactive network with a large degree of emergent self-organisation in that a small external signal can rapidly produce a large effect (much coming from little) (Holland 1998). The effect produced must be controlled by negative feedback loops and inhibitory signals to prevent excessive activation and system disruption, which may ultimately lead to system failure (chaos) (Lewin 1999; Theise & d'Inverno 2004).

Complex adaptive systems must have a certain degree of unpredictability or non-deterministic behaviour (flexibility) to be able to rapidly alter intrinsic regulation and network function. This unpredictability, however, must be controlled to prevent pathological disruption. Low-level engraftment from other tissues has the power to alter network function and may therefore introduce a certain degree of unpredictability into the system. Less complex systems are more determined in their response and do not have this flexibility or self-organisation to rapidly adapt to response. They are not emergent and need a larger stimulation and more time to exert an effect (Theise & d'Inverno 2004).

1.6.3. **Communication**

The individual components of a network are not aware of the larger organisation or requirements of the system and therefore must interact or communicate with each other and the external environment to maintain and adapt their function as necessary (Rasmussen 1991). Environmental factors from cells and the extracellular matrix (ECM), such as hormones (cytokines, growth factors and metabolites), chemokines, adhesion factors, neurotransmitters and other signalling molecules, in addition to direct cell-cell and cell-ECM contact, act in accordance with each other to determine cell behaviour (Theise & d'Inverno 2004). Each cell is therefore continuously being instructed by extracellular signals from the surrounding microenvironment (Dunkley & Ralston 1992; Flake 2004).

a) **Extracellular communication**

There are various forms of extracellular communication. Extracellular signals, secreted by various cells can either travel long distances through the bloodstream (e.g. hormones) to exert a cellular effect (endocrine interactions) or can be locally acting (local hormones) either on the same cell that secreted it (autocrine interaction) or / and on neighbouring cells (paracrine interactions) (Dunkley & Ralston 1992). They exert a cellular response (e.g. proliferation, differentiation, contraction, secretion or metabolism) by binding to receptors that convert the extracellular signal to one or more intracellular messengers within a particular signalling pathway that changes the function of the cell (Berridge 1991). Receptors therefore mediate communication between the extracellular environment and target enzymes or specific DNA sequences within the cell to induce a specific set of responses. Ligand binding and receptor activation provides cells with information about their surrounding environment and allows them to react appropriately to the demands of the body (de Fougerolles & Koteliansky 2002).

Integrins are heterodimeric transmembrane receptors. They not only transduce extracellular signals across the membrane, but also anchor the actin cytoskeleton of a cell to components of the extracellular matrix (adhesion), allowing for alteration in shape and structural organisation (Danen & Sonnenberg 2003). Binding occurs at a low affinity so that the cells can readily detach and migrate when necessary (Rosendaal & Krenacs 2000).

b) **Intracellular communication**

Intracellular mediators greatly amplify the extracellular signal to induce an adequate cell response through the use of signalling cascades (Dunkley & Ralston 1992). There are three known classes of cell-surface receptor proteins that initiate signalling systems within the cell:

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ion channel-linked, enzyme-linked and G-protein-linked receptors. Ion-channel linked receptors are involved in rapid synaptic signalling between electrically excitable cells, whilst enzyme-linked receptors either function directly as enzymes or activate other enzymes to exert an effect. G-protein-linked receptors are the most common and activate a plasma membrane bound GTP-binding protein (G-protein), which in turn phosphorylates and activates another plasma membrane bound protein such as an enzyme or ion channel. The enzyme (serine or threonine kinases) acts to increase the concentration of one or more intracellular mediators, which exert an effect on the cell by activating further proteins. The opening of an ion channel allows for the influx of a particular ion to increase intracellular concentrations to exert an effect.

The two most commonly used intracellular mediators are cyclic AMP (cAMP) and calcium. Concentrations of cAMP are controlled by the G-protein activation of adenylyl cyclase and can have numerous effects on the cell depending on the initial receptor-ligand interaction (Alberts et al. 1994). Calcium has numerous cellular effects depending on the cell type including secretion, motility, glycogen metabolism, muscle contraction, cellular proliferation and differentiation (Cheung 1991). Its concentration within the cytosol is kept considerably lower than the extracellular fluid and endoplasmic reticulum by the actions of a calcium-ATPase pump. Upon stimulation, which can be depolarisation of the membrane in electrically excitable cells or G-protein activation in non-excitable cells, calcium can rapidly influx into the cell through activated ion channels to cause an effect (Alberts et al 1994).

An important intracellular calcium receptor, which mediates many of the functions of calcium is calmodulin, which has four calcium binding domains. Following an influx of calcium, a calcium-calmodulin complex is formed, which activates various intracellular proteins by phosphorylation, catalysed by calcium-calmodulin-dependent protein kinases. One of these protein kinases is the myosin light chain kinase, which activates smooth muscle contraction (Alberts et al 1994; Bygrave 1992).

Gap junctions are another method for coordinating the activities of neighbouring cells. They connect the cytoplasm of joined cells together, allowing for the rapid passage of small soluble molecules or ions (nutrients, cAMP or calcium <1kDa in size) between cells. This type of intercellular communication allows for groups of cells in contact with one another to function in a rapid and coordinated manner, for example, during myocardial contraction (Montecino-Rodriguez & Dorshkind 2001). Gap junctions also create physiological compartments by their ability to only form between specific cell types (Gilbert 2003).

BMdSCs are known to communicate via gap junctions as demonstrated by dye transfer experiments, but the formation of gap junctions between stromal cells and haemopoietic cells is still controversial. Connexin 43, one of the most constitutively expressed gap junctions, has been detected on the stromal processes that envelope haemopoietic cells, and gene expression for gap junction proteins has been shown in haemopoietic cells, suggesting that they may form gap junctions with each other and with stromal cells. Rosendaal (Rosendaal et al. 1991) demonstrated an approximate 10% dye transfer frequency between stromal cells and haemopoietic cells whilst Montecino-Rodriquez's group (Montecino-Rodriguez et al. 2000) estimated that 0.1% of haemopoietic cells form gap junctions with the stroma. Therefore, gap junctions may form between the haemopoietic and mesenchymal systems within the bone marrow but as a rare event (Montecino-Rodriguez & Dorshkind 2001).

1.7. Aims of Investigation

For future use of BMdSCs in cell therapeutic techniques it is likely that they will need to be grown and possibly manipulated down specific lineages *in vitro* before implantation into a recipient. This investigation aimed to determine the optimal conditions for growth by comparing various media and calcium concentrations, and assessing the importance of hydrocortisone in long-term cultures. Simple methods for quantitating growth were devised that did not exhaust the culture. The cell surface receptor expression of BMdSCs was characterised as a function of passage and in comparison to the literature. The BMdSCs were also assessed by cytogenetic analysis to ensure that they remained karyotypically stable as a function of passage. The optimal time and conditions for inducing osteogenesis and adipogenesis in BMdSC cultures were also assessed and the markers of differentiation measured using various biochemical, immunohistochemical and real time PCR techniques.

BMdSCs closely interact with haemopoietic cells *in vivo* and *in vitro* within long-term cultures in the regulation and survival of haemopoiesis. It is therefore highly likely that abnormalities within one stem cell system will affect the other. This investigation aimed to look for abnormalities within the BMdSC compartment of patients with haematological malignancies that may be a contributing factor to the disease. Normal ranges were established from healthy donors (chapters 3 & 4) to compare with the growth and differentiation of BMdSCs from patients with various malignant disorders (chapter 5).

Chapter 2

Materials and Methods

Materials and Methods

2.1. Ethical Approval and Consent

Full ethical approval for the use of excess biological material in this study was granted by the Bro Taf Local Research Committee (Ref: 01/4274); and the study was approved by the Research and Commercial Office of the University of Wales, College of Medicine. Samples of the patient consent forms are in Appendix I.

2.2. Materials

2.2.1. Chemicals

General laboratory chemical reagents were ordered from GIBCO, Sigma or Fisher Biosciences unless otherwise stated.

2.2.2. Buffers

Diethyl pyrocarbonate (DEPC) water: 1/1000 dilution in distilled water, incubated overnight at 37°C and autoclaved.

Phosphate buffered saline (PBS, 1x) (Oxoid) 1 tablet added to 100 ml water for injection BP.

Tris Borate EDTA buffer (TBE, 5x) was made up from TBE buffer in 1 litre of distilled water.

Tris EDTA buffer (TE, 1x): 10mM Tris HCL and 1mM EDTA in 1L sterile, distilled, DNase free water, titrated to pH 7.5 with 5N HCl.

2.2.3. Consumables

Tissue culture consumables such as flasks, graduated plastic pastettes (Alpha laboratories), bijous, universal containers, Nalgene nylon filtration units (pore size: 0.45μm) for filtering large amounts of liquid, and nylon Whatman filters (pore size: 0.45μm) for small amounts of liquid, were obtained from Bibby Sterilin or Fisher Biosciences unless otherwise stated.

2.2.4. Cell lines

Human Caucasian foetal foreskin fibroblast cell line with a finite life-span, derived from a 14 – 16 week old foetus (ECACC, code HFFF2)

2.3. Cellular Biology

All cell culture work was carried out aseptically in a tissue culture laboratory using class II microflow cabinets, which were wiped with water and sterilised using 70% ethanol before and after each session. Periodically the cabinets were also sterilised with formalin vapour

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overnight. Labcoats and gloves were worn at all times. All disposable consumables, for example, universal containers, flasks and plastic pipettes, were obtained sterile ready for use, whilst media, PBS and other liquids used with cell culture work were sterilised by filtration (Whatman filters: 0.45μm) before use. Culture supernatants, cell suspensions (in sealed containers) and all disposable consumables contaminated with human material were placed into biohazard bags within autoclave bins and sterilised before being placed into yellow bags for incineration according to the Cardiff and Vale NHS trust waste policy. Sharps (glass, needles, pipette tips) were placed into plastic sharps boxes whilst all other waste, including gloves, were transferred straight to yellow bags for incineration.

2.3.1. Isolation and expansion of BMdSC

Bone marrow aspirates were taken from the iliac crest of normal bone marrow harvest donors and from untreated patients with haematological malignancies after informed consent. The bone marrow mononuclear cell (MNC) population was separated by density centrifugation with Hypaque-1077, a solution of polysucrose and sodium diatizoate adjusted to a density of 1.077 ± 0.001 g/ml. Froth from shaken FCS was layered over 5 ml Hypaque-1077 in 20 ml universal containers (UCs) and the bone marrow was very carefully layered on top. PBS was used to rinse the bone marrow sample containers and added to the UCs to ensure all the bone marrow was collected. The layered bone marrow was centrifuged at 450g (1800 rpm) for 30 minutes at room temperature. During centrifugation the erythrocytes and the granulocytes were aggregated by polysucrose and sedimented to the bottom whilst the lymphocytes and other mononuclear cells remained at the plasma-Histopaque interface. The supernatant, containing the MNC fraction and Hypaque-1077, was removed to a clean UC using a plastic Pasteur pipette, pelleted at 1000g (2,500 rpm) for 20 minutes and the liquid phase discarded. The MNC pellet was resuspended in PBS and counted in white blood cell counting fluid (1% acetic acid in water with a drop of Giemsa stain) in a plastic counting chamber (2.4.2. 'Cell quantitation')

2.3.2. Primary long-term bone marrow culture (LTC)

The MNCs were plated at 20×10^6 cells per 25 cm^2 flask (Nunc) into 10 ml HEPES buffered Alpha MEM (with or without hydrocortisone), HEPES buffered Dulbecco's minimal Eagle's medium (DMEM) or modified (low glucose) McCoy's 5A based medium (with or without glutamine and hydrocortisone) (2.3.4. 'Medium') as primary long-term cultures (LTCs) (3.3. 'Long-term culture BMdSCs'). Where MNC numbers were limited, cultures for stromal cell growth were set up with less than 20×10^6 cells in 25 cm^2 flasks. Cultures for colony-forming

unit-fibroblast (CFU-F) assays (2.4.4. 'CFU-F') were set up in the same media at $1-2 \times 10^6$ MNC per 25 cm^2 flask.

The flasks were incubated at 37°C in a dry incubator (this reduced the risk of contamination, which often occurred with humidified incubators) for 7 days, demi-fed, and then moved to a 33°C dry incubator (Dexter et al. 1977). The cultures were demi-fed (4.5 ml conditioned medium replaced with 5 ml fresh medium) weekly for 5-6 weeks and then detached, but their lifespan could be increased further if necessary.

Haemopoietic activity *in vitro* was assessed only in cultures established with ~20×10^6 cells per 25 cm^2 flask (3.3.3. 'Effect of HC on non-adherent cells and CFU-GMs'). During demi-feeding, 0.5 ml of recovered culture supernatant was diluted in 10 ml isoton and the number of non-adherent haemopoietic cells (Size: >8 µm) were determined (Beckman Coulter, Z2). The remaining supernatant was centrifuged at 1150g (2,500 rpm) for 20 minutes and the pH tested. The cell pellet was resuspended in 0.5 ml PBS for clonal CFU-GM assay.

a) CFU-GM assay

CFU-GM (Colony forming unit – granulocyte macrophage) cells are myelomonocytic stem cells (progenitor cells to the granulocyte / monocyte lineages) within the haemopoietic system that exist in the bone marrow. Their number in culture was determined by counting the colonies produced in a CFU-GM assay. Triple strength Alpha MEM (16 ml) was added to melted agar gel (4 ml) (melted in a boiling water bath for 15 minutes), mixed well and placed in a 40°C water bath for 20 minutes. 1 ml aliquots of the agar mixture were added to 0.1 ml 5637-conditioned medium (Quentmeier et al. 1997), 0.5 ml distilled water and 0.5 ml FCS. This mixture was added to a 0.5 ml cell suspension and mixed before being aliquotted (1 ml volumes) into petri dishes (NUNC, 35mm diameter) prepared in duplicate. These were incubated for 14 days at 37°C and stained with diluted giemsa stain (1/100 in PBS). The numbers of cloned CFU-GM colonies were then counted using a dissecting microscope (Tennant & Jacobs 1989).

2.3.3. Passaged BMdSC culture

The bone marrow derived stromal layers within the long-term bone marrow cultures were detached using the Accutase detachment protocol (2.3.5. 'Detachment protocol') and replated to form first passage BMdSC cultures.

BMdSCs were passaged in Alpha MEM, modified McCoy's based medium (with and without glutamine / hydrocortisone) or DMEM (2.3.4. 'Medium') (3.4. 'Passaged Stromal Culture Growth of BMdSCs'). BMdSCs were sub-cultured during log-phase growth by detaching the adherent cells and replating them at fairly low inoculum densities (1-3x10^4 cells per 25 cm^2 flask). Cultures were also set up with ~10x cell dilutions for CFU-F assays (2.4.4. 'CFU-F'). The stromal cultures were fed twice weekly (all conditioned medium replaced with fresh medium) and incubated in sealed flasks at 37°C in a dry incubator.

2.3.4. Medium

a) Alpha-modified minimal essential medium eagle (Alpha MEM):

BMdSCs were grown in a commercial preparation of Alpha MEM (200 mg/l CaCl$_2$). BMdSCs were induced in a laboratory preparation of Alpha MEM (100 mg/l CaCl$_2$) (Stanners C.P et al. 1971). These basic 1x preparations (table 2.1) were supplemented with 5000 mg/l HEPES and sodium HEPES to a pH of 7.3-7.4, 86 mg/l Benzyl penicillin (Crystapen, Britannia), 29 mg/l Gentomycin (Cidomycin Adult, Roussel) and 143 mg/l Streptomycin sulphate (Cell Tech); 10% horse serum (HS) and 10% foetal calf serum (FCS) (Labtech / Biowest). The HS and FCS were batch selected for either LTC or passaged stromal growth. The medium was made up with or without hydrocortisone (0.1 μM). Osmolarity: 290-300 mOsmol/Kg.

b) Dulbecco's modified Eagle medium (DMEM):

BMdSCs were grown in a commercial preparation of DMEM (200 mg/l CaCl$_2$). BMdSCs were induced in a laboratory preparation of DMEM (100 mg/l CaCl$_2$) (table 2.1) (Dulbecco R & Freeman G 1959; Smith J.D et al. 1960). These basic 1x preparations were supplemented with 5000 mg/l HEPES and sodium HEPES to a pH of 7.3-7.4, 86 mg/l Benzyl penicillin (Crystapen, Britannia), 29 mg/l Gentomycin (Cidomycin Adult, Roussel) and 143 mg/l Streptomycin sulphate (Cell Tech); 10% HS and 10% FCS. Osmolarity: 290-300 mOsmol/Kg.

c) Modified low-glucose McCoy's 5A based medium:

BMdSCs were grown in a laboratory prepared modified McCoy's 5A based medium (200 mg/l CaCl$_2$) (table 2.1) (Tennant et al. 2000a). This 1x preparation was supplemented with 5000 mg/l HEPES and 5750 mg/l sodium HEPES to a pH of 7.3-7.4, 86 mg/l Benzyl penicillin (Crystapen, Britannia), 29 mg/l Gentomycin (Cidomycin Adult, Roussel) and 143 mg/l Streptomycin sulphate (Cell Tech); 10% HS and 10% FCS. The medium was made up with or

without glutamine (table 2.1) and hydrocortisone (HC) (0.1 μM). Osmolarity: 290 - 300
mOsmol/Kg.

Table 2.1. Medium composition (1x strength)

Component	Alpha MEM (mg/L)	Dulbecco's MEM (mg/L)	McCoys 5A (mg/L)
Ferric Nitrate 9H$_2$O		0.1	
Magnesium Sulphate (anhyd)	97.7	97.7	200
Potassium Chloride	400	400	400
Sodium Chloride	6800	4400	
Sodium Phosphate Monobasic	122	109	650
Bacto-peptone			600
Galactose			750
Glutathione (reduced)			1.5
L-Alanine	25		13.4
L-Arginine HCl	126	84	35
L-Asparagine H$_2$O	50		45
L-Aspartic acid	30		20
L-Cysteine HCl H$_2$O	100		35
L-Cystine 2HCl	31.3	62.6	
L-Glutamic acid	75		22
L-Glutamine	292	584	220
Glycine	50	30	7.5
L-Histadine HCl H$_2$O	42	42	21
L-Hydroxyproline			20
L-Isoleucine	52	105	39.4
L-Leucine	52	105	39.4
L-Lysine HCl	72.5	146	36
L-Methionine	15	30	15
L-Phenylalanine	32	66	16.5
L-Proline	40		17.5
L-Serine	25	42	26.3
L-Threonine	48	95	18
L-Tryptophan	10	16	3.1
L-Tyrosine 2Na 2H$_2$O	51.9	103.8	18.1
L-Valine	46	94	17
L-Ascorbic acid	50		0.5
Biotin	0.1		0.2
Choline Chloride	1	4	10
Folic Acid	1	4	10
myo-Inositol	2	7.2	36
p-aminobenzoic acid			1
Niacinamide	1	4	0.5
D-Pantothenic acid	1	4	0.2
Pyridoxal HCl	1	4	0.5
Riboflavin	0.1	0.4	0.2
Thiamine HCl	1	4	0.2

Vitamin B_{12}	1.36		2
Adenosine	10		
Cytidine	10		
2'Deoxyadenosine	10		
2'Deoxycytidine HCl	11		
2'Deoxyguanosine	10		
Glucose	1000	4500	342
Guanosine	10		
Phenol Red Na	11	15.9	
Pyruvic acid Na	110		75
DL-6,8-Thioctic acid	0.2		
Thymidine	10		
Uridine	10		

2.3.5. Detachment protocol

Two detachment agents were used: Accutase (PAA) and Trypsin-EDTA (<0.5 g porcine trypsin and 0.2 g EDTA 4Na/l Hanks Balance Salt Solution). Accutase is a relatively mild protease solution, and was routinely used to detach long-term bone marrow cultures and passaged stromal cultures for extended growth experiments. Trypsin-EDTA was used when a more powerful detachment agent was required.

The culture supernatant was discarded and the cells were briefly washed in PBS, removing any traces of medium that inhibited the action of the detachment agent.

Accutase: 2 ml Accutase (diluted 1/4 in PBS) was added to the adherent stromal cell layer and incubated at 37°C for 30 minutes. Any remaining attached cells were released by tapping the flask onto a hard surface.

Trypsin-EDTA: 2 ml Trypsin-EDTA was added to the adherent cell layer and incubated at 37°C. Incubation times varied depending on the cell layer being detached.

The reaction (using Accutase or Trypsin-EDTA) was stopped with 2 ml medium (10% FCS and 10% HS) and the cell suspension was transferred to a sterile container. Any remaining cells in the flask were rinsed out with a further 2 ml fresh medium and added to the cell suspension. The cells were counted using a disposable plastic counting chamber (2.4.2. 'Cell quantitation'). Cells detached with Accutase were re-inoculated into medium in sterile flasks for regrowth. The detachment agent was diluted at least 1/30 in medium during cell inoculation obviating the need to wash the cells of detachment agent before replating.

2.4. Quantitating BMdSC Growth

2.4.1. BMdSC growth

Stromal cell proliferation was monitored weekly in LTCs and twice weekly in passaged stromal cultures by counting and averaging the number of adherent cells in 5 random cell fields using a phase contrast microscope (10x lens, 1.5x light path magnification).

2.4.2. Cell quantitation

Detached cells were counted using a disposable plastic counting chamber (Immune Systems Ltd BVS100).

Cell count per ml = (total counts / number of 4x4 grids counted) x 10^4.

(4x4 grid = 1 mm x 1 mm x 0.1 mm depth giving a volume of 0.1 mm^3)

The cell counts needed to be multiplied by the dilution factor when using white blood cell counting fluid to count MNCs.

2.4.3. Cell doubling

There were two ways to monitor cell doubling: 1) by counting the number of cells recovered from a culture (2.4.2. 'Cell quantitation') and relating it to the number of cells inoculated into the culture (Bruder et al. 1997; Phinney et al. 1999), and 2) by counting and averaging the number of cells per microscopic field at two time points during the culture (2.4.1. 'BMdSC growth').

Cell doubling = (Log_{10} final cell count – Log_{10} initial cell count) / Log_{10} 2.

Neither of these methods were accurate. Both methods assumed that all cells were dividing at an equal rate and therefore, did not give an absolute quantitation of cell doubling (Appendix VI). Using the first method it was difficult to establish stromal cell doublings in passage 1 because of an unknown number of contaminating haemopoietic cells within the inoculum. Using the second method, cell doubling could be quantitated at passage 1, but cell counts became less accurate as the cultures became more confluent. Contaminating macrophages that adhered to the flask could be identified by their rounder more refractile appearance and be excluded from the cell count.

2.4.4. CFU-F

BMdSCs were set up with 10% of the original inoculum for CFU-F assays. This allowed for discrete colonies to form, each colony representing the clonal expansion of a single CFU-F or

stem / progenitor cell (Still & Scutt 2001). CFU-F colonies were assayed at day 14 for both LTCs and passaged stromal cells using phase contrast microscopy (4x lens, 1.5x light path magnification). Seven to ten strips across the flask (width) were viewed and the number and sizes of colonies recorded. The width of a 4x field is 2.5 mm and the flask length is 60 mm, so to determine the number of colonies per flask, the average number of colonies per strip were multiplied by 24. The colonies were sub-divided into three categories: large colonies (>100 cells), medium colonies (25-100 cells) and small colonies (6-25 cells). CFU-F values were recorded as CFU-F / 10^6 MNC inoculated in LTCs and CFU-F / 10^4 stromal cells in passaged cultures.

2.4.5. Time lapse

Rapidly dividing cultures were selected for time-lapse photography at distinct time periods during culture (e.g. 3, 7, 10, 14 days). The flasks were placed onto an inverted microscope stage (Nikon) within an enclosed plexiglass incubator, maintained at about 36°C with forced heated air (Rickard et al. 2003). A x10 phase contrast objective was used to study the cells. Cell activity was recorded using a colour JVC CCD video camera attached to the microscope. Image signals were digitalised and captured using Pinnacle System DV500 plus DVCAM. The images were then visualised, manipulated and stored using Adobe Premier, version 6.0. A graticule slide with an engraved circle 2mm in diameter with cross-hairs was used to assess cell size and motility (3.6. 'Time lapse photography').

2.5. Cell Characterisation

2.5.1. Cytogenetic analysis

Cytogenetic analysis of BMdSCs was carried out by the staff of the Cytogenetics Laboratory, Haematology department, University Hospital of Wales, Cardiff, using standard G-banded analysis on a minimum of 20 metaphases, defined according to the International System for Human Cytogenetic Nomenclature (ISCN 1995) (3.5.3. & 5.6. 'Cytogenetics'). The technique was modified from Czepulkowski (Czepulkowski 2001) and Mandahl (Mandahl 2001) for use with BMdSCs.

a) Reagents

i. For harvesting the cells

- KaryoMax Colcemid (10 μg/ml) in Hank's balanced salt solution (Gibco, 15210-040)

- Complete medium: McCoy's (Gibco) containing 20% FCS, 2mM glutamine, 0.1 mg/ml streptomycin and 100 u/ml streptomycin/penicillin.
- Trypsin-EDTA (2.3.5. 'Detachment protocol')
- KCl: Potassium chloride (0.75M)
- Fixative: 75% methanol and 25% Analar acetic acid

ii. For banding

- Solution of Worthington's trypsin (183 u / mg P): 5 µg / 100 ml PBS
- Lieshmans stain: (Eosin-polychrome methylene blue) 2 g/l
- Leishmans buffer: pH 6.8
- PBS

b) Method

i. Incubation with colcemid

Fresh medium was added to three to seven day old cultures and incubated at 37°C for 24 hours. Colcemid (0.1 ml) was added to the medium and incubated overnight at 37°C. The Colcemid prevented dividing cells from progressing past metaphase within their cell cycle, appearing as small, round, refractile cells in culture.

ii. Harvesting the cells

The culture supernatant, into which some of the metaphase-restricted cells had released, was collected into a UC. The BMdSCs were detached using the Trypsin-EDTA detachment protocol (5 minutes at 37°C) and the cell suspension was added to the previously collected supernatant.

The cells were transferred to a conical based centrifuge tube and centrifuged at 500 g for 10 minutes. The supernatant medium was discarded, leaving 1 ml to resuspend the pellet, and 9 ml 0.75 M KCl was added and incubated for 10 minutes at room temperature. The cells were then centrifuged at 500 g for 10 minutes and the KCl discarded, leaving 1 ml over the pellet to resuspend. A volume of 1 ml fixative was added slowly, drop wise, to the cell suspension whilst constantly agitating, then a further 9 ml fixative was added more quickly and mixed by inversion. The cell suspension was centrifuged for 10 minutes at 500 g and the fixative discarded. Fresh fixative (10 ml) was added to the pellet and centrifuged at 500 g for 10 minutes and the supernatant was discarded. The pellet was resuspended in a mixture of 50%

fresh fixative and 50% distilled water and immediately centrifuged before discarding the supernatant. The pellet was immediately resuspended with fresh fixative and either stored at 4°C for future analysis or used to make slides.

iii. Slide preparation and banding

The pellet was resuspended in enough fresh fixative to give a slightly turbid suspension. One drop of the cell suspension was placed onto a clean wet slide, which was manipulated to spread the cell suspension evenly. The slides were allowed to dry on a hotplate and left to age at room temperature for 5 to 10 days before being analysed.

iv. Analysis

The aged slides were placed into a coplin jar containing a trypsin solution for up to 1 minute (digestion) and washed twice in PBS before being stained in a ¼ dilution of Leishmans stain (in buffer) for 1 to 2 minutes. The slides were rinsed in tap water and dried on a hotplate. The slides were then mounted in XAM for G-banding analysis.

2.5.2. Cell markers

Each cell-type within the body has a unique set of epitopes (antigens) to which monoclonal antibodies can be raised. Fluorochromes can be conjugated to the monoclonal antibodies, which can be detected using the flow cytometer when bound to specific epitopes on the cells. The BMdSCs were analysed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) (3.5. 'BMdSC Characterisation').

a) Flow cytometry
i. Basic principles

The flow cytometer operates using four basic systems, a fluidic system, an illumination system, an optical and electronic system and a data storage and computer control system (Radcliff & Jaroszeski 1998). Within the fluidic system, the pressure of the sheath fluid (hydrodynamic focusing) directs the cells to the centre of the laminar flow in single file to precisely bisect the analytical laser light source within the illumination system. As the cell intercepts the laser beam, a pulse of scattered light is produced, which is proportional to the size of the cell. For example, a smaller pulse of light indicates a smaller cell. The light scattered in a forward angle direction (forward scatter) is related to the size of the cell, whilst the light scattered in a side angle direction (side scatter) is related to the refractile properties of the cell (granularity). These two parameters provide important cellular information and enable particular cell populations to

be selected for analysis. The scattered light is directed by a series of mirrors and filters to detectors within the optical and electronic system, which convert the light into information the computer control system can process (described later).

ii. **Fluorescence**

When cells specifically labelled with a fluorescent tag-conjugated antibody bisect the laser light source, light is absorbed by the fluorochrome and the electrons are excited to a higher energy state. As the electrons return to their original state, fluorescent light is emitted. Two commonly used fluorochromes are fluorescein isothiocyanate (FITC) and phycoerythrin (PE), which maximally emit light at 530 nm and 578 nm respectively. Peridinin-chlorophyll-a (PerCP) maximally emits light at 680 nm and Allophycocyanin (APC) emits maximally at 660 nm.

iii. **Detecting fluorescence**

A series of dichroic mirrors and filters enable the various fluorescence emissions (different wavelengths) to be measured by specific detectors of the optical and electronic system called photomultiplier tubes (PMTs) (figure 2.1).

The argon-ion laser produces 488 nm light, sufficient for most cells to be entirely illuminated within the beam. The forward (low angle) scatter is detected directly in the laser light path. The side scatter is collected by the 90 degree collection lens and split by a collection of dichroic mirrors into a series of optical filters. The first mirror (560 short pass), which reflects longer wavelengths, allows shorter wavelengths to pass through to the FL1 (FITC) PMT with a 10% fraction split off to the side scatter detector (PMT). The reflected light from the first mirror is focused to the second mirror (640 long pass), which reflects wavelengths between 560 nm and 640 nm to the FL2 (PE) PMT, and wavelengths >640 nm to the FL3 (PerCP) PMT.
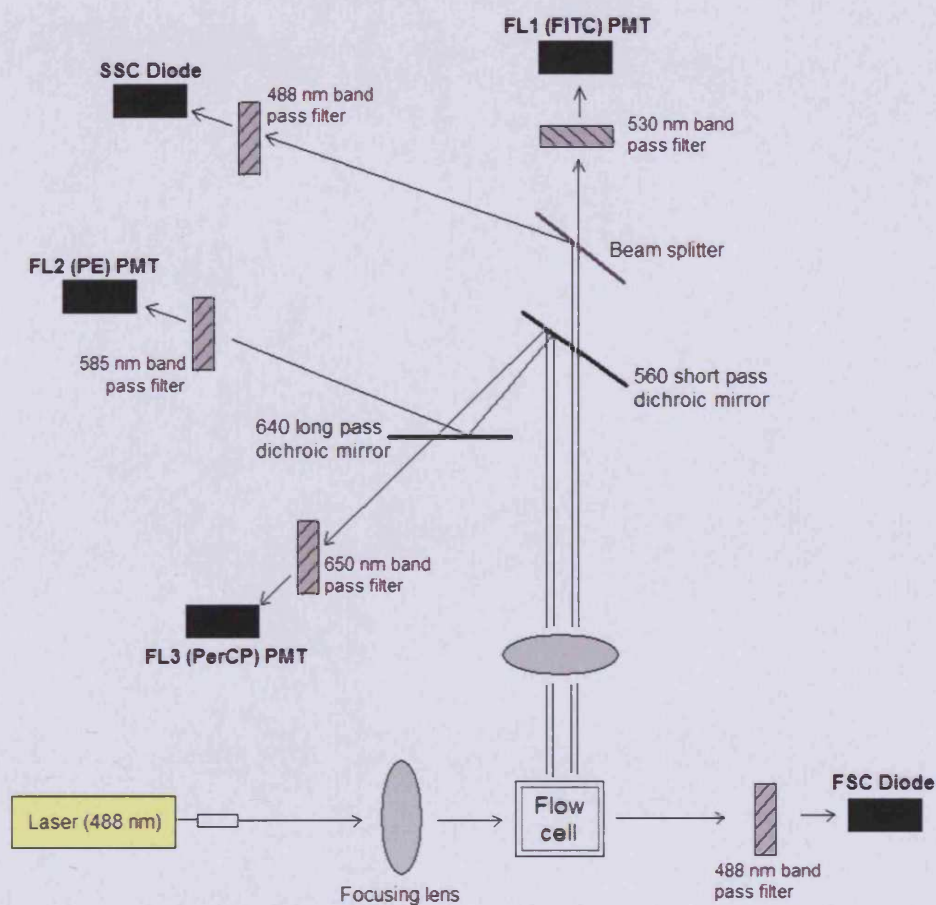
Figure 2.1. Schematic diagram showing the flow cytometric measurement of forward scatter, side scatter, FITC and PE fluorescence (Becton Dickinson 1996).

iv. **Analysis**

The electronics system converts optical signals to electronic signals, which are converted to digital values and sent to a computer. FSC optical signals are converted to electronic signals by a photodiode, whilst SSC and fluorescent optical signals are converted into electronic signals by PMTs (Becton Dickinson 1996).

b) **BMdSC characterisation by flow cytometry**

The aim was to characterise BMdSCs in comparison to equivalent cells in the literature to determine if the expression profile was retained as a function of passage (i.e were epitopes down-regulated or lost through sub-culture techniques or during extended culture?) (3.5.2.b. 'Antigenic expression as a function of passage'), and to characterise BMdSCs from patients with haematological malignancies in comparison to normal cell populations (5.5. 'Passaged BMdSC characterisation by cell markers').

Table 2.2. Antibody panel and associated conjugates, indicating the volumes used for each antibody and relevant incubation times.

Antibody	Product	Specificity	Conjugate	Volume (µl)	Incubation Time (mins)
CD34	BD 345804	Haemopoietic cell marker	APC	2	15 mins
CD45	BD 345809	Haemopoietic cell marker	PerCP	5	15 mins
CD117	BD 332785	c-Kit: Stem cell factor receptor	PE	5	15 mins
CD133	MACS 808-01	Stem cell marker	PE	5	15 mins
CD64	Dako R7219 Clone 10.1	Monocyte / macrophage	PE	3	15 mins
CD11c	Dako F0713 Clone KB90	β-integrin	FITC	3	15 mins
CD49b	Immunotech I425	β-integrin; VLA-α2	FITC	10	15 mins
CD106	DAKO M7106	VCAM-1, Stro-1	Unconjugated 2° Ab: RAM-PE	5	15 mins with 1° and 15 mins with 2°
CD105	BD 555690	TGF-β, SH-2 receptor	Unconjugated 2° Ab: RAM-PE	5	15 mins with 1° and 15 mins with 2°
CD166	BD 559263	ALCAM, SB-10	PE	5	15 mins
CD44	BD 550989	HCAM-1	PE	3	15 mins
CD90	Beckman Coulter IM3600	Thy-1	PE	2	15 mins
IgG$_1$ γ$_1$/γ$_1$ FITC/PE	BD 349526	Control	FITC/PE	3	15 mins
IgG$_1$ γ$_1$ Percy P	BD 345817	Control	PerCP	3	15 mins
IgG$_1$ γ$_1$ APC	BD 345818	Control	APC	2	15 mins
IgG$_1$ RAM-PE	BD 340270	2° Antibody (rat anti mouse)	RAM-PE	10	15 mins

BD: Becton Dickinson
MACS: Miltenyi Biotec GmbH

i. Method

Passaged BMdSCs were grown to sub-confluence and detached using the Trypsin-EDTA detachment protocol (5 minute incubation at 37°C). The cells were counted, pelleted at 1150 g (2,500 rpm) for 20 minutes and resuspended in PBS. Approximately $0.3-0.6 \times 10^5$ cells in a volume of 100 µl were incubated with the antibodies listed in table 2.2 for 15 minutes and washed in PBS (containing 1% BSA and 0.05% sodium azide) to remove excess antibody.

After washing, CD105 and CD106 required an additional 15 minute incubation with a secondary antibody (RAM-PE). Excess secondary antibody was removed in another PBS wash. The cells were resuspended in 500 μl PBS (with BSA and sodium azide) for analysis using the FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). Approximately 10,000 events were collected and the BMdSCs were gated into three sub-populations by their size (forward scatter: FSC) and granularity (side scatter: SSC) (figure 3.30).

Results were analysed using WinMDI (version 2.8) (Joseph Trotter, Scripps Institute, California) and the level of expression quantitated from the ratio of the log geometric means of the test over isotype control samples ($\log_{10}$ G mean of the test sample / $\log_{10}$ G mean of the isotype control).

2.5.3. Cell cycle

Proliferative activity in culture is dependent on the speed of the cell cycle, controlled by a series of growth hormones and survival factors, and the proportion of cells committed to the cell cycle (Hughes & Mehmet 2003). There are two main events within the cell cycle: DNA replication (S-phase) and mitosis or cell division (M-phase) and these are separated by checkpoints known as G1 (precedes S-phase) and G2 (precedes M-phase), which control a cell's passage through the cell cycle. Not all the cells are actively cycling (can be resting, awaiting apoptosis, or are waiting to enter the cell cycle) and these exist in the quiescent G0 phase of the cell cycle.
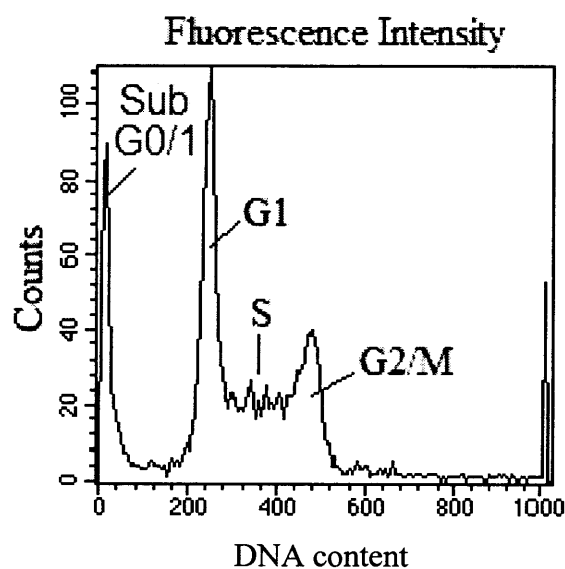
Figure 2.2. DNA histogram showing a subG0/1 peak, G1, S and G2/M phase.

DRAQ5™ (5mM) (Biostatus Ltd) is a red fluorescent cell-permeable probe, which binds to DNA within the cells and can be used to ascertain the DNA content of cells and determine where the cells are within the cell cycle. Cells in G1 will have 1 set of chromosomes (2n). Cells in S phase (replication) and G2 phase will have 2 sets of chromosomes (4n) in preparation for division in M phase and the cycle begins again (figure 2.2).

a) Cell cycle analysis

DNA content and cell cycle analysis of BMdSCs was determined using DRAQ5 (3.5.1. 'Cell cycle analysis') with the flow cytometer (2.5.2.a 'Flow cytometry').

i. Method

Passaged BMdSCs were detached at 3 to 7 days using the Trypsin-EDTA detachment protocol (5 minute incubation at 37°C). The cells were counted, pelleted at 1150g (2,500 rpm) for 20 minutes and resuspended in PBS. A 50 μl cell suspension (~0.3-0.5x10^5 cells) was added to 3 μl DRAQ5 and 450 μl PBS (with BSA and sodium azide) in a flow cytometry test tube. No incubation was necessary before analysis. Three populations of cells were gated according to their forward and side scatter parameters (figure 3.30) and the cell cycle was assessed within each population (3.5.1. 'Cell cycle analysis'). Results were analysed using WinMDI and Cylchred (Joseph Trotter, Scripps Institute, California & Terry Hoy, School of Medicine, Cardiff).

2.6. BMdSC Differentiation

2.6.1. Culture preparation

Passaged stromal cells were inoculated (2-4x10^4 cells) into 25 cm^2 sealed flasks with 5 ml HEPES buffered Alpha MEM (200 mg/l CaCl$_2$, 10% HS and 10% FCS) and incubated at 37°C in a dry incubator. The medium (5 ml/flask) was changed every 3-4 days.

2.7. Osteogenic Differentiation

The stromal cells were grown to sub-confluence (Goldstein 2001; Stanford et al. 1995) and the medium was changed to either DMEM or Alpha MEM. Uninoculated control flasks were set up in parallel (4.4. 'Part I: Osteogenic Induction').

2.7.1. Osteogenic induction

Inducing agents were made up in distilled water at 100x concentration, filtered and stored at 2-8°C:

- Ascorbic acid-2-phosphate, 50 μM (1x): 100x solution prepared by adding 25.6 mg to 10 ml distilled water

- Sodium β-glycerophosphate, 10 mM (1x): 100x solution prepared by adding 3150 mg to 10 ml distilled water. Dissolved at 37°C

- Dexamethasone, 0.1 μM (1x): 100x solution prepared from a 10,000x stock solution by adding 30 mg to 5 ml distilled water and diluting 1/100 in distilled water

(Bruder et al. 1998; Pittenger et al. 2000)

a) Standard 21-day induction protocol

Eight stromal cultures were set up in parallel (2.6.1. 'Culture preparation') and grown to sub-confluence. Four cultures were induced (2.7.1. 'Osteogenic induction') by adding 50 μl of each induction agent (100x concentration) to 5 ml fresh medium every 3-4 days for 21 days. Four control stromal cultures were fed with fresh medium every 3-4 days for 21 days (no induction agents).

b) Time series induction

A series of seven parallel BMdSC cultures were induced (2.7.1.a. 'Standard 21-day induction protocol') and assayed at 0, 3, 7, 10, 14, 17 and 21 days post induction for osteogenic markers of differentiation. Parallel control cultures were also analysed.

2.7.2. Problems encountered with osteogenic induction

A problem initially encountered during osteogenic differentiation was that in Alpha MEM (200 mg $CaCl_2$/l medium), the extracellular matrix overlying the cells rapidly mineralised causing the cells to break up. This released RNA and RNases into the culture supernatant resulting in a net loss of RNA from the cells as the mineralisation increased. The rate of mineralisation *in vitro,* therefore, needed to be limited to enable RNA extraction for ≥21 days, whilst allowing for measurable amounts of calcium to deposit. The rate of mineralisation was limited by reducing the calcium concentration within the medium. BMdSCs were osteogenically induced in Alpha MEM (calcium free) with added 0, 75, 100, 150 and 200 mg/l $CaCl_2$ (4.4.3. 'Limiting the rate of mineralisation').

2.7.3. Analysis and quantitation of osteogenic differentiation

a) Medium calcium utilization by cells

Calcium concentration was measured in the conditioned medium to establish the amount of calcium being removed from the medium during the process of mineralisation. The calcium concentration was measured using a modified O'cresolphthalein complexone technique (Cowley et al. 1986). Uninoculated control flasks were run in parallel to provide a base-line reading (Balint et al. 2001) (4.6.1.a. 'Calcium removed from the medium during 21 days of induction').

i. Reagents

- Colour Reagent: 24 mg (0.48 g/l) O'cresolphthalein complexone and 250 mg (5 g/l) Quinolinol dissolved in 50 ml distilled water.

- AMP buffer: 16.8 ml AMP (2-amino-2-methylpropanol) buffer (density: 0.93 g/ml) dissolved in 34 ml distilled water

- Calcium standards: 50 mg/l, 100 mg/l and 150 mg/l

ii. Method

Conditioned media was removed from the cultures during feeding and frozen at -70°C for analysis.

Distilled water (0.14 ml), AMP buffer (0.73 ml) and colour reagent (0.1 ml) were mixed together. Aliquots of 1 ml were pipetted into 1.5 ml cuvettes, and 0.026 ml blank (distilled water), sample, control (FCS) or standard were added to each cuvette and incubated for 3 minutes at room temperature. The optical density was determined at 575 nm using a spectrophotometer blanked to zero with water.

iii. Calculation

Calcium concentration (mg/l) =

   (ΔA Sample or control / ΔA Standard) x Standard concentration

Calcium removed from medium during mineralisation (mg/l) =

   Sample calcium concentration – Control calcium concentration

Aliquots of FCS (lot 1786) and Alpha MEM were run in parallel with each batch of tests with a coefficient of variation of 9% (n=11) and 13.6% (n=9) respectively.

b) Mineralisation (Alizarin Red S staining)

To morphologically assess the extent of mineralisation, induced and control cultures were stained with Alizarin red S, which stains calcium deposit a dark red colour (Gori et al. 1999; Majors et al. 1997; Stanford et al 1995).

i. Reagents

Alizarin red S: 13.8 g/l in nanopure water, pH adjusted to 4.2 with ammonium carbonate (10 g/l in distilled water)

ii. Method

Cultures were rinsed in PBS and fixed in ice-cold 70% ethanol for a minimum of 1 hour. They were then rinsed in nanopure water and immersed in Alizarin red S solution for 10 minutes at room temperature. Excess stain was removed and the cultures were washed several times in nanopure water. The cultures were then soaked in PBS for 15 minutes to remove all non-specific staining and viewed by phase contrast microscopy.

c) Demineralisation

To quantitate the extent of mineralisation, the Alizarin red S stained cultures were demineralised in 2 ml 0.6 N HCl at 4°C for 24 hours. The optical density of the acidified extract was measured at the optimal wavelength of 420 nm (figure 2.3).

Initially, the acidified extracts were measured on the spectrophotometer (SP 8-100 UV/VIS, Pye Unicam) at 420 nm and stored at 4°C for the determination of calcium concentration by atomic absorption in Medical Biochemistry, UHW. The acidified extracts were diluted 1/24 in lanthanum chloride and analysed by atomic absorption (Spectr AA 220 optimised to 422.7 nm). A serial dilution of calcium atomic absorption standards provided a standard curve from which the calcium concentrations were determined (Stanford et al 1995). The relationship between the absorbance of the acidified extract at 420 nm and the calcium concentration as determined by atomic absorption was then determined (figure 2.4).

Wavelength scan of Alizarin red S (1:200 dilution) in HCl

Figure 2.3. Wavelength scan for acidified extract from demineralisation procedure following Alizarin Red S staining of calcium deposition using 1 cm path length.
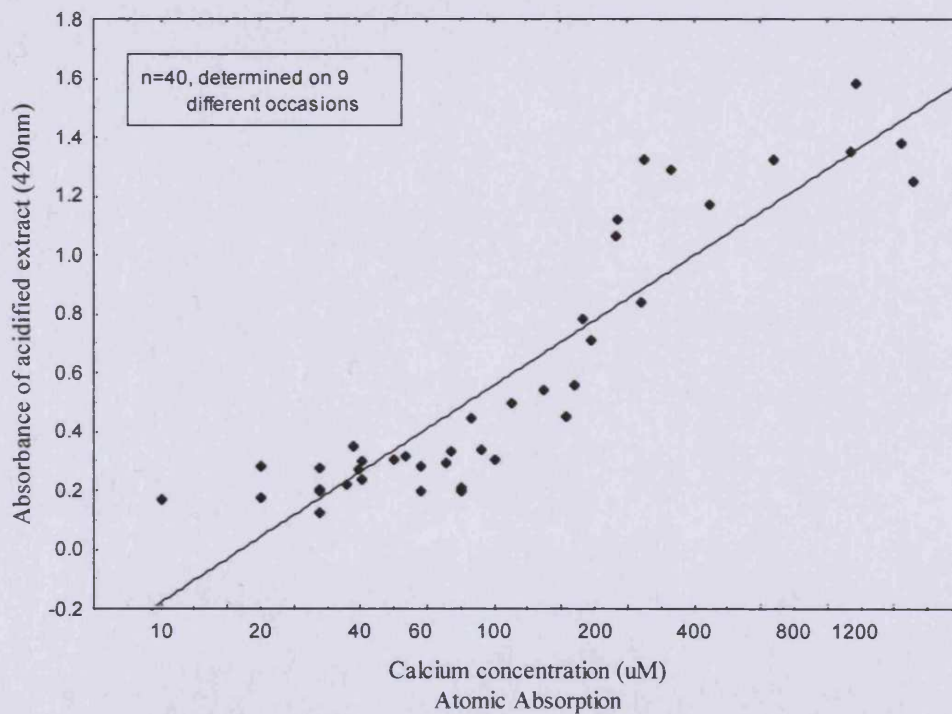
Figure 2.4. Relationship between the absorption of the acidified extract and calcium concentration as measured by atomic absorption. Calcium (μM) = $10^{(A420nm +1.24)}$ (r=0.95)

Absorbance of the acidified extract correlated well with calcium concentration by atomic absorption (r=0.95), enabling the calcium concentration to be determined from the optical density of the acidified extract using the equation:

$$\text{Calcium concentration } (\mu M) = 10^{\wedge} (A_{420 \, nm} + 1.24).$$

Values were normalised to cells per field at induction (2.7.3.g. 'Data normalisation') (4.6.1. 'Calcium deposition').

Alizarin red S (MW = 342.3) was ascertained by to be 70% pure, a 150 μM solution having an average absorbance value of 0.720, which is equal to a calcium concentration by atomic absorption of 91.2 μM. Therefore 150 μM Alizarin red S binds 91.2 μM calcium or 1.64 μM Alizarin binds 1 μM calcium. In contrast, Stanford (Stanford et al 1995) determined that 2 molecules of calcium bound to 1 molecule of Alizarin red S dye in solution.

d) Collagen I

Collagen I was found within the extracellular matrix overlaying the BMdSC layer, which was measured in induced and control cultures using an immunocytochemical technique involving a primary antibody, a biotinylated secondary antibody and the ABC (Avidin-Biotin Complex) reaction (figure 2.5) (4.6.3. 'Collagen I').

i. Mode of action

Avidin has four high affinity binding sites for biotin, and biotin has one binding site for avidin and multiple binding sites for various antibodies, fluorophores or enzymes, which makes the ABC technique very sensitive. A biotinylated secondary antibody binds to the primary antibody, which is specifically bound to the antigen of interest, in this case, collagen I. The avidin and biotin are incubated together to form ABC complexes, which then bind to the biotinylated secondary antibody (figure 2.5).

| Primary antibody binds | Biotinylated secondary antibody binds | Avidin and Biotin form complexes | ABC binds to biotinylated secondary antibody |
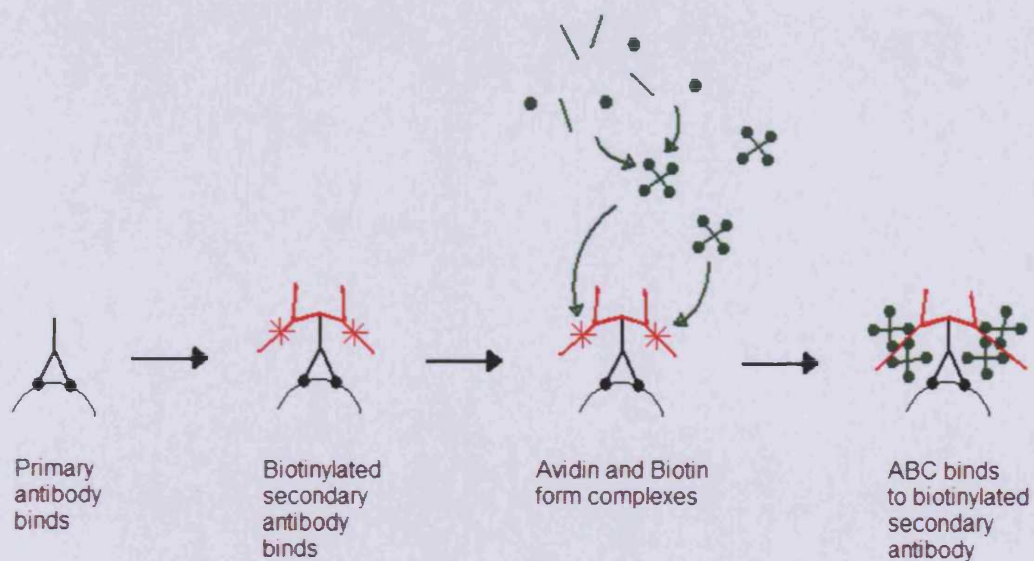
Figure 2.5. The ABC reaction

ii. Reagents

- Primary antibody: Anti Collagen type I. Clone COL-1, Mouse, IgG_1. 1/1000 dilution in PBS (with 1% BSA and 0.05% sodium azide).

- Detection System: Peroxidase Vectastain elite ABC Kit (Universal). (Vector Laboratories). Kit includes normal horse serum (HS), biotinylated secondary antibody and ABC reagents (A&B) in 50 μl dropper bottles.

  - ➤ Blocking reagent: 1 drop HS in 5 ml PBS
  - ➤ Secondary antibody: 2 drops HS and 2 drops biotinylated secondary antibody in 5 ml PBS
  - ➤ ABC reagent: 2 drops reagent A and 2 drops reagent B in 5 ml PBS. Allowed to stand for 30 minutes prior to use

- Enzyme Substrate Kit: Peroxidase Enzyme substrate Kit: DAB (3,3' diaminobenzidine) (Vector Laboratories): 2 drops buffer, 4 drops DAB, 2 drops hydrogen peroxide and 2 drops Nickel in 5 ml distilled water, carried out in fume hood.

iii. Method

Cultures, previously stained with Alizarin red S and demineralised in HCl, were rinsed with PBS. Endogenous peroxidase activity was blocked with 2 ml normal HS for 20 minutes at room temperature. The HS was removed and 2 ml anti-collagen, type 1, added for 30 minutes. After washing in PBS to remove excess primary antibody, 2 ml biotinylated secondary antibody

was added and incubated for 30 minutes. The cultures were washed again and 2 ml ABC reagent was added for 30 minutes. After washing the cultures in PBS to remove any unbound reagent, the enzyme substrate (DAB) was added for 10 minutes to create a visible reaction that could be quantitated and the cultures were washed in tap water to blue the nuclei.

iv. Analysis

The extent of collagen I staining in induced and control cultures were quantitated using a flask reader and normalised to the number of cells per field at induction (2.7.3.g. 'Data normalisation'). The flask reader was devised on a similar principle to plate readers but on a larger scale to measure the amount of light passing through a flask after staining. The denser the staining, the less light passes through with a concomitant increase in absorbance.

e) Cellular Alkaline phosphatase (ALP) activity

ALP activity is increased during osteogenesis. It can be detected indirectly by measuring the hydrolysis of p-nitrophenyl phosphate to p-Nitrophenol within the cells (Calzyme laboratories) (Stinson 1993) (4.6.2. 'Cellular alkaline phosphatase activity').

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{p-Nitrophenol} + \text{phosphate}$$
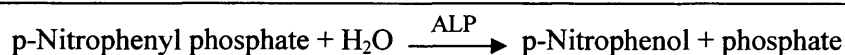
i. Reagents

- Magnesium chloride stock solution, 1 mM (1x): 100x solution prepared by adding 2.03 g to 100 ml distilled water
- Zinc sulphate stock solution, 0.1 mM (1x): 100x solution prepared by adding 0.28 g to 100 ml distilled water
- Buffer: 9.7 ml (10.6 g) Diethanolamine buffer (1M), 1 ml magnesium chloride solution and 1 ml zinc sulphate solution in 100 ml distilled water
- Substrate: 167 mg p-Nitrophenyl phosphate, disodium, hexahydrate in 0.5 ml distilled water

ii. Method

Adherent cells were detached using the Trypsin-EDTA detachment protocol, pelleted (20 minutes at 1150g) and solubilised using 2 ml 1% Triton X-100 in PBS. The solubilised cells were stored at -80°C until analysis.

A working solution of 0.05 ml substrate and 2.9 ml buffer was made up immediately before use.

A spectrophotometer, with the cuvette carrier warmed to 30°C (Stinson 1993), was set to

405 nm and blanked to zero with water. The working solution was incubated at 30°C for 5

minutes and 0.1 ml of sample was added and mixed. An initial absorbance reading was taken

and the sample was incubated in the cuvette carrier at 30°C for 5 minutes before taking a

second absorbance reading. The change in absorbance per minute was determined and

normalised to cells per field at induction (2.7.3.g. 'Data normalisation').

$$\text{ALP index} = \Delta A_{405nm} / \min / \text{cells per field at induction}$$
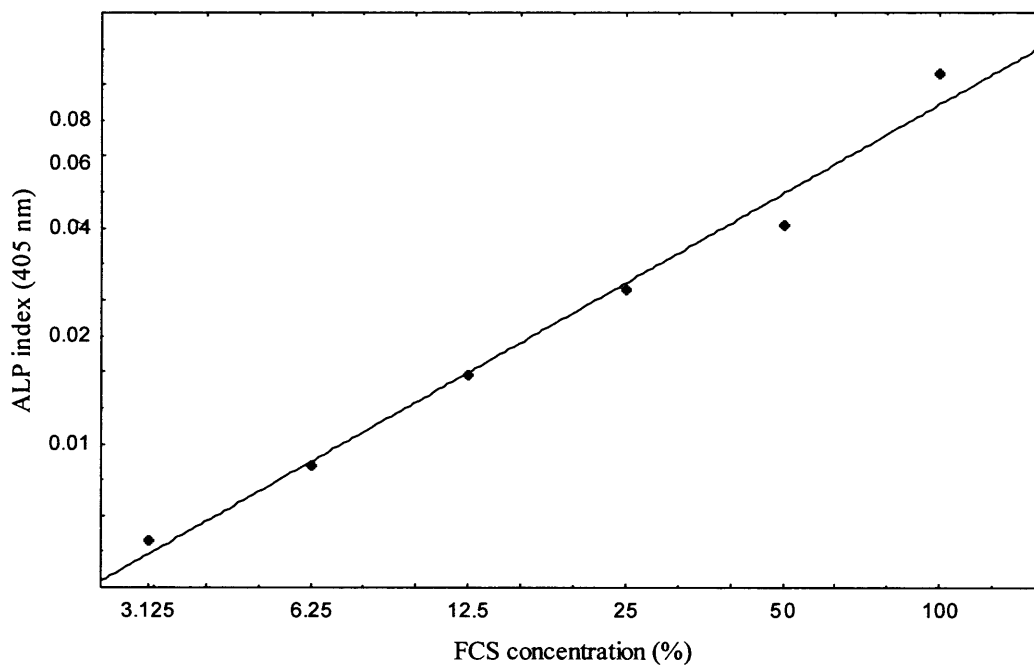
Figure 2.6. Standard curve for ALP determination using dilutions of FCS in buffer. (r=0.981)

There was a log-linear relationship between the change in absorbance per minute (ALP index)
and concentration (figure 2.6). Aliquots of FCS (lot 1786) were assayed in parallel with each
batch test run with a coefficient of variation of 4.5% (n=5).

f) Intracellular staining of ALP

Intracellular ALP activity was directly identified within the adherent BMdSCs using a staining
procedure involving naphthol AS-MX phosphate (Dacie & Lewis, 1984) (4.5.2.c. 'Intracellular
staining of ALP').

i. Reagents

- Fixative: 10 ml formalin (40% formaldehyde) and 90 ml methanol. Stored at -20°C

- TRIS buffer: 36.35 g Tris (hydroxymethyl) aminomethane dissolved in 250 ml water with 7.5 ml 1 N HCl, and made up to 1 litre, pH 9

- Stock Substrate: 30 mg Naphthol AS-MX phosphate dissolved in 0.5 ml NN dimethyl formamide and made up to 100 ml in 0.3 M tris buffer, pH 9. Stored at 4°C

- Incubation mixture: 10 mg Fast blue BB salt (4-Benzoylamino-2,5 diethoxybenzenediazonium chloride hemi – zinc chloride salt) added to 10 ml stock substrate solution

ii. Method

Cultures were rinsed in PBS, fixed in ice-cold fixative for 30 seconds and briefly washed under running tap water. The excess water was removed and the cultures were allowed to air dry. The incubation mixture was filtered and 2 ml volumes were added to each culture and incubated for 15 minutes at room temperature. Cultures were washed in tap water and viewed using a phase contrast microscope. ALP positive cells stained a dark blue colour.

g) Data normalisation

Data normalisation enabled the results to be standardised to cellular material. Normalising mineralisation to total protein content involved the determination of not only cellular protein but also extracellular matrix protein (Balint et al 2001). Normalising to DNA content was a more accurate portrayal of cellular material and could be determined using Pico Green (binds to double-stranded (ds) DNA) and a fluorometer (Molecular Probes Inc 2003).

i. Measuring DNA content

ii. Reagents

- TE buffer (2.2.2 'Buffers')

- Pico Green dsDNA quantitation reagent concentrated in DMSO (Molecular probes): Diluted 1/200 in TE buffer (10 μl in 2 ml TE). Prepared immediately before use in a plastic container (reagent may adsorb to glass) and wrapped in aluminium foil to protect it from the light

- λ DNA standards (Molecular probes): 1, 10 and 100 ng/ml preparations in TE buffer

iii. Method

Cells were detached using the Trypsin-EDTA detachment protocol and diluted 1/40 in TE buffer (100 µl cells in 4 ml TE buffer). Samples were stored at -20°C for analysis. The frozen cell suspensions were thawed and sonicated for 15 seconds to release DNA from the cells. Sonication did not affect the double stranded DNA (Stanford et al 1995). Control samples (Trypsin-EDTA and respective medium, no cells) were also diluted 1/40 in TE buffer and sonicated for 15 seconds.

The standards, blank (TE buffer) and samples were diluted 1/2 in Pico Green in borosilicate glass tubes to a final concentration of 100 µl. Sample fluorescence (excitation ~480 nm, emission ~520 nm) was measured in a TD-700 Fluorometer (Turner Designs). To minimise the effect of photobleaching the time for each measurement was kept constant. The concentration of dsDNA was determined from the standard curve (figure 2.7) and the control (background fluorescence from the medium) was subtracted from the test samples. The results were expressed as ng dsDNA / culture.
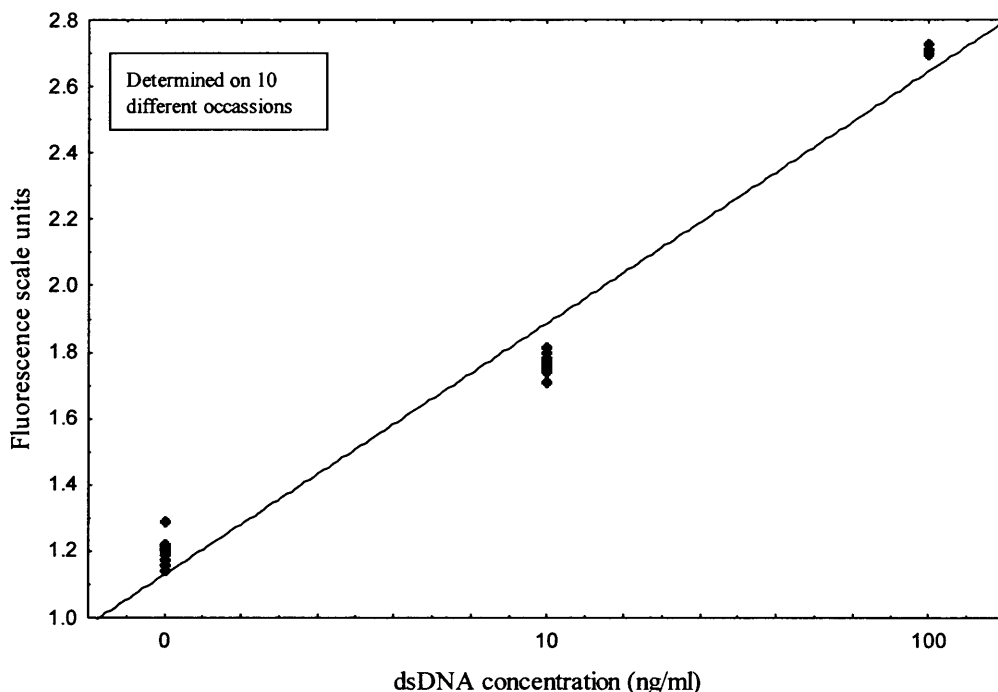
Figure 2.7. Standard curve for determining dsDNA concentration. (r=0.98)

iv. Problems encountered with using DNA content for standardisation

Unfortunately the technique for measuring DNA content was not optimised early enough to normalise the osteogenic markers of differentiation. Therefore, results for osteogenic induction were normalised to cell counts at induction.

Normalising markers of osteogenic differentiation analysed at 21 days post induction to cell counts taken at the time of induction was not ideal because it assumed that the cellular content remained constant during induction. Some cultures may have continued to proliferate whilst others may have started to break up by 21 days post induction, but it was impossible to estimate cell counts in mineralised confluent cultures. The method of normalisation (DNA or cells per field) did not alter the statistical analysis for adipogenesis.

2.8. **Adipogenic Differentiation**

Passaged stromal cells were set up as described (2.6. 'Culture preparation') and grown to confluence. The stromal cells were maintained at confluence for 3-4 days (Pittenger et al 1999) in Alpha MEM ($CaCl_2$ 200 mg/l) before being adipogenically induced in DMEM or Alpha MEM ($CaCl_2$ 100 mg/l).

2.8.1. **Adipogenic induction**

Inducing agents were made up in distilled water at 100x concentration, filtered and stored at 2-8°C:

- Dexamethasone, 1 µM (1x): 100x solution prepared by adding 12 mg to 20 ml distilled water

- 3-Isobutyl-1-methylxanthine (IBMX) 0.5 mM (1x): 100x solution prepared by adding 111 mg to 10 ml distilled water. To dissolve the IBMX 1 small pellet of NaOH was added, and the solution was titrated with 0.5 N HCl to approximately pH 9

- Indomethacin (1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid), 60 µM (1x): 100x solution prepared by adding 43 mg to 16ml distilled water and 4 ml DMSO

- Hypurin bovine Insulin, 10 µg/ml (1x): 100x solution prepared by diluting the insulin 1/4 in distilled water (1 ml insulin in 3 ml water)

- Hydrocortisone (HC) sodium succinate solution (20 mg/ml) (Solu-Cortef, Pharmacia) 0.5 µM (1x): HC was diluted 1/100 in distilled water (0.2 mg/ml). 100x solution prepared by adding 0.9ml of 1/100 dilution to 9.1 ml distilled water.

(Digirolamo et al. 1999; Pittenger, Mosca, & McIntosh 2000)

a) **Standard 21 day induction protocol**

Two parallel cultures were induced for 21 days in dexamethasone, IBMX, insulin, indomethacin and HC by adding 50 µl (100x solution) to 5 ml fresh medium every 3-4 days. Control cultures (no induction agents) were run in parallel.

b) **Cyclic induction protocol**

Two cultures were induced on a cyclic basis involving 3-4 days of incubation with dexamethasone, IBMX, insulin, indomethacin and HC (induction medium), followed by 3-4 days of incubation with insulin and HC (maintenance medium).

- **Induction agents**: Dexamethasone, IBMX, insulin, indomethacin and HC
- **Maintenance agents**: Insulin and HC

The cyclic induction protocol was carried out for 3 cycles (21 days). Control cultures (no induction agents) were run in parallel (Janderova et al. 2003; Pittenger et al 1999).

2.8.2. Analysis and quantitation of adipogenic differentiation

a) **Oil red O staining**

Cultures were stained with oil red O, which stains intracellular lipid droplets within multidroplet fat cells and adipocytes an intense red colour. Modified from Janderova (Janderova et al 2003) (4.8.2. 'Multidroplet cell cluster formation').

i. **Reagents**

- Formalin: 10% solution prepared in PBS
- Isopropanol: 60% solution (60 ml 100% isopropanol in 40 ml distilled water) and 100%
- Oil Red O: A 0.5% stock solution was prepared in 100% isopropanol and stored at room temperature. A working solution was prepared immediately before use by adding 12 ml stock solution to 8 ml distilled water and incubating at room temperature for 10 minutes. The working solution was filtered using Whatman (No 1) filter paper before use.

ii. **Method**

Cultures were rinsed in PBS and fixed for >5 hours in 10% buffered formalin at room temperature. They were then washed in PBS and rinsed in 60% isopropanol before being immersed in a working solution of oil red O for 30 minutes at room temperature. Cultures were washed in tap water to remove excess stain and viewed using phase contrast.

iii. **Analysis**

Adipocytes usually formed in microscopically visible colonies, which were counted using a low power dissecting microscope. All colonies visible using the low power dissecting microscope were counted.

iv. Quantitation

Cultures were destained in 100% isopropanol for 15 minutes at room temperature. The optical density was determined at the optimal wavelength of 500 nm (figure 2.8) (Janderova et al 2003).
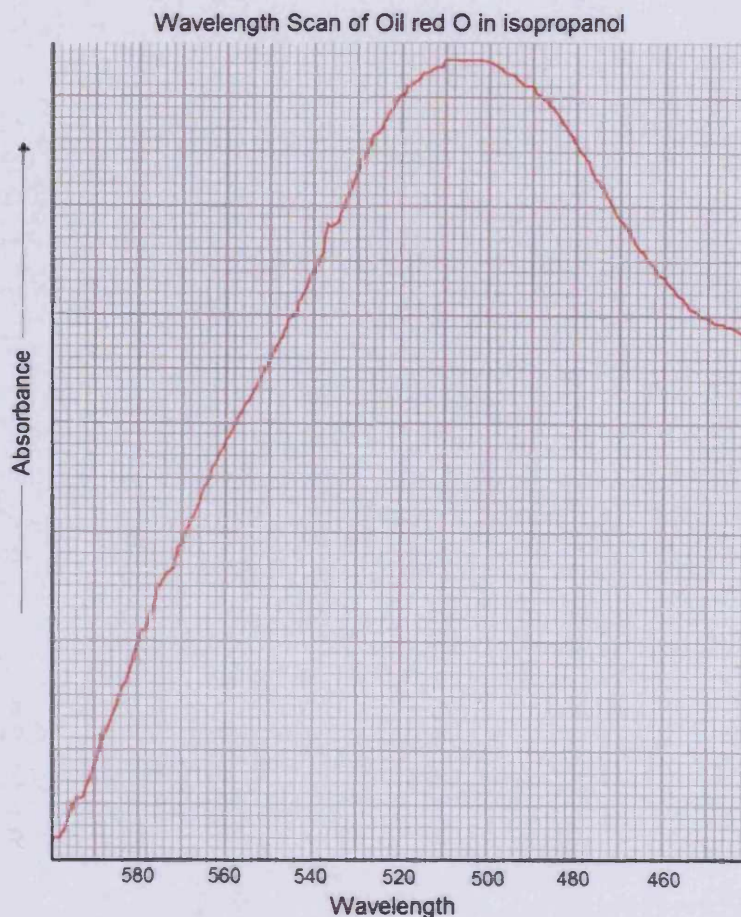
Figure 2.8. Optimal wavelength scan for Oil Red O in isopropanol, established to be 500 nm.

The Oil red O staining properties of Olive oil, Rape-seed oil, Sunflower oil and Vegetable oil were compared in a series of standard curves (figure 2.9). These were prepared by dissolving 1 ml Oil in 5 ml isopropanol, adding 5 ml filtered Oil red O solution (0.5% w.v in isopropanol), and incubating for 2 hours at room temperature. Water (15 ml) was added and the solution was centrifuged at 2500 rpm for 20 minutes. The supernatant was removed leaving the oil on the walls of the UC, which was redissolved in 10 ml isopropanol. Water was added (15 ml) and the solution was centrifuged again at 1000g. The supernatant was removed and the oil attached to the walls of the UC was redissolved in 20 ml isopropanol, from which a serial dilution in isopropanol was prepared.

There was a linear relationship between concentration and absorbance (500 nm) from 0.2 to 10 mg/ml, and the combined regression was good (r=0.94). However, the staining characteristics of the different oils were variable and the results were expressed as lipid index (4.8.3.'Lipid index').
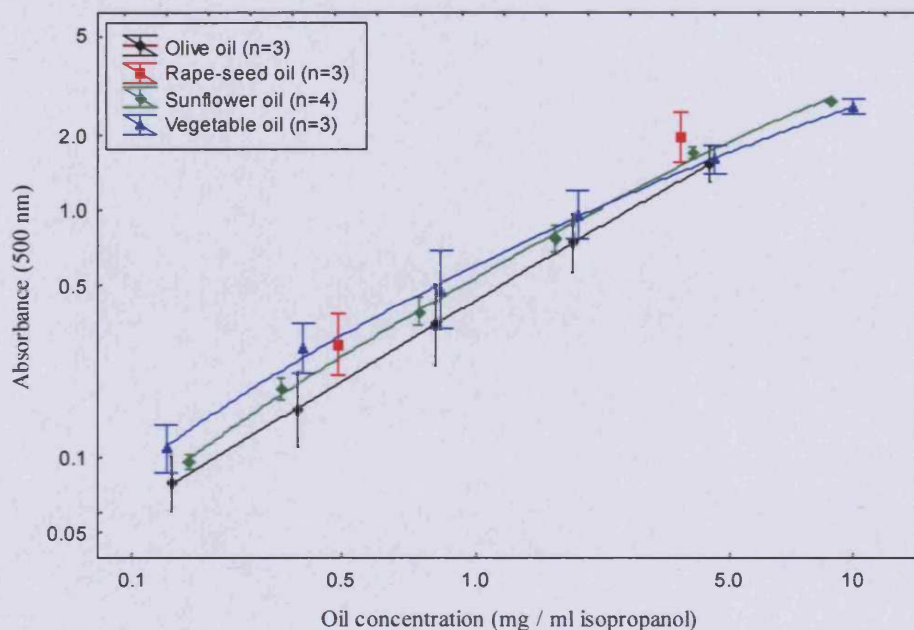
Figure 2.9. Standard curves for lipid concentration, prepared from Olive oil, Rape-seed oil, Sunflower oil and Vegetable oil.
Combined: Oil concentration (mg/ml) = $10^{((\log10 \text{ Abs } 500nm + 0.2864) / 0.734)}$ (r=0.94)

b) **Data normalisation**

Data was normalised to DNA content (2.7.3.g. 'Data normalisation').

2.9. **Analysis of Gene Expression**

In eukaryotic cells, DNA is transcribed to an RNA molecule (messenger RNA (mRNA)), which is then translated into one or more protein sequences specific to the original genetic code (Turner et al. 2000). By extracting the mRNA from the cells and converting it back into DNA (complementary DNA (cDNA)) using reverse transcriptase, a PCR (polymerase chain reaction) can be performed using a pair of complementary oligonucleotide primers and DNA polymerase to amplify the specific sequence of DNA. If performed quantitatively, for example, using real time PCR (LightCycler system (Roche)), it is possible to determine the amount of cDNA in the original test sample and thereby quantitate the level of gene expression. The level of gene expression, however, is not always proportional to the amount of protein transcribed.

2.9.1. RNA extraction

RNA was extracted from adipogenically and osteogenically induced and control BMdSC cultures at 21 days post induction or at distinct time-series points (1, 3, 7, 10, 14, 17 & 21 days) during the induction period.

a) Osteogenically induced BMdSCs

Cultures were detached using the Trypsin-EDTA detachment protocol and the cells pelleted (1150g for 20 minutes). The supernatant was discarded and the cell pellet immediately frozen on dry ice and stored at -80°C for RNA extraction. The cell pellet was removed from the -80°C freezer and allowed to thaw until the pellet could be dislodged. RLT lysis buffer (350 µl) (2.9.1.c.i.'Reagents') was added and vortexed to break up any clumps. The samples were homogenised by being passed through a 20-G needle attached to a 2 ml syringe five times, and 350 µl 70% ethanol was added.

b) Adipogenically induced BMdSCs

Cultures had to be lysed *in situ* by the direct addition of 700 µl RLT lysis buffer (2.9.1.c.i.'Reagents') to the cell layer because fat cells could not be pelleted during centrifugation. Following addition of the lysis buffer, a cell scraper was used to release the cells from the flask surface, which were collected into a UC and stored on ice. Lysed BMdSCs were vortexed and homogenised as described above, before being stored at -80°C for RNA extraction. The cells in RLT lysis buffer were removed from the -80°C freezer for RNA extraction and allowed to thaw before adding 350 µl 70% ethanol.

c) RNeasy RNA extraction protocol

The RNeasy mini kit utilised a high salt-buffer, which allowed up to 100 µg of RNA (longer than 200 bases) to bind to the silica-gel membrane inside the mini spin column. The kit enriches mRNA, and other RNA molecules such as ribosomal RNA or transfer RNA that are also greater than 200 bases long.

i. Reagents

- RNeasy mini kit (Qiagen): Contains mini spin columns with a silica-gel membrane and the following reagents:

> ➤ RLT lysis buffer: Addition of 10 μl β-Mercaptoethanol (β-ME) to 1 ml RLT buffer (composition unknown, however, stated to contain guanidine isothiocyanate to denature ad inactivate RNases). Stable for one month at room temperature.

> ➤ RW1 buffer: Composition unknown. Stated to contain a guanidine salt

> ➤ RPE buffer: (Composition unknown) Addition of 44 ml ethanol (>96%)

- RNase-free DNase kit (Qiagen): The DNase I stock solution (kit component) was dissolved in 550 μl of RNase free water. Then 10 μl was added to 70 μl RDD buffer (kit component, composition unknown)

- 70% ethanol

ii. Method

The lysed and homogenised sample (700 μl) was added to an RNeasy mini spin column and microcentrifuged for 15 seconds at 10,000 rpm to bind the RNA to the silica-gel membrane within the column (ethanol was used to promote the selective binding of RNA to the silica-gel membrane). Buffer RW1 (350 μl) was added as a wash and the column microcentrifuged for 15 seconds at 10,000 rpm. The RNA was then treated with RNase-free DNase I in RDD buffer (80 μl) for 15 minutes at room temperature to digest any remaining DNA. The DNase I was washed away during a second wash with buffer RW1 (350 μl) and 500 μl buffer RPE was added to the column and microcentrifuged for 15 seconds at 10,000 rpm. A second aliquot of 500 μl buffer RPE was added and the column was microcentrifuged for 2 minutes at 13,000 rpm to remove all residual traces of ethanol from the membrane. The RNA was eluted in 30 μl RNase free water.

iii. RNA quantitation:
The RNA was diluted 1/20 (5 μl in 95 μl RNase-free water) and analysed at 260 nm and 280 nm. RNA quantity (ng/μl) was determined by multiplying A_{260} by the dilution factor and the RNA calibration factor (1 A_{260} unit of RNA = 40 ng/μl).

$$RNA\ (ng/\mu l) = A_{260} \times 20 \times 40$$

The A_{260} to A_{280} ratio was used to assess the purity of the RNA (pure RNA regarded as having a ratio of ~2). No RNA was extracted with a purity below 1.5 and all relevant RNA was used for real-time gene expression.

2.9.2. Reverse transcriptase reaction

The RNA extracted from the cultured cells (2.9.1. 'RNA extraction') was converted to cDNA by reverse transcription (RT) in a reaction volume of 20 μl, involving DEPC water (2.2.2.

'Buffers'), 1 µg RNA and reagents from GeneAmp RNA PCR kit (Applied Biosystems): 4 µl

$MgCl_2$ (25 mM), 2 µl 10x PCR buffer II (with no MgCl), 2 µl of each oligo dNTPs (dTTP,

dGTP, dCTP, dATP: 10 mM), 1 µl RNase inhibitor (20 u/µl), 1 µl Random Hexamers (50 µM)

and 1 µl MuLV (cloned Murine Leukemia Virus) Reverse Transcriptase. The RT reaction

involved 60 minutes at 37°C and 5 minutes at 75°C. The cDNA was then quantitated by real-

time PCR using the LightCycler system.

2.9.3. **LightCycler: principles and application**

The Roche Applied Science LightCycler system enables PCR
reactions to be carried out more rapidly than conventional PCR
techniques. The amplification of PCR products can be
visualised in real time and quantitated during the exponential
phase of PCR.

The LightCycler system
(Roche Applied Science 2002)

a) **Operating System**

The LightCycler controls the various temperatures required during a PCR reaction by using a

heating coil and a fan, which evenly distributes the warm air around the reaction capillaries

within the thermal chamber (figure 2.10). The capillaries are made out of borosilicate glass and

have a high surface to volume ratio, allowing for a rapid equilibration between the air

temperature and the reaction components.

An LED (Light-emitting diode) focuses blue light (470nm) onto the tip of the capillary to excite

the fluorophore (e.g. SYBR Green I, described later). The fluorescent light emitted by the

fluorophore is directed by a series of mirrors and filters to a detector within the optical unit.

The fluorescence is measured at pre-programmed intervals (usually once per cycle) during the

PCR reaction to monitor the increase in PCR product formation.
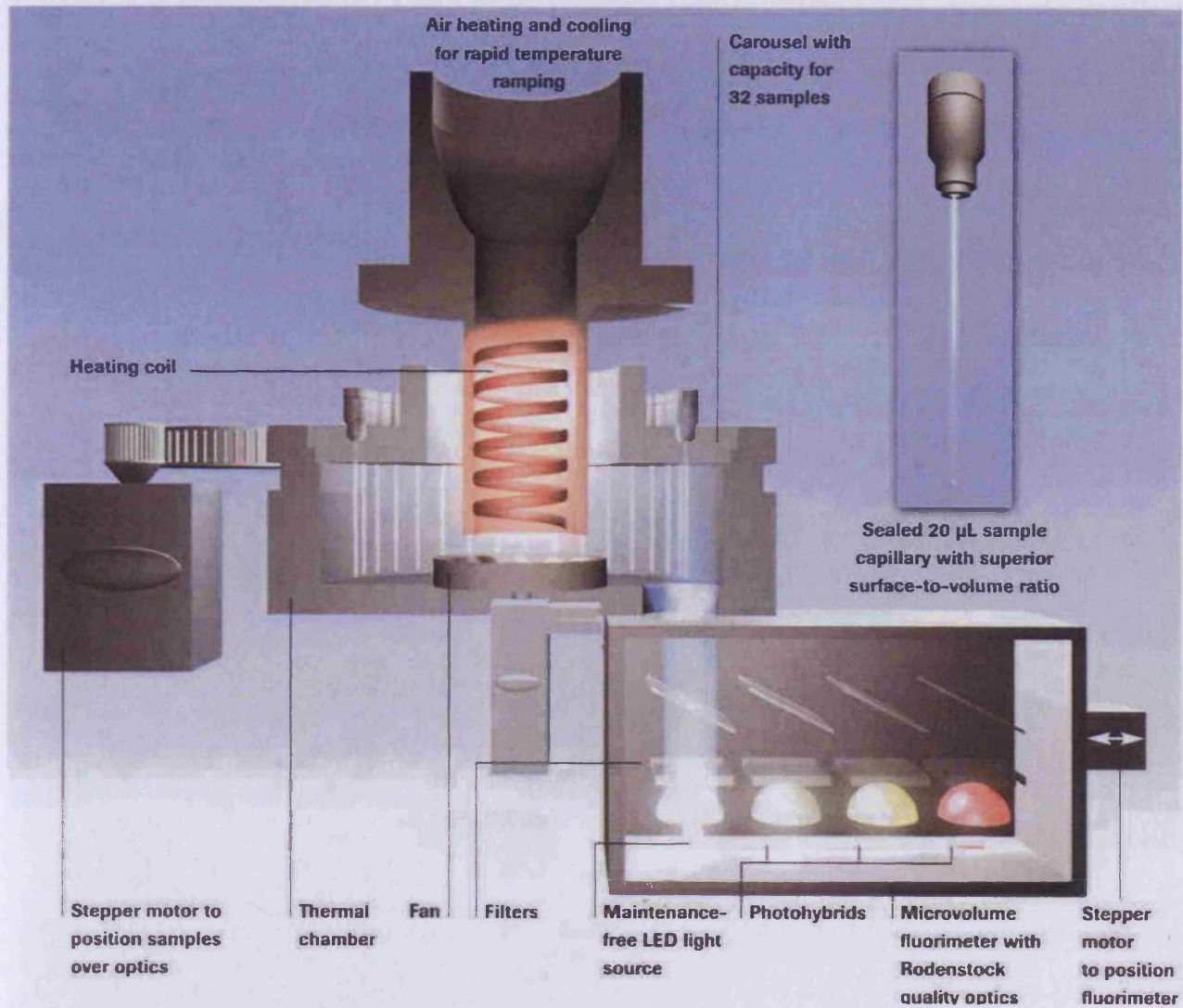
Figure 2.10. The LightCycler operating system (Roche Applied Science 2002)

b) **Sybr Green I**

Sybr Green I only fluoresces when bound to the minor groove of double stranded DNA (figure 2.11), therefore, as the number of copies of dsDNA increase during the PCR reaction, the fluorescence intensity increases.

During the first stage of PCR, the cDNA is denatured (single-stranded DNA) so the intensity of fluorescence is low because SYBR Green I cannot bind.

As the first cycle begins, the primers anneal to the target DNA sequence allowing small amounts of SYBR Green I to bind increasing fluorescence.

As the primers extend to create a complementary strand of DNA, more and more SYBR Green I molecules bind.

Eventually the DNA becomes double-stranded with the maximum amount of dye bound.

Figure 2.11. The action of Sybr Green I (Roche Applied Science 2002)

c) Analysis and quantitation

With each cycle, the number of PCR products double resulting in a log-linear increase in fluorescence, from which the starting concentration of target sequence can be extrapolated. During the log-linear phase, the differences in signal intensity between samples can be observed (figure 2.12) and the background signal can be eliminated (Ginzinger 2002). The cycle threshold (CT), derived from the second derivative of the rate of change during this log-linear

phase, is proportional to the amount of starting material and can be used for comparative purposes to determine relative gene expression (2.9.4. 'Real time analysis').
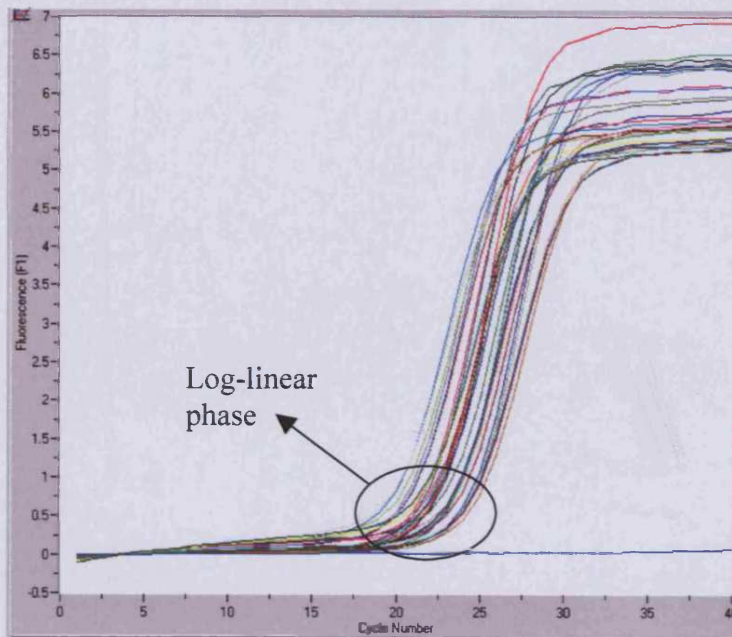
Figure 2.12. Logarithmic increase in fluorescence during the PCR.

d) **Melting curve analysis**

Each dsDNA molecule has a specific melting temperature (T_m) at which 50% of the DNA becomes single stranded. Following the PCR reaction, the temperature within the thermal chamber rises, causing the dsDNA to denature, releasing the SYBR Green I molecules. The reduction in SYBR Green I fluorescence is measured and plotted against the increasing temperature to produce a melting curve (figure 2.13). The T_m can be identified by taking the first negative derivative ($d(F1)/dT$) of the melting curve to produce a peak (Roche Applied Science 2002).
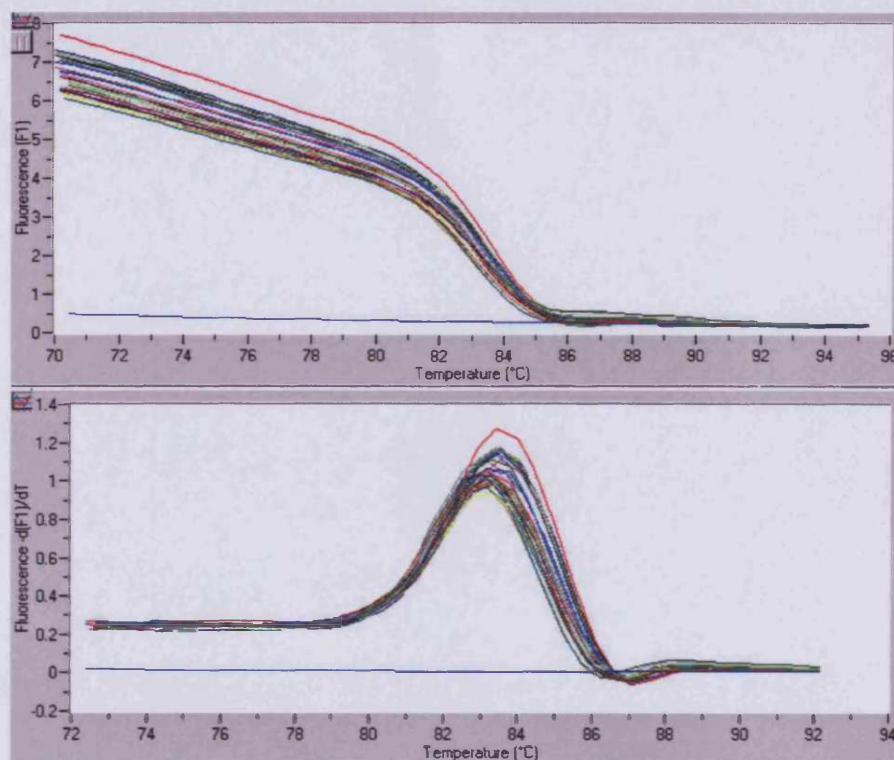
Figure 2.13.   Melting curve analysis

This peak can then be compared to the peaks of other samples (figure 2.13).   Each dsDNA molecule within one reaction should have the same T_m value with varying levels of fluorescence intensity depending on the level of gene expression.   Figure 2.13 shows the melting curve for alkaline phosphatase in osteogenically induced BMdSCs.   The horizontal blue line was water (blank).

2.9.4. Real time analysis

a) **Reagents**

- LightCycler FastStart DNA Master SYBR Green I kit (Roche) contained water, $MgCl_2$ (25mM) and SYBR Green I (10 μl enzyme (provided) added to SYBR Green I).

- Primers (Appendix II)

b) **Method**

Only one gene-specific primer (Appendix II) was used per LightCycler run. A mastermix of 5.8 μl water, 1.2 μl $MgCl_2$ (prepared to a concentration of 4 mM) and 1 μl SYBR green I was prepared with 0.5 μl of the relevant forward and reverse primers (50 ng/μl) (Appendix II). To each capillary 1 μl of sample was added to 9 μl of mastermix (prepared in duplicate) and

capped. A negative control template (H_2O) was run with every gene-specific primer. The capillaries were centrifuged in a microcentrifuge for 5 seconds at 2,500 rpm to push the reaction into the capillary tip before loading them into the LightCycler. The amplification program involved 1 cycle at 95°C (600 seconds), followed by 40 to 50 cycles (depending on the gene) of 95°C (3 seconds) for denaturation, 60°C (5 seconds) for annealing, and 72°C (12 seconds) for extension. A single acquisition mode recorded the level of fluorescence at every cycle. The amplification program was followed by a melting curve analysis (95°C for 0 seconds, 70°C for 15 seconds and 95°C for 0 seconds) with a continuous acquisition mode, and a cooling program.

The CT values were established by the LightCycler and used in the following equation to compare sample gene expression normalised to the housekeeping gene (S14):

$$\text{Index of gene expression} = 2^{(\text{Housekeeping CT} - \text{Test CT})}.$$

The same housekeeping gene was used for both osteogenic (4.6.5. 'Gene expression related to osteogenic induction') and adipogenic differentiated cells (4.8.4. 'Gene expression related to adipogenic induction').

2.9.5. Gel electrophoresis

The PCR reaction products were extracted from the LightCycler capillaries by placing them upside down in 1.7 ml ependorph tubes and centrifuging them for 5 seconds at 2,500 rpm. Equivalent reaction products were pooled (6 capillaries with 10 µl reactions = 60 µl) and electrophoresed on a 1% agarose gel (w/v) with ethidium bromide (1 mg/ml). This enabled the PCR product to be isolated on a gel, excised, the DNA extracted (2.9.6. 'DNA extraction'), quantitated and sequenced for gene confirmation (2.9.7. 'Automated sequencing').

a) Method

Ethidium bromide (3 µl) was added to 20 ml mixed agarose gel (melted slowly in a microwave oven until the agarose had melted), poured into a gel block (with comb insert at negative electrode) within an electrophoresis tank and allowed to set. TBE buffer (1x) was added to the tank and allowed to cover the gel. Blue/Orange (6x) Loading dye (Promega) was added to the samples (10 µl loading dye in 60 µl sample) and to the pGEM marker (1/10 dilution in distilled water) (2 µl loading dye in 10 µl pGEM) and mixed well. The comb was removed from the gel and the samples (50 µl, large wells) and pGEM marker (6 µl, small well) were added. The gel was run for approximately 45 minutes at 50 volts (~25 mA). The ethidium bromide stained the

PCR products, which created a band that could be visualised under UV. The size of the PCR product was identified by comparison to the pGEM marker.

2.9.6. DNA extraction

The relevant sized DNA fragment (established from the pGEM marker) was excised from the agarose gel with a clean sharp scalpel and weighed in a 1.7 ml ependorph. DNA was extracted from the gel using the QIAquick gel extraction kit (Qiagen), which contained buffer QG, buffer PE and elution buffer (unknown composition). Three volumes of buffer QG were added to 1 volume of gel (100 mg = 100 μl) and incubated at 50°C for 10 minutes, vortexing every 2-3 minutes to dissolve the gel. 1 volume of isopropanol was added, and the DNA was bound to the silica membrane within the spin column. Buffer QG was added to the column and centrifuged to ensure all traces of the agarose had been removed and the DNA was washed with buffer PE (centrifugation step). The membrane was dried by centrifugation to remove all residual traces of ethanol and the DNA was eluted in 30 μl elution buffer. The DNA was diluted 1/20 (5 μl in 95 μl distilled water) and analysed at 260 nm and 280 nm. DNA quantity (ng/μl) was calculated by multiplying A_{260} by the dilution factor and the DNA calibration factor (1 A_{260} unit of DNA = 50 ng/μl): DNA (ng/μl) = A_{260} x 20 x 50

2.9.7. Automated sequencing

The gene products amplified by real time PCR were run on a 1% agarose gel (2.9.5. 'Gel electrophoresis'), the relevant sized DNA fragment was excised and the DNA extracted (2.9.6. 'DNA extraction'). The PCR product was confirmed using the ABI PRISM™ BigDye terminator ready reaction cycle sequencing kit (Applied Biosystems). The ready reaction mix included dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase FS, (no 5'-3' nuclease activity), magnesium chloride and buffer. The cDNA template, 2 mM primer (one direction only) and RNase/DNase free water was added to the ready reaction mix in a reaction volume of 20 μl. Immersion oil was used to cover the reaction mixture to prevent evaporation. The cDNA template was amplified in 25 cycles involving a denaturing step (96°C for 5 minutes), annealing (50°C for 15 seconds) and extension time (60°C for 4 minutes). Unincorporated dye terminators were removed from the sequencing reaction using a DyeEx spin column (Qiagen) containing prehydrated gel-filtration resin, carried out as per manufacturers instructions (Appendix III & IV).

2.10. **Statistical Analysis**

Within this investigation, the graphics and statistical analysis were carried out using Statistica, release 6.0. Statistical comparison between two independent groups was carried out using the t-test (logarithmic transformation was used to normalise the data) or non-parametric statistics such as the Mann-Whitney where normality was uncertain.

a) **Distribution of the data**

Graphically, results can be shown as scatter plots that show the individual values for comparison between multiple data sets. The population limits can be estimated from sample groups by calculating the 95% confidence intervals using mean $\pm$ 1.96 Standard Deviations (only 1 in 20 samples would be expected to fall outside these limits). This shows the variability of the data.

b) **Comparison of the means**

Many results were expressed graphically as mean $\pm$ Standard Error (SE), which gives an indication of the accuracy of the mean of a population, which is influenced by data variability and sample size. The mean of a larger sample group is more likely to be closer to the true population mean than with a smaller group, therefore, the SE bars will be smaller for larger groups. This investigation was more interested in comparing the changes within the mean values than with the variability within the data sets. Mean $\pm$ SE gives a good visual indication of where the fitted plot lies and shows no statistical difference when the error bars overlap.

Chapter 3

Bone Marrow derived Stromal Cell Growth

Bone Marrow derived Stromal Cell Growth

3.0. Aims of Chapter 3

To investigate the growth properties of BMdSCs including the effect of different medium, calcium, hydrocortisone and glutamine concentrations on cell confluence, cell doubling and CFU-F formation at the primary LTC and passaged stromal culture stages of *in vitro* growth. To characterise BMdSC surface receptor expression as a function of passage in relation to the literature. To relate cell proliferation (cell doubling) and migration (time lapse photography) to ECM collagen I formation during a culture period of 14 days.

INTRODUCTION

3.1. Bone Marrow derived Stromal Cells

a) BMdSC source

BMdSCs can be isolated from the bone marrow or compact bone and demonstrate a limited proliferative capacity. Phenotypically similar stromal cells with a greater proliferative capacity and multipotentiality *in vitro* have also been isolated from early foetal peripheral blood (until approximately twelve weeks gestation) (Campagnoli et al. 2001; Javazon, Beggs, & Flake 2004), and more controversially from cord blood (Erices et al. 2000; Lee et al. 2004) and amniotic fluid (Tsai et al. 2004). The presence of stromal cells within the post natal peripheral circulation is very rare with only a small number of researchers reporting isolation from G-CSF-mobilised peripheral blood (Fibbe & Noort 2002; Zvaifler et al. 2000). Evidence for their existence within the peripheral circulation largely comes from the demonstration of BMdSCs homing and engrafting into multiple tissues of the body (Krause et al 2001). Efforts to directly isolate primitive BMdSCs are hampered by their presence in tiny numbers and non-specific cell surface markers for identification (Roufosse et al 2004).

b) BMdSCs isolation

In long-term cultures (LTC), a proportion of the bone marrow mononucleated cell population, isolated by Ficoll Hypaque density centrifugation, adhere to the base of the flask, elongate and divide to form the heterogeneous bone marrow derived stromal cell layer (LTC BMdSCs). LTC BMdSCs include a wide variety of adherent cells found within the heterogeneous layer including stromal fibroblast-like cells, reticular cells, smooth muscle cells, endothelial cells, macrophages, osteoblasts and adipocytes. The diverse layer also includes cells at different stages of differentiation from the larger more differentiated cuboidal and polygonal cell types

(photo 3.1) to the smaller spindle shaped cells (photo 3.2) (Javazon, Beggs, & Flake 2004). The photos were taken from this investigation.
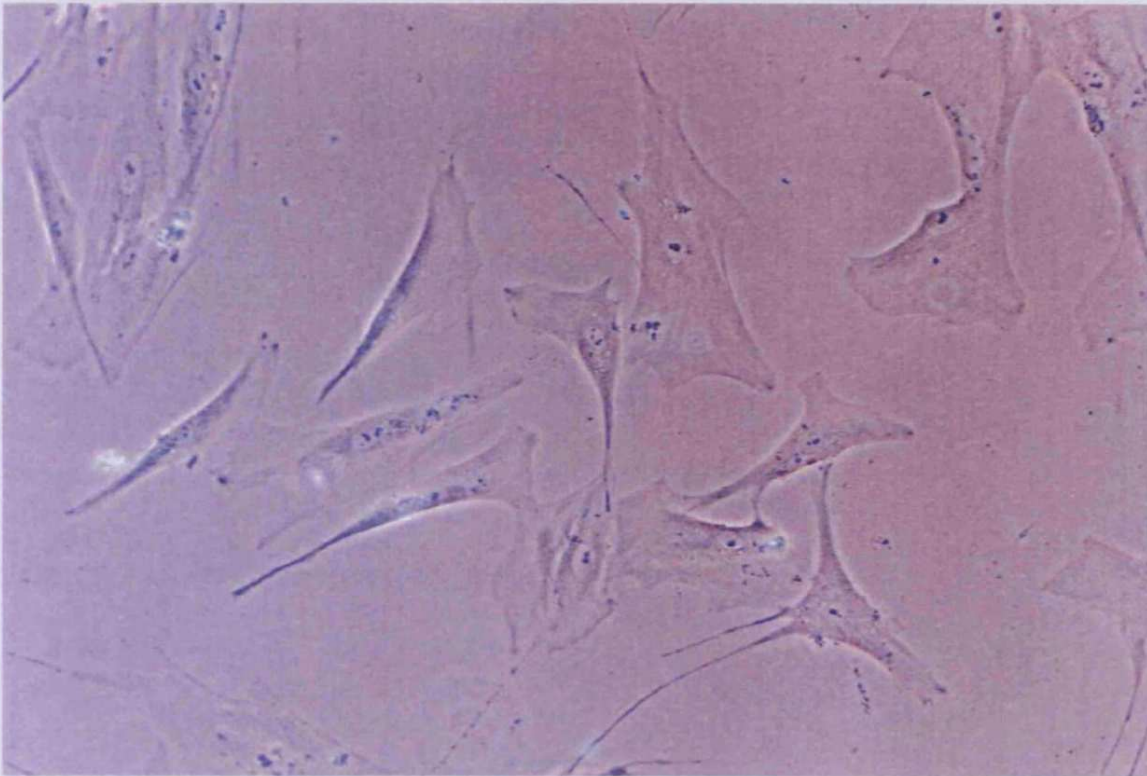
Photo 3.1. Low power magnification (x10) of large cuboidal and polygonal BMdSC types in a non-proliferating 18 day old culture

Photo 3.2. Low power magnification (x10) of more spindle shaped BMdSCs at day 3 of a rapidly proliferating culture

The LTC BMdSCs form a support matrix for the regulation of haemopoietic proliferation and differentiation. The haemopoietic progenitor cells adhere to niches, defined by Spradling (Spradling et al. 2001) as a 'subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo*', within the stromal cell layer. The niches that exist *in vitro* are unlikely to mimic the niches that exist *in vivo* because self-renewal has not been attained indefinitely *in vitro*. The colonies take on the microscopic appearance of cobblestone areas (photos 3.3 & 3.4 from this investigation) with small dark coloured granulocytes. One cell type associated with cobblestone areas were termed blanket cells by Allen & Dexter (Allen & Dexter 1984) because they formed a blanket over the tightly packed macrophages and granulocytes, and may aid in cellular migration. They are large, alkaline phosphatase (ALP) positive cells, which are difficult to see in dense areas. The dark coloured granulocytes exist beneath this blanket layer and gradually become much larger and more refractile as they extrude through and release into the supernatant (figure 3.1). With a confluent BMdSC layer, active haemopoiesis can be maintained in Dexter-type LTCs for many weeks under appropriate culture conditions. However, *in vitro* conditions do not mimic the *in vivo* environment and the LTCs eventually lose much of their haemopoietic capacity.
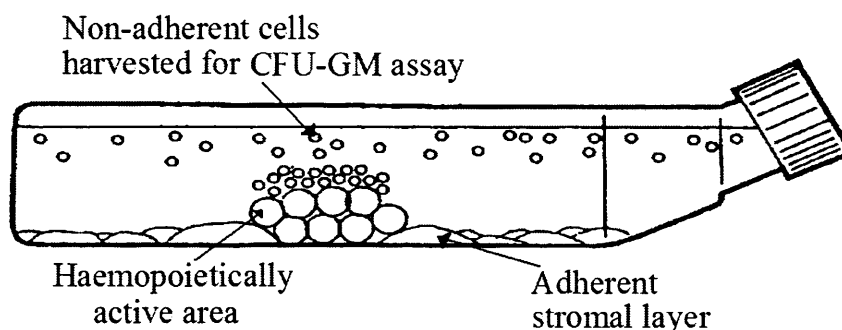
Figure 3.1. Long-term culture in which haemopoietic cells adhere to niches within the stromal matrix

The inoculum size used to seed a culture is likely to have a large effect on the morphology and growth rate of a culture. Smaller seeding densities result in well spread cells with a rapid growth rate and high mobility, whilst larger seeding densities result in the formation of cell clusters with limited mobility and proliferative potential (Goldstein 2001). However, with very small inoculum sizes growth is limited, possibly due to a low concentration of survival and growth factors, although many of these are provided by the serum (foetal calf or horse serum) within the medium. Reports have shown that in the absence of serum or when antagonising chemicals such as platelet-derived growth factor, TGF-β, basic fibroblast-growth factor or

epidermal growth factor in culture, the colony forming capacity is significantly decreased (Kuznetsov et al. 1997).
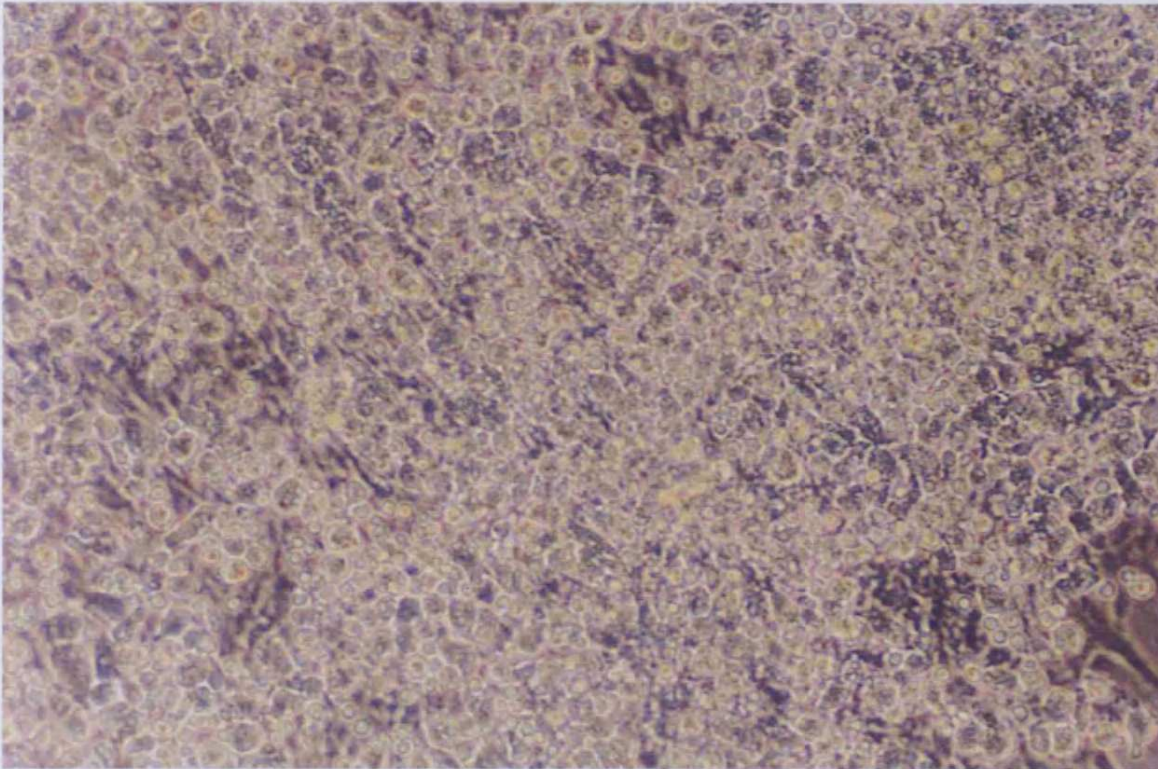
Photo 3.3. Low power magnification (x10) of an active area of haemopoiesis within a normal LTC in modified McCoy's medium with hydrocortisone
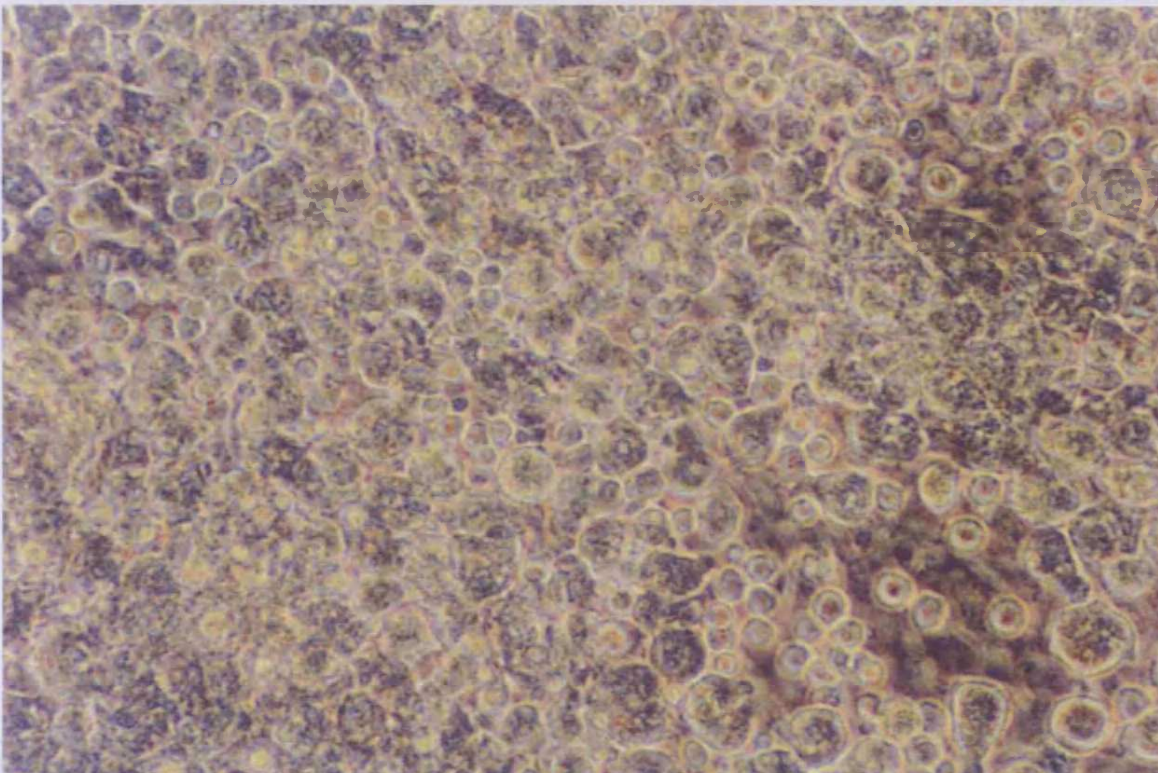
Photo 3.4. High power magnification (x20) of an active area of haemopoiesis. Refractile granulocytes above the blanket layer and darker cells beneath the blanket cells.

c) BMdSC colony formation

Primitive BMdSCs were originally referred to as colony forming unit-fibroblasts (CFU-F) because they produced colonies of adherent, non-phagocytic, fibroblast-like cells *in vitro* (Friedenstein et al. 1970). Each colony is a clone produced from the proliferation of a single precursor cell or CFU-F, which when plated at low density can form isolated colonies that can be counted (assesses number of CFU-F in culture) and assessed for size (assesses the immaturity of the CFU-F) (Kuznetsov, Friedenstein, & Robey 1997). These colonies usually include a heterogeneous population of cells at different stages of lineage commitment and vary widely in terms of growth potential, morphology and differentiation (Digirolamo et al 1999; Javazon, Beggs, & Flake 2004). Assuming that the colonies are not contaminated with other cells (cells not derived from the CFU-F), the variation in cell types found (spindle, flat or cuboidal) indicate a common progenitor cell (CFU-F) with multilineage capability (McIntyre & Bjornson 1986).

The size of the colony indicates the primitiveness of the CFU-F, for example, smaller colonies are likely to be derived from more committed precursor cells with less proliferative capacity, and consist mainly of larger more differentiated cell-types. Larger more dense CFU-F colonies are likely to be derived from more primitive cells with a greater proliferative potential and consist mainly of smaller spindle shaped cells (Digirolamo et al 1999). Progenitor cells represent a spectrum of cells with a declining capacity for proliferation, the progenitor cells (CFU-F) higher in the hierarchical model forming larger colonies (Owen 1998). If self-renewal is not maintained in culture the number and size of the colonies will decline with passage.

CFU-F colony assays are able to provide quantitative information about the stromal precursor compartment, assuming that each colony is formed from a single stem or progenitor cell. The numbers of colonies within the LTC provide information about the initial number of primitive cells within the original bone marrow sample, and the numbers of colonies within the passaged stromal cultures indicate the number of primitive cells recovered from the previous passage. This gives an indication of the loss of the stromal precursor compartment as a function of passage.

d) BMdSC extracellular matrix

Through cell to cell and cell to ECM interactions by cell surface receptors BMdSCs are ideally adapted for cell migration, adherence and cell signalling (communication). These interactions are usually mediated by integrins, which bind components of the ECM such as fibronectin,

collagens or / and laminin, whilst also binding to cytoskeletal proteins within the cells. Integrins can also be responsible for initiating intracellular signalling pathways (Majumdar et al. 2003). The ECM is mainly composed of proteoglycans, fibronectins, collagens, elastins and laminin, which are secreted by the cells to create a microenvironment immediately surrounding the cells. Proteoglycans are responsible for the gel-like character of connective tissues and provide protection against pressure damage in addition to binding to various growth hormones for the activation of signalling cascades. Fibronectins are large glycoproteins that interact with cell adhesion proteins, such as integrins. The collagens are large insoluble glycosylated fibrous proteins that are organised into bundles that cross the ECM and provide the tensile strength of tissues such as cartilage, bone and skin. The elastins impart elasticity to tissues through their none glycosylated cross-linked meshes. Laminin is responsible for separating tissues and adhering to ECM components such as the integrins to provide a substrate over which cells can move (Arias & Stewart 2002a).

Cellular survival and function (secretion, proliferation, differentiation) are controlled within the environment through the actions of growth hormones and cytokines, secreted by neighbouring cells (paracrine), distant cells (endocrine), or in an autocrine fashion. They bind to cell receptors to initiate signalling cascades, which bring about a cellular response (Herzog, Chai, & Krause 2003).

3.1.2. Cell Motility

Cell shape and motility is regulated by changes in the actin filaments within the cytoskeleton. Actin fibres associate in bundles or networks to form strong but flexible structures. They interact with myosin, actin binding proteins and GTPases to generate both intracellular movement and cellular migration (Arias & Stewart 2002a).

During adhesion (for example, BMdSC adhesion to plastic), cells develop a strong attachment to the substrate using 'focal adhesion plaques' (figure 3.2). During cell migration, the actin-based stress fibres are rearranged allowing for the leading edge of the cell to emit spikes (filopodia) or expansions of the membrane (lamellipodia), which probe the immediate environment beyond the cell. These are stabilised by more focal adhesion plaques, at which integrins provide an interaction between the ECM and the cytoskeleton. The tension provided by the actin-based stress fibres allow the cell to project itself forward in the direction of the protrusions, which weakens the focal adhesion plaques at the rear end of the cell. Some of these

are recycled by the cell for future plaque formation, whilst others are left adhered to the substrate (Arias & Stewart 2002a).

a) Process of cellular migration

Formation of focal adhesion plaques to adhere the cell to the substrate

Filopodia and lamellipodia protrude at the leading edge of the cell, secured by adhesion plaques

Actin-based stress fibres provide tension across the cell, which allow the cell to project itself forward

The focal adhesion plaques are weakened at the tail end of the cell, some of which are recycled and others left behind

Figure 3.2.   Cell migration (Arias & Stewart 2002a)

RESULTS

3.2. Normal BMdSC Samples

BMdSCs were derived from the mononuclear cell population of normal healthy donors, aged between 18 and 49 years, and were grown in a long-term culture (LTC) system (2.3.2. 'Primary long term bone marrow culture').
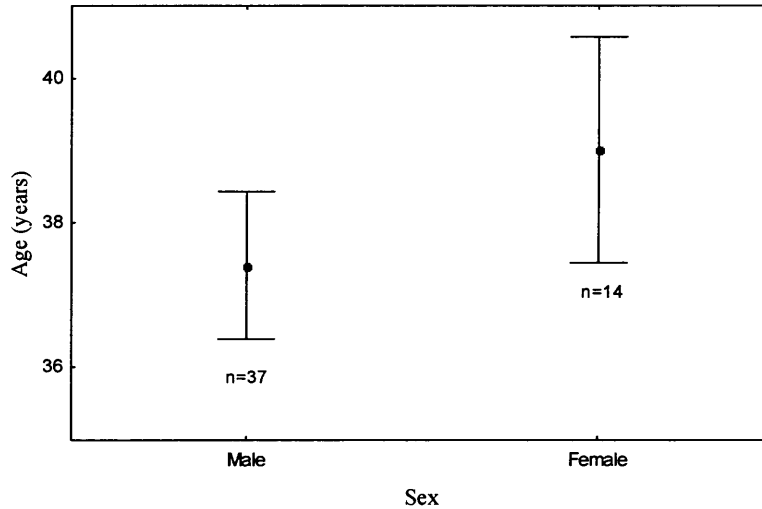
Figure 3.3. The age and sex distribution for normal bone marrow samples. Mean ± SE

37 male donors and 14 female donors were received with comparable age ranges (figure 3.3). The larger number of male donors reflects a nationwide trend. Male donors are preferentially chosen over women because they generally provide more bone marrow material (Roy Bailey-Wood, Department of Haematology, Cardiff University, personal communication).

3.3. Long-term Culture (LTC) BMdSCs

Mononuclear cells (MNCs) were isolated from bone marrow samples (2.3.1. 'Isolation and expansion of BMdSC') and used to establish LTCs (2.3.2. 'Primary long-term bone marrow culture') in Alpha MEM, DMEM or modified McCoy's 5A based medium (with or without glutamine) (2.3.4. 'Medium'). The inoculums varied between $\sim 1 \times 10^6$ to 20×10^6 MNC depending on the number of cells available.

3.3.1. Effect of inoculum size on LTC BMdSC growth

The effect of inoculum size ($\leq 11 \times 10^6$ MNC & $>11 \times 10^6$ MNC) on LTC BMdSC growth was assessed in Alpha MEM and modified McCoy's medium (figure 3.4).
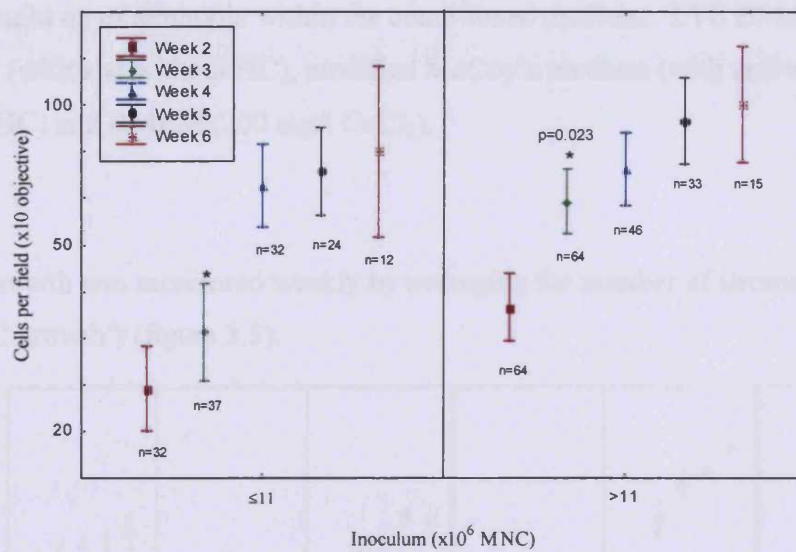
Figure 3.4. Effect of inoculum size on BMdSC growth. Mean ± SE

Initially the larger inoculum size had a greater growth rate with significantly more cells at week 3 (n=64, n=37; p=0.023), however, by week 4 cell counts were comparable. The following analysis was carried out at all inoculum sizes.

3.3.2. Effect of hydrocortisone (HC) and glutamine on LTC BMdSC Growth

a) **Hydrocortisone** (HC) is a glucocorticoid, which is reported to be important for the development and maintenance of a functional adherent LTC cell layer necessary for supporting haemopoiesis such as granulocyte / macrophage colony formation (Suda & Dexter 1981). It has been documented that *in vitro* haemopoiesis only occurs in culture medium supplemented with glucocorticoids (Fernandez & Minguell 1997). However, HC has been reported to be involved with adipogenic differentiation (Croisille et al. 1994; McIntyre & Bjornson 1986) and as the aim was to maintain the BMdSC in an undifferentiated state, the effect of omitting HC was determined.

b) **Glutamine** is vital to most mammalian cell cultures as a major metabolic energy source and protein constituent. It spontaneously degrades in culture medium to ammonia and pyrrolidonecarboxylic acid (Heeneman et al. 1993) and is metabolically catabolised by the cells within the culture (Capiaumont et al. 1995) to produce ammonia by the removal of the amido group to yield glutamate and the subsequent transfer of the α-amino group to yield α-ketoglutarate (Schneider et al. 1996). Ammonia has been found to be toxic to some mesenchymal cells at concentrations as low as 2 – 3 mM (Schneider, Marison, & von Stockar 1996), therefore, it was hypothesised that the presence of glutamine indirectly inhibited BMdSC

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growth by the build up of ammonia within the conditioned medium. LTC BMdSCs were grown in Alpha MEM (with and without HC), modified McCoy's medium (with and without glutamine and HC) and DMEM (200 mg/l CaCl$_2$).

i. Growth

LTC BMdSC growth was monitored weekly by averaging the number of stromal cells per field (2.4.1. 'BMdSC growth') (figure 3.5).
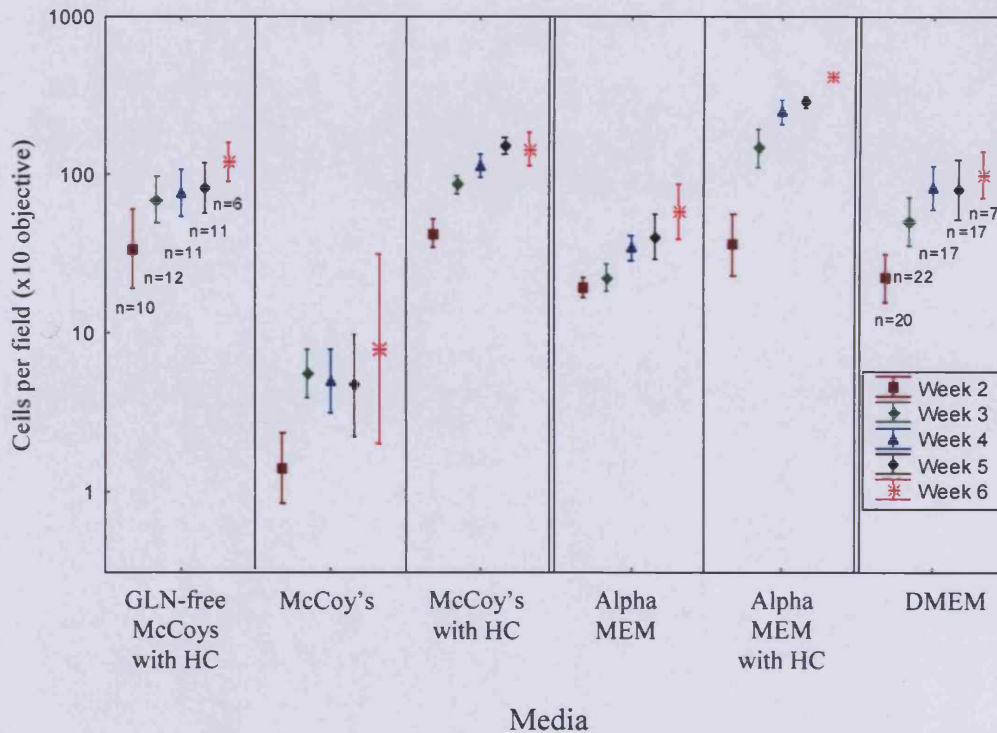
Figure 3.5. Effect of Alpha MEM (with and without HC), modified McCoy's medium (with and without glutamine and HC) and DMEM on LTC BMdSC growth. Mean ± SE. See table 3.1 for data. (GLN-: glutamine free)

Table 3.1. Effect of HC on LTC BMdSC growth: mean number of cells per field, number of cultures and significant differences between McCoy's and Alpha MEM with and without HC.

Week in culture	McCoy's	McCoy's with HC	P-value	Alpha MEM	Alpha with HC	P-value
2	n = 22 1.4 cells	n = 46 42.66 cells	0.000001	n = 55 20.4 cells	n = 8 30.2 cells	
3	n = 24 5.6 cells	n = 51 85.1 cells	0.000001	n = 57 22.9 cells	n = 7 107.2 cells	0.00089
4	n = 21 5 cells	n = 38 112.2 cells	0.000001	n = 49 32.4 cells	n = 4 229 cells	0.0036
5	n = 19 4.7 cells	n = 38 141.3 cells	0.000001	n = 49 35.5 cells	n = 4 269.2 cells	0.049
6	n = 6 7.9 cells	n = 9 144.5 cells	0.026	n = 14 57.5 cells	n = 1 416 cells	

In the presence of HC, LTC BMdSC growth was significantly greater in both modified McCoy's medium and Alpha MEM (table 3.1). Cell doubling (2.4.3. 'Cell doubling') (week 3 to week 5) was also significantly improved in modified McCoy's medium in the presence of HC (n=38, n=19; p=0.023) (not shown).

Glutamine concentration had little effect on LTC BMdSC growth with glutamine free McCoy's medium (with HC) having comparable cell counts to modified McCoy's medium and Alpha MEM (with HC). The standard glutamine concentrations of each culture medium and the concentrations of ammonium (measured using the Sigma diagnostics kit 171-UV) measured in the conditioned media of LTCs at week 2 and week 5 are shown in table 3.2.

Table 3.2. Medium glutamine, and ammonium concentrations in the conditioned medium at week 2 & 5 in modified McCoy's (with and without glutamine), Alpha MEM and DMEM. Mean ± SD

	Glutamine free McCoy's	**McCoy's**	**Alpha MEM**	**DMEM**
[Glutamine] added (mg/l)	0	220	292	584
[Ammonium] at week 2 (mg/l)	4.22 ± 0.94	18.76 ± 5.83	24.26 ± 3.85	27.7 ± 1.13
[Ammonium] at week 5 (mg/l)	5.09 ± 0.05	17.46 ± 3.03	24.17 ± 7.69	27.18 ± 1.58

The ammonium concentration in the conditioned media increased with glutamine concentration, but there was no dose-related inhibition of LTC BMdSC growth (figure 3.5). No significant differences in LTC BMdSC growth were observed between glutamine-free McCoy's medium and DMEM despite DMEM having >5 times more ammonium in the conditioned medium than glutamine-free McCoy's (FCS may have provided trace amounts of glutamine). There was no build up of ammonium concentration in the conditioned medium from week 2 to week 5, indicating that the demi-feeding regimes were sufficient to keep waste products to a minimum.

ii. CFU-F colony formation

The number of CFU-F colonies within the LTCs and the estimated number of cells per colony were counted at week 2 to establish the number of stromal precursors (CFU-Fs) within the MNC fraction of the bone marrow sample (2.4.4. 'CFU-F'). The CFU-F colonies were expressed as large (>100 cells, red), medium (25-100 cells, green) or small (<25 cells, blue) and recorded as CFU-F per 10^6 MNCs (figure 3.6).
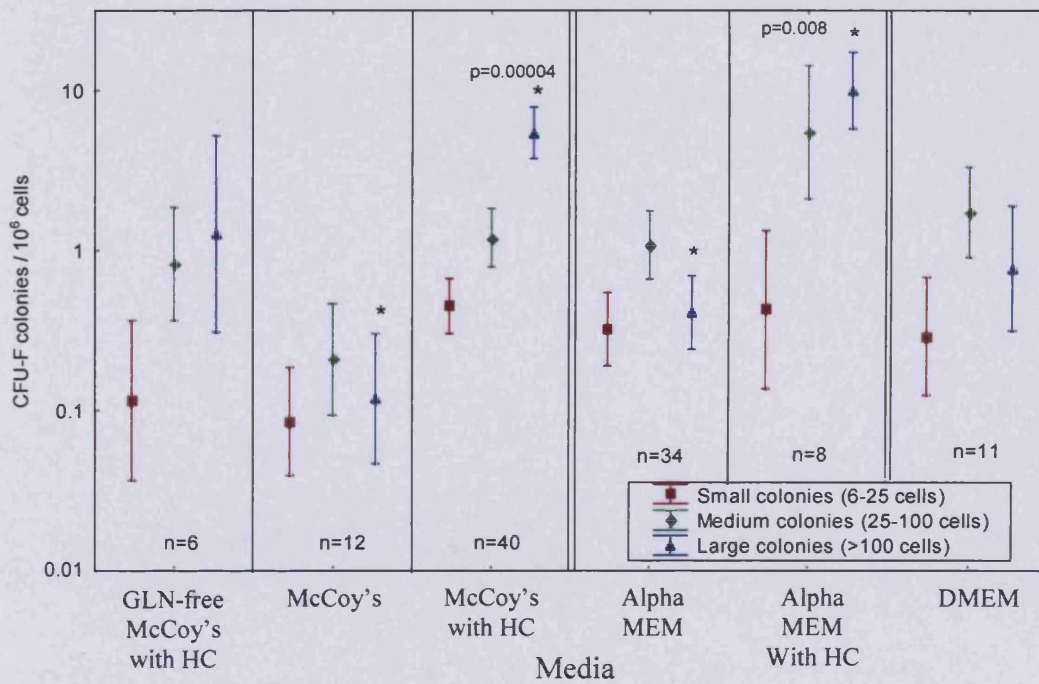
Figure 3.6. Effect of Alpha MEM (with and without HC), modified McCoy's medium (with and without glutamine and HC) and DMEM on LTC CFU-F colony formation. Mean ± SE
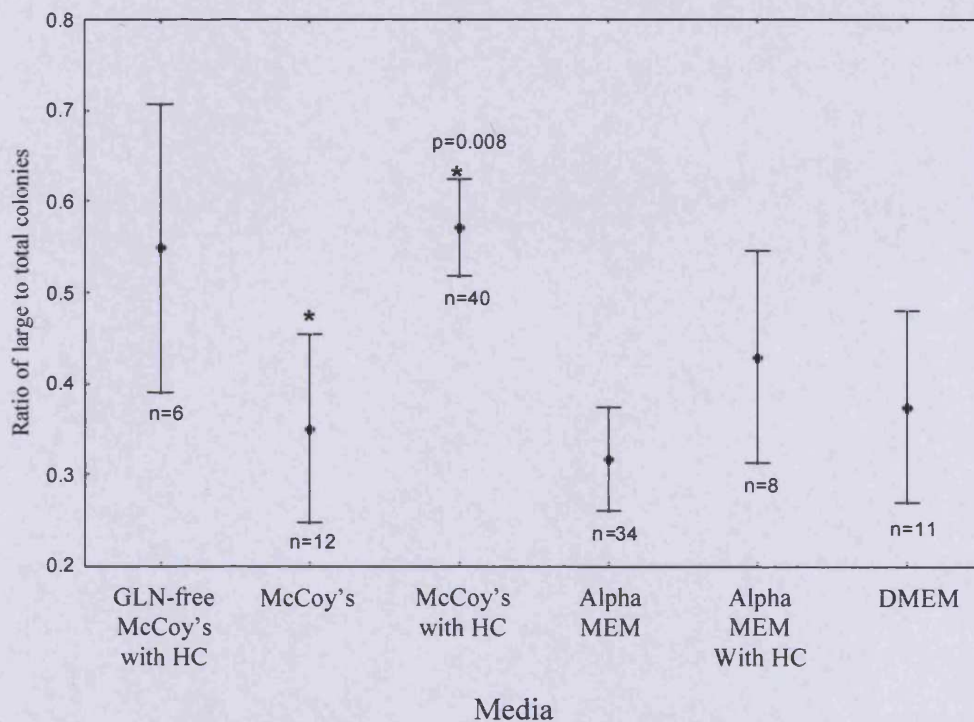
Figure 3.7. Ratio of large to total CFU-F colonies in long-term BMdSC cultures in Alpha MEM (with and without HC), modified McCoy's (with and without glutamine and HC) and DMEM. Mean ± SE

In the presence of HC, the formation of large CFU-F colonies in LTCs were significantly greater in both modified McCoy's medium (n=12, n=40; p=0.00004) and Alpha MEM (n=34,

n=8; p=0.008) (figure 3.6). The ratio of large to total colonies was also significantly increased in modified McCoy's medium with HC (n=12, n=40; p=0.008) (figure 3.7). Glutamine concentration, and therefore, ammonium concentration, had little effect on LTC CFU-F colony formation in modified McCoy's medium. The numbers of large colonies within DMEM were significantly reduced in comparison to modified McCoy's medium with HC (n=11, n=40; p=0.027) and Alpha MEM with HC (n=8; p=0.043). The addition of HC to DMEM would likely significantly increase the number of large colonies formed by week 2, suggesting an effect on the more primitive stromal cells.

The mean number of large colonies within the HC-containing media was established to be 5.5 colonies per 10^6 MNC (0.055 primitive precursor cells in 10^4 MNCs) (table 3.5). This is lower than the estimated 0.3 in 10^4 MNC by Wexler (Wexler et al 2003), but CFU-F numbers are likely to vary depending on culture conditions. The mean number of total colonies was estimated to be 16 CFU-F per 10^6 MNC (1 in 62,000 MNC) (table 3.5).

iii. Summary
LTC BMdSC growth and CFU-F colony formation were significantly increased in the presence of HC in modified McCoy's medium and Alpha MEM. There were no significant differences in LTC BMdSC growth between the HC-containing media. HC was never added to DMEM, which showed a significantly reduced number of large colonies in comparison to HC-containing media. The results suggest that HC had a stimulatory effect on the more primitive cells within the culture.

Glutamine, and therefore, ammonium concentration, showed no inhibitory effect on LTC BMdSC growth and CFU-F colony formation.

LTC BMdSC growth was assessed within various age groups (18-28, 29-38 and 39-49) in HC-containing media (not shown), but no significant differences in cell counts, cell doubling (weeks 3 to 5) or CFU-F colony formation were observed. There were also no significant differences in BMdSC growth between the male and female donors (not shown).

3.3.3. Effect of HC on non-adherent cells and CFU-GMs
Non-adherent cells (NACs) and CFU-GMs (progenitors of the granulocyte, macrophage lineage) were assessed weekly in the supernatants of LTCs inoculated with >16x10^6 MNCs

(2.3.2.a. 'CFU-GM assay').   This gave an indication of the haemopoietic activity of the cultures.
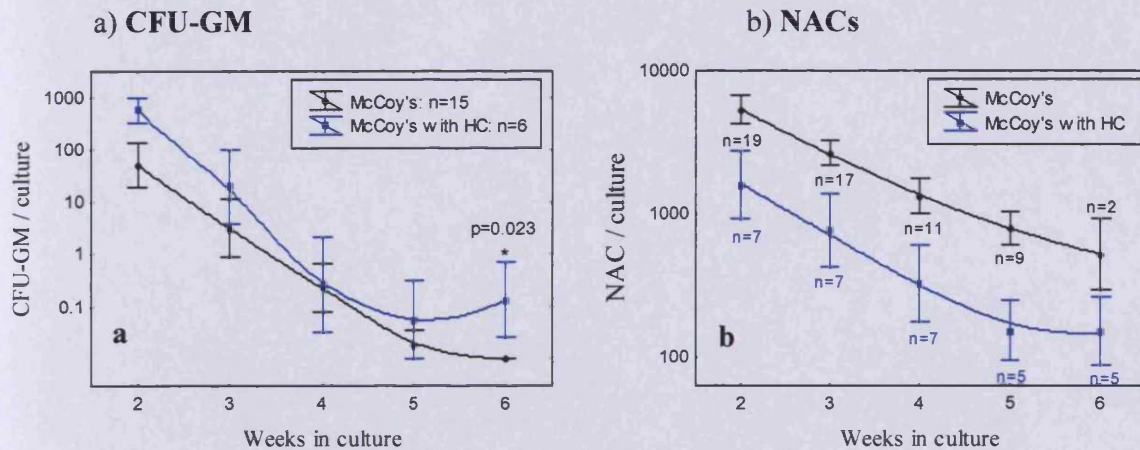
Figure 3.8. Effect of HC on a) CFU-GM formation and b) NAC in modified McCoy's 5A based medium. Mean ± SE

Areas of haemopoietic activity (cobblestone areas, photo 3.3) were only established in cultures containing HC, however, it was clear that the LTCs were not maintaining haemopoietic activity past 6 weeks, which may have been due to the inadequate serum used in the cultures. In modified McCoy's medium (with glutamine) the cultures containing HC had significantly more CFU-GM colonies at week 6 (n=6, n=15; p=0.023), but no CFU-GMs were detected beyond week 6 (figure 3.8.a). In the absence of HC (black), the NACs significantly declined from week 2 (n=19) to week 3 (n=17; p=0.033), 4 (n=11; p=0.0008), 5 (n=9; p=0.00005) and 6 (n=2; p=0.0023). In the presence of HC (blue), NACs declined significantly from week 2 (n=7) to week 5 (n=5; p=0.012) and week 6 (n=5; p=0.015) (figure 3.8.b). The HC-containing media demonstrated more CFU-GM colonies and fewer NAC during the 6 week period, suggesting an effect on the progenitor population. This needs to be further assessed in both modified McCoy's media and Alpha MEM.

3.4. Passaged Stromal Culture Growth of BMdSCs

Passaged BMdSCs, derived from LTCs, (2.3.3. 'Passaged BMdSC culture') were grown in modified McCoy's 5A based medium, Alpha MEM (without HC), or DMEM (200 mg/l $CaCl_2$). Their growth was assessed by cell counts (2.4.1. 'BMdSC growth), cell doublings (2.4.3. 'Cell doubling') and CFU-F colony formation (2.4.4. 'CFU-F). The inoculums varied between ~1×10^4 to 10×10^4 cells per flask depending on the number of cells available.

3.4.1. Effect of inoculum size on passaged BMdSC growth

BMdSC growth was assessed over 17 days in Alpha MEM at various inoculum sizes.
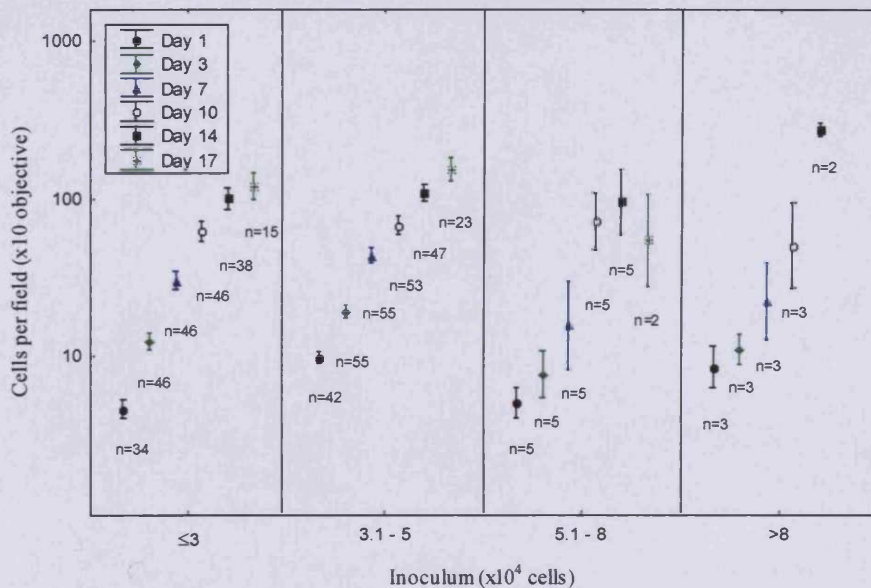
Figure 3.9. Effect of inoculum size on passaged BMdSC growth in Alpha MEM. Mean ± SE

Inoculum size did not affect the growth rate of passaged BMdSCs (figure 3.9). It was unclear why the day 1 cell counts did not increase with inoculum size. Perhaps the higher seeding densities affected BMdSC adherence, the cells adhering in clusters rather than as individual cells.

3.4.2. Effect of added calcium on passaged BMdSC growth

The effect of calcium concentration (calcium added to a calcium free laboratory preparation of Alpha MEM) on passaged BMdSC growth was ascertained in Alpha MEM (figure 3.10).
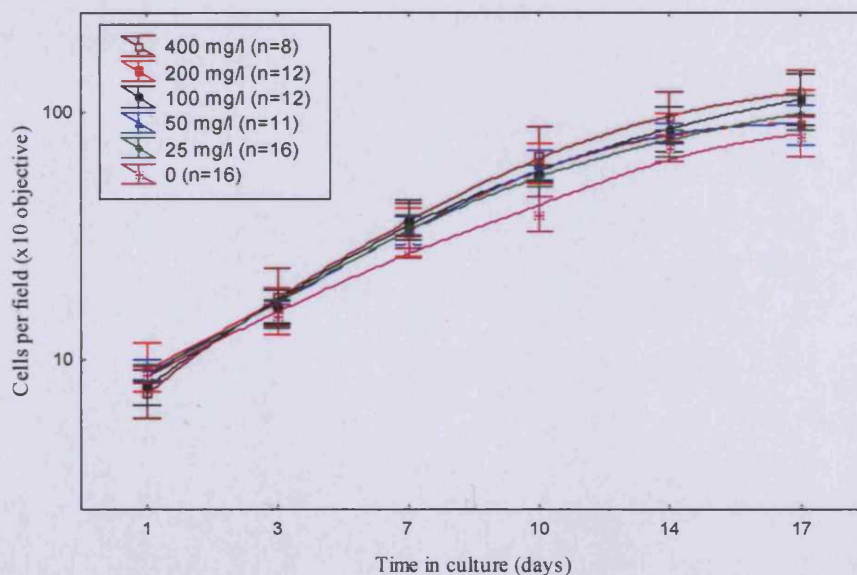
Figure 3.10. Effect of added $CaCl_2$ on BMdSC growth in Alpha MEM. Mean ± SE

There were no statistical differences in BMdSC growth between $CaCl_2$ concentrations in Alpha MEM (figure 3.10), also reflected by the cell doublings (not shown). Precipitation occurred above 400mg $CaCl_2$ /l medium, therefore a medium $CaCl_2$ concentration of 200 mg/l was chosen for BMdSC growth, as is used in commercial medium (2.3.4. 'Medium').

3.4.3. Effect of different media on passaged BMdSC growth

a) **Growth**

BMdSC growth was assessed over 17 days in Alpha MEM, modified McCoy's medium and DMEM by twice weekly averaging the number of cells per field (2.4.1. 'BMdSC growth').
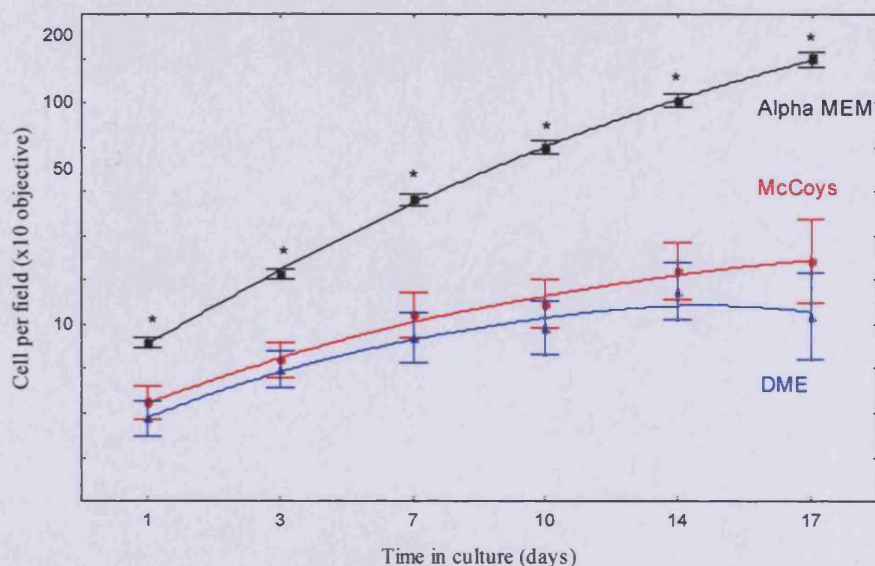
Figure 3.11. Passaged BMdSC growth in modified McCoy's medium, Alpha MEM and DMEM. See table 3.3 for data. Mean ± SE

Table 3.3. Comparison between Alpha MEM and modified McCoy's or DMEM at 1, 3, 7, 10, 14 and 17 days in culture

Days in culture	Alpha MEM	McCoy's	DMEM
1	n = 84	n = 12; p = 0.015	n = 20; p = 0.00079
3	n = 109	n = 14; p= 0.00012	n = 21; p = 0.000007
7	n = 109	n = 14; p = 0.000006	n = 21; p = 0.000001
10	n = 107	n = 15; p = 0.000001	n = 21; p = 0.000001
14	n = 92	n = 11; p = 0.000001	n = 17; p = 0.000001
17	n = 40	n = 8; p = 0.000003	n = 8; p = 0.000001

Passaged BMdSCs grew significantly better in Alpha MEM in comparison to modified McCoy's medium and DMEM (figure 3.11 and table 3.3).

b) Cell doubling

The BMdSC counts were significantly higher in Alpha MEM from day 1, therefore, measuring the cell doubling instead (2.4.3. 'Cell doubling') of the confluency of the culture gave a more accurate portrayal of growth (figure 3.12). Growth rates for passaged stroma were determined from averaged cell counts over 11 days (day 3 to day 14) using a x10 objective under phase contrast.
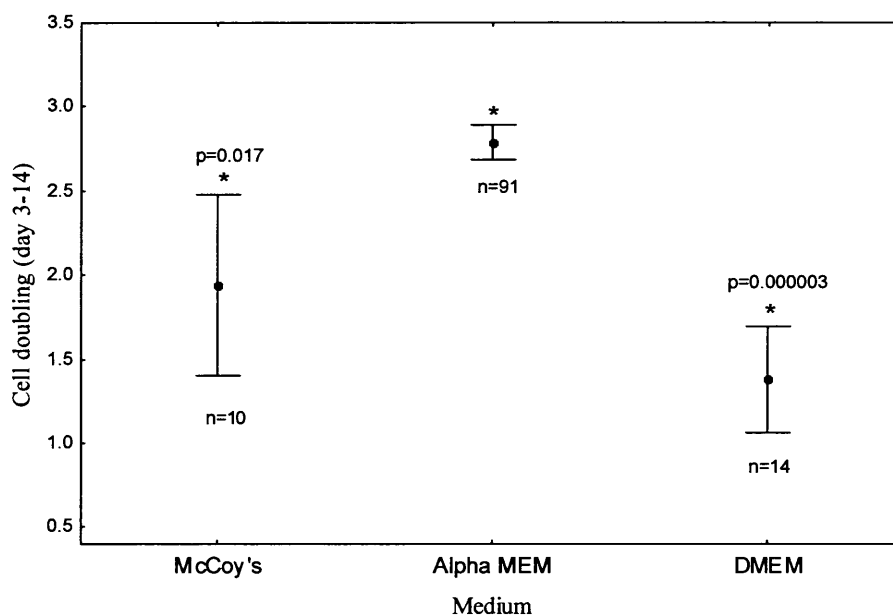
Figure 3.12. Passaged stroma: Cell doubling (days 3 to 14) in modified McCoy's medium, Alpha MEM and DMEM. Mean ± SE

BMdSC doubling was significantly greater in Alpha MEM (n=91) than McCoy's media (n=10; p=0.017) and DMEM (n=14; p=0.000003) (figure 3.12).

c) CFU-F colony formation

The number and size of CFU-F colonies within passaged stroma were determined at day 14 in modified McCoy's medium, Alpha MEM and DMEM. The CFU-F colonies were divided into large (>100 cells, blue), medium (25 – 100 cells, green) and small colonies (<25 cells, red).
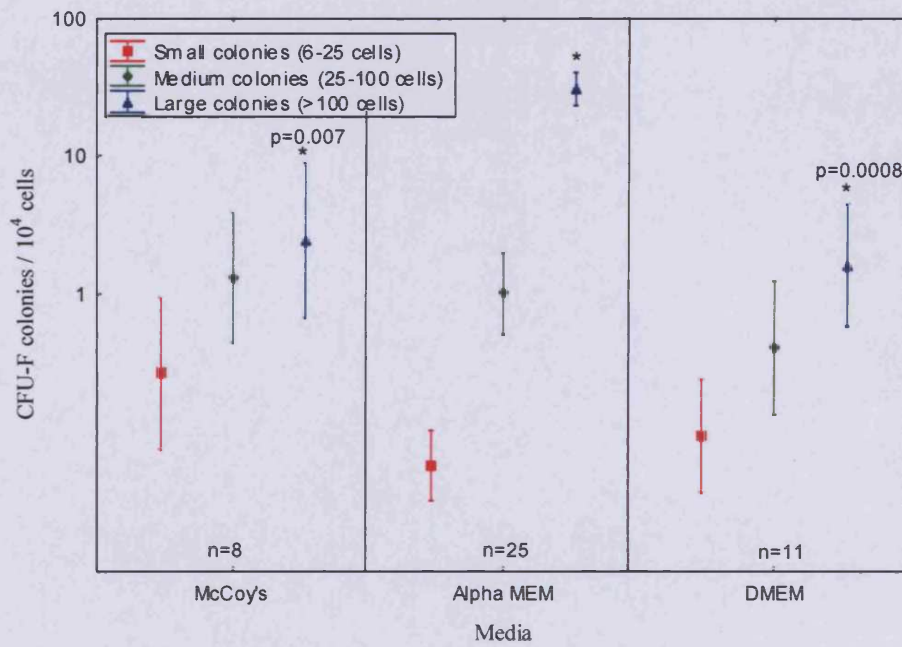
Figure 3.13. Passaged stroma: CFU-F colony formation in modified McCoy's medium, Alpha MEM and DMEM. Mean ± SE

There were significantly more large CFU-F colonies (n=25) in Alpha MEM in comparison to modified McCoy's medium (n=8; p=0.007) and DMEM (n=11; p=0.0008) (figure 3.13).
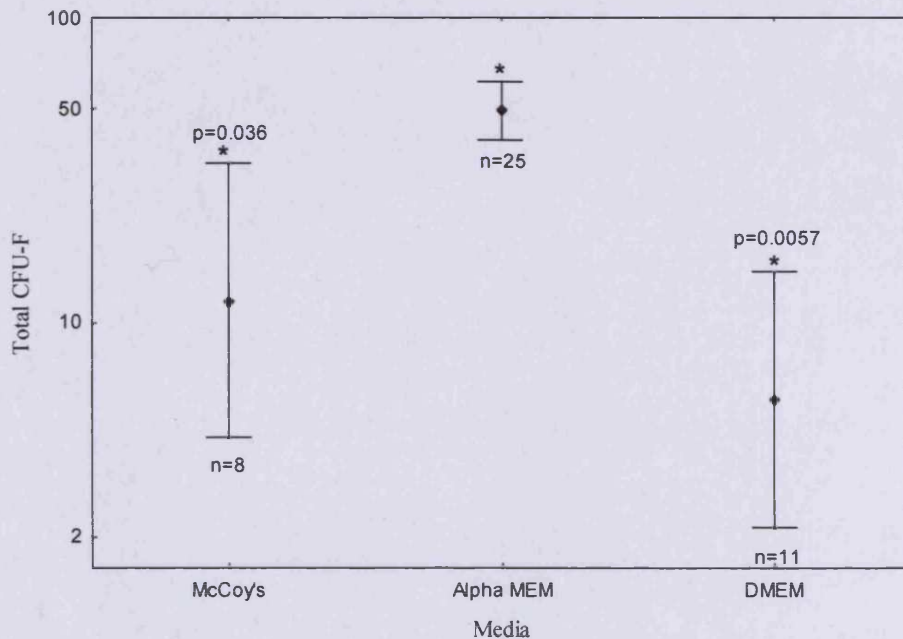
Figure 3.14. Passaged stroma: total CFU-F colony formation in modified McCoy's medium, Alpha MEM and DMEM. Mean ± SE

There were significantly more total colonies in Alpha MEM (n=25) in comparison to modified McCoy's medium (n=8; p=0.036) and DMEM (n=11; p=0.0057) (figure 3.14). These results suggested that Alpha MEM contained ingredients, absent from DMEM and modified McCoy's

that stimulated the more primitive cells within the passaged BMdSC cultures (Alpha MEM contained deoxyribonucleic acids and a wider variety of amino acids).

c) **Summary**

Passaged BMdSC growth was significantly improved in Alpha MEM in comparison to modified McCoy's medium and DMEM (significant increase in cell counts, cell doubling and CFU-F colony formation). Inoculum size and $CaCl_2$ concentration had no significant effect on passaged BMdSC growth.

3.4.4. **Effect of HC on passaged BMdSC growth**

The presence of HC in LTCs significantly improved BMdSC confluence, cell doubling and CFU-F colony formation in modified McCoy's medium, however, the presence of HC in passaged stromal cell cultures (modified McCoy's medium) did not have the same effect.

a) **Growth**
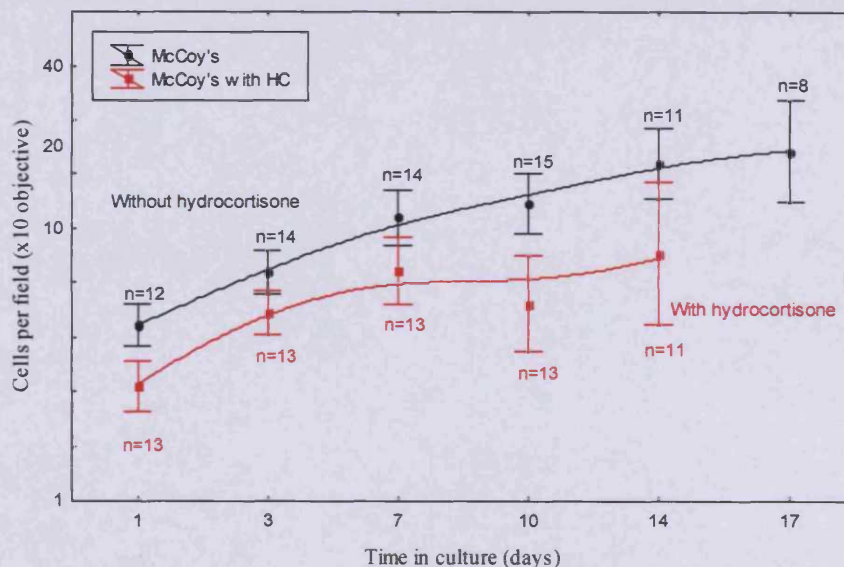
Figure 3.15. Passaged BMdSC growth in modified McCoy's medium with and without HC. Mean ± SE

No significant differences in passaged BMdSC growth (cell counts) were observed between cultures incubated with or without HC in modified McCoy's medium (figure 3.15).

b) Cell doubling
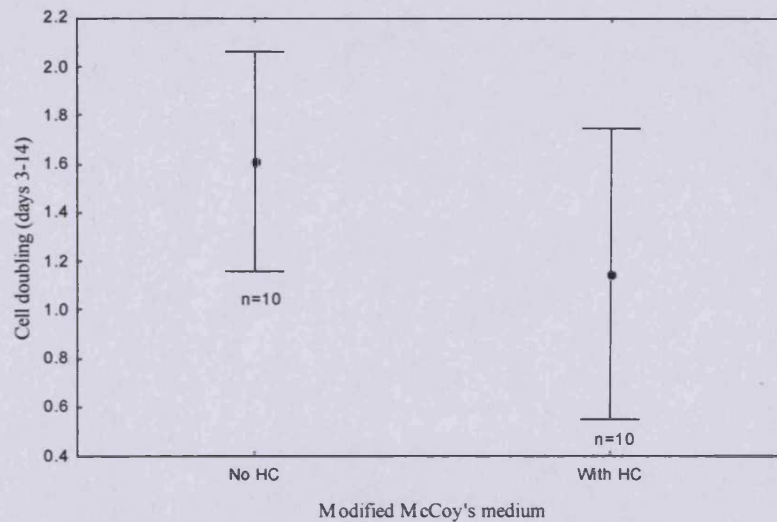
Figure 3.16. Passaged stroma: Cell doubling (days 3 to 14) in modified McCoy's medium with and without HC. Mean ± SE

No significant differences in passaged BMdSC doubling were observed between cultures incubated with or without HC in modified McCoy's medium (figure 3.16).

c) CFU-F colony formation
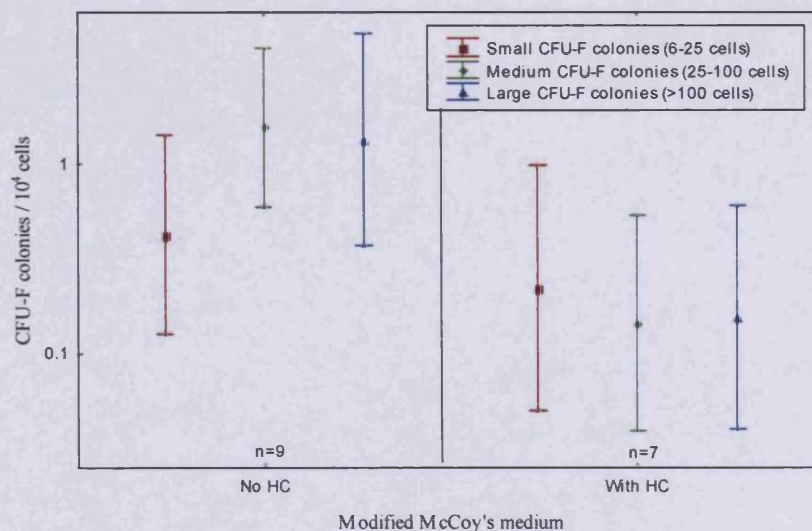
Figure 3.17. Passaged stroma: CFU-F colony formation in modified McCoy's medium with and without HC. Mean ± SE

HC did not significantly increase CFU-F colony formation (figure 3.17) or the proportion of large to total colonies (not shown) in passaged BMdSC cultures.

d) **Summary**

Table 3.4 summarises the effect of HC in LTCs and passaged stromal cultures in modified McCoy's medium (containing glutamine).

Table 3.4. Effect of HC on LTC and passaged BMdSCs in modified McCoy's medium.

Culture	Cells per field	Cell doubling	Large CFU-F	Total CFU-F	Large / total CFU-F
LTC	SI		SI	SI	SI
Passaged	R	R	R	R	R

SI: Significantly increased in the presence of HC ($p<0.05$)
R: Reduced in the presence of HC (not significant)

HC appeared to stimulate the more primitive BMdSCs in the LTCs with significantly more cells per field and large CFU-F colonies, but had no direct effect on passaged BMdSCs.

3.4.5. Passaged BMdSCs derived from LTCs grown with or without HC

The ability of HC to maintain the primitive progenitor cells in LTC (in Alpha MEM and modified McCoy's) was assessed in subsequent passaged stromal cultures in Alpha MEM (no HC). Growth (cell counts), cell doubling and CFU-F colonies were determined in passaged BMdSCs derived from LTCs grown with or without HC.

a) **Growth**

BMdSC growth was analysed in cultures derived from LTCs grown with (red) and without HC (black) (figure 3.18).
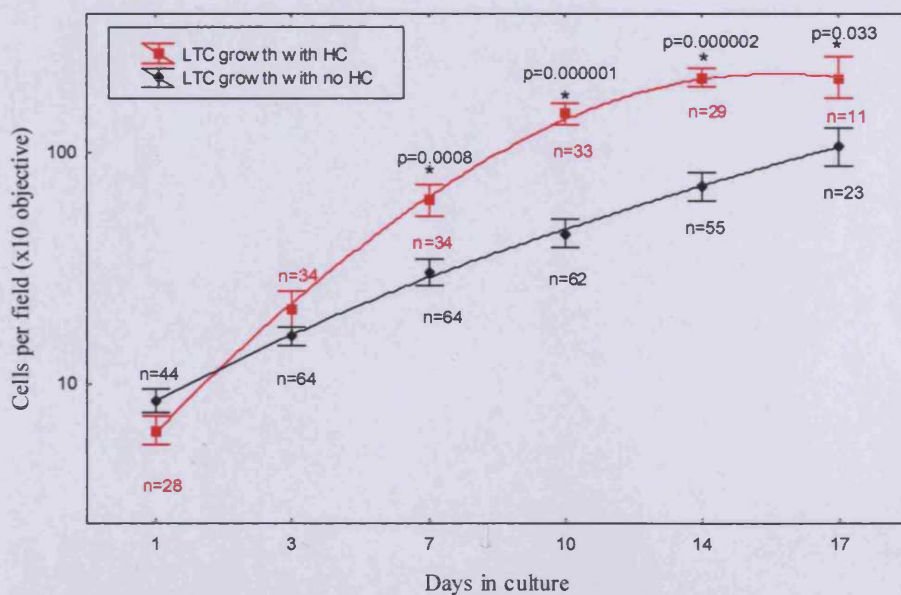
Figure 3.18. Passaged BMdSCs grown in Alpha MEM (without HC), derived from LTCs grown with (red) or without (black) HC. Mean ± SE

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BMdSCs (all passages), derived from LTCs grown with HC, had significantly higher cell counts at day 7 (n=34, n=64; p=0.0008), 10 (n=33, n=62; p=0.000001), 14 (n=29, n=55; p=0.000002) and 17 (n=11, n=23; p=0.033) (figure 3.18).
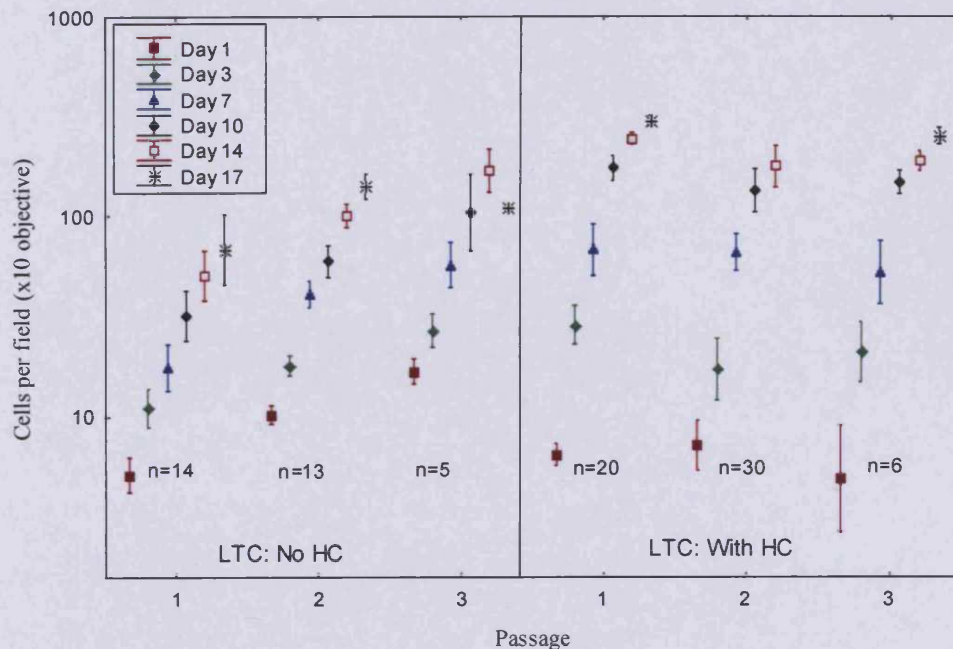
Figure 3.19. BMdSC growth at passages 1, 2 and 3 in Alpha MEM, derived from LTCs grown with (right) and without (left) HC. Mean ± SE

Looking more specifically at passage 1 (figure 3.19), BMdSCs derived from LTCs grown with HC had significantly more cells per field at day 3 (n=14, n=20; p=0.0068), 7 (p=0.0022), 10 (p=0.00005), 14 (p=0.00008) and 17 days (n=6, n=9; p=0.012), suggesting that HC maintained the more primitive (more proliferative) cells within the LTC. This effect was lost in subsequent passages.

b) **Cell doubling** (day 3 to 14)
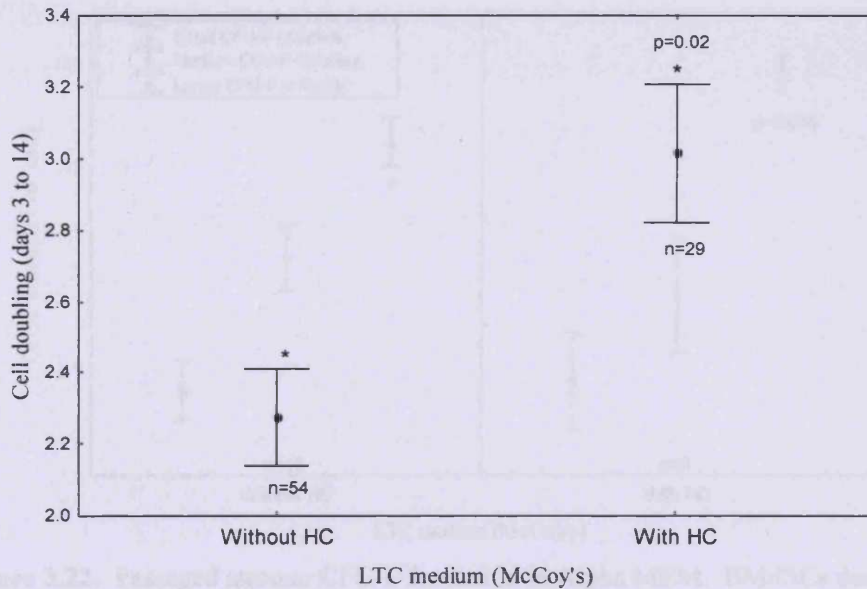
Figure 3.20. Passaged stroma: BMdSC doubling (days 3 to 14) in Alpha MEM derived from LTCs grown with and without HC. Mean ± SE

BMdSCs (all passages) derived from LTCs grown with HC had a significantly higher cell doubling (n=29, n=54; p=0.02) (figure 3.20). Passage I showed a higher cell doubling in BMdSCs derived from LTCs grown with HC (results not significant) (figure 3.21).
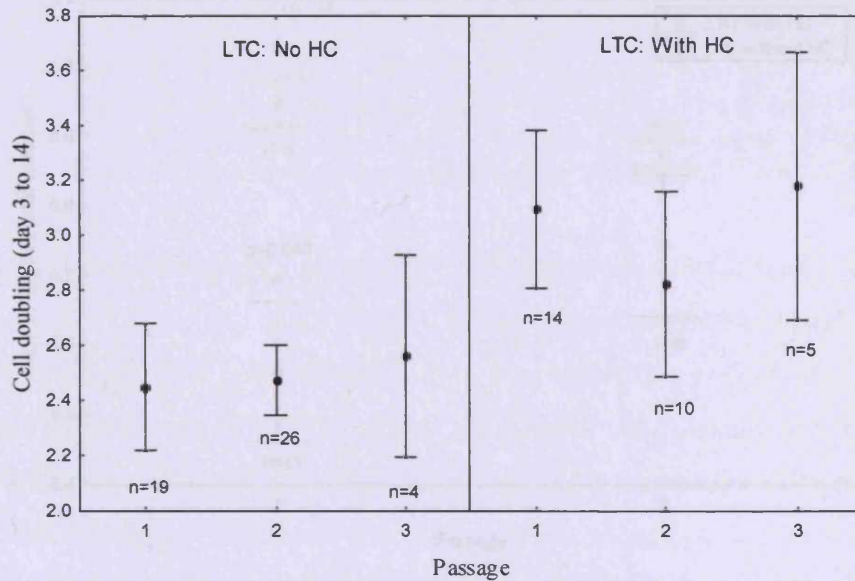
Figure 3.21. Passaged stroma: BMdSC doubling at passages 1, 2 and 3 in Alpha MEM derived from LTCs grown with (right) and without HC (left). Mean ± SE

c) CFU-F colony formation
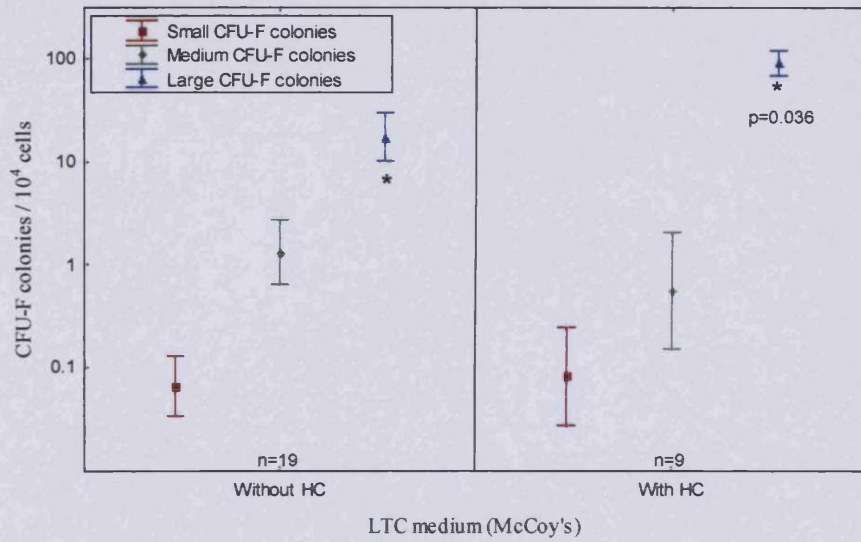
Figure 3.22. Passaged stroma: CFU-F formation in Alpha MEM. BMdSCs derived from LTCs grown with (right) or without (left) HC. Mean ± SE

BMdSCs (all passages), derived from LTCs grown with HC, had significantly more large CFU-F colonies (n=9, n=19; p=0.036) (figure 3.22). The passage 1 cultures had a greater proportion of large colonies in relation to total colonies when derived from LTCs grown with HC (n=4, n=11; p=0.043) (figure 3.23).
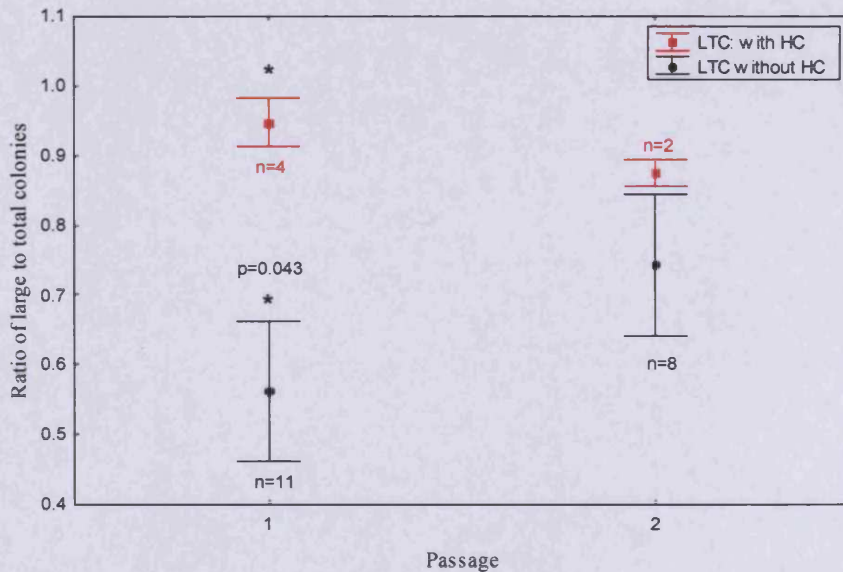
Figure 3.23. Passaged stroma: Ratio of large to total CFU-F colonies in Alpha MEM as a function of passage. BMdSCs derived from LTCs grown with (red) and without (black) HC. Mean ± SE

d) **Summary**

More primitive cells were recovered from LTCs grown in the presence of HC with passage 1 cultures demonstrating greater growth and CFU-F colony formation in Alpha MEM. This effect, however, was not shown beyond passage 1.

3.4.6. Culture growth kinetics of passaged BMdSCs

Cell doubling was assessed at various times during 17 days of passaged stromal cell growth in Alpha MEM (1 - 3 days, 3 - 7, 7 - 10, 10 - 14 and 14 - 17 days). This was then plotted in parallel with BMdSC growth (cell counts) in Alpha MEM.
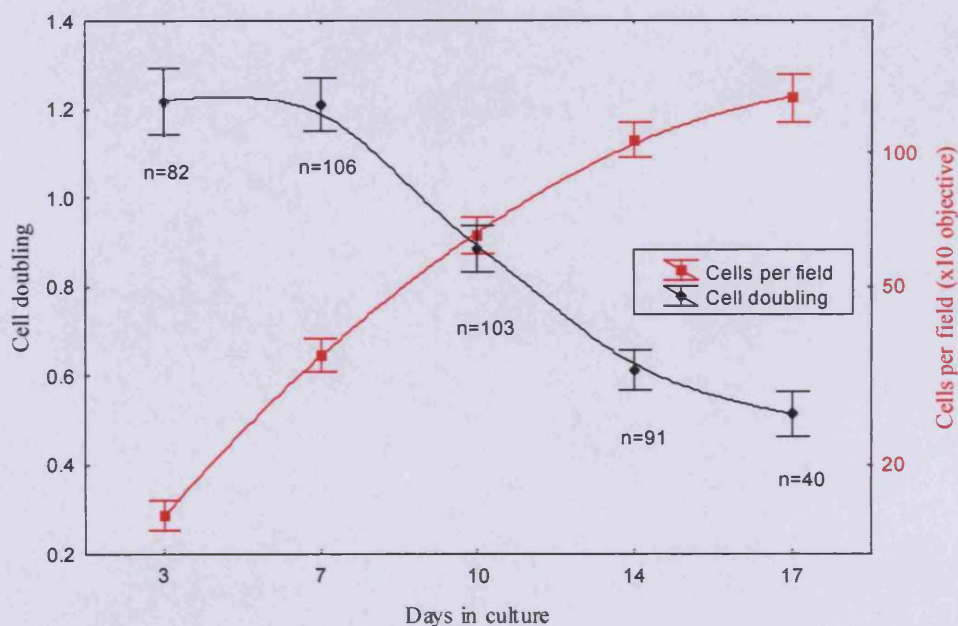
Figure 3.24. Passaged stroma: Cell doubling from 1 to 3 days (3), 3 to 7 days (7), 7 to 10 days (10), 10 to 14 days (14) and 14 to 17 days (17) (black circle) in Alpha MEM. Cell counts per field at 3, 7, 10, 14 and 17 days during culture (red square). Mean ± SE

Cell doubling plateaued for about 7 days before declining as the level of confluence increased (figure 3.24). Days 1 - 3 (n=82) and 3 - 7 (n=106) had a significantly higher cell doubling than days 7 - 10 (n=103; p=0.011; p=0.02 respectively), 10 - 14 (n=103; p=0.000001) and 14 - 17 (n=40; p=0.000009). By the 10th day in culture, the BMdSCs had reached approximately 50 cells per field and the cell doubling had reduced significantly. This indicated that increasing confluence made conditions for growth less favourable and suggested that the best time to subculture BMdSCs was within the first 7 days. These cultures were maintained for a minimum of 14 days to assess CFU-F formation or were grown to sub-confluence / confluence for bone or lipid induction respectively.

As a function of passage, cell doubling (determined from cell counts taken at 3 and 14 days)

remained constant for three passages (n=42, n=50 & n=22 respectively) and declined during passages 4 (n=3; p=0.009) and 5 (n=3; p=0.00008) (figure 3.25).
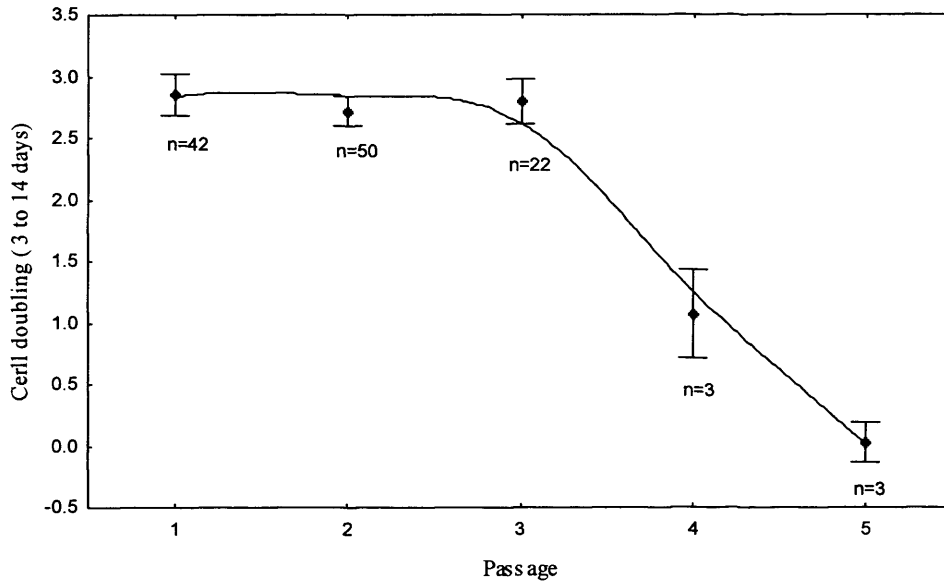
Figure 3.25. Passaged stroma: Cell doubling (days 3 to 14) as a function of passage in cultures consistently passaged at 14 to 17 days. Mean ± SE

In a parallel investigation, cultures were consistently passaged by day 10 before reaching subconfluence. Reducing the passage time and preventing the cells from reaching sub-confluence extended the growth to passage 10 with no loss in cell doubling by passage 4 (figure 3.26).
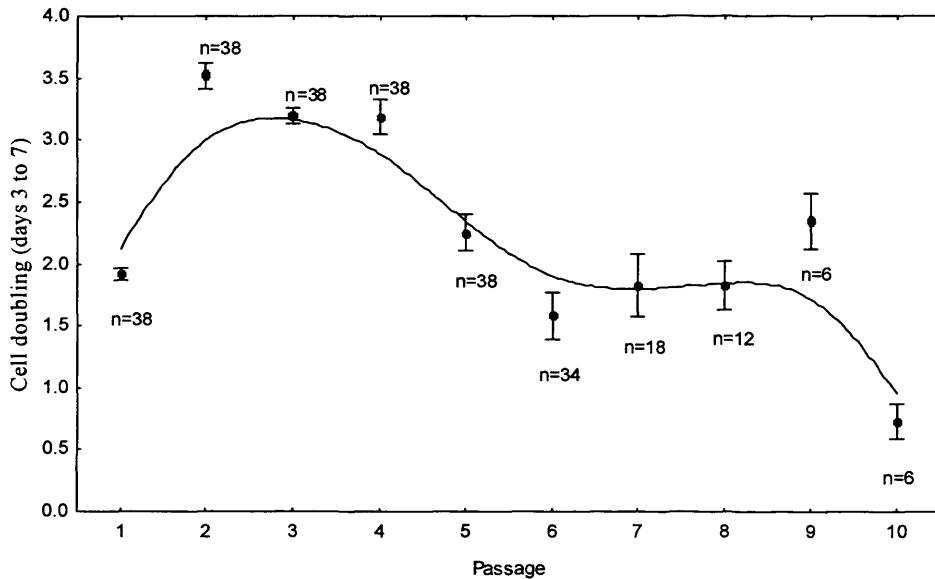
Figure 3.26. Passaged stroma: Cell doubling (days 3 to 7) in Alpha MEM as a function of passage in cultures consistently detached at 7 days. Mean ± SE

The cell doubling at passage 1 was significantly reduced (n=38; p=0.000001), indicating a lag growth phase whilst the BMdSCs adjusted to their new environment. This was not apparent

when detaching cultures at 14 to 17 days, suggesting that BMdSCs needed longer in passage 1 to adjust to their environment. Post passage 4, the cell doubling significantly declined (p=0.00013), representing a loss in the proliferative potential of the progenitor cell population.

The cumulative doublings were compared between cultures consistently detached before, or after 10 days in culture (figure 3.27).
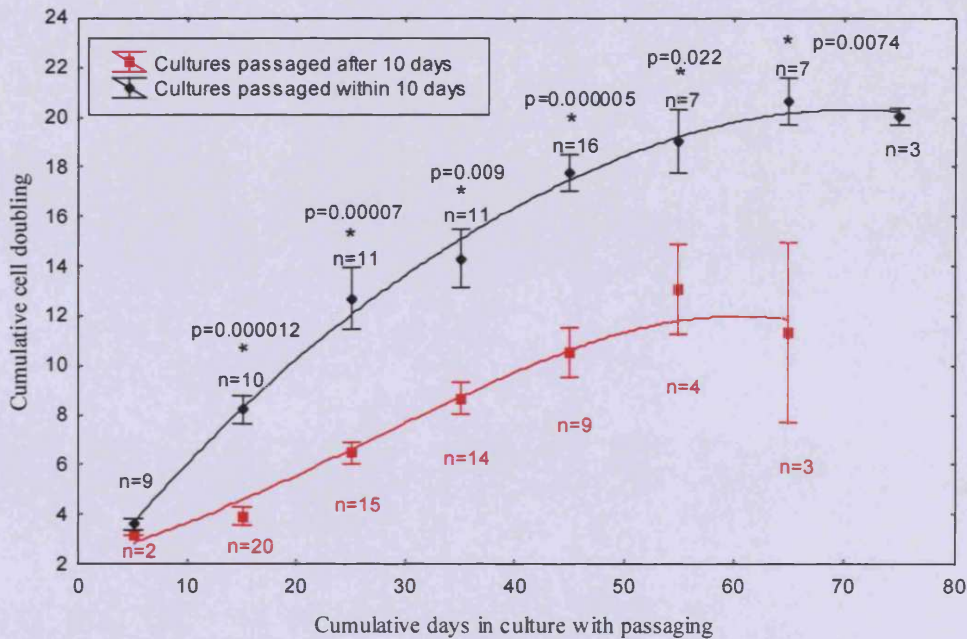
Figure 3.27. Cumulative cell doublings for BMdSCs consistently detached before (black circle) or after (red square) 10 days in Alpha MEM. Mean ± SE.

The cumulative doublings for cultures consistently passaged before 10 days (red) was significantly poorer (data shown on figure 3.27) with proliferation reaching a plateau at approximately 45 days (~12 doublings). The cumulative cell doublings for cultures detached within 10 days continued to increase until approximately 65 days in culture (~20 doublings). The cumulative cell doubling does not include cell doubling within the LTCs.

3.4.7. BMdSC growth as a function of passage

Cell growth and CFU-F colony formation in Alpha MEM were determined over 14 to 17 days as a function of passage.

a) **Growth**
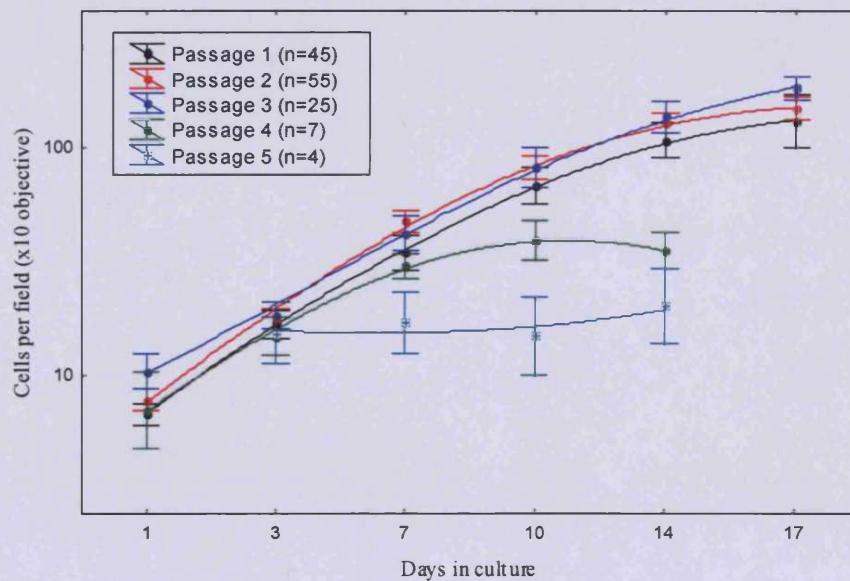
Figure 3.28. Passaged BMdSC growth in Alpha MEM as a function of passage. Mean ± SE

No significant differences were observed between passages 1 to 3 in Alpha MEM. By passage 4 most cultures were failing to reach confluence, with significantly fewer cell counts at day 10 (n=7, n=55; p=0.042) and day 14 (p=0.0023) in comparison to passage 2 and 3 (p=0.006). Passage 5 (n=4) showed no growth with significantly poorer cell counts from day 7 (p=<0.032).

b) **CFU-F colony formation**
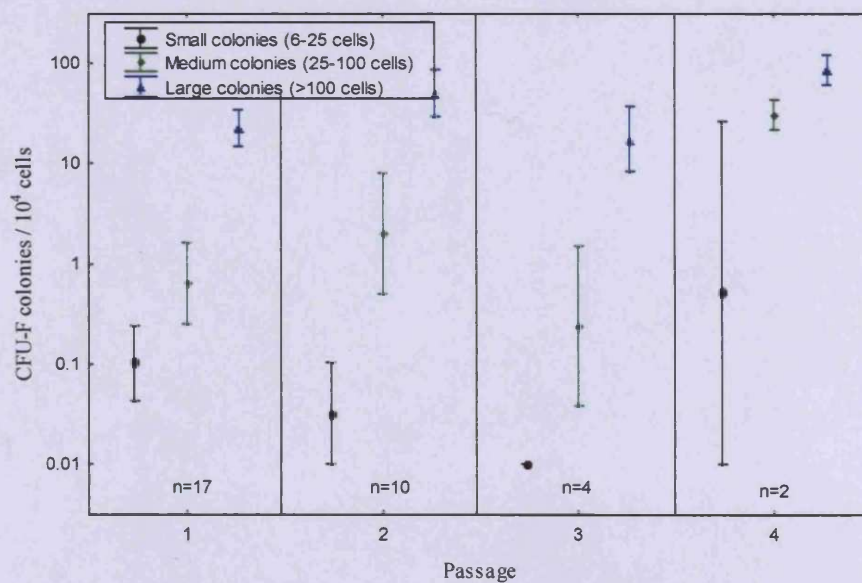
Figure 3.29. Passaged stroma: CFU-F colony formation in Alpha MEM as a function of passage. Mean ± SE

There were no significant differences in CFU-F size between passages 1 to 4 (figure 3.29) or between the proportions of large to total colonies (not shown), however, by passage 4 only two cultures formed CFU-F colonies, so the results may be falsely high. No CFU-F colonies were formed within passage 5. Table 3.5 shows the number of progenitor cells within the original sample (LTC CFU-F) and the number recovered from the LTC (passaged stroma).

Table 3.5. Number of CFU-Fs within the LTC (HC-containing media) and passaged cultures (Alpha MEM)

Culture	Primitive CFU-F (large colonies) (per 10^4 cells)	Total CFU-F (per 10^4 cells)
LTC	0.055	0.16
Passaged stroma (Averaged P1 - 4)	28	49

There were a very low number of proliferating progenitor cells within the LTC. The higher number of CFU-Fs within the passaged cultures suggested that many of the stem / progenitor cells remained quiescent within the LTC and / or that the progeny from the LTC CFU-Fs became colony forming cells (CFU-Fs) within the passaged cultures.

c) Summary

When consistently detaching the passaged cultures at 14 to 17 days and allowing the cells to become confluent, the long-term proliferative potential was reduced. However, by increasing the frequency at which the cultures were passaged, the growth rate could be maintained for up to 10 passages with comparable growth rates for passages 2 to 4. No significant differences in passaged BMdSC growth or CFU-F colony formation were observed between age groups (18-28, 19-38 and 39-49 years) or between male and female donors as a function of passage (not shown).

3.5. BMdSC Characterisation

From the FSC (forward scatter) by SSC (side scatter) plot (figure 3.30) three potential sub-populations of cells (R1, R2 and R3) were identified.
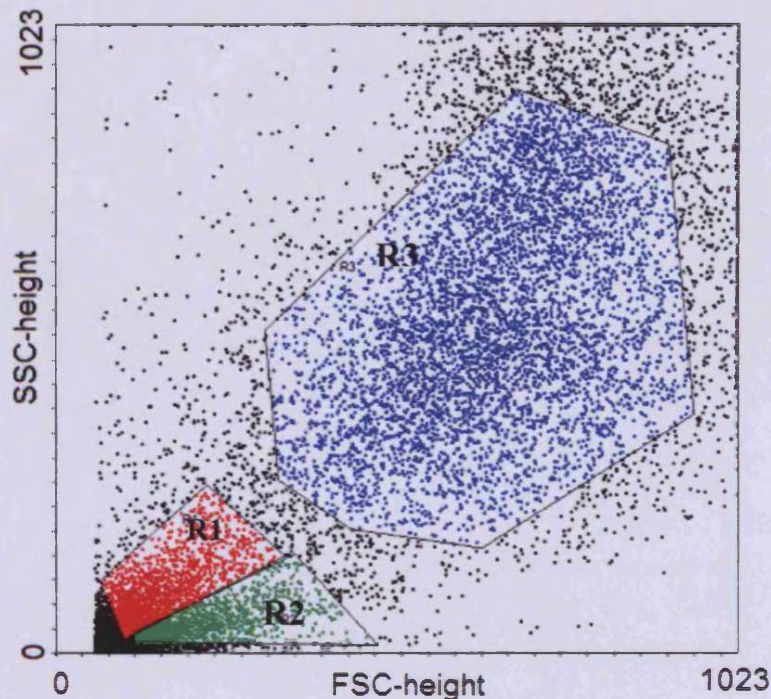
Figure 3.30. Forward versus side scatter plot with gated sub-populations: R1 (red), R2 (green) and R3 (blue)

R1 (red) & R2 (green) had low FSC and SSC parameters, whilst R3 (blue) had a much higher FSC and SSC. It was hypothesised that the larger granular R3 population encompassed the more mature or differentiated cell types, whilst the smaller R1 and R2 populations included less granular and agranular cells respectively and represented the more immature progenitor-like cells (Javazon et al. 2001). The R1 and R2 populations were also likely to contain a large amount of debris material. To analyse this hypothesis the cell cycle within each population was assessed using DRAQ5 to measure DNA content (2.5.3. 'Cell cycle'). Cell cycle analysis was determined using WinMDI 2.8 and Cylchred.

3.5.1. Cell cycle analysis

The results for each cell population were similar for passage 1 to 3. The following results were determined from passage 2.

a) **The R1 population**

In reference to figure 2.2 the R1 cell population had a large sub G0/1 peak with only a small G1 peak (figure 3.31) and a tiny G2/M peak, but S phase was not apparent. Figure 3.32 (FL3-width v area) identified the different sub-populations of cells according to their position within the cell cycle (R4 showing the senescent/apoptosing cells and debris, R5 showing the cells in the G1 phase of the cell cycle, and R6 showing the cells in the G2/M phase) and excluded doublets from the analysis. The cells in S phase, identified between R5 and R6, were not clear in the R1 population.

Figure 3.31 is back-gated from figure 3.32 to exclude any doublets found on the right of figure 3.32. Doublets are clumped cells that are analysed as single cells, two cells in G1 having the same amount of DNA as one cell in G2/M, giving a false impression of cycling cells.
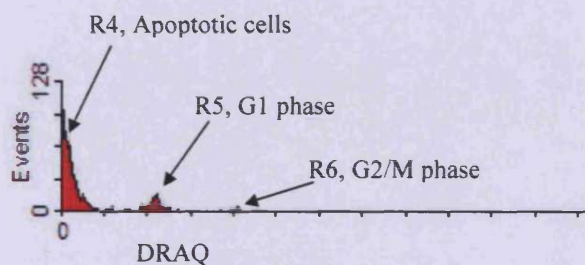
Figure 3.31. Cell cycle analysis of the R1 sub-population with a large sub G0/1 peak, small G1 peak and tiny G2/M peak.
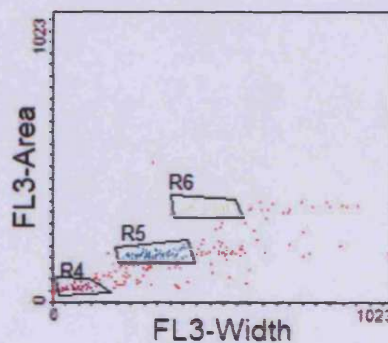
Figure 3.32. FL3 – width v area: identified cells in sub G0/1 (R4), G1 (R5) and G2/M (R6)

A large sub G0/1 peak indicated that the R1 population mainly consisted of debris (fragmenting / budding cells), but the G1 and G2/M phases of the cell cycle showed the presence of a small population of actively dividing cells, providing that all doublets were excluded.

b) **The R2 population**

The R2 population of cells had a very poor cell cycle with only a tiny G1 peak and a very large sub G0/1 peak (figure 3.33).
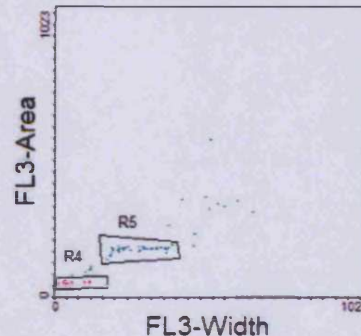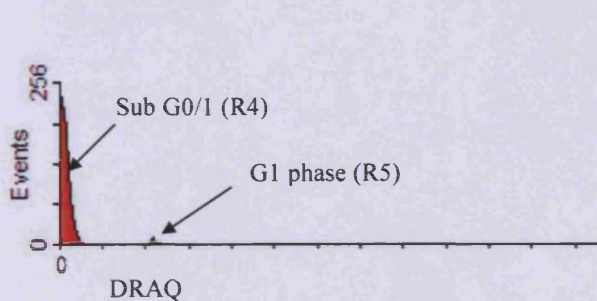
Figure 3.33. Cell cycle analysis of the R2 population   **Figure 3.34.** FL3 – width v area
with a large sub G0/1 peak and a tiny G1 peak

This cell population appeared to be largely composed of debris material. There was a tiny G1 peak, but it was often difficult to clearly isolate R1 from R2 so there may have been an overlapping population of cells.

c) **The R3 population**

The R3 sub-population was very different from the R1 and R2 sub-populations. The cells were larger (larger FSC) and more refractive (larger SSC). Without back gating from the FL3-width vs area plot, the individual phases of the cell cycle were not clear. The initial peak in figures 3.35 & 3.36 was likely to be the sub G0/1 peak, but this occurred further along the scale in comparison to the sub G0/1 peak of the R1 and R2 sub-populations.   The second peak was wide and may represent a very active population of cycling cells (G1 phase merging into S phase and G2/M).
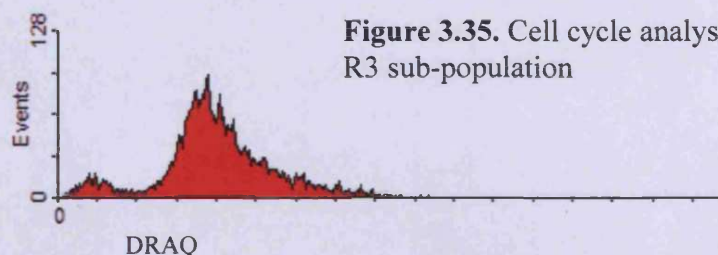
Figure 3.35. Cell cycle analysis for the R3 sub-population

When back gating from the FL3-width vs area plot, the individual phases became clearer.

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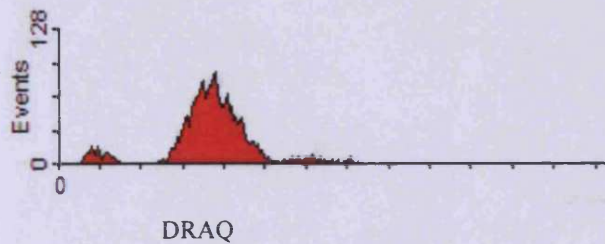
Figure 3.36. Cell cycle analysis of the R3 sub-population showing active proliferation and a small sub G0/1 peak
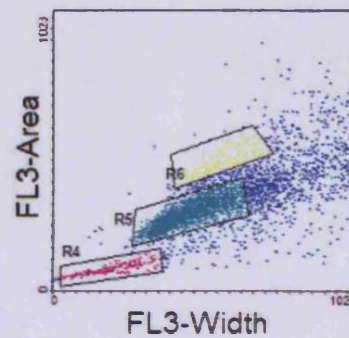
Figure 3.37. FL3 – width v area

R3 was the most active sub-population and involved much less debris material, as identified by the smaller G0/1 peak. Flow sorting also backed up this conclusion. The BMdSCs in suspension were sorted into two populations (R1-R2 & R3) according to their FSC, SSC parameters and placed into flasks at low cell concentrations ($<1 \times 10^4$ cells). This was repeated several times but the only sorted population to lead to the formation of CFU-F colonies was R3 (not shown).

These results are in accordance with Colter (Colter et al. 2000) who identified a large granular population (represented by our R3) and a much smaller agranular population (represented by our R2). He demonstrated that 98% of the cells in both populations were viable but showed with the cell-cycle specific antigen K_i-67 that the smaller agranular population was not in the cell cycle unlike the large granular population when inoculated at high seeding densities. However, when inoculating cultures at lower seeding densities, a new more granular (represented by our R1) population appeared that was found to be actively cycling. This indicated that plating at lower inoculums promoted expansion of the progenitor population, which would be shown by a dwindling R1 sub-population and an increasing R3 sub-population through culture. If the R1 and R2 sub-populations contained progenitor-like cells, which expanded at low cell densities as Colter suggested, larger CFU-F colonies would be expected to grow when the flow sorted R1 / R2 cells were plated at low cell densities. We attempted to prove this by flow sorting the BMdSCs into two populations (R1-R2 & R3) according to their FSC, SSC parameters and inoculating them into flasks at low cell concentrations ($<1 \times 10^4$ cells). This was repeated several times but the only sorted population to lead to the formation of CFU-F colonies was R3 (not shown). It may be that the concentration of viable cells

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amongst the debris material within the R1 and R2 populations were very small, or that the cells present within these populations were fragile and breaking up during the sorting process. This would suggest that the larger more refractive cells in the R3 population were tougher and resisted the stresses the flow sorter subjected them to.

3.5.2. Cell markers

Having assessed the cell cycle within each gate, R3 was determined to be the most viable, active cell population, therefore, antigen expression was only analysed in R3. Cultures were detached at sub-confluence (2.5.2.b. BMdSC characterisation by flow cytometry).

a) Quantitative antigenic expression

For comparative purposes, the level of antigenic expression was quantitated from the G-mean ratio of the test (red) over the isotype control (black outline).

i. Adhesion molecules

Antibody	G-mean Ratio
90	2.80
44	2.43
105	1.92
166	1.91
49b	1.49
106	1.34
11c	1.09
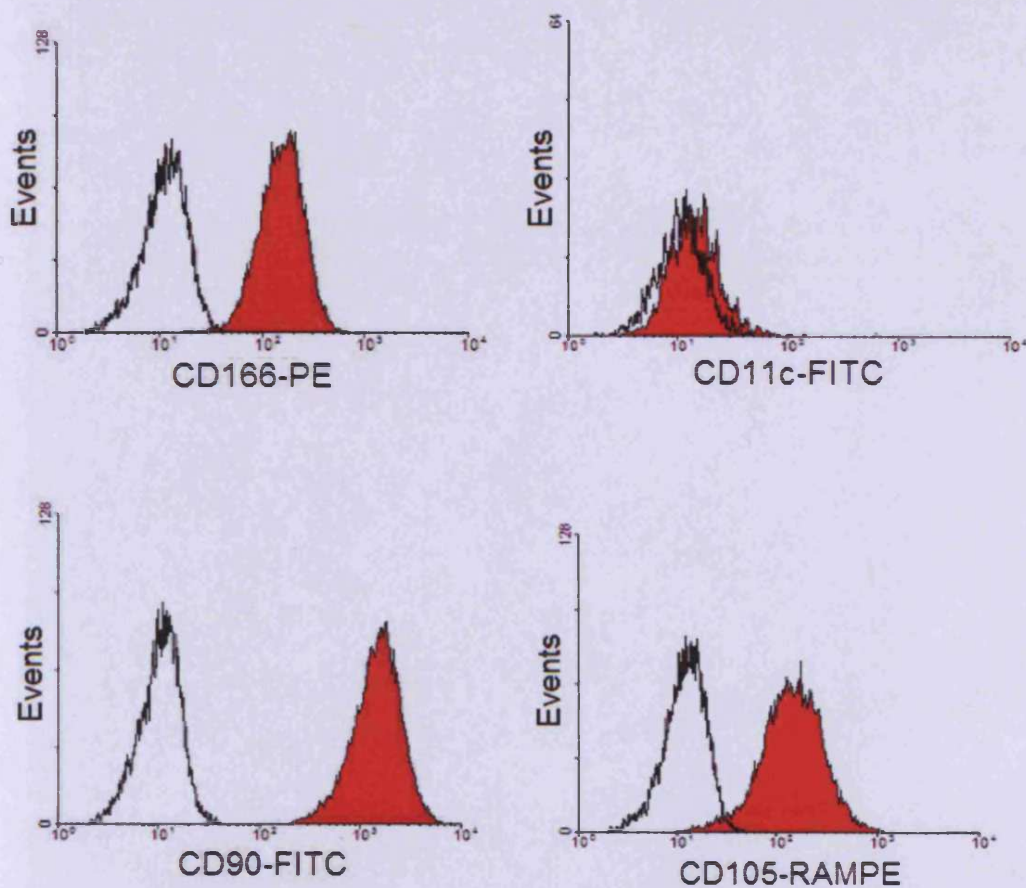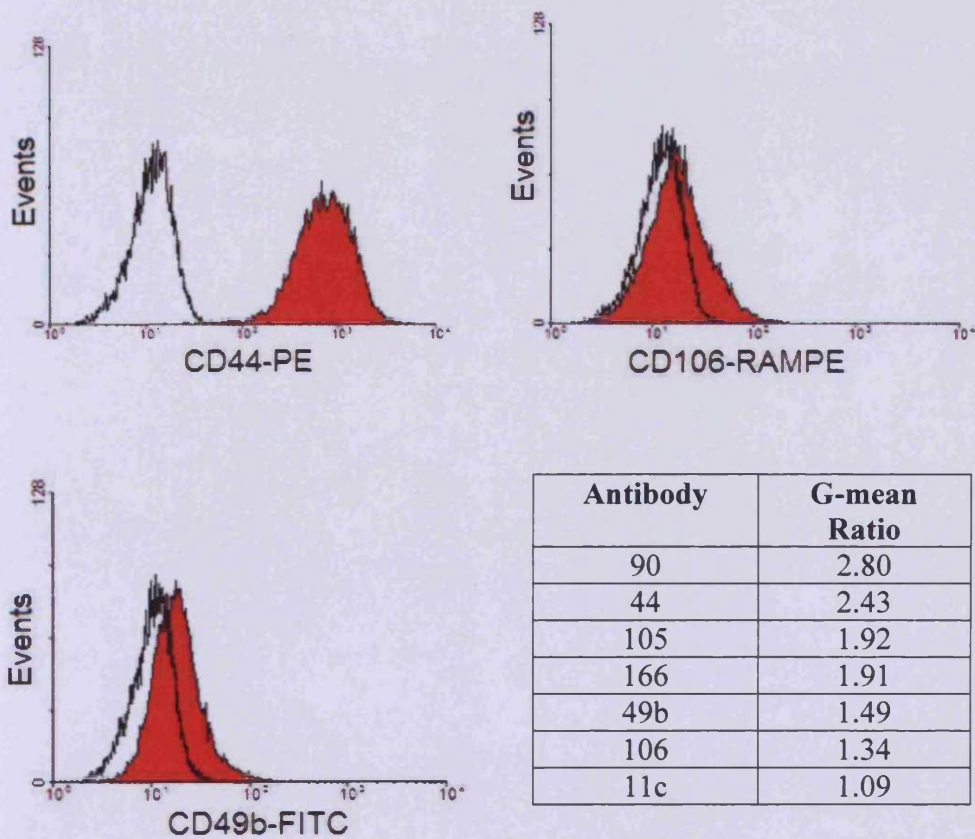
Table 3.6. G-mean ratios (test / isotype control)

ii. **Stem cell markers**

Antibody	G-mean ratio
117	1.11
133	1.04
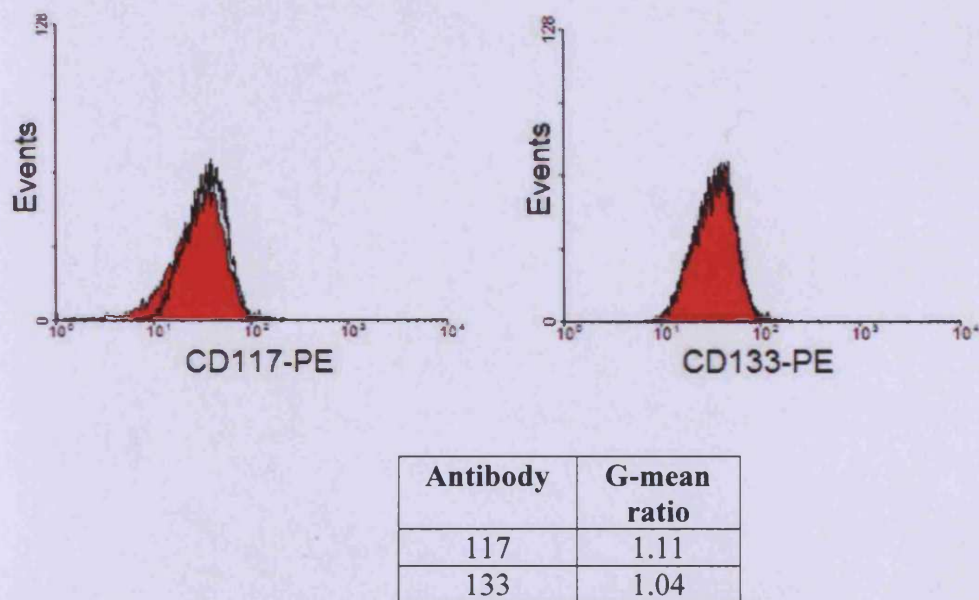
Table 3.7. G-mean ratios (test / isotype control)

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The BMdSCs were negative for stem cell markers CD133 and c-Kit (117), a stem cell factor receptor.

iii. **Haemopoietic markers**

CD45-PerCP

CD34-APC

CD64-PE
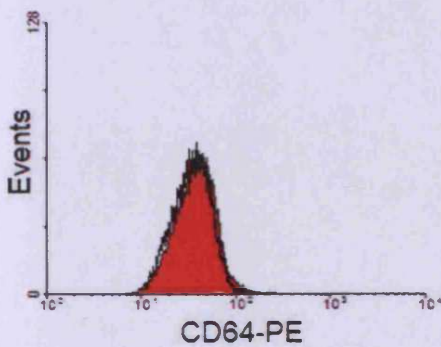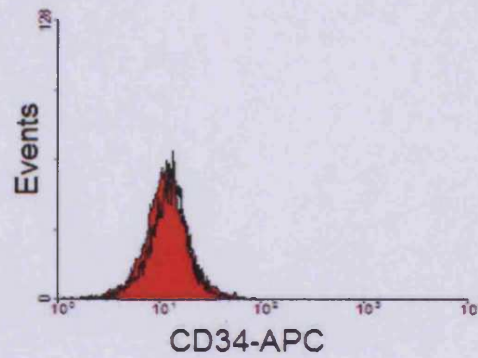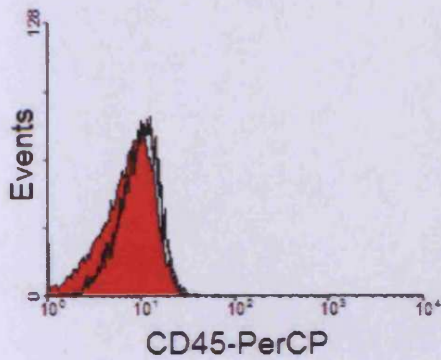
Antibody	G-mean Ratio
34	1.11
64	0.93
45	0.89

Table 3.8. G-mean ratios (test / isotype control)

The BMdSCs were negative for the haemopoietic markers CD64 (monocyte / macrophages), CD34 (stem / progenitor cells), and CD45 (receptor tyrosine phosphatase).

The ratios were used to compare the antigenic expression of normal BMdSCs. A ratio below 1.2 was considered negative.
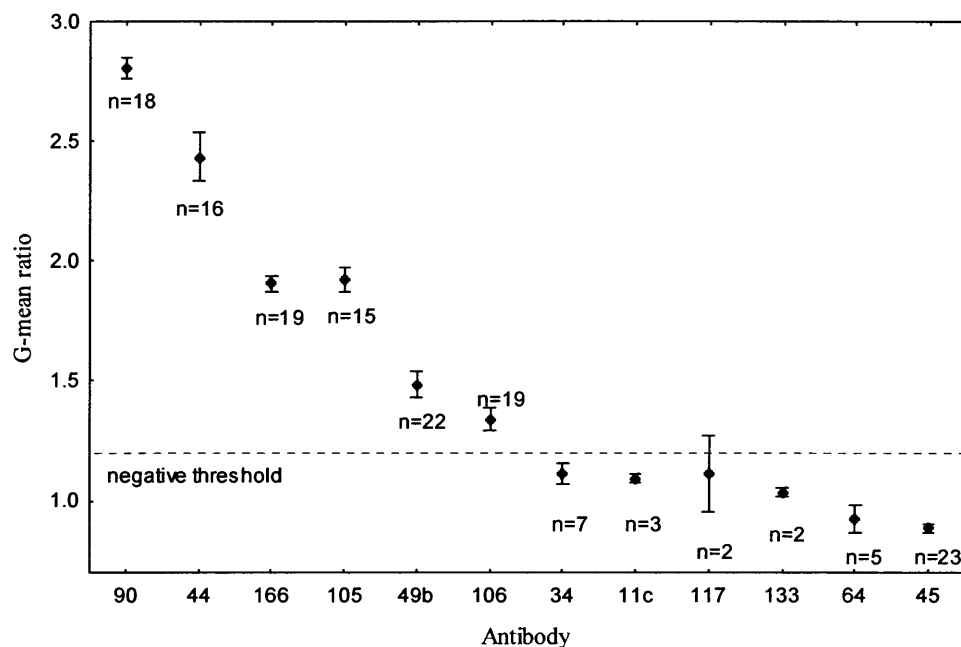
Figure 3.38. Antigenic expression of BMdSCs grown in Alpha MEM. Mean ± SE

The BMdSCs were strongly positive for CD90 (Thy-1), CD44 (homing-associated cell adhesion molecule), CD166 (activated leukocyte cell adhesion molecule) and CD105 (component of the receptor complex for TGF-β). They were weakly positive for adhesion molecules CD49b (integrin α subunit that forms a heterodimers with β1 integrin) and CD106 (vascular cell adhesion molecule, otherwise known as STRO-1), and negative for some adhesion molecules such as 11c (β integrin receptor), the haemopoietic markers, CD34, CD64 and CD45, and the stem cell markers CD117 and CD133.

Expression varies in the literature depending on the BMdSC source and nature of the cell layer, but the results from this investigation are largely in accordance with other published data (table 3.9). Our aim was to quantitate the levels of antigenic expression on BMdSCs for comparative purposes, but the literature generally reports expression as positive (+), negative (-) or weakly positive (+/-).

Table 3.9. Ratios of antigenic expression from this investigation in comparison to the literature

Antibody	Ratio	Detection	(Deans & Moseley 2000)	(Devine 2002)	(Horwitz 2001)	(Vogel et al 2003)	(in 't Anker et al. 2003)
CD90	2.8	+	+	+ / -	+	+	+
CD44	2.43	+	+	+	+		+
CD105	1.92	+	+	+	+	+	+
CD166	1.91	+	+	+			+
CD49b	1.49	+	+	+			
CD106	1.34	+	+	+	+ / -		-
CD34	1.11	-	-	-	-	-	
CD117	1.11	-		+ / -		-	
CD11c	1.09	-	-	-	-	-	
CD133	1.04	-		-		-	
CD45	0.89	-	-	-	-	-	-

b) **Antigenic expression as a function of passage**

Antigenic expression (CD45, CD49b, CD166, CD90, CD44, CD105 and CD106) was determined as a function of passage to establish any variation in expression during culture.
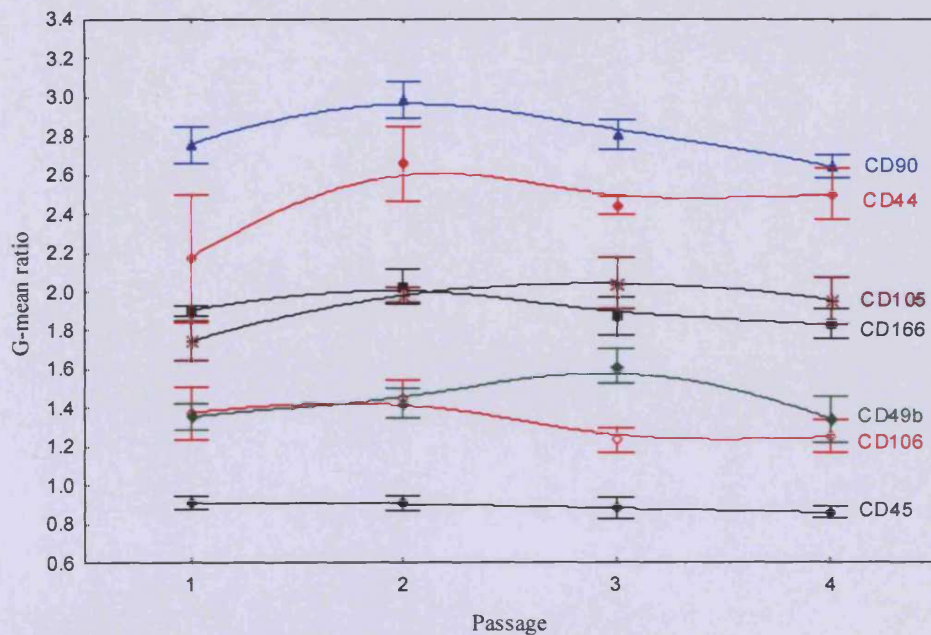
Figure 3.39. Antigenic expression as a function of passage. Mean ± SE

Table 3.10. Number of BMdSC cultures analysed at each passage for each antibody

Antibody	Passage 1	Passage 2	Passage 3	Passage 4
CD90	6	4 p=0.022	4	4 p=0.022
CD44	4	3	3	4
CD166	6	4	4	4
CD105	5	5	3	2
CD49b	6 p=0.047	7	5 p=0.047	4
CD106	5	6	4	4
CD45	6	8	5	3

No significant differences were observed between passages with the exception of CD90, which had a significant decline in expression from passage 2 to 4 (n=4; p=0.022), and CD49b, which had a significant increase in expression from passage 1 to 3 (n=6, n=5; p=0.047) (table 3.10). There was no significant change in CD166 expression, which was reported to increase in culture expanded BMdSCs (Stewart et al 2003), and a slight increase in CD44 (not significant), which may become upregulated during culture for adhesion. Wexler (Wexler et al 2003) reported a stable expression of CD44, CD90 and CD45 through passaged culture.

3.5.3. **Cytogenetics**

Cytogenetic analysis, using standard G-banded analysis, as defined according to the International System for Human Cytogenetic Nomenclature (ISCN 1995), was determined in normal passaged BMdSCs as a function of passage to establish if the BMdSC karyotype transformed during culture.

Table 3.11. Karyotype analysis of BMdSC cultures as a function of passage

Origin	Passage	BMdSC Cytogenetics	Cells analysed
YH	1	46XX: add(16)(q22)	Found in 6 / 33 cells
	2	46XX: add(16)(q22)	Found in 4 / 20 cells
AAF	1	46XX: 16qh+	17
	2	46XX: 16qh+	10
	3	46XX: 16qh+	2
	4	46XX: 16qh+	1
B37	2	46XX: 16qh+	11
	3	46XX: 16qh+	20
887	1	46XY	20
	2	46XY	20
	3	46XY	15
ABI	1	46XX	10
AC5	2	46XX	15
AC7	1	46XY	10
880	2	46XX	20
B33	3	46XY	10

Table 3.11 outlines the karyotypes that were found in normal BMdSCs. The clinical significance of karyotype add(16)(q22) (discovered in 'YH') was unknown with no literature supporting these results. All cells examined in 'AAF' and 'B37' contained a large distinctive heterochromatic region on one copy of chromosome 16 and additional chromatin on other chromosomes. Heterochromatic variation is a known polymorphism, occurring in 1% of the population, and is not considered to be of clinical significance (Hsu et al. 1987). Unexpectedly the karyotype was found in 2 out of 9 normal samples (22%) during this investigation. The

abnormalities were found in consecutive passages with no signs of transformation during culture.

3.6. Time Lapse Photography

Time lapse photography was carried out during passaged culture to determine the migratory activities of BMdSCs and to follow through the actions of a cell during division (2.4.5. 'Time lapse').

3.6.1. Cell division

The following time lapse sequence was taken from a highly proliferative passage 2 culture, 3 days after plating, and shows the cell sizes, motility and time taken for division to occur (time shown at bottom right of each photo). The time sequence follows 2 labelled cells through the division process, but other cells can also be seen to divide. Not all cells are proliferating. A graticule slide with an engraved circle 2mm in diameter with cross-hairs was used to assess cell size and motility, from which a scale of 1mm was determined, shown on each photo. This time lapse movie sequence is provided on CD with this investigation, which can be viewed with windows media player or Adobe premier (2 frames per minute).

Photo 3.5. Cells labelled 1 and 2 are small, elongated spindle shaped cells with slightly refractile edges.
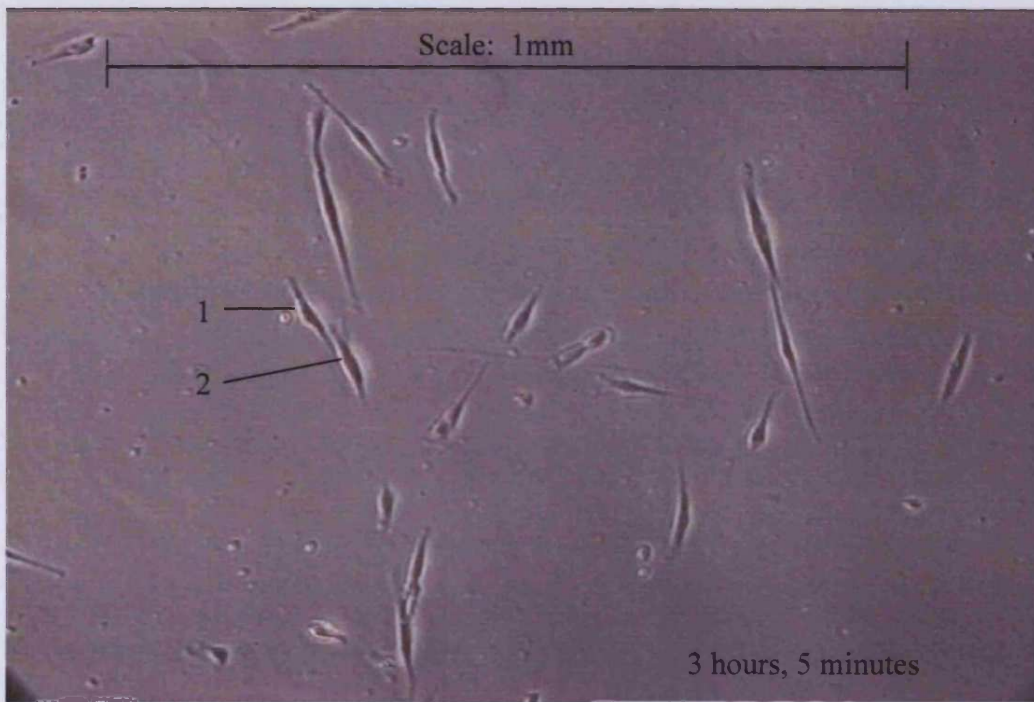
Photo 3.6. At 3 hours, 42 minutes, cell 1 contracted into a refractile sphere for division, remaining adhered to the flask surface by focal adhesion plaques.
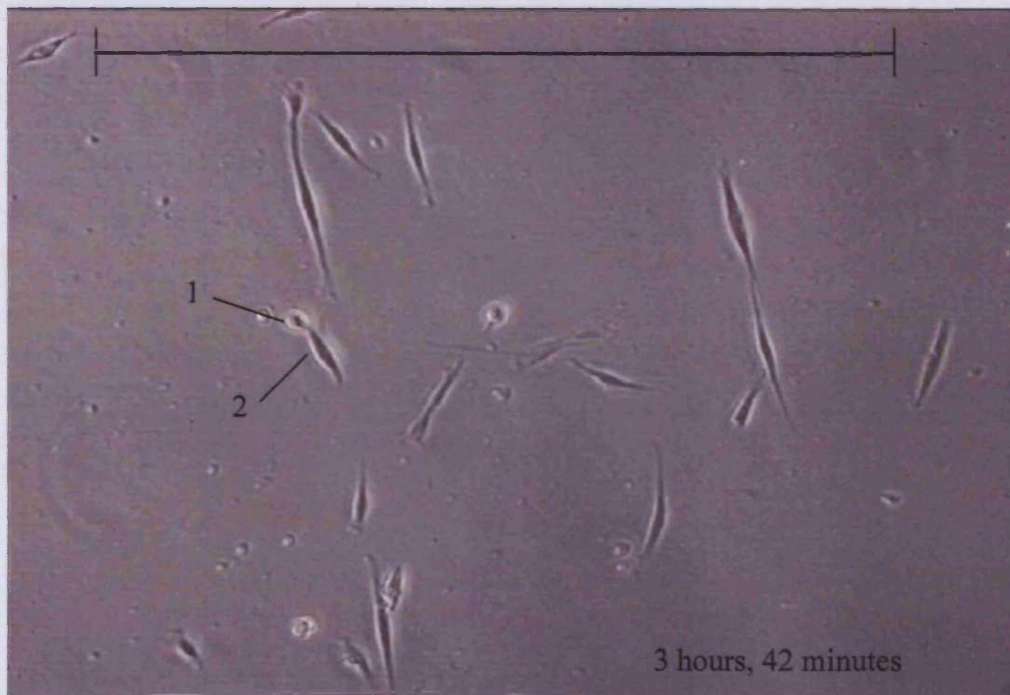
Photo 3.7. At 3 hours 56 minutes, cell 2 contracted into a sphere for division.

3 hours, 56 minutes

Photo 3.8. At 4 hours and 2 minutes, cell 1 divided into 1a and 1b.
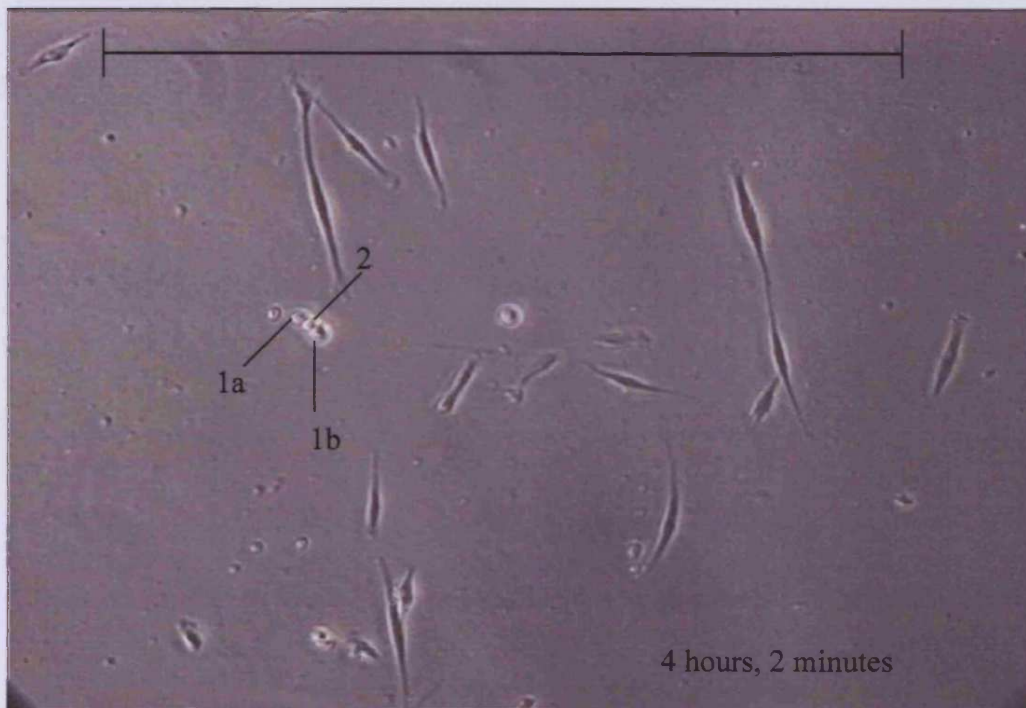
4 hours, 2 minutes

Photo 3.9. At 4 hours, 47 minutes, 1a and 1b lost their refractile properties and spread across flask to form elongated spindle-shaped cells. Cell 2 divided.

1a

2

1b

4 hours, 47 minutes
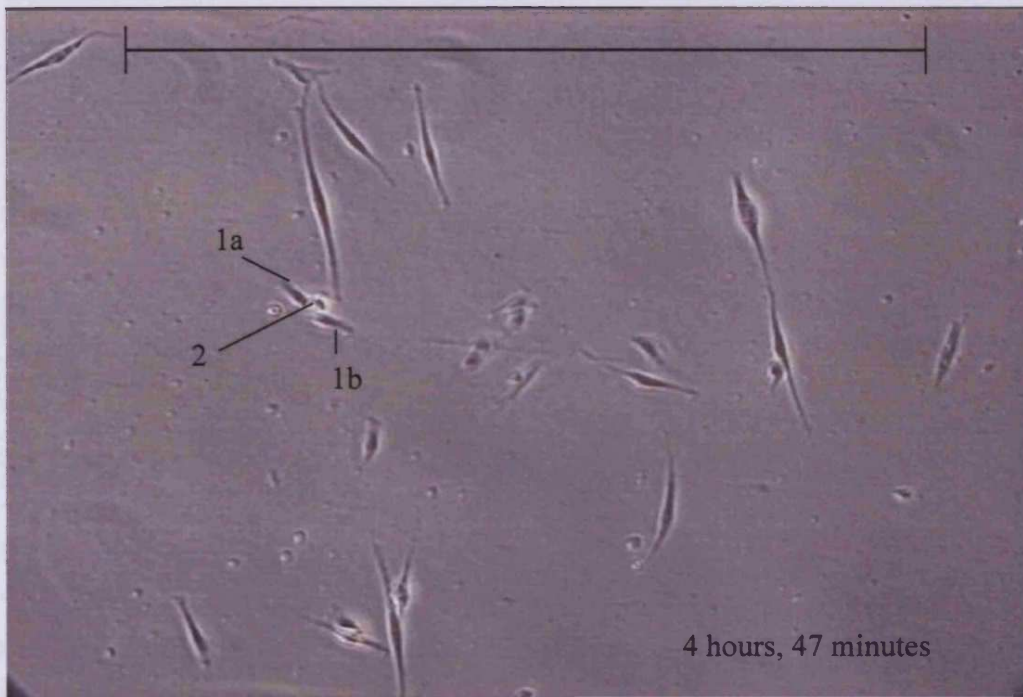
Photo 3.10. At 5 hours, 16 minutes, cells 1a and 1b migrated away, breaking contact from each other. Cell 2 began to spread across the flask surface.

1a

2

1b

5 hours, 16 minutes
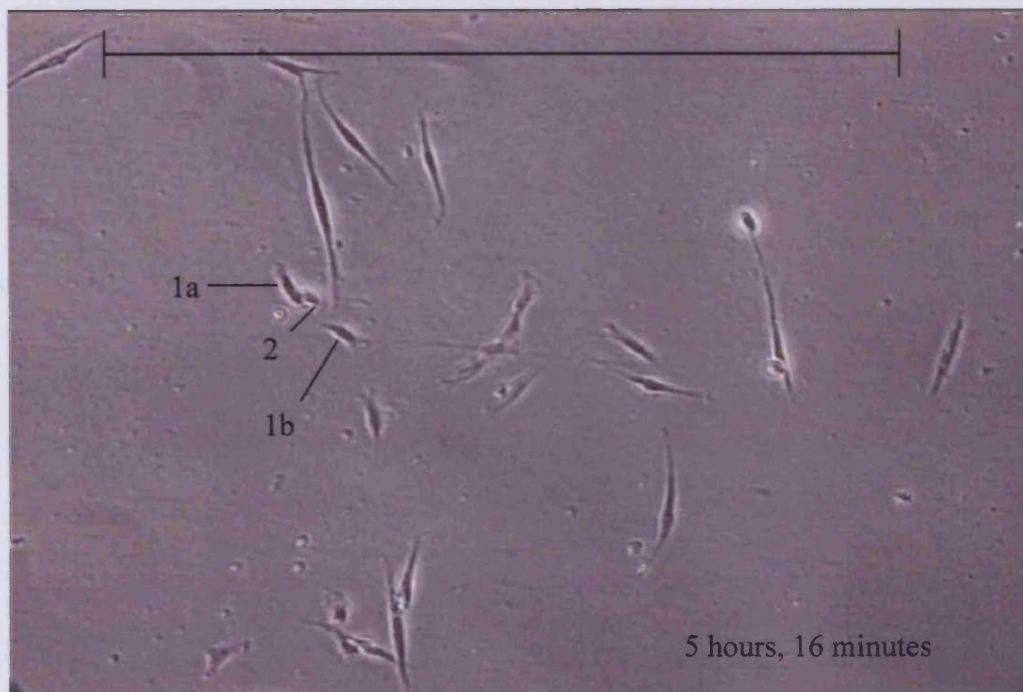
Photo 3.11. By 6 hours, 9 minutes, cells 2a and 2b had spread across the flask and migrated away from each other.
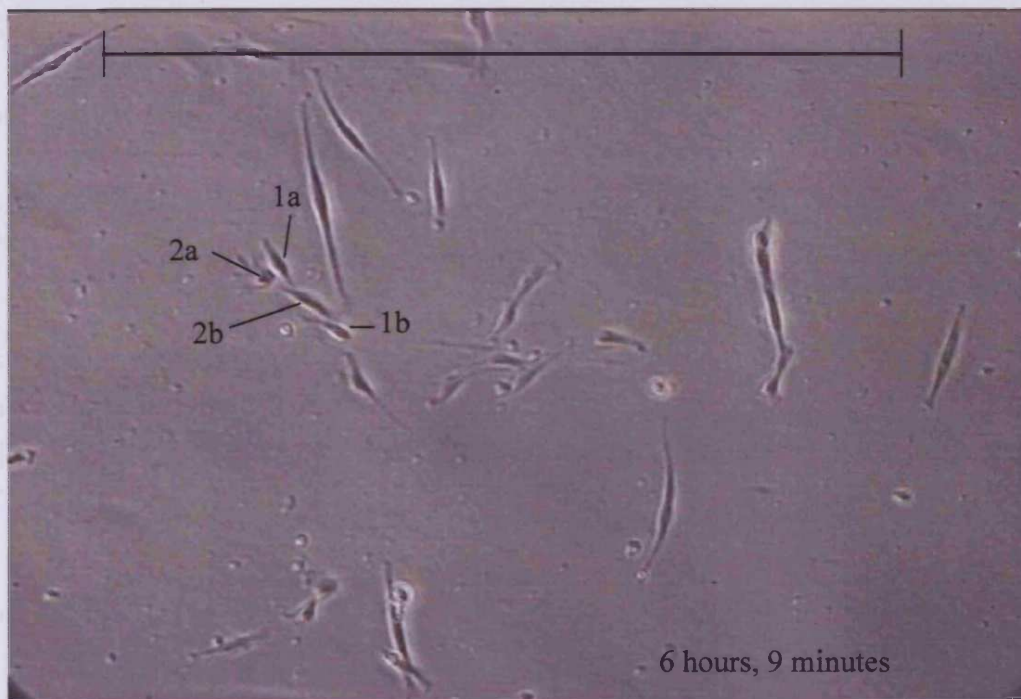
6 hours, 9 minutes

Photo 3.12. By 9 hours, 9 minutes, the cells have relinquished contact with each other and proceed to migrate across the flask, forming processes with other cells.

9 hours, 9 minutes
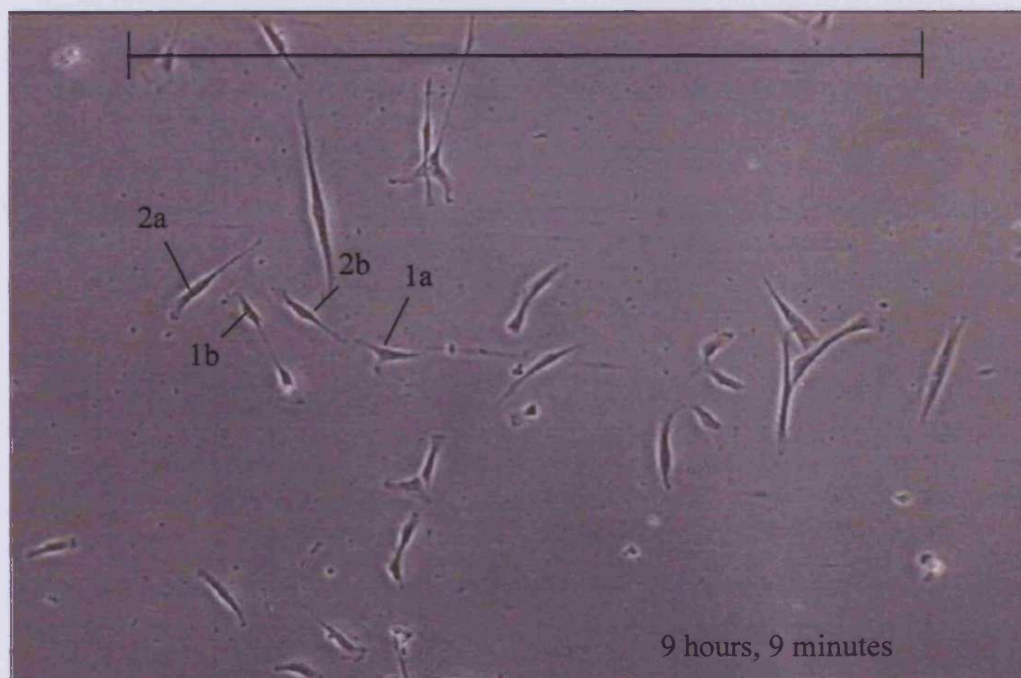
Photo 3.13. By 16 hours, 58 minutes, cell 1a and 2b have migrated east and attached to a cell, which undergoes mitosis.
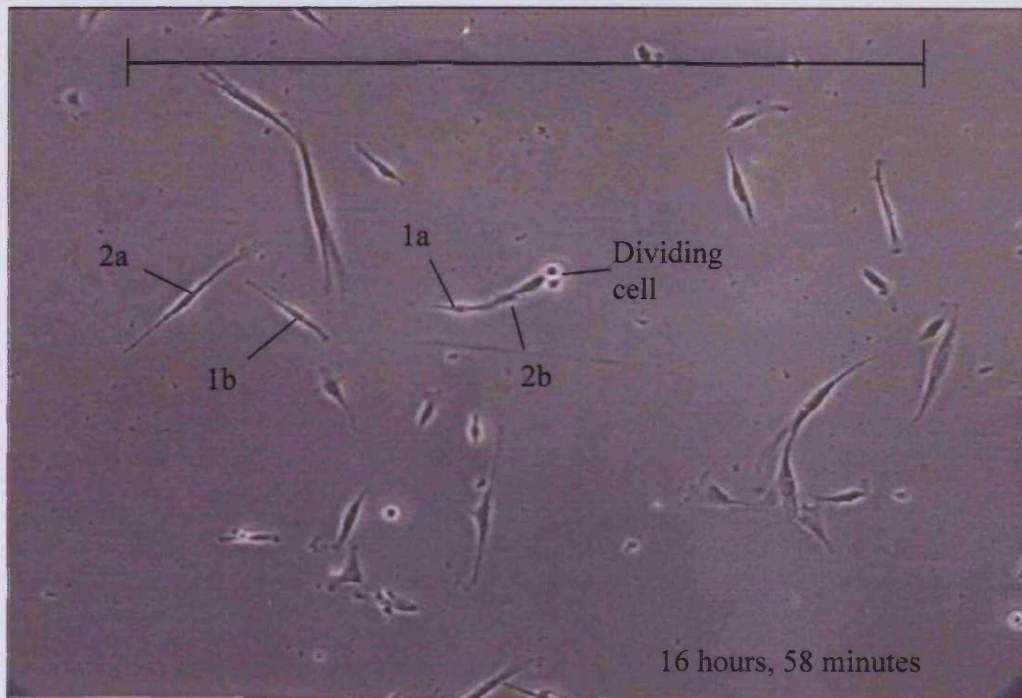
Photo 3.14. About 15 hours after cell 1 divided to produce a daughter cell, cell 1a contracts into a sphere for replication.
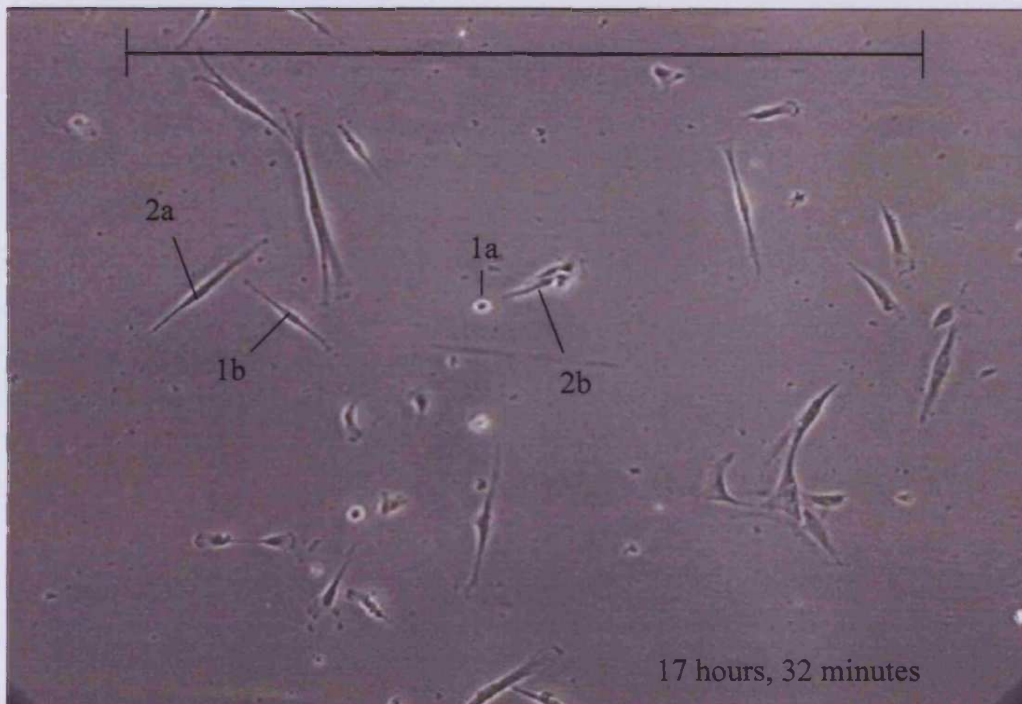
Photo 3.15. 30 minutes later, cell 1b contracts into a sphere for division as cell 1a divides to produce a new daughter cell.

18 hours
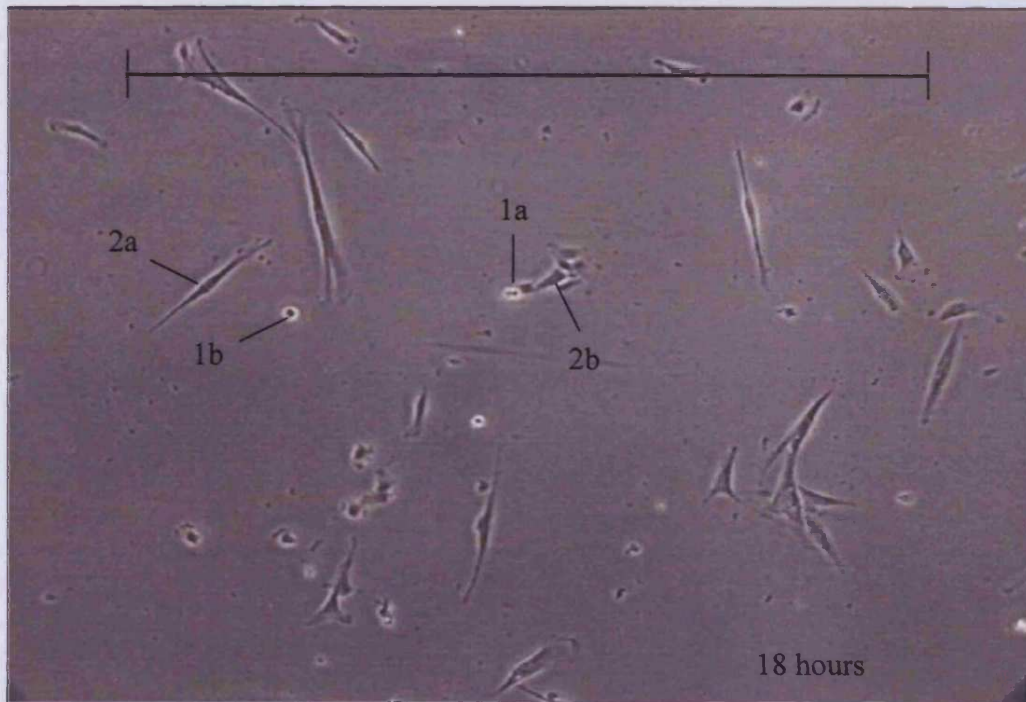
Photo 3.16. Both 1a and 1b divided to produce 2 new daughter cells. Cells 2a and 2b have not moved.

18 hours, 27 minutes

Photo 3.17. By 20 hours, 23 minutes, $1a_1$, $1a_2$, $1b_1$ and $1b_2$ have re-adhered, spread and migrated across the flask. Although 2b formed processes with other cells, it did not divide again within the time lapse period.
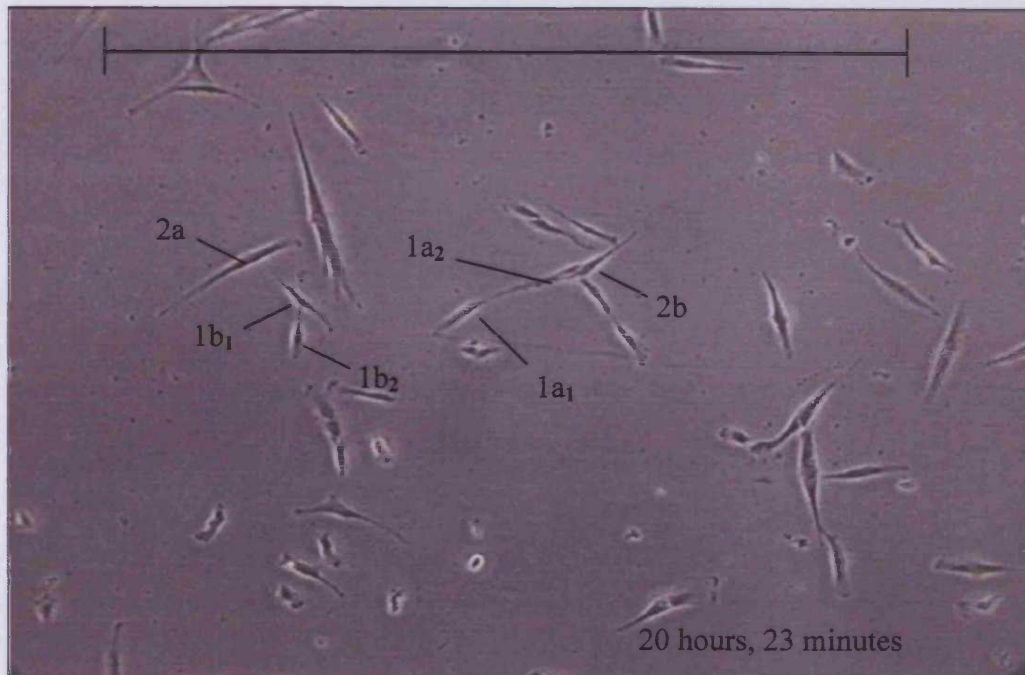
a) **Summary of events**

Table 3.12. Time sequence of events during time lapse photography

Time taken	Description of events	Photo
0	Adhered elongated cells with slightly refractile edges	3.5
51 minutes	Both cells (1 & 2) contract into spheres for division	3.7
57 minutes	Division occurs	3.8
1 hours, 42 minutes	Cells 1a and 1b readhere, spread and migrate	3.9
3 hours, 4 minutes	Cells 2a and 2b readhere, spread and migrate	3.11
15 hours, 22 minutes	Second division of 1a and 1b.	3.16

3.6.2. Migratory capacity of BMdSCs during culture

The time taken for a cell to travel 100 µm (flask surface) was measured and recorded during time lapse photography at 3, 7, 10 and 14 days during culture. After 14 days proliferative cultures were usually too confluent to measure cell movement. Cells were constantly changing shape making it difficult to follow the leading or trailing edge of the cell, therefore the nucleus was chosen as the focal point. Cells usually moved in a haphazard fashion, presumably in

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response to particular chemical signals so this technique only measured cells moving in a straight line in one direction.
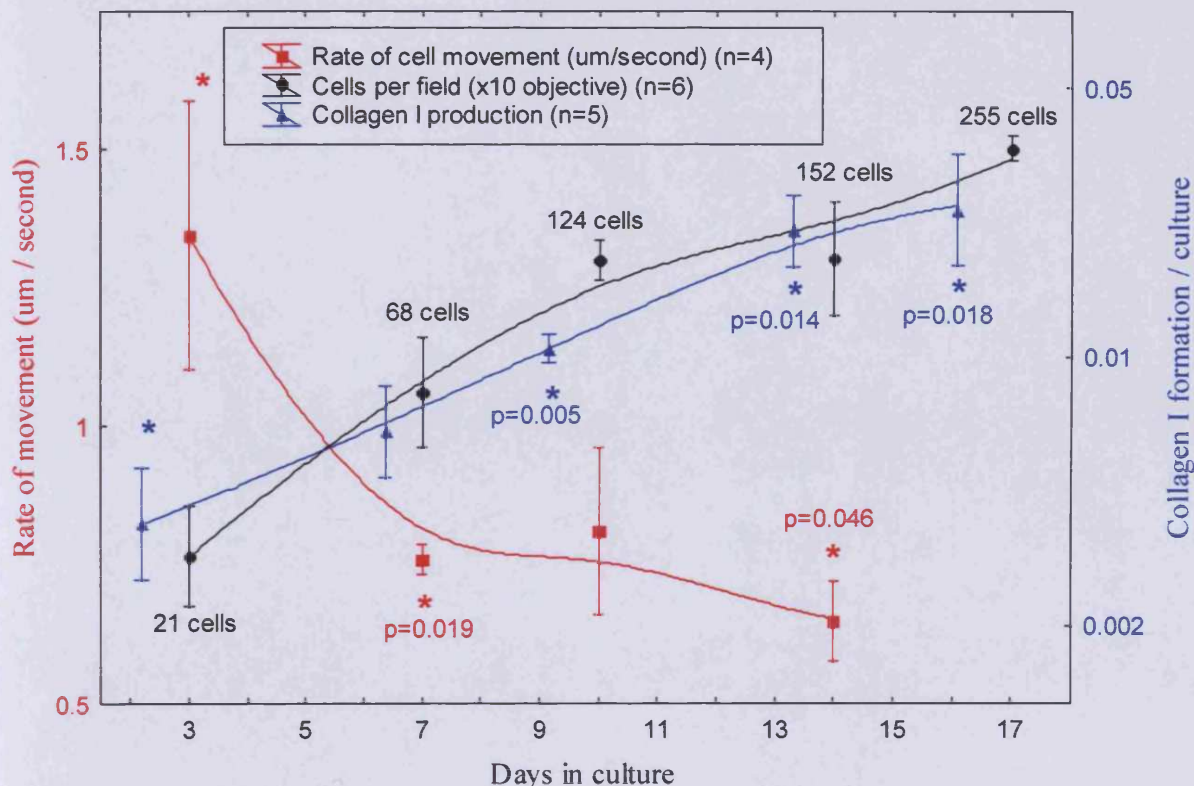
Figure 3.40. Rate of movement (red), collagen I production (blue) and cells per field (black) from 3 to 17 days in Alpha MEM. Mean ± SE

The cultures that were assessed by time lapse photography all grew to confluence within 17 days (approximately 255 cells per field). Single, non-dividing, elongated and well spread cells that did not leave the microscopic field during the time lapse recording and only moved in a forward direction across 100 μm were chosen for measurement to exclude the interference of cell-cell interactions. The motile behaviour of individual cells was highly variable. Some BMdSCs did not migrate; others moved only a short distance and remained stationary, whilst others were constantly moving without crossing 100 μm in any direction. When BMdSCs were stationary, their cellular membranes were constantly moving over the surface of the flask in an amoeboid, probing fashion.

Early in culture (day 3), cells rapidly migrated across the flask (mean: 1.34 μm/second). This migratory capacity significantly reduced to day 7 (mean: 0.76 μm/second, n=4; p=0.019) and day 14 (mean: 0.65 μm/second; p=0.046) as the cultures became more confluent and produced more collagen I. Collagen I formation per culture was significantly greater at day 10 (n=5; p=0.005), 14 (p=0.014) and 17 (p=0.018) than at day 3.

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Early in culture, the cells were very prolific, as demonstrated by the high doubling rates (figure 3.24), and demonstrated an increased migratory capacity, but this doubling rate and migratory capacity significantly declined through culture. The loss in migratory capacity may simply represent a loss in flask surface due to increasing confluence and ECM formation (collagen production) but it was unclear if there was a relationship between cell doubling and migration. Was migration necessary for cellular proliferation? It is likely that migration is vital for cells to assess their immediate environment for division. They may have to come into contact with other cells or move into areas concentrated with growth signals to induce cell division.

DISCUSSION

3.7.1. Culture variation

There were often variations between cultures from the same donor and from different donors, resulting in large standard error bars. Some cultures grew poorly at the LTC level and failed by passage 1, others senesced by passage 2 or 3 and others reached passage 5 before senescing. This may reflect variations in the numbers and quality of stromal stem cells within the original sample (Sekiya et al. 2002), which were determined by CFU-F analysis in the LTCs. It may also reflect variation in bone marrow composition among samples given the heterogeneity of primary cultures (Phinney et al 1999). Why variations occurred between passaged cultures derived from the same donation was unclear. It may reflect slight differences in when cultures were sub-cultured or how long they were out of the incubator for during feeding regimes. The variations were unlikely to reflect age or gender (Phinney et al 1999) with no significant differences in LTC BMdSC growth between normal male and female donors or between different age groups (not shown) (Digirolamo et al 1999; Phinney et al 1999).

3.7.2. Quantitation

The aim was to simply quantitate cellular markers of proliferation, without exhausting a culture, by analysing cell counts, cell doubling and CFU-F colony formation.

a) Cell counts

Cell growth was quantitated twice weekly for passaged stromal cultures and weekly for LTCs by counting the number of cells in several random microscopic cell fields (2.4.1. 'BMdSC growth'). Occasionally, the cells were not separated well during inoculation and adhered as small clusters, which were difficult to count. In these instances, the cell counts were averaged from more microscopic fields. Averaging the number of cells per field in cultures that grew to

confluence (>200 cells per field) was not practical. Cell counts at this level were only estimates.

b) Cell doubling

There were two ways of calculating cell doubling (2.4.3. 'Cell doubling'), 1) by averaging the number of adhered cells per microscopic field from, for example, 3 to 14 days, or 2) by utilising the inoculation and recovery values from each culture. The first method enabled the cell doubling to be determined at various times within a culture (figure 3.24) and at every passage including passage 1 (figures 3.25 & 3.26). Contaminating haemopoietic cells (e.g. macrophages) could be excluded from the count by their spherical shape. The main disadvantage was that quantitating adherent cells at day 14 in confluent cultures was inaccurate giving an approximate cell doubling value. The second method subtracted the inoculum size from the number of cells recovered from the culture, but the inoculum included an unknown number of haemopoietic cells from the LTC that did not adhere at passage 1. Also, not all stromal cells within the inoculum may have adhered to the flask. If the culture had poor growth, it was possible to get a negative cell doubling value, which falsely indicated that cells were dying and detaching during culture. For these reasons, the first method was utilised for cell doubling during this investigation.

A large problem with using cell doubling to quantitate growth was that the calculation assumed that all the cells within the culture were proliferating at the same rate, but in truth, only a small proportion of cells within the BMdSC cultures were actively proliferating (3.6.1. 'Cell division') (Appendix VI). Cell doubling, therefore, was an index of growth rather than an absolute quantitation of growth. If cell doubling was only determined for the proliferating CFU-F cells (approximately $49 / 10^4$ cells in passaged cultures), the doubling rates would likely be many times higher.

c) CFU-F colony formation

CFU-F colony formation was quantitated at day 14 (LTCs and passaged stromal cultures) using a phase contrast microscope. The numbers of colonies in seven to ten strips across the flask were counted and the number of cells per colony estimated (2.4.4. CFU-F). The numbers of colonies (number of CFU-Fs) were then related back to the number of cells inoculated into the flask (10^6 cells in LTCs and 10^4 cells in passaged stromal cultures) and expressed as small (<25 cells), medium (25 – 100 cells) or large (>100 cells) colonies. The size of the colony gave an indication of the primitiveness of the CFU-F. In the literature the commonest way of

quantitating CFU-F colonies was to stain them with 0.5% Crystal Violet in methanol (Colter et al. 2001; Digirolamo et al 1999; Sekiya et al 2002), but this would have terminated the culture. In some cases it was difficult to ascertain the correct number of cells to inoculate into the CFU-F flasks ($<2x10^6$ cells per LTC and $<0.5x10^4$ cells per passaged culture) because different cultures had different rates of growth. Some cultures inoculated at this level did not grow (poor CFU-F colony formation), whilst others proliferated rapidly (unable to distinguish between colonies). This meant that many of the rapidly proliferating cultures were excluded from the analysis. CFU-F analysis was carried out with the x4 objective (1.5x light path magnification), which was necessary to visualise the entirety of a large colony and enabled the size of the colony to be estimated.

3.7.3. Effect of passaging BMdSC growth

The high doubling rates (figure 3.24) and rapid migration (figure 3.40) showed that BMdSCs were most active within the first 7 days of culture. After 7 days, the cell doubling and migratory capacity reduced as cultures gradually became more confluent with a greater degree of ECM formation (increase in collagen I deposition). It is likely that the increasing confluence and ECM formation were responsible for the reduced migratory capacity and proliferation rates. Hartmann-Petersen (Hartmann-Petersen et al. 2000) noted that plating cells (L929 cell line) at a high density ($1.2x10^4$ cells/cm^2) caused a significant decrease in motility compared to control cultures (plated at $0.3x10^4$ cells/cm^2). They also noted that 3 hours after plating, cells migrated approximately 40% slower than the cells at 6 hours, after which time a steady state was achieved up to 48 hours, indicating a short lag phase whilst cells adapted to their new environment. They did not measure motility beyond 2 days. Migration was not determined in this investigation until 72 hours in culture.

It was unclear if migration was necessary for division. BMdSCs were constantly migrating across the surface of the flask in young (3-7 days) cultures, forming and de-forming processes with each other. Perhaps the cells have to move into an area concentrated with growth signals, or come into close contact with other cells to induce mitosis. The increasing formation of stable junctions between cells may also help explain the reduction in motility during confluence (Rickard et al 2003).

The level of confluence at the time of subculture appeared to affect the proliferation of BMdSCs within subsequent passages (figure 3.27). The overall lifespan of the culture was reduced when continually passaging subconfluent to confluent cultures with reduced doubling rates

(maximum passage 5 with ~12 cumulative cell doublings). When consistently passaging cultures with high doubling rates (within 7 – 10 days), and before sub-confluence, the lifespan of the culture was increased (passage 10, with ~20 cumulative cell doublings, excluding LTC cell doublings). As the cultures became more confluent, the cells may have down regulated receptors required for cell growth and eventually exited from the cell cycle. Passaging the cultures may not always have stimulated them back into the cell cycle.

3.7.4. LTC growth of BMdSCs

LTC BMdSCs were grown in Alpha MEM, DMEM and modified McCoy's medium with or without glutamine and HC (figure 3.5). It was found that medium glutamine concentration, and therefore ammonium concentration within the supernatant, was not inhibitory to LTC BMdSC growth, and no build up of ammonia took place within the cultures over 5 weeks (table 3.2). Hydrocortisone was found to be vital for LTC BMdSC growth with a significant increase in growth (figure 3.5) and CFU-F colony formation (figure 3.6). Only cultures that were incubated with HC became haemopoietically active (photos 3.3 & 3.4), but haemopoiesis could not be maintained in culture (continued CFU-GM production and release of NACs) beyond 6 weeks (figure 3.8). This was likely to be due to the use of inadequate serum batches. HC appeared to stimulate the proliferation of progenitor cells, whilst maintaining their immaturity (query self-renewal).

3.7.5. Passaged BMdSC growth

Passaged BMdSCs were grown in Alpha MEM, DMEM and modified McCoy's medium with glutamine, but growth (figure 3.11) and CFU-F colony formation (figure 3.13) was significantly improved in Alpha MEM. Alpha MEM contained amino acids and deoxyribonucleic acids that were not present in DMEM and modified McCoy's medium. Further studies would be to analyse the effect of these components on passaged BMdSC growth in DMEM and modified McCoy's medium.

The effect of HC on passaged BMdSC growth was briefly assessed in modified McCoy's medium (derived mostly from LTCs grown without HC) and was shown to have no effect on proliferation (figure 3.15) or CFU-F colony formation (figure 3.17). The long-term effect of HC on passaged BMdSCs derived from LTCs grown with HC was not assessed. Passage 1 cultures were found to have a significantly higher proportion of large colonies when derived from LTCs grown with HC (figure 3.23), suggesting that HC maintained the more primitive cells within the LTC. It may be that with continued HC treatment (LTC and passaged stroma),

the primitive progenitor cells are maintained in culture (query self-renewal) for longer periods of time, extending the life-span of the culture. Further studies would be to analyse the long-term effects of HC (LTC and passaged stroma) in both Alpha MEM and modified McCoy's medium.

3.7.6. Effect of hydrocortisone on BMdSC growth

The exact mechanisms of action for HC are not known. Since significantly larger CFU-F colonies were formed in LTCs grown with HC (figure 3.6), and BMdSCs derived from LTCs grown with HC grew significantly better at passage 1 (figure 3.19), it was suggested that HC stimulated the progenitor cells to proliferate whilst maintaining their immaturity in culture. Croiselle (Croisille et al 1994) reported that HC blocked LTC-IC differentiation towards the granulocytic pathway and resulted in an accumulation of primitive progenitor cells. Preliminary data suggested that HC did not have a direct effect on passaged BMdSCs (figure 3.15), which indicates a secondary mechanism of action. The main differences between LTCs and passaged stromal cultures are outlined in table 3.13.

Table 3.13. Differences between LTC BMdSC and passaged BMdSC culture environments

LTC BMdSCs	Passaged BMdSCs
Interaction with haemopoietic cells	No interaction with haemopoietic cells other than the occasional carry over in passage 1 and associated macrophage adherence.
Demi feeding weekly	Twice weekly feeding
7 days at 36-37°C followed by incubation at 33°C (dry incubator)	Incubation at 36-37°C (dry incubator)
More heterogeneous population of BMdSCs including osteocytes, adipocytes, reticular cells, endothelial cells, smooth muscle cells	More homogeneous population. The more proliferative cells being selected for and cloned.

Hydrocortisone may have operated via an intermediate cell such as a haemopoietic cell (e.g. macrophage) within the LTCs, which may have been stimulated to produce chemical signals that either directly acted upon stromal growth receptors or upregulated BMdSC receptors specific for HC. BMdSCs are vital for the survival, proliferation and differentiation of haemopoietic cells so it is possible that the haemopoietic cells had a positive feedback on their own survival by stimulating BMdSC proliferation in the presence of HC.

McIntyre & Bjornson (McIntyre & Bjornson 1986) found that in Dexter-type cultures (LTC), maximal colony size was found at a HC concentration of approximately 10^{-7} to 10^{-6} M (10^{-7} M used in this investigation). It was unclear if HC directly affected the CFU-GM cells, or

increased CFU-GM proliferation by indirectly stimulating BMdSC growth. There is growing evidence that glucocorticoids, such as HC, modulate the ECM (secreted by BMdSCs) for improved interaction between stromal cells and haemopoietic cells by upregulating relevant adherent molecules, which may explain the improved CFU-GM formation in the presence of HC. One example is CD44, a homing associated cell adhesion molecule (HCAM), which has multiple ECM ligands including fibronectin, osteopontin, collagen I and IV and hyaluronate, and can bind to immature haemopoietic cells (Turner et al. 1998). The upregulation of CD44 by HC may also stimulate cytokine production to influence adhesion and activation (Reyes et al. 2001). Unfortunately the expression of stromal cell adherent molecules was not ascertained in both LTCs and passaged stromal cultures. There is also evidence that HC alters the production of stromal proteoglycans, which participate in haemopoietic stem cell homing and in the induction of the fibronectin-matrix assembly for haemopoiesis (Fernandez & Minguell 1997).

Other more unlikely differences that may account for the variable effects of HC observed in LTCs and passaged BMdSC cultures were that LTCs were demi fed weekly whilst passaged cultures were fed twice weekly. Demi feeding may allow for a greater build up of cytokines and survival factors in the LTCs, which in the presence of HC, could exert a greater effect on the BMdSCs, however, this is unlikely because demi-feeding prevented the toxic accumulation of ammonium in cultures (table 3.2). The slight differences in temperature are unlikely to have a significant effect on BMdSC growth, and the effect of the heterogeneous composition of the BMdSC layer in LTCs (endothelial cells, smooth muscle cells, osteocytes and so on) are unknown. It is possible that the intermediate cell is not haemopoietic.

3.7.7. BMdSC characterisation

Characterisation of passaged BMdSCs (3.5. 'BMdSC characterisation') confirmed that these cells were not haemopoietic and did not contain contaminating macrophages (negative for CD34, CD45, CD64). Passaged BMdSCs strongly expressed CD90 (Thy-1), a member of the immunoglobulin supergene family, but expression was not specific to BMdSCs, also being found on neurons, fibroblasts, stromal cells, endothelial cells and connective tissue. CD44 was strongly expressed on passaged BMdSCs, but expression is also common to haemopoietic cells including T and B-cells, monocytes and macrophages suggesting its importance in ECM interactions. CD44 is also found on fibroblasts, keratinocytes, endothelial cells, osteoclasts and chondroblasts (Turner et al 1998). CD105, a component of the transmembrane glycoprotein receptor complex for TGF-β, is involved with cellular proliferation, differentiation, ECM formation, migration and modulation of the immune response (Lastres et al. 1996). Its

importance in haemopoietic cell – stromal cell interactions are shown by its expression on activated monocytes and macrophages, endothelial cells, pre-B cells and erythroid precursors (Barbara et al. 1999; Goldsby, Kindt, & Osborne 2000). CD166, an activated leukocyte cell adhesion molecule (ALCAM), is a member of the immunoglobulin superfamily of cell adhesion molecules (CAM) (Bruder et al 1998). It is not specific to BMdSCs being expressed on subsets of activated leukocytes (activated T cells and monocytes), fibroblasts, neurons and epithelial cells, but it is thought to play a role in osteogenesis because expression is down-regulated once the cells embark upon the osteogenic pathway (Barry & Murphy 2004). Antigenic expression during osteogenesis was not assessed for in this investigation. CD49b, an integrin $\alpha 2$ subunit of the VLA-2 receptor, forms a heterodimer with the β_1 integrin, which is important in BMdSC adhesion, being expressed on both haemopoietic cells and BMdSCs. Reyes (Reyes et al 2001) demonstrated that fibronectin and interacting β-integrin signalling were necessary to maintain the early progenitor cells in culture. CD106, a vascular cell adhesion molecule (VCAM), is a transmembrane glycoprotein characterised by seven C2 binding domains, which binds to VLA-4 (on haemopoietic cells) to promote adhesion and extravasation (migration) of haemopoietic cells through the vascular endothelium. Interactions between the β_1 integrins (VLA-4 and VLA-5) and VCAM are the principle interactions between haemopoietic cells and stromal cells (Duhrsen & Hossfeld 1996) and CD106 has previously been used to isolate the more primitive stromal cells from the MNC population (Gronthos et al 1994; Stewart et al 2003). CD106 is also commonly expressed on activated neurons, endothelial cells, smooth muscle, fibroblasts and dendritic cells.

3.8. Final Summary

This investigation optimised the conditions for LTC and passaged BMdSC growth and assessed simple methods for quantitating growth without exhausting a culture (cell counts, cell doubling and CFU-F colony formation). Detaching LTCs and feeding passaged stromal cultures twice weekly resulted in a loss of haemopoietic cells, as shown by antigenic characterisation. The BMdSCs remained karyotypically stable as a function of passage. The antigenic expression profiles within this investigation were comparable to those described by others in the field.

Chapter 4

Bone Marrow derived Stromal Cell Differentiation

Mesenchymal Differentiation

4.0. Aims of Chapter 4

To investigate the osteogenic and adipogenic induction of bone marrow derived stromal cells *in vitro*. Part I (osteogenesis) investigates the optimal calcium concentration for inducing mineralisation and the quantitation of mineral deposit during a 21 day induction period; the differences between the cultures that did and did not mineralise; the quantitation of markers related to osteogenesis including collagen and ALP (protein and mRNA), osteopontin and osteocalcin mRNA, and the difference between Alpha MEM and DMEM in osteogenic induction. Part II (adipogenesis) quantitates the markers related to adipogenic differentiation including multidroplet fat cell production, aP2, LPL and PPAR-γ2 gene expression, and compares differentiation in Alpha MEM and DMEM.

INTRODUCTION

4.1. Mesenchymal Differentiation

BMdSCs are known to differentiate down various mesenchymal lineages including bone (osteogenesis), fat (adipogenesis), cartilage (chondrogenesis), tendon (tendogenesis), muscle and haemopoietic-supporting stromal cells (Horwitz 2003) (figure 1.10), although the process of differentiation is not clear. It is thought that all mesenchymal lineages are derived from a common stem cell (a mesenchymal stem cell), so given the right environmental conditions, a single stem cell (CFU-F) should be able to produce all mesenchymal lineages (1.4.2.b. 'The bone marrow stromal matrix'). Whether less proliferative progenitor cells (also CFU-Fs) maintain this multilineage capability is not clear. If differentiation occurs down a discrete pathway with no ability to switch lineages (1.3. 'Adult stem cell plasticity'), a lineage committed precursor cell (CFU-F) should only produce a colony with one cell type (figure 4.1.a). However, mesenchymal differentiation is likely to be much more complex with interacting and reciprocal pathways (e.g. osteogenesis and adipogenesis) (figure 4.1.b & c) for more control in an adaptive environment (1.6.2. 'Adaptation') (Gimble et al 1996).
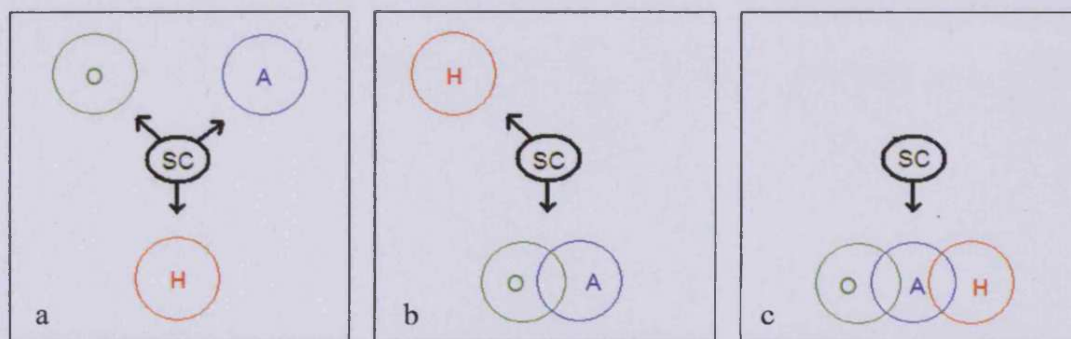
Figure 4.1. Three examples of mesenchymal lineages from the stem cell (SC): Osteoblast (O), Adipocyte (A) and Haemopoietic supporting stromal cell (H). Modified from Gimble (Gimble et al. 1996)
a)   Each lineage is a distinct pathway
b)   Overlap between the osteogenic and adipogenic lineages
c)   Overlap between all lineages

4.2. Part 1: Osteogenesis

Osteogenesis has been poorly defined but it is thought to progress from a stem cell to an

osteoprogenitor cell to a preosteoblast before terminally differentiating into an osteocyte

(Bodine, Trailsmith, & Komm 1996).

4.2.1. Induction

The extracellular matrix in bone tissue is rich in collagen I and osteogenic inducing cytokines

required to stimulate bone formation and mineralisation. Many chemicals have been used *in*

vitro to stimulate osteogenesis, but ascorbic acid-2-phosphate, dexamethasone and β-

glycerophosphate are the commonest. Ascorbic acid acts as a cofactor in the hydroxylation of

lysine and proline residues, which are required for the secretion and processing of type I

procollagen in the synthesis of a collagenous bone extracellular matrix (Coelho & Fernandes

2000; Gundle 1998; Jaiswal et al 1997). The deposition of type I collagen induces the

bone/liver/kidney isoenzyme of alkaline phosphatase (ALP) and osteocalcin gene expression,

which are required for mineralisation (Shiga et al. 2003). Ascorbic acid-2-phosphate is used *in*

vitro because it is more stabile in solution than ascorbic acid.

Dexamethasone changes the morphology of the cells from a spindle to a more cuboidal shape

(Cheung et al. 1994). Coelho (Coelho & Fernandes 2000) reported that dexamethasone, alone

or in combination with ascorbic acid-2-phosphate and β-glycerophosphate, increased cell

growth (determined by MTT reduction and total protein content) and induced ALP activity,

increasing the rate of mineralisation. Cheung (Cheung et al 1994) reported that dexamethasone

inhibited the proliferation of human BMdSCs (determined by thymidine incorporation and cell

numbers), but induced ALP activity, osteopontin and osteocalcin mRNA expression. Dexamethasone is insufficient to cause mineralisation alone, but its absence has been reported to reduce the rate of mineralisation (in the presence of ascorbic acid-2-phosphate and β-glycerophosphate) (Herbertson & Aubin 1995; Ter Brugge & Jansen 2002).

The exact mechanisms that initiate mineralisation have not been determined, but a source of phosphate ions, calcium, a confluent cell layer and mature collagenous extracellular matrix appear to be important (Hsu et al. 1993). The phosphate ions are mainly provided by the hydrolysis of β-glycerophosphate by ALP (Coelho & Fernandes 2000). Both β-glycerophosphate and ALP are reported to be important in the initiation of mineralisation in embryonic chick and foetal rat osteoblasts in Alpha MEM, but are not required to maintain it (Bellows et al. 1992). Coelho & Fernandes (Coelho & Fernandes 2000) and Gori (Gori et al 1999) also reported the necessity of β-glycerophosphate, alone or in combination with ascorbic acid and dexamethasone, for mineralisation *in vitro*. A confluent cell layer was deemed necessary because cultures seeded as dense clusters were found to have an increased ALP activity and mineralisation in comparison to dispersed cells, although proliferation was significantly reduced with dense seeding (Goldstein 2001; Stanford et al 1995). Stanford (Stanford et al 1995) also reported that confluence was important for mineralisation with respect to the extracellular matrix, but showed that the extracellular matrix alone was insufficient to induce mineralisation. Jaiswal (Jaiswal et al 1997) reported similar findings but the densely seeded cultures usually detached during culture. Hsu (Hsu et al 1993) suggested that matrix vesicles were the initial site of calcification in rachitic rat epiphyseal cartilage growth plates by showing membrane phosphatidylinositol linked ALP, but these vesicles were not visible in human BMdSC cultures under phase contrast. The *in vitro* mineral deposit within induced BMdSC cultures was not associated with particular loci but distributed throughout the culture making identification of the initiation sites difficult (Jaiswal et al 1997).

The following photos were taken from this study and show the lack of mineralisation in the absence of induction agents (photo 4.1), the scattered refractile mineral deposit forming by day 10 of culture (unstained) (photo 4.2) and the extensive Alizarin red S positive calcium deposit by 21 days post induction (photo 4.3).
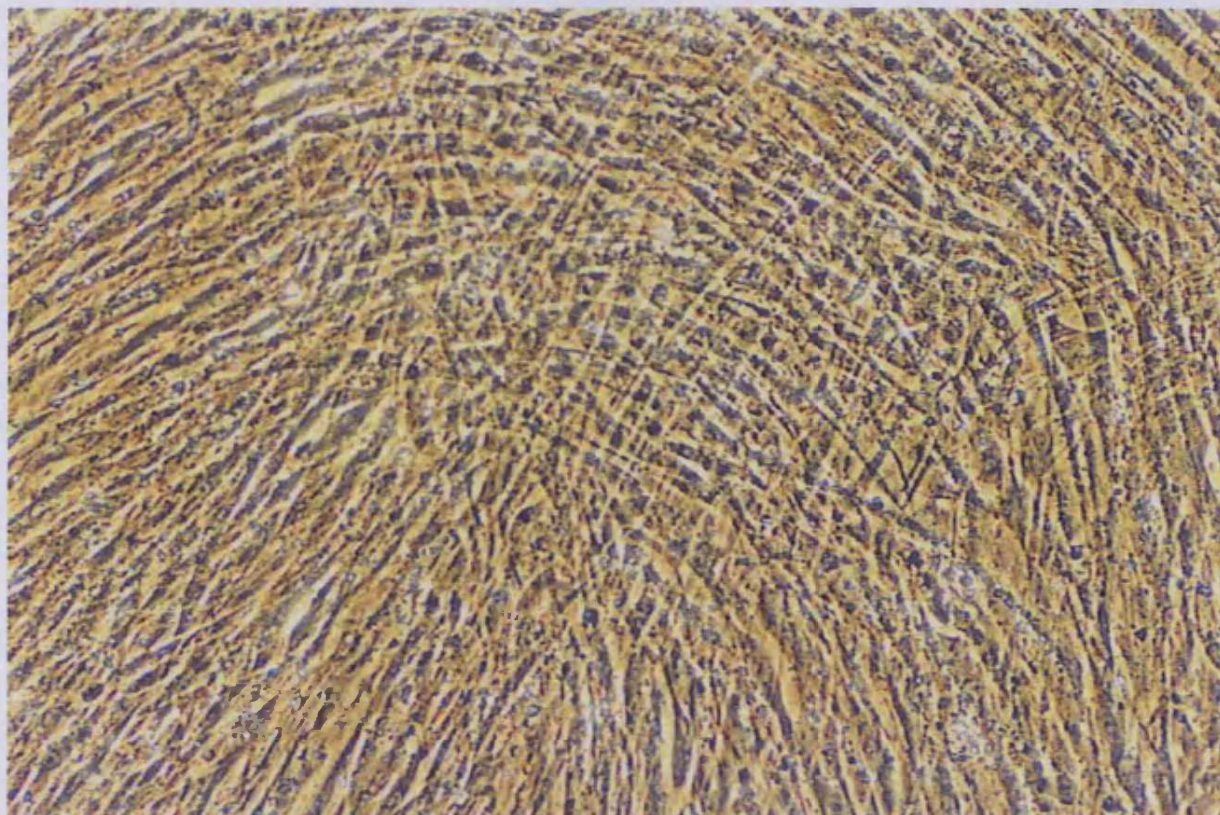
Photo 4.1. Control (not induced) culture stained with Alizarin red S, shows no mineralisation at 21 days post induction.
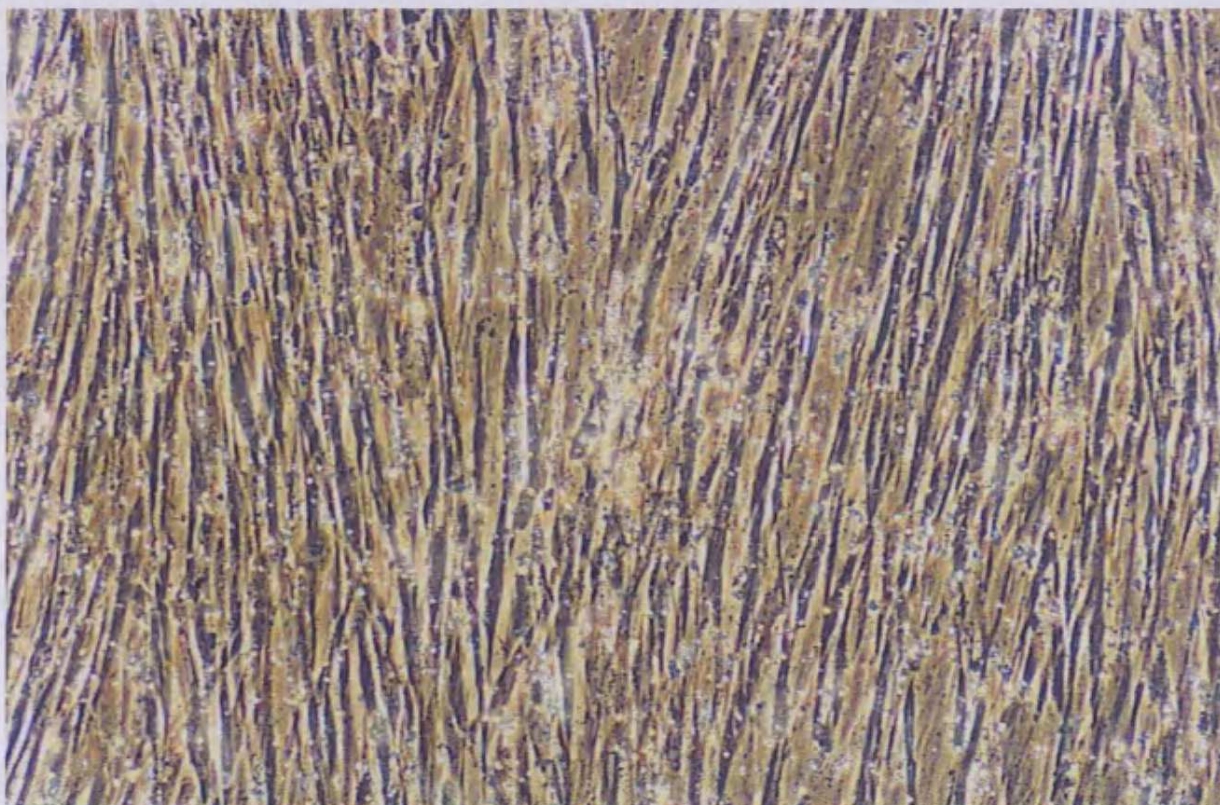
Photo 4.2. Scattered refractile mineral deposit across the cell layer at day 10 post induction (unstained). Unable to determine the original foci of the mineralisation
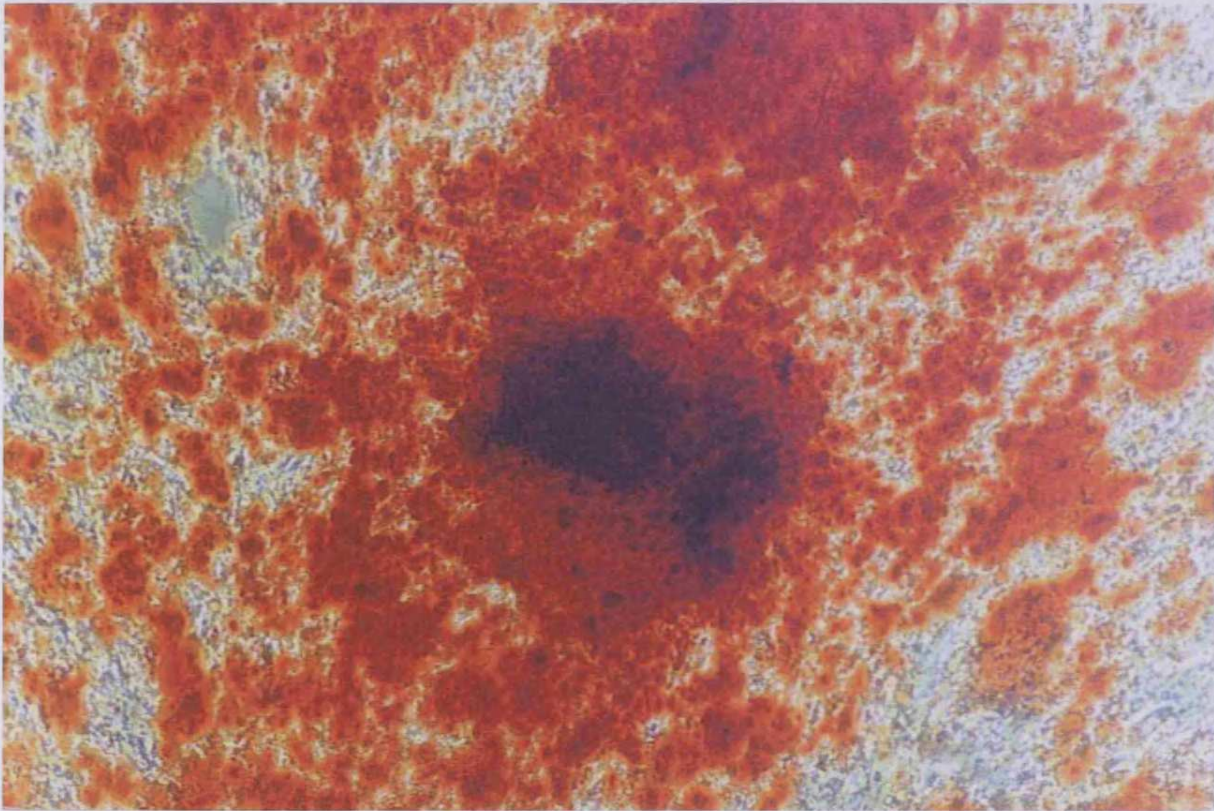
Photo 4.3. Osteogenically induced culture in Alpha MEM (200 mg/l CaCl$_2$) at 21 days post induction. Cells visibly breaking up beneath the mineralised extracellular layer.

4.2.2. **The process of osteogenesis**

Osteogenesis is commonly divided into three phases, proliferation, maturation (involving the synthesis and deposition of a collagenous extracellular matrix) and mineralisation (Coelho & Fernandes 2000), although the three phases are unlikely to occur one after the other. The proteins involved in osteogenesis may not be unique to bone, but their coordinated production is largely responsible for the various stages of bone formation (Malaval et al. 1994). Type I collagen mRNA, fibronectin, ALP (activity and mRNA) and osteopontin mRNA (early markers of osteogenesis) increase in concentration with the confluence of the culture and maturation of the ECM (Bodine, Trailsmith, & Komm 1996). Collagen I and osteopontin mRNA are then reported to maintain low basal levels throughout the mineralisation process (Stein & Lian 1993). Osteopontin binds to various extracellular molecules including type I collagen, fibronectin and osteocalcin to provide physical strength to the ECM. Reduced osteopontin mRNA has been found in patients with reduced bone formation (e.g. osteoporosis) (Frank et al 2002).
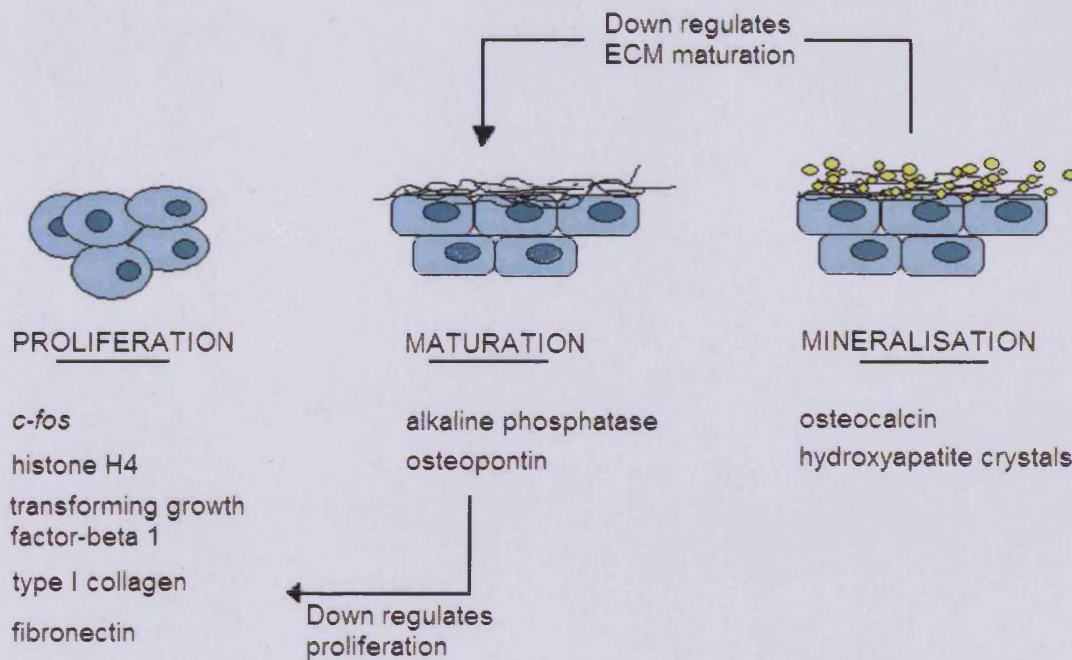
Figure 4.2. Three stages of osteogenesis, proliferation characterised by an increase in transcription factors and markers of proliferation; maturation associated with a decline in proliferation markers and an increase in ALP and osteopontin expression; and mineralisation characterised by an up-regulation in osteocalcin and the formation of hydroxyapatite crystals with declining ALP and osteopontin. Modified from Bodine (Bodine et al. 1996) and Stein & Lian (Stein & Lian 1993)

With the down-regulation of proliferation, ALP activity is increased further to begin the process of mineralisation by hydrolysing β-glycerophosphate (Bellows, Heersche, & Aubin 1992). This increases the local concentrations of inorganic phosphate ions available to bind with calcium, largely provided by the medium, to form hydroxyapatite crystals, which become embedded in the ECM. The time taken for phosphate concentrations to reach the threshold value for hydroxyapatite formation accounts for the slow onset of mineralisation following induction (Bellows, Heersche, & Aubin 1992; Coelho & Fernandes 2000). Once mineralisation is initiated, ALP has been reported to decline (Bodine, Trailsmith, & Komm 1996; Hsu et al 1993) (differentiated osteocytes are weakly positive for ALP) or remain unchanged (Chak et al. 1995). Osteocalcin (calcium binding protein) and collagenase (responsible for collagen I turnover) are reported to increase (Bodine, Trailsmith, & Komm 1996; Hsu et al 1993), whilst conflicting reports show an increase (Stein & Lian 1993) or a decline (Malaval et al 1994) in osteopontin mRNA following mineralisation.

4.2.3. Alpha MEM and DMEM

Coelho (Coelho et al. 2000) reported significantly more ALP mRNA and an earlier onset of mineralisation following osteogenic induction in Alpha MEM in comparison to DMEM. This

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may be attributed to a higher concentration of ascorbic acid, which stimulates collagen I formation to increase ALP activity for hydrolysis of β-glycerophosphate. Jaiswal (Jaiswal et al 1997) reported more mineralisation in DMEM despite there being a lower ALP activity per cell.

DMEM is not as rich in amino acids, vitamins or nucleotides as Alpha MEM, but the concentrations of amino acids present within DMEM are generally much higher (2.3.4. 'Medium'). Perhaps the most important difference is the presence of ascorbic acid in Alpha MEM, which is known to stimulate collagen I production, however, ascorbic acid is also known to be labile in culture medium, so the effects of medium concentrations on BMdSC differentiation is unclear.

4.3. **Part II: Adipogenesis**

4.3.1. **Adipocyte function**

Fat cells (white adipose tissue cells) occupy spaces between areas of haemopoiesis in the bone marrow and may have a supporting role for haemopoietic proliferation / differentiation. They also act as local energy stores in lipid metabolism by storing and clearing triglycerides (Gimble et al 1996). Fatty acids form small micelles in water with their hydrophilic tails on the inside and hydrophilic heads on the outside (figure 4.3). Three fatty acid molecules link to a glycerol molecule to form a triacylglycerol molecule (figure 4.4), which accumulate in the cytoplasm as spherical droplets. Triacylglycerol molecules are hydrolysed to release free fatty acids when energy is required (Slavin 1985).
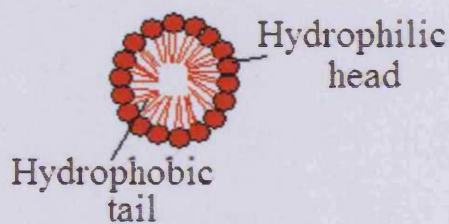
Figure 4.3. Fatty acids form small micelles in water with hydrophilic heads on the outside and hydrophobic tails inside
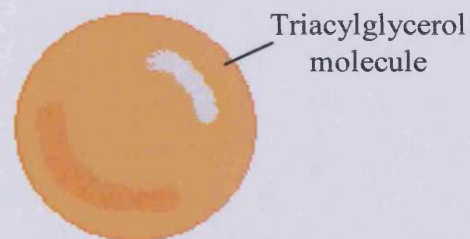
Figure 4.4. Three fatty acid molecules join to a glycerol molecule to form large spherical triacylglycerol molecule, which accumulate in the cell's cytoplasm

4.3.2. Induction

Adipogenesis is most commonly induced or accelerated *in vitro* by the addition of fatty acids (provided *in vitro* by horse serum or foetal calf serum), 3-isobutylmethylxanthine, insulin, indomethacin and dexamethasone. These regulate the transcription factors CCAAT/enhancer binding proteins (C/EBP α,β,δ) and peroxisome proliferator activated receptors (PPAR α, γ2, δ) to bring about the initial stages of adipocytic differentiation (Gregoire et al. 1998). Following induction, there is an increase in the transcription factors C/EBP β and C/EBP δ, which induce PPAR-γ2 gene expression (Janderova et al 2003; Scavo et al. 2004), the most adipocyte specific PPAR of the type II nuclear hormone receptor family. PPAR-γ2 interacts with the retinoic acid receptor X to induce C /EBP α production (Scavo et al 2004), which, in accordance with other transcription factors, regulates other genes such as lipoprotein lipase (LPL), adipocyte-specific lipid binding protein (aP2) and FAS (fatty acid synthetase) (Shi et al. 2000) to bring about lipid accumulation in cells (Ahdjoudj et al 2001; Gimble et al 1996; Gregoire, Smas, & Sul 1998; Janderova et al 2003). LPL is associated mainly with capillaries and arteries and is responsible for hydrolysing triacylglycerol molecules in lipoproteins in the production of free fatty acids (Cryer 1981; Goldberg 1996). It is involved in BMdSC lipid accumulation, although its expression is not adipocyte specific (also expressed by macrophages) and it occurs spontaneously at confluence during *in vitro* culture (Gregoire, Smas, & Sul 1998).

Individual adipogenic induction agents are unlikely to have a large effect on BMdSC differentiation, but together they can significantly influence gene expression and lipid accumulation during *in vitro* induction (Ahdjoudj et al 2001). 3-isobutylmethylxanthine, a cAMP phosphodiesterase inhibitor, stimulates the cAMP dependent protein kinase pathway to accelerate adipogenesis (Ntambi & Young-Cheul 2000) by increasing C/EBP-β and PPAR-γ2 expression (Gregoire, Smas, & Sul 1998). Insulin has been shown to increase the number of multidroplet fat cells in BMdSC cultures with dexamethasone, IBMX and indomethacin (Gregoire, Smas, & Sul 1998) by stimulating the insulin-like growth factor-1 (IGF-1) signalling pathway (Janderova et al 2003; Ntambi & Young-Cheul 2000).

4.3.3. The process of adipogenesis

Adipogenesis requires cellular proliferation, ECM maturation, commitment to the adipocyte lineage and cellular growth arrest before lipid accumulation and terminal differentiation. Following proliferation, fibronectin, tenascin and osteogenic related markers (type I collagen, osteopontin and osteocalcin expression) are reported to be reduced, whilst CD44 (hyaluronate),

CD36 (involved in fatty acid transport), collagen IV, aP2, LPL and PPAR-γ2 mRNA are increased (Gimble et al 1996; Gregoire, Smas, & Sul 1998).
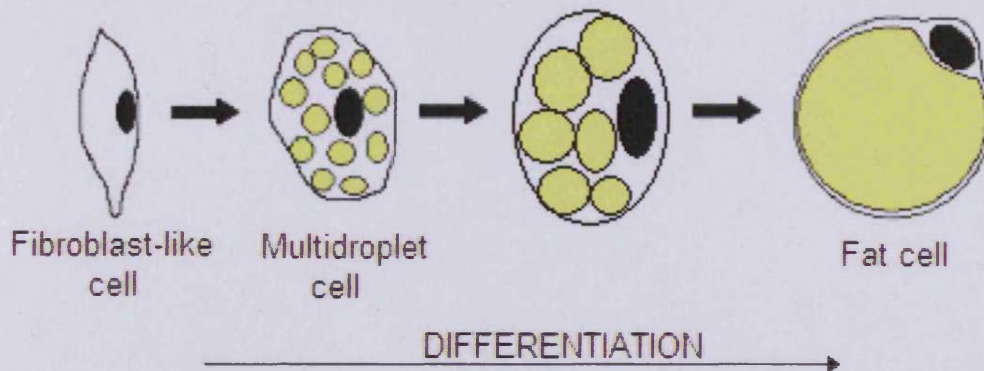
Figure 4.5. Adipogenesis from a fibroblastic-like spindle-shaped immature preadipocyte, to a more committed cuboidal cell, which develops multiple fat droplets, to a more spherical terminally differentiated fat cell (adipocyte). Modified from (Alberts et al. 1998)

BMdSCs morphologically change following induction from a fibroblast-like spindle shape to a more cuboidal shape for lipid accumulation to a more spherical adipocyte shape (figure 4.5). Multiple small triglyceride droplets, which can be stained using Oil red O, begin to accumulate within the cells' cytoplasm to form multidroplet cells (photo 4.5). These droplets gradually increase in size and fuse together to form a more spherical shaped cell (photo 4.6). Eventually, all the droplets fuse to form one large droplet, pushing the cell nucleus to the edge of the cell (photo 4.7). This rarely occurs in human bone marrow stromal cultures, and is frequently seen in mouse Dexter-type cultures (Dexter 1979b).

The following photos are from this study but show the time sequence of events from the development of granular cells beginning lipid accumulation to the formation of multidroplet fat cells and eventually more spherical globular adipocytes.
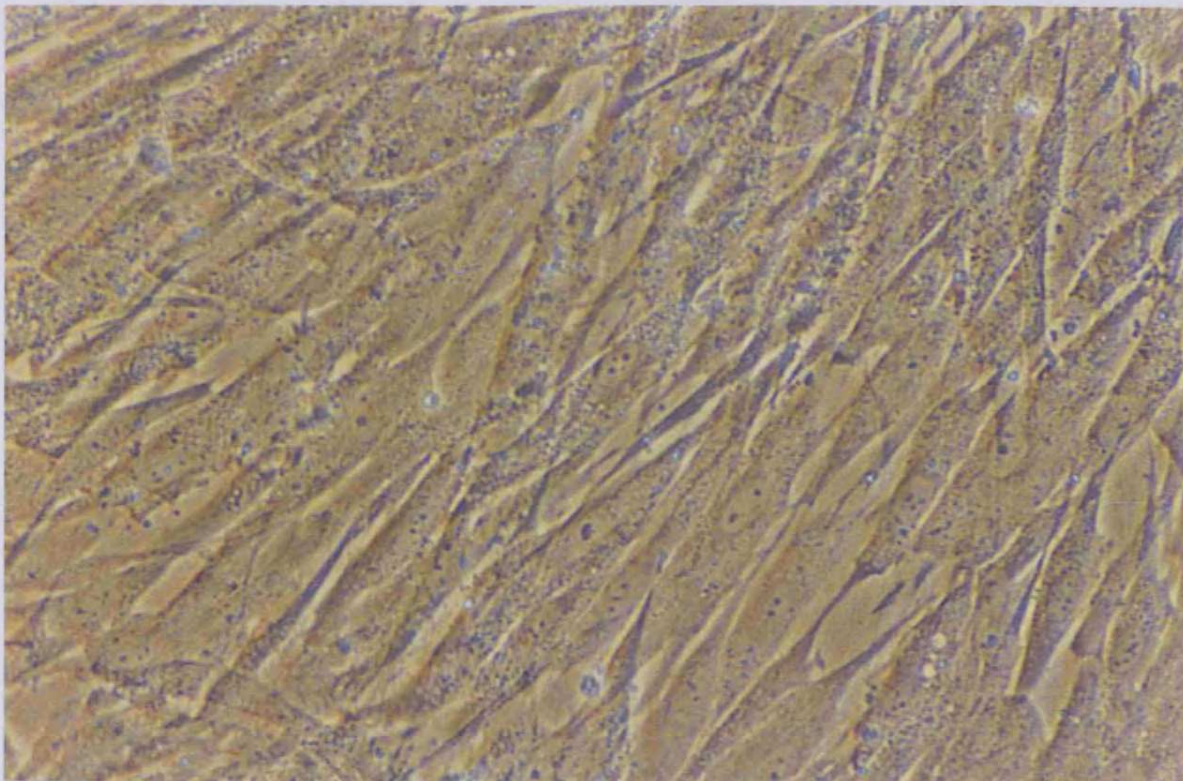
Photo 4.4. 1st cycle of the cyclic induction protocol. Cells becoming granular, possibly beginning lipid accumulation.
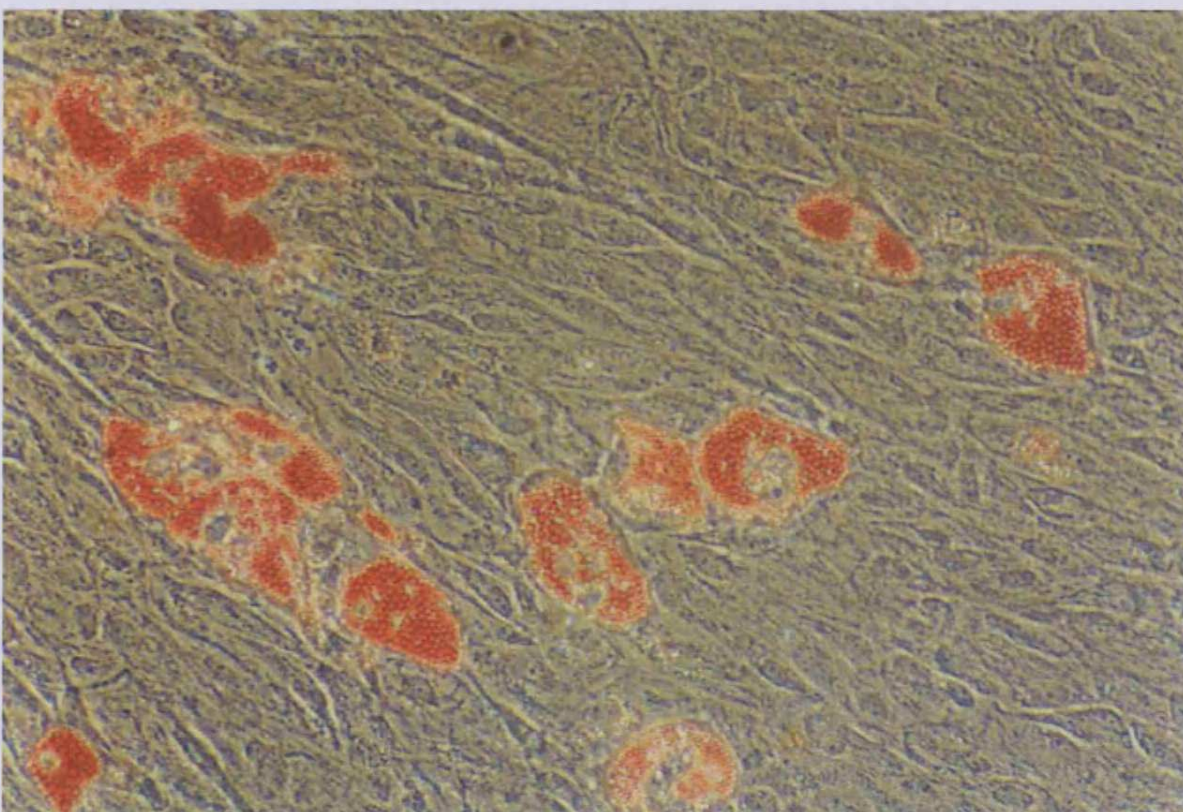
Photo 4.5. High power magnification of multidroplet fat cells stained with Oil red O
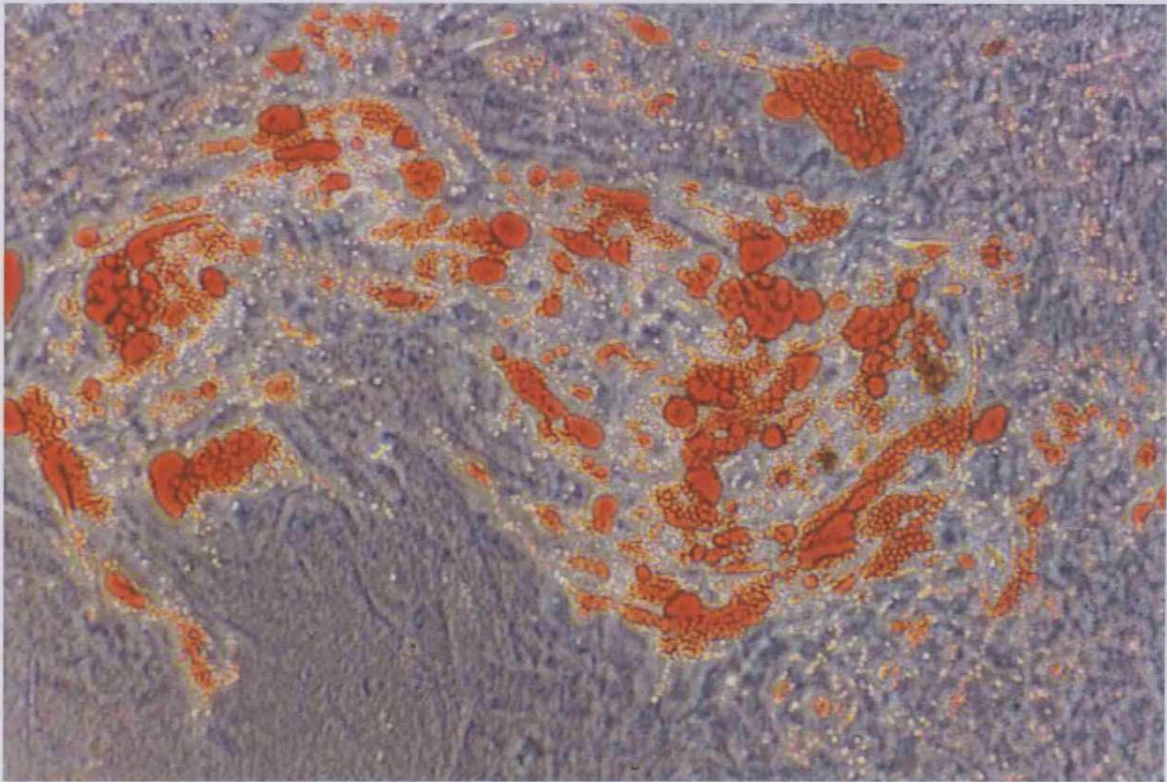
Photo 4.6. Low power magnification of a multidroplet fat cell cluster stained with Oil red O. Lipid droplets are varying in size and the cells are becoming more spherical. Loosely scattered weakly stained tiny droplets scatter the flask around the cluster
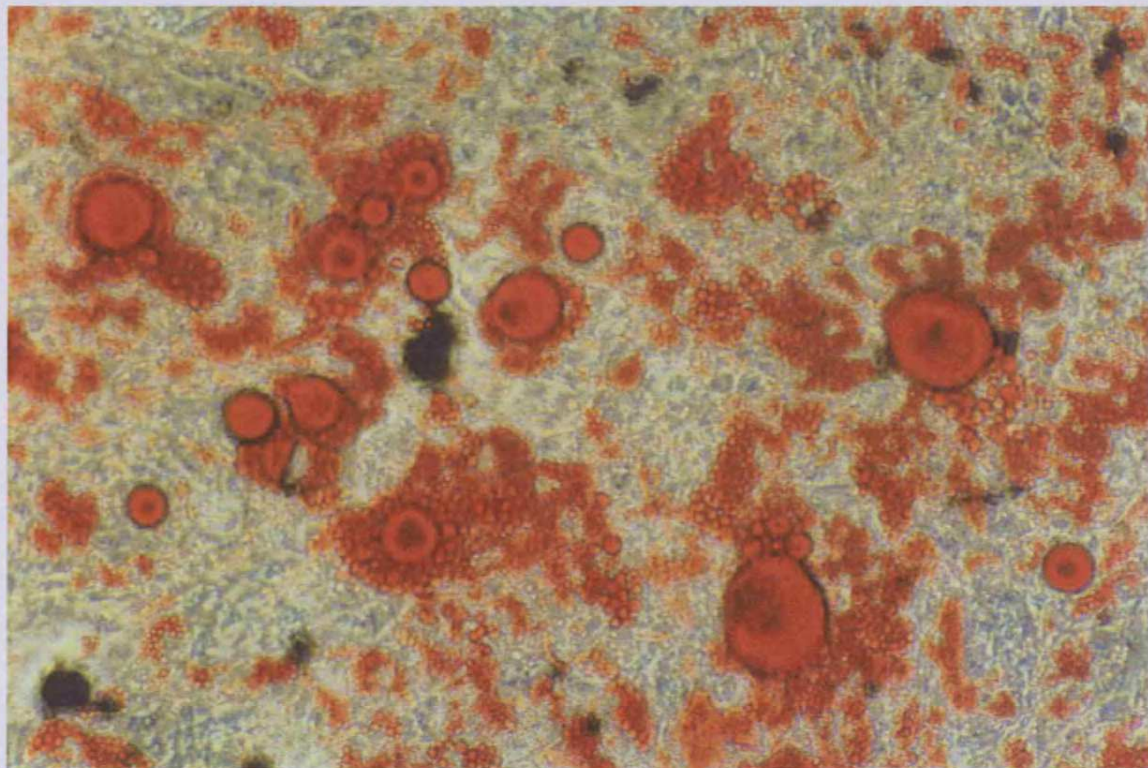
Photo 4.7. Formation of large spherical adipocytes. These are not as big as the ones seen in mouse cultures (Dexter 1979b). Only occurred in one culture.

The multidroplet fat cells appear to form in clusters within the BMdSC layer and can be easily visualised *in vitro* by staining with Oil Red O. Cell to cell contact and adherence are reduced during adipogenesis as the cells become more spherical and refractile, therefore, the ECM must play a large part in maintaining tissue structure. Gregoire (Gregoire, Smas, & Sul 1998) hypothesised that interactions between the ECM and adherent cells allowed for the cells to become more spherical whilst maintaining their adherence and keeping them in close proximity for maximal exposure to induction signals and for maintaining tissue structure.

Preadipocytes (spectrum of stem-like cells to more differentiated progenitor cells committed to the adipocyte lineage) eventually undergo growth arrest and withdraw from the cell cycle for terminal differentiation and lipid accumulation (Janderova et al 2003). Ntambi & Young-Cheul (Ntambi & Young-Cheul 2000) reported this to happen 24 hours post induction, although during *in vitro* differentiation this largely suggests that committed preadipocytes were already present within the cell layer before induction. Induction stimulates further differentiation towards growth arrest and lipid accumulation. Growth arrest is not thought to be induced by cell to cell contact during confluence (Gregoire, Smas, & Sul 1998). The presence of proliferating preadipocytes within the cell layer may account for the formation of fat cell clusters.

There are variations in reports as to when lipid accumulation begins during the process of adipogenesis. Scavo (Scavo et al 2004) reported a relatively late onset with no lipid droplets being detected in human bone marrow stromal cells within the first 48 to 72 hours during a cyclic induction protocol (2.8.1.b. 'Cyclic induction protocol'), and only being rarely observed within 96 hours. They detected small and sparse lipid droplets within the second induction cycle (5 – 9 days), with abundant lipid accumulation between the end of the second maintenance phase and third induction phase. Janderova (Janderova et al 2003) reported lipid accumulation soon after the onset of differentiation with the most pronounced change occurring during the second induction cycle.

RESULTS

4.4. **Part I: Osteogenic Induction**

Mineralisation could be seen microscopically as a scattered refractile layer overlying the top of the stromal cells in association with the extracellular matrix (photo 4.2). The mineral deposit stained positively for Alizarin red S as an intense red colour (2.7.3.b 'Mineralisation') (photo

4.3). The extent of mineralisation was quantitated by demineralising the cultures in HCl and measuring the absorbance of the acidified extract (2.7.3.c 'Demineralisation').

4.4.1. **Mineralisation at 200 mg added CaCl$_2$ per litre Alpha MEM**

The extent of mineralisation in cultures induced at 200 mg/l CaCl$_2$ was measured at distinct time points within an induction period of 21 days (figure 4.6).
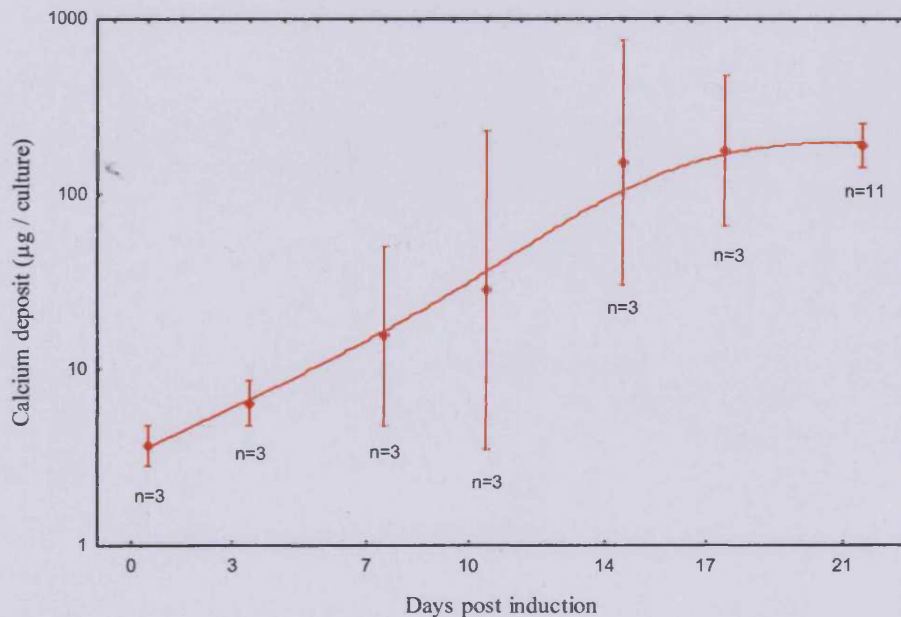
Figure 4.6. Mineral deposited during osteogenic induction in Alpha MEM, 200 mg/l CaCl$_2$. Results not normalised. Mean ± SE

The amount of calcium deposited at 200 mg/l CaCl$_2$ increased significantly during induction until approximately day 14 where the curve plateaus. Significantly more calcium was deposited at day 21 (n=11) than at day 0 (n=3; p=0.00006), day 3 (n=3; p=0.00036) and day 7 (n=3; p=0.0095) in Alpha MEM. Day 17 also had significantly more mineralisation than day 0 (p=0.02) and day 3 (p=0.033). By day 21 a dense mineralised matrix had formed, which often caused the cells to break up, limiting the amount of RNA that could be extracted (2.9.1. 'RNA extraction'). Photo 4.8. shows a culture at 21 days post induction in which the cells had broken up with the release of mineral deposit into the supernatant.
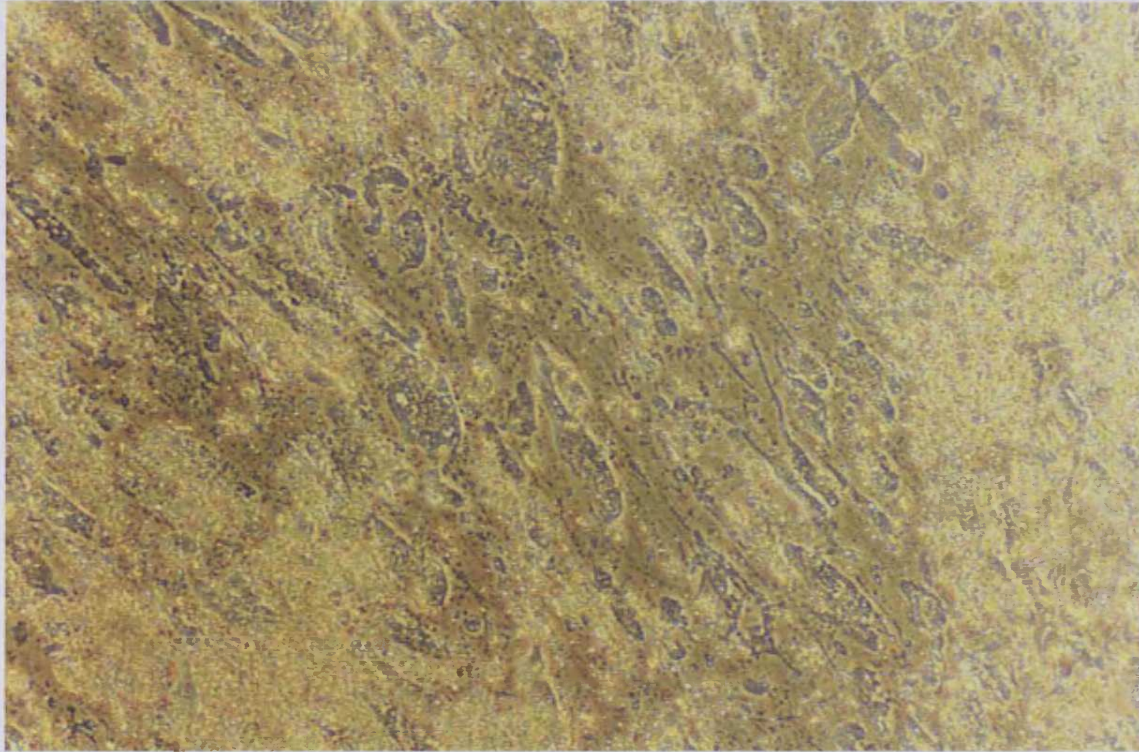
Photo 4.8. Culture induced in Alpha MEM (200 mg/l $CaCl_2$). Cells broken up beneath the refractile mineralised layer (unstained). The mineral deposit is releasing from the ECM.

4.4.2. **RNA extraction at 200 mg added $CaCl_2$ per litre Alpha MEM**

RNA was extracted from osteogenically induced BMdSC cultures at distinct time points within the 21 days of induction and quantitated.
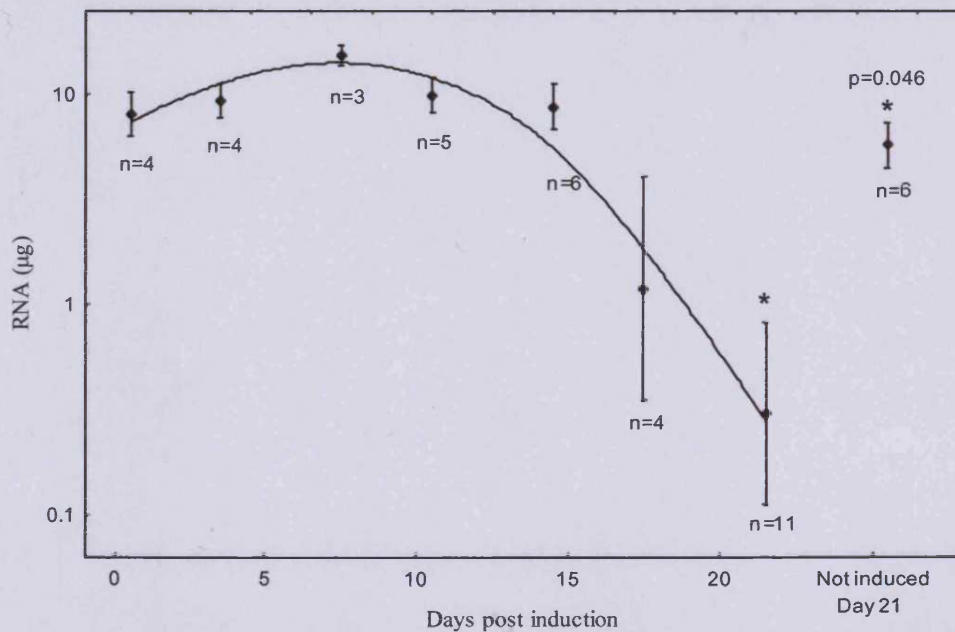
Figure 4.7. RNA extracted from osteogenically induced BMdSCs at 200 mg/l $CaCl_2$ during 21 days of induction. Mean ± SE

The amount of extractable RNA increased initially to 7 days post induction, which may represent proliferating BMdSCs. As the culture began mineralising, the amount of extractable RNA reduced significantly to day 21 (n=11) from day 0 (n=4; p=0.046), day 3 (n=4; p=0.017), day 7 (n=3; p=0.0086), day 10 (n=5; p=0.0097) and day 14 (n=6; p=0.006). Significantly more RNA was extracted from non-mineralised control cultures (not induced) at 21 days post induction (n=6, n=11; p=0.046).

4.4.3. Limiting the rate of mineralisation

Insufficient RNA was extracted from osteogenically induced BMdSCs at 21 days post induction (at 200 mg/l $CaCl_2$) for analysis of gene expression. Therefore, the rate of mineralisation needed to be limited to prevent loss of RNA, whilst still allowing for measurable amounts of mineral deposit to form. Reducing the medium calcium concentration reduced the rate of hydroxyapatite formation. BMdSC cultures were osteogenically induced in $CaCl_2$ concentrations ranging from 0 to 200 mg/l in Alpha MEM. At 21 days post induction the extent of mineralisation was analysed and the total amount of extractable RNA determined (figure 4.8).
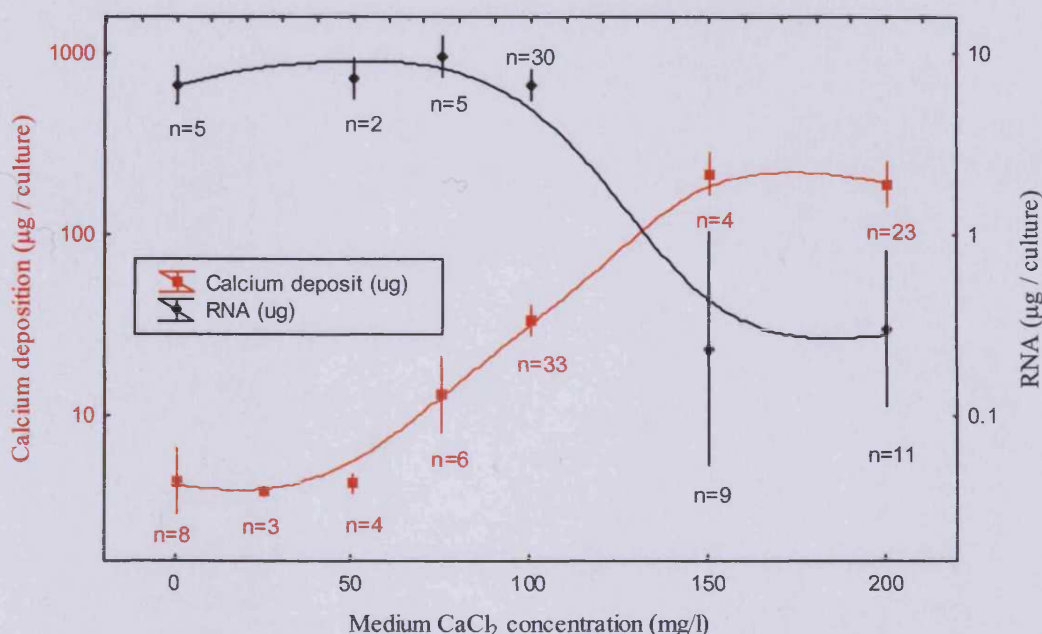
Figure 4.8. Mineralisation (red square) and RNA extraction (black circles) from osteogenically induced BMdSCs at various $CaCl_2$ concentrations at 21 days post induction. Mean ± SE

Medium calcium concentrations 200 mg/l (n=23) and 150 mg/l (n=4) had significantly more calcium deposition (p=0.00018 & p=0.005 respectively) and significantly less extractable RNA at 21 days post induction (p=0.038 & p=0.008 respectively) than 100 mg/l. The $CaCl_2$

concentration 100 mg/l (n=33) deposited significantly more calcium by 21 days post induction than 0 mg/l (n=8), 25 mg/l (n=3) and 50 mg/l (n=4; p=<0.004), and significantly more RNA was extracted than at 150 mg/l (n=4; p=0.000001) and 200 mg/l (n=9; p=0.00004). Alizarin red S staining at 0, 75 and 100 mg/l $CaCl_2$ is shown in photo 4.9.
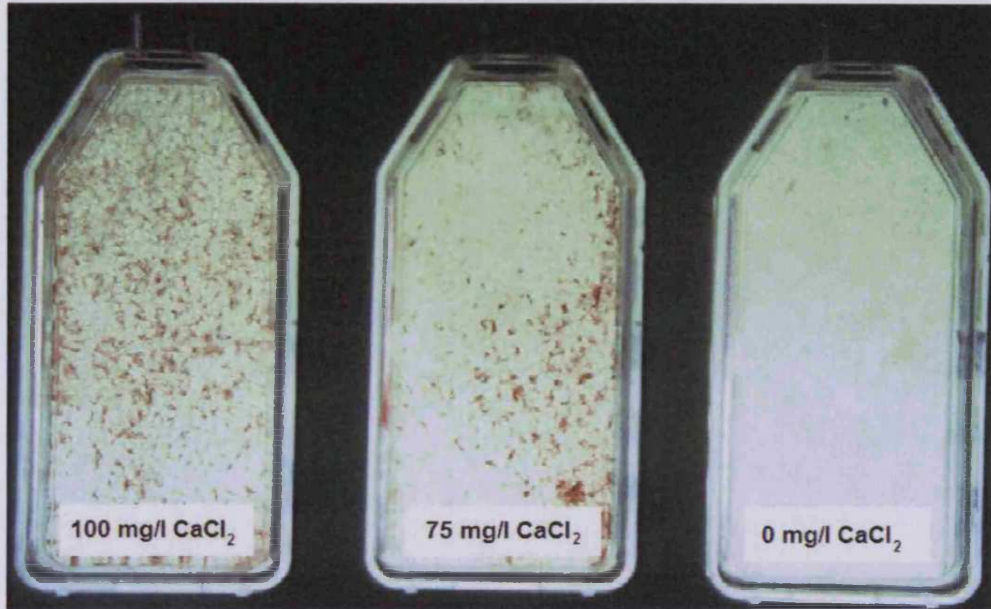
Photo 4.9. Alizarin Red S staining at 21 days post induction in BMdSC cultures induced at 0, 75 and 100 mg/l $CaCl_2$.

4.4.4. RNA extraction at 100 and 200 mg added $CaCl_2$ per litre Alpha MEM

RNA extraction was compared between 100 mg/l and 200 mg/l $CaCl_2$ at 0, 10, 14, 17 and 21 days post induction (figure 4.9).
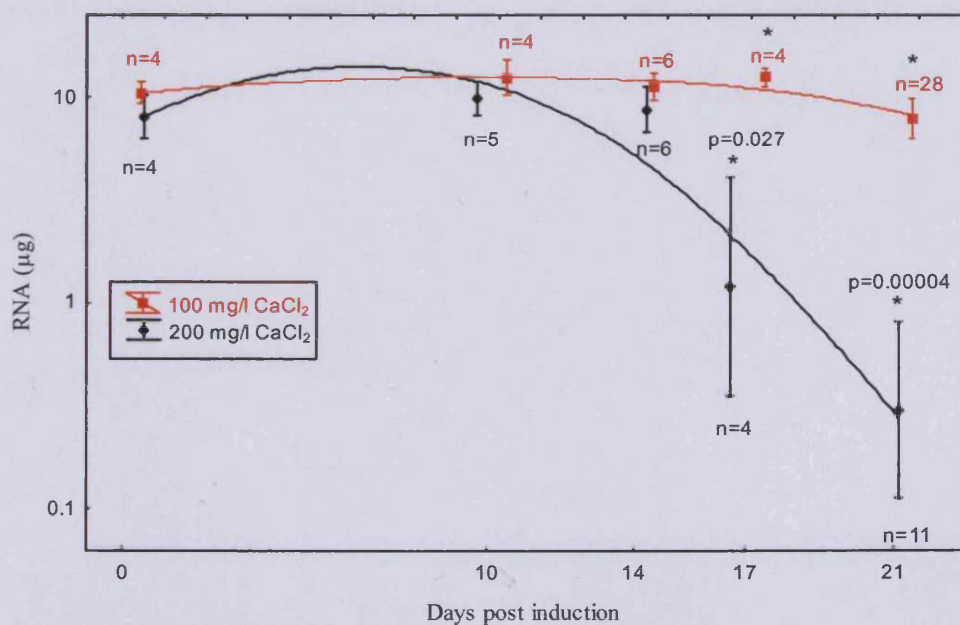
Figure 4.9. RNA extracted from osteogenically induced BMdSCs at 100 mg/l (red square) and 200 mg/l (black circle) $CaCl_2$ in Alpha MEM during 21 days post induction. Mean ± SE

There was no significant loss of RNA using 100 mg/l $CaCl_2$ (red square) by 21 days post induction (figure 4.9). Significantly more RNA was extracted at 17 (n=4, n=4; p=0.027) and 21 days (n=28, n=11; p=0.00004) post induction using 100 mg/l $CaCl_2$ than 200 mg/l $CaCl_2$.

4.4.5. Summary

Calcium concentration in Alpha MEM had little effect on passaged BMdSC growth (figure 3.10), however, cultures osteogenically induced at 200 mg/l $CaCl_2$ showed significantly more calcium deposition by 21 days post induction than at 100 mg/l $CaCl_2$ (n=11, n=21; p=0.00018). Cultures osteogenically induced at 200 mg/l $CaCl_2$ also resulted in a significant loss of RNA by 21 days post induction. The optimum $CaCl_2$ concentration for osteogenic induction was established to be 100 mg/l allowing for measurable mineral deposit to form and sufficient RNA to be extracted.

4.5. Mineralised and Non-mineralised Cultures

During osteogenic induction, BMdSC cultures mineralised at different rates, and not all BMdSC cultures mineralised (assessed microscopically by phase contrast) within 21 days of induction. The following tables (4.1 & 4.2) summarise the cultures that did and did not mineralise at 200 mg/l and 100 mg/l $CaCl_2$, with the aim of establishing the reasons why not all cultures mineralised within 21 days of induction.

Table 4.1. Mineralised cultures at 100 mg/l and 200 mg/l CaCl$_2$ in Alpha MEM.

Culture	Passage	[CaCl$_2$] (mg/l)	Origin Sex / Age	Age of LTC (wks)	Mineralisation: Days post induction	Culture age at induction (days)	Cells / field at induction
S2	2	200		3	14	14	110
S6	2	200		5	7	10	140
S7	2	200	846	5	7	10	170
SA	3	200	F / 36	3	14	14	100
SY	3	200		6	10	10	120
N6	3	200	JV M / 25	13	10	11	80
U1	2	200		8	7	14	110
U1	2	100	853	8	14	14	110
UB	3	200	F / 30	8	10	14	100
UB	3	100		8	14	14	100
SR	2	200	847	7	10	9	150
T3	2	200	M / 36	7	14	13	80
SX	2	200	848	7	10	11	120
SX	2	100	M / 36	7	21	11	120
YY	2	200		5	7	15	60
YY	2	100		5	21	15	60
ZA	2	200	WP	6	7	14	120
ZA	2	100	F / 37	6	7	14	120
ZQ	3	100		6	14	15	120
YS	2	100		5	10	13	230
UH	3	100	857	5	10	14	100
TP	1	100	M / 33	5	10	14	100
TP	1	200		5	7	14	100
Z7	1	100	876	3	10	10	230
AAK	2	100	M/32	6	10	9	280
ZD	2	100		3	14	12	230
AHH	2	100	886	9	10	6	200
AHP	2	100	M/39	5	10	7	150
AJR	3	100		6	10	9	200
ZU	3	100	XD F/33	5	10	9	220
AFD	4	100		7	10	19	200
AB4	3	100	880	4	10	10	170
ABL	3	100	F/39	4	17	15	115
AB9	2	100		4	21	13	120
PL	3	100		4	10	7	110
PC	2	100	O4	4	7	9	250
P7	1	100	F / 38	4	7	7	210
P9	1	100		4	7	7	100
AHV	2	100	888 M / 21	5	10	9	200

The LTC, age of the LTC, passage at which the culture was induced or calcium concentration did not affect the rate of mineralisation, for example, five cultures derived from the LTC labelled '846' mineralised between 7 and 14 days post induction regardless of the passage, age of the culture at induction or age of the LTC at detachment. Also cultures derived from LTC 'WP' were induced at both 100 and 200 mg/l $CaCl_2$ and mineralised from 7 to 21 days post induction.

Table 4.2. Non-mineralised cultures at 100 mg/l and 200 mg/l $CaCl_2$ in Alpha MEM

Culture	Passage	[CaCl₂] (mg/l)	Origin Sex / Age	Culture age at induction (days)	Cells / field at induction
SI	2	200	849 M / 40	17	90
N4	3	200	JV	13	100
ON	3	200	M / 25	14	50
TT	2	200	853	11	80
UA	3	100	F / 30	10	100
WL	2	200	UE	10	100
WM	2	100	M / ?	10	110
YX	2	100	WP	15	60
Z1	2	100	F / 37	15	90
Z2	2	100		15	100
AAU	2	100	876 M / 32	20	150
OR	2	200	NC F / 46	14	100

The reason why some cultures mineralised and others did not was unclear. Mineralisation was unlikely to be influenced by the original bone marrow or LTC because culture 'N6' mineralised (table 4.1) whilst 'N4' did not (table 4.2) (both passage 3 and both derived from LTC 'JV'). Also cultures 'U1' and 'UB' mineralised (table 4.1) whilst 'UA' and 'TT' (table 4.2) did not (passages 2 & 3, all derived from LTC '853'). One possible explanation was that the rate of growth and confluency of the cultures at induction influenced mineralisation. Most mineralised cultures (table 4.1) had microscopic cell counts ≥100 cells per field at induction with the exception of 'N6', 'T3' and 'YY', which had <100 cells at induction. The cultures induced earlier with higher cell counts were more likely to mineralise than those with lower cell counts (<100 cells) (figure 4.10).
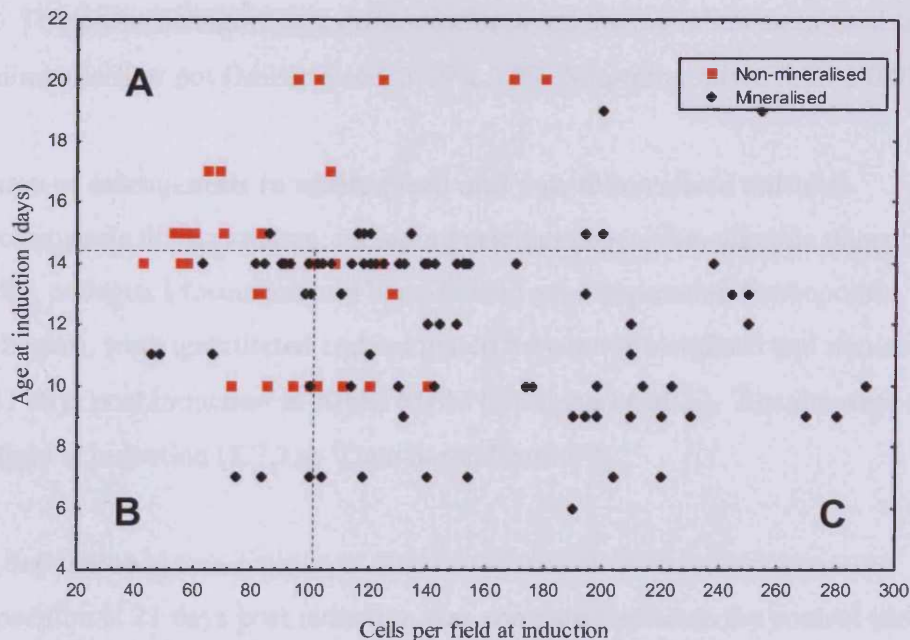
Figure 4.10. Culture age at induction against cell number at induction

The red square plots show the cultures that did not mineralise and the black circle plots show the cultures that mineralised. The data was taken for each individual culture, rather than averaged data for parallel cultures as shown in tables 4.1 and 4.2. The mineralised cultures were found to have significantly higher cell numbers at induction (mean: 151.8 cells, n=89) than the non-mineralised cultures (mean: 96.64 cells, n=41; p=0.000001). The age of the culture at induction was also found to be significantly lower in mineralised cultures (mean: 11.9, n=90) compared to non-mineralised cultures (mean: 14, n=42; p=0.00019) (figure 4.10). Section A shows mostly non-mineralised cultures that were induced after 14 days. Section B shows both mineralised and non-mineralised cultures induced with poorer cell counts before 14 days, and section C shows mostly mineralised cultures induced before 14 days with good cell counts. There was a significant difference between sections A and C (p=0.00001) and between sections B and C (p=0.0001), but not between sections A and B. This shows that cultures with good cell counts, induced before 14 days, were more likely to mineralise within 21 days of induction. Cultures with poorer cell numbers at induction and slow growth (induced after 14 days) were less likely to mineralise.

4.5.1. Summary

With the mineralised cultures having a significantly higher cell count and a significantly younger age at induction than the non-mineralised cultures, it was possible to conclude that the cultures with a more rapid growth rate were more likely to mineralise than those with poorer

growth rate. There was no significant difference between the inoculum sizes used for cultures that either mineralised or not (Mineralised: 3.19 ± 0.99, Non-mineralised 3.08 ± 0.92).

4.5.2. Markers of osteogenesis in mineralised and non-mineralised cultures

Markers of osteogenic differentiation, including calcium deposition, alkaline phosphatase (ALP) activity, collagen I formation and bone-related gene expression (osteopontin, osteocalcin, ALP and collagen), were quantitated and compared between mineralised and non-mineralised cultures at 21 days post induction in Alpha MEM (≥ 100 mg/l $CaCl_2$). Results were normalised to cells per field at induction (2.7.3.g. 'Data normalisation').

a) Calcium deposition

Calcium deposition at 21 days post induction was compared between the control (not induced) (blue triangle), mineralised (black circle) and non-mineralised cultures (red square) (figure 4.11).
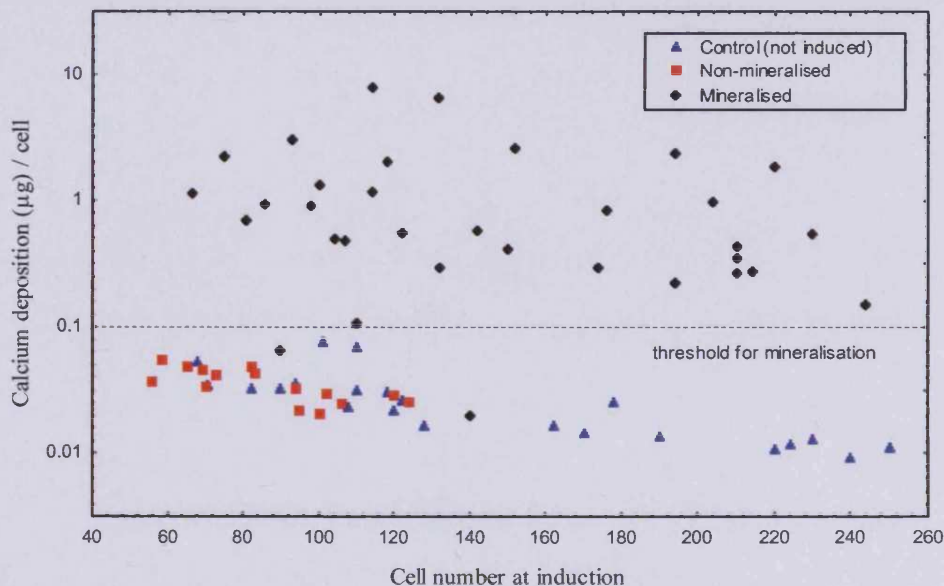
Figure 4.11. Calcium deposition at 21 days post induction per cell number at induction. Control (blue triangle), mineralised (black circle) and non-mineralised (red square) cultures

With the exception of two cultures, all the mineralised cultures had >0.1 µg calcium deposition per cell (figure 4.11). The control cultures and non-mineralised cultures had <0.1 µg calcium deposition, therefore, all cultures with less than 0.1 µg calcium deposited per cell at 21 days post induction were considered negative for mineralisation. The pattern was very similar for calcium deposition per culture (figure 4.12), which showed the amount of Alizarin red S staining in the absence of cells (dotted line along the bottom).
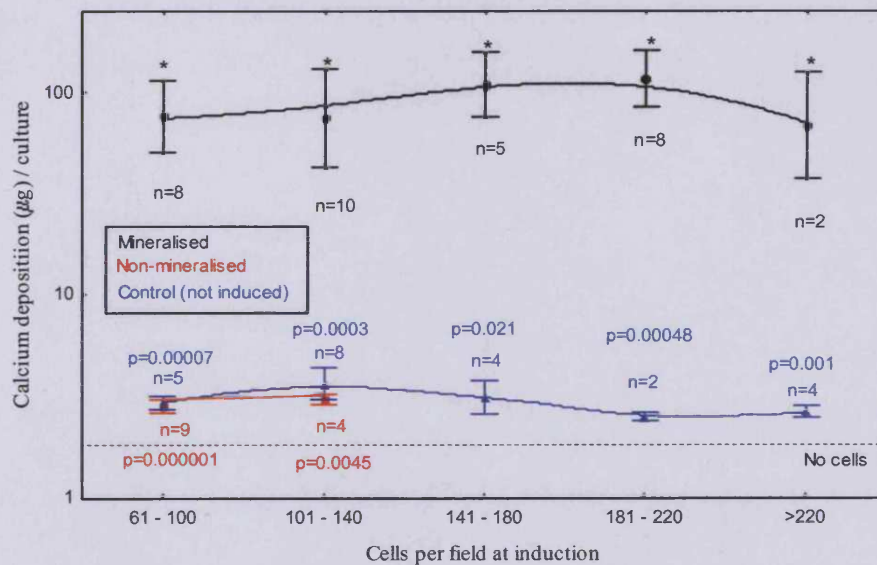
Figure 4.12. Mineralisation at 21 days post induction in Alpha MEM at $CaCl_2$ concentrations ≥ 100 mg/l. Mean $\pm$ SE

b) Cellular alkaline phosphatase activity

Cellular ALP activity was assayed by sonicating permeabilised cells and measuring the change in absorbance per minute following the addition of p-Nitrophenyl phosphate (2.7.3.e. 'Alkaline phosphatase').
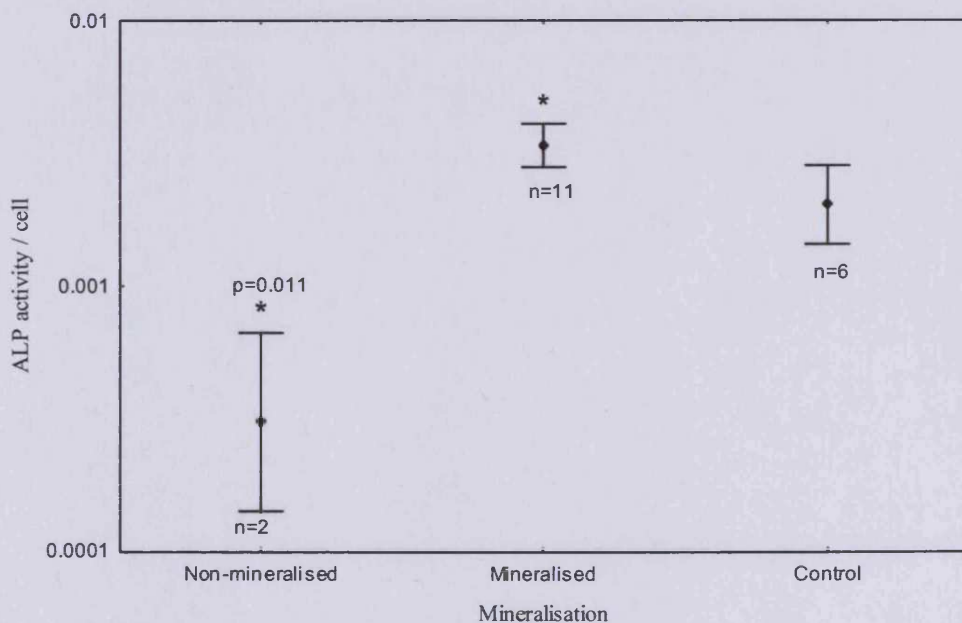
Figure 4.13. Cellular ALP activity at 21 days post induction in Alpha MEM ($CaCl_2$: ≥ 100 mg/l). Non-mineralised, mineralised and control cultures. Mean $\pm$ SE

Mineralised cultures had significantly more ALP activity per cell at 21 days post induction than non-mineralised cultures (n=11, n=2; p=0.01) (figure 4.13) indicating that ALP had an

important role in the mineralisation process. There was no significant difference between mineralised and control (not induced) cultures.

Normalising ALP activity to cell number assumed that all cells were equally positive for ALP, however, intracellular staining for ALP (4.5.4.c. 'Intracellular staining of ALP') shows this to be not the case (photos 4.10 & 4.11). ALP activity per cell must produce sufficient activity per culture to react with β-glycerophosphate within the medium to stimulate mineralisation (Bellows, Heersche, & Aubin 1992). In this respect ALP activity per culture may be a more accurate representation of its importance in mineralisation, although the pattern and statistical analysis for ALP activity per cell and per culture were similar. A small number of cells producing a small amount of ALP activity was unlikely to stimulate mineralisation, whereas a small number of cells producing a larger amount of ALP, and hence a larger amount of ALP per culture, was sufficient to promote mineralisation. Because not all the cells were actively producing ALP, it was unclear if production was increased per cell, or whether a larger proportion of synthesising cells were responsible for the increased production. Although the β-glycerophosphate was available in the non-mineralised cultures at the same concentration as the mineralised cultures, the ALP activity per cell and per culture was significantly less, which may be responsible for the lack of mineralisation.

The control cultures had comparable ALP activity to the mineralised cultures but did not mineralise due to the absence of β-glycerophosphate. This indicated that the induction agents were not responsible for stimulating ALP activity, although it was unclear what was responsible (proliferation, collagen I and ECM formation or other unknown factors?).

c) Intracellular staining of ALP

Intracellular ALP could be stained directly using a method outlined in Dacie & Lewis (Dacie & Lewis 1984) (2.7.3.f 'Intracellular staining of ALP'). No method was established for quantitating intracellular ALP staining, however, photos 4.10 & 4.11 show that ALP activity was scattered throughout the culture in many, but not all, cells.
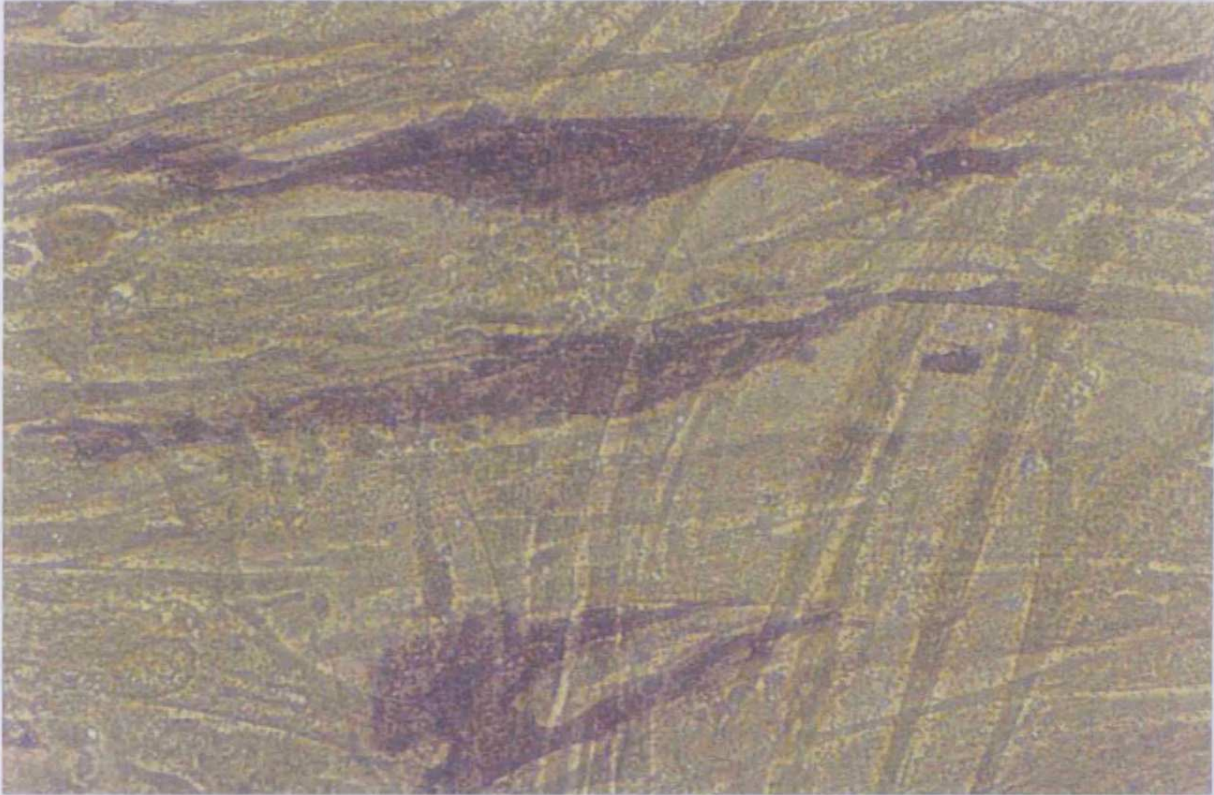
Photo 4.10. High power magnification (x20) of intracellularly stained ALP positive BMdSCs.
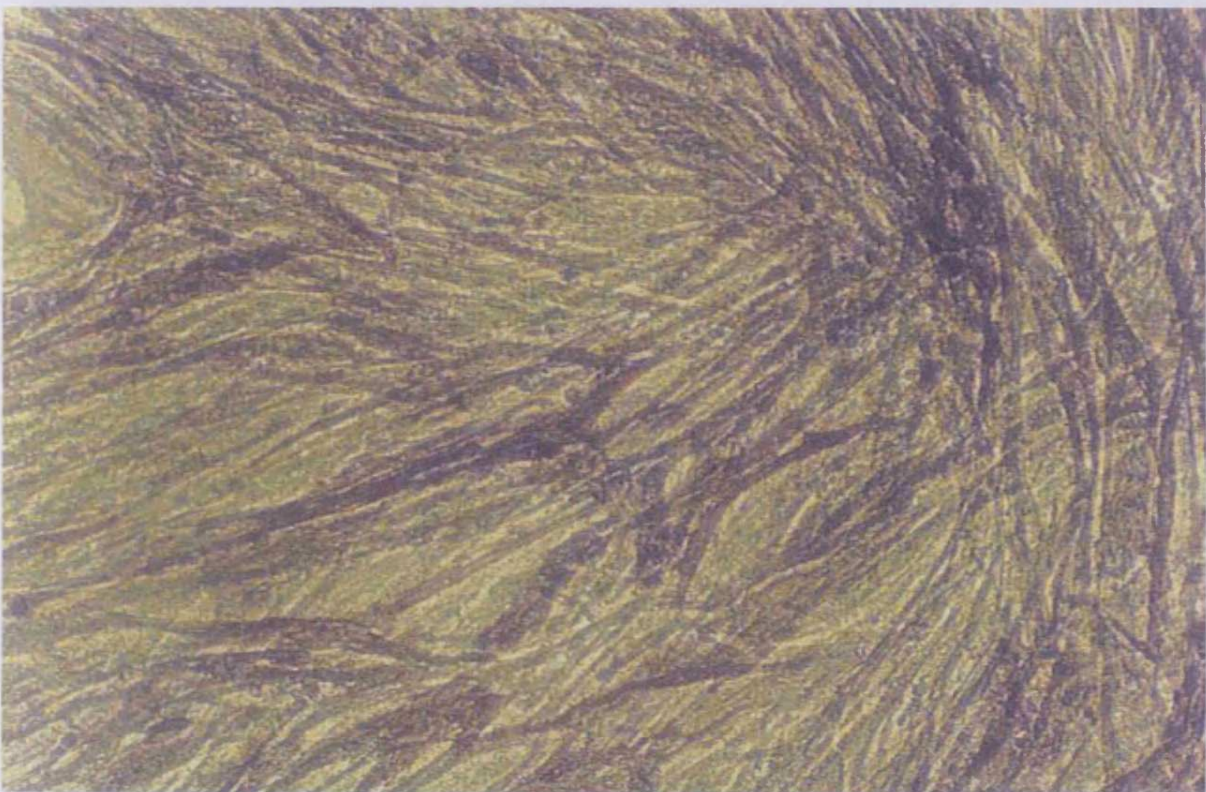
Photo 4.11. Low power magnification (x10) of a collection of ALP positive BMdSCs

d) Collagen I

Collagen, type I, was stained at 21 days post induction using an immunocytochemical technique involving the ABC reaction, and quantitated using the flask reader (2.7.3.d. 'Collagen I'). The stained collagen I appeared as a fibrous mesh on top of the cells, which could be patchy or dense across the flask (photo 4.12). Not all cells within the BMdSC layer were likely to be actively producing collagen I.
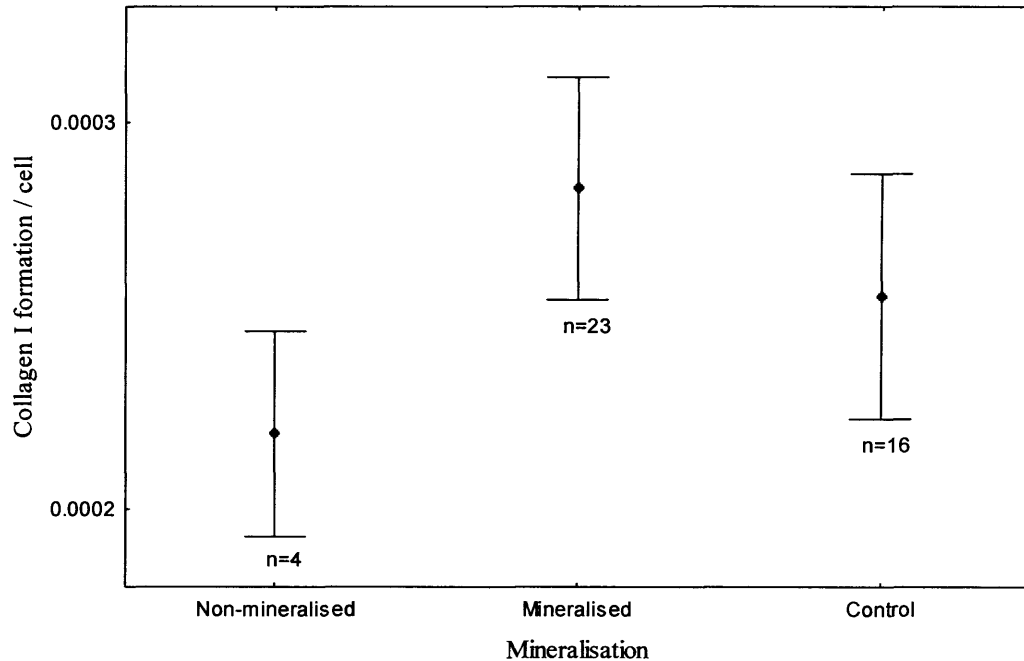
Figure 4.14. Collagen I formation per cell at 21 days post induction in Alpha MEM (CaCl$_2$: $\geq$100 mg/l). Non-mineralised, mineralised and control cultures. Mean ± SE

No significant differences in collagen I production per cell were observed between non-mineralised, mineralised and control cultures at 21 days post induction (figure 4.14). When collagen I was quantitated per culture (figure 4.15), the non-mineralised cultures were found to have significantly less collagen I formation than the mineralised cultures (n=4, n=23; p=0.017) at 21 days post induction. The control cultures were comparable to the mineralised cultures, suggesting that the induction agents had no effect on collagen I formation.
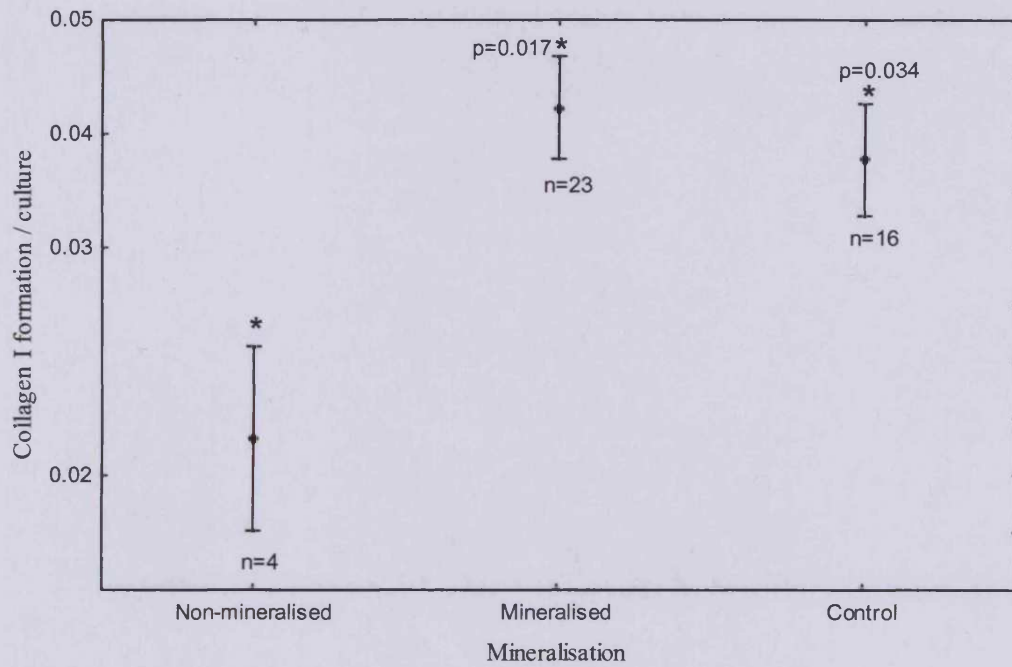
Figure 4.15. Collagen I staining per culture at 21 days post induction in Alpha MEM (CaCl$_2$: ≥100 mg/l). Non-mineralised, mineralised and control cultures. Mean ± SE
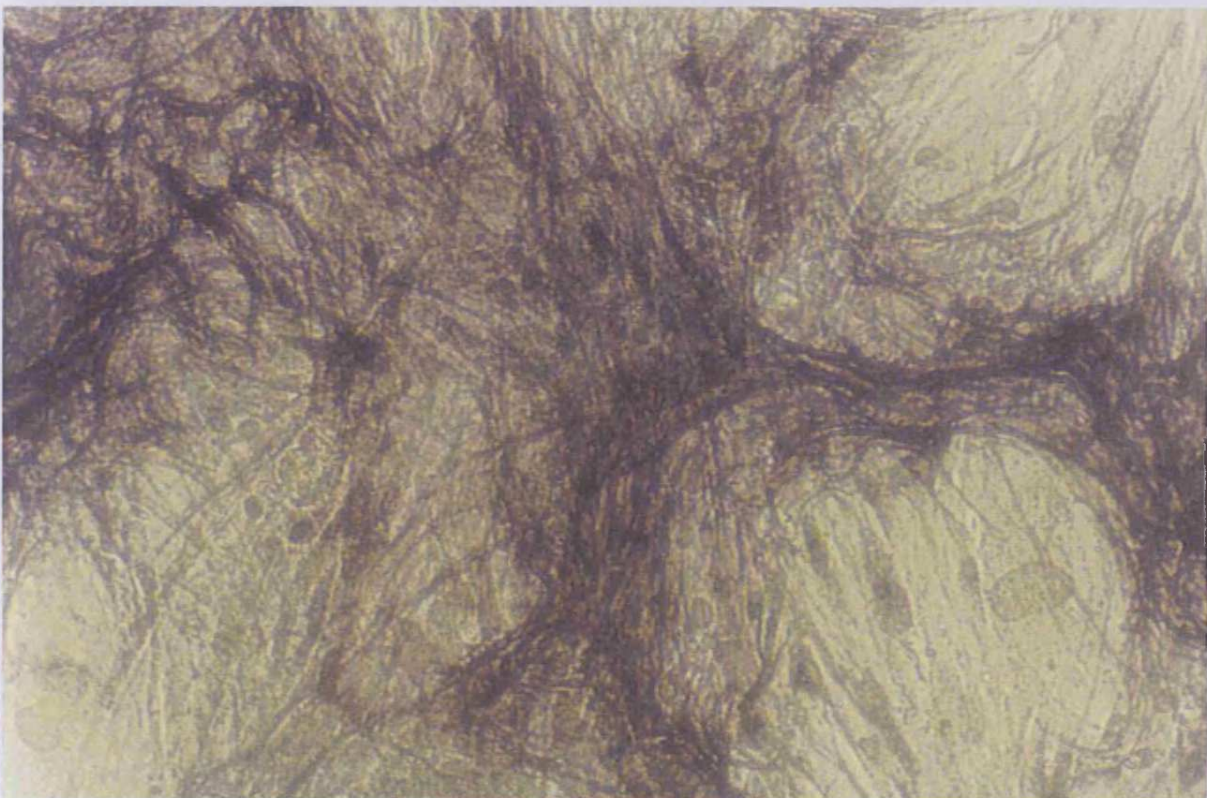
Photo 4.12. Collagen I staining at 21 days post induction in an osteogenically induced culture. Collagen I forms as a fibrous mesh across the flask

The mineralised group in figure 4.14 included 18 cultures that grew well (≥ 100 cells at induction) and 5 cultures that grew poorly (<100 cells at induction). Collagen I production per cell was significantly greater in the 5 mineralised cultures with low cell counts at induction than the 4 non-mineralised cultures (p=0.027) (not shown). A small number of cells producing large amounts of collagen I were able to create culture levels of collagen I sufficient to stimulate mineralisation (figure 4.16).
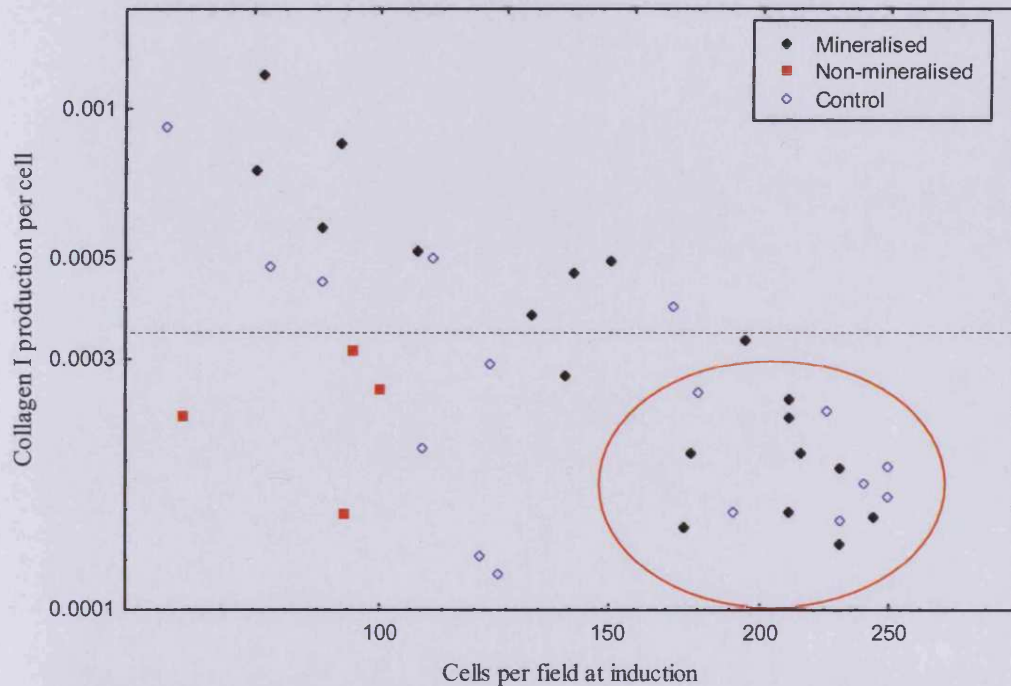
Figure 4.16. Collagen I production per cell in relation to cell number at induction. Mineralised (black circles), non-mineralised (red squares) and control cultures (blue circles open)

There were two mineralised populations of cells (black circles) (figure 4.16). One population, identified by the red ring, had a low collagen I production per cell and a larger number of cells at induction (high collagen I production per culture), whilst the other population above the dotted line had a high collagen I production per cell and a smaller number of cells at induction (high collagen I production per culture). Both populations produced sufficient levels of collagen I per culture to induce mineralisation. The non-mineralised cultures (red squares) produced insufficient levels of collagen I per culture to initiate mineralisation with a low cell count and a low collagen I production per cell.

Within the mineralised population, there was a negative correlation between collagen I production per cell and cell number (r=0.62) (figure 4.16). A larger number of cells could produce less collagen I per cell and still reach the culture threshold value for mineralisation, whereas a smaller number of cells needed to produce much more collagen I per cell to reach the

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threshold value. This indicated that collagen I producing cells within a more confluent cell layer received a greater negative feedback on synthesis.

In summary, threshold values of collagen I and ALP activity per culture appeared to be necessary for mineralisation to occur. There appeared to be a relationship between collagen I production and ALP activity but due to the poor number of samples analysed for ALP activity, no correlation was seen. It was unclear if collagen I concentration influenced ALP activity or vice versa, or whether synthesis occurred simultaneously, although Shiga (Shiga et al 2003) reported that deposition of type I collagen induced the bone/liver isoenzyme of alkaline phosphatase. Also Coelho (Coelho, Cabral, & Fernandes 2000) reported that ascorbate was related to a dose-dependent synthesis of collagen, type I, and that the resulting increase in extracellular matrix was associated with a higher ALP activity and ability to form a mineralised matrix. It is possible that the same cells were responsible for both collagen I and ALP production.

e) Gene expression

Osteopontin, osteocalcin, collagen I and ALP gene expression (normalised to the housekeeping gene S14) were assessed in mineralised, non-mineralised and control (not induced) cultures at 21 days post induction in Alpha MEM (≥ 100 mg/l $CaCl_2$).

i. Osteopontin
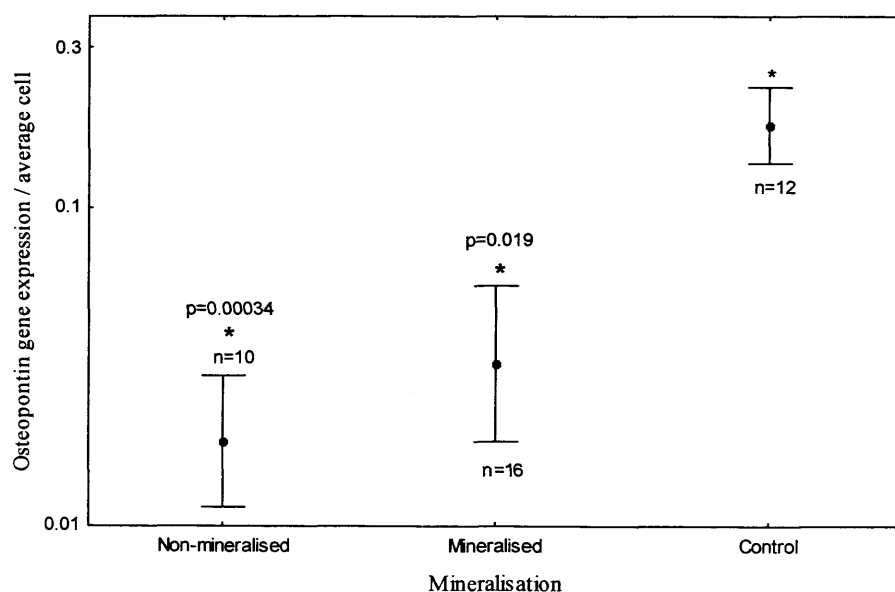
Figure 4.17. Osteopontin gene expression in non-mineralised, mineralised and control cultures at 21 days post induction in Alpha MEM ($CaCl_2$: ≥ 100 mg/l). Mean $\pm$ SE

The control cultures had significantly more osteopontin gene expression per average cell than both the mineralised (n=12, n=16; p=0.019) and non-mineralised cultures (n=10; p=0.00034).

ii. **Osteocalcin**
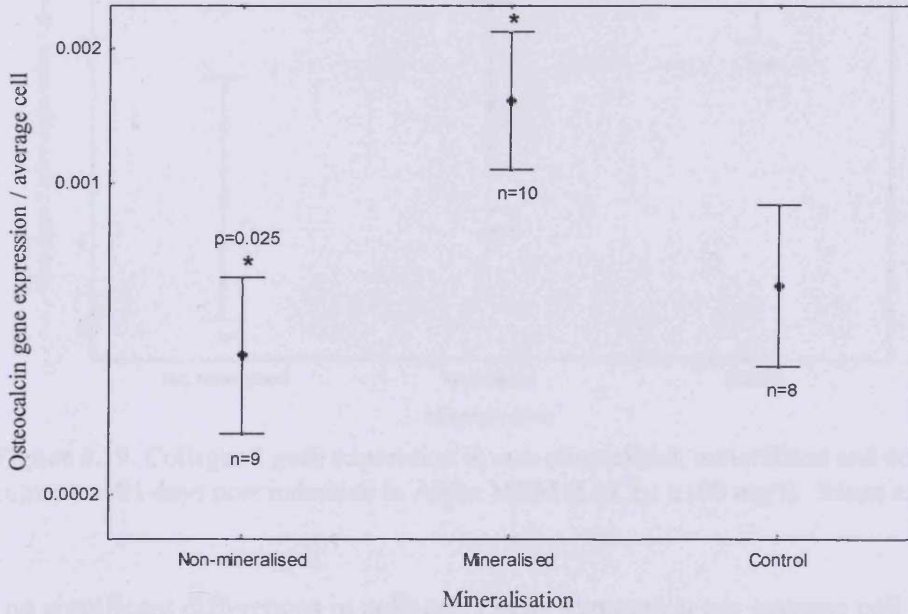
Figure 4.18. Osteocalcin gene expression in non-mineralised, mineralised and control cultures at 21 days post induction in Alpha MEM (CaCl$_2$: ≥100 mg/l). Mean ± SE

The mineralised cultures had significantly more osteocalcin gene expression per average cell at 21 days post induction in Alpha MEM (CaCl$_2$: ≥100 mg/l) than the non-mineralised cultures (n=10, n=9; p=0.025). There was no significant difference between the mineralised and control cultures.

iii. Collagen I
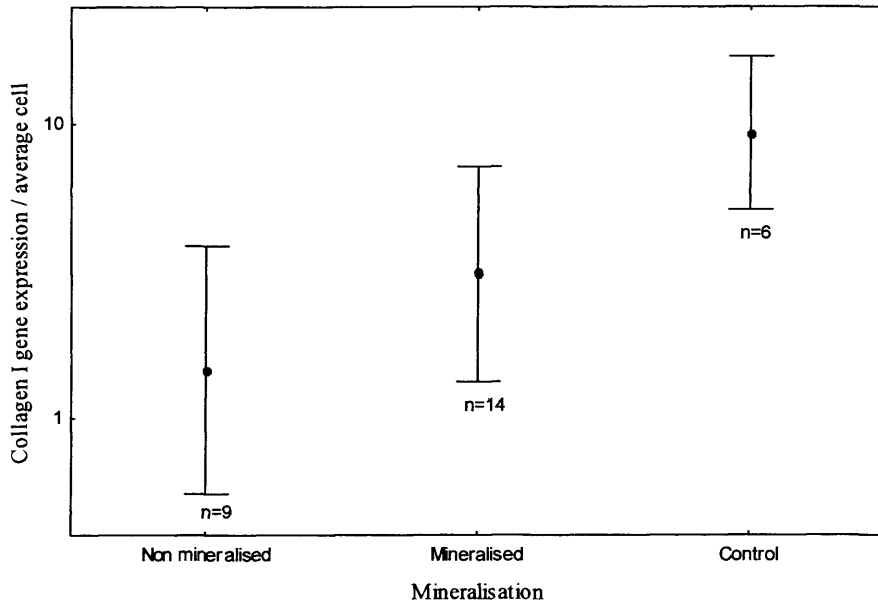
Figure 4.19. Collagen I gene expression in non-mineralised, mineralised and control cultures at 21 days post induction in Alpha MEM ($CaCl_2$: ≥100 mg/l). Mean ± SE

There were no significant differences in collagen I gene expression per average cell or per culture at 21 days post induction in non-mineralised, mineralised and control cultures. This corresponds to collagen I formation per cell (figure 4.14) and may become significant if only analysed in the mineralised cultures that grew poorly (<100 cells).

iv. Alkaline phosphatase
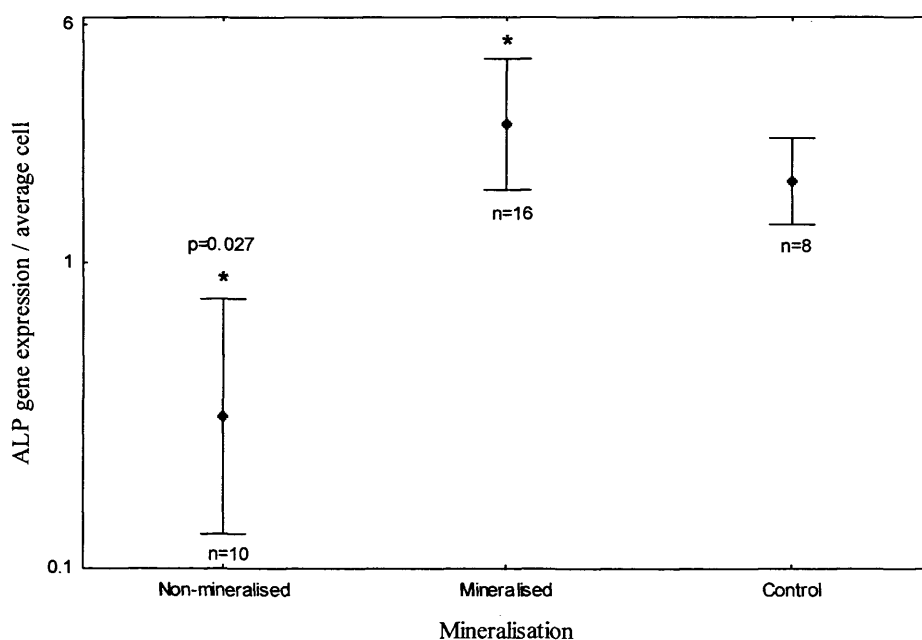
Figure 4.20. ALP gene expression in non-mineralised, mineralised and control cultures at 21 days post induction in Alpha MEM ($CaCl_2$: ≥100 mg/l). Mean ± SE

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The mineralised cultures had significantly more ALP gene expression per average cell than the non-mineralised cultures (n=16, n=10; p=0.027) and were comparable to the control cultures (figure 4.20). This data corresponds to ALP activity per cell and per culture (figure 4.13).

There was a positive relationship between the relative gene expression levels of collagen I and ALP (r=0.9) (figure 4.21) with an increase in collagen I expression corresponding to an increase in ALP.
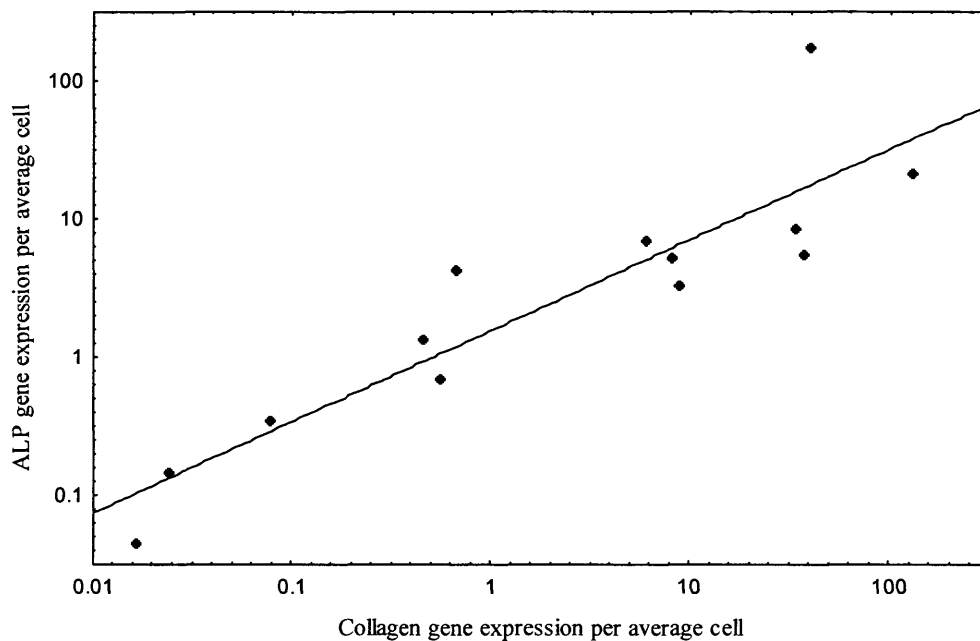
Figure 4.21. Relationship between collagen gene expression and ALP gene expression. $(10^{\wedge}(\log_{10}\text{ALP}$ gene expression $= 0.1895 + 0.6587*x))$ $(r = 0.9)$

This relationship was not clear with collagen I staining and cellular ALP activity due to the poor number of samples analysed. The relationship indicated a close interaction between the cells responsible for collagen I and ALP expression. It is possible that the same cells were stimulated to produce both collagen I and ALP gene expression.

f) Summary

Younger cultures with higher cell counts at induction were more likely to mineralise within 21 days of induction in Alpha MEM. The mineralised cultures had significantly more calcium deposition, ALP (activity and mRNA) and collagen I formation per culture, and significantly more osteocalcin gene expression per average cell at 21 days post induction than the non-mineralised cultures. The control cultures were comparable to the mineralised cultures for ALP (activity and mRNA), collagen I (protein and mRNA) and osteocalcin gene expression, which

indicated that the induction agents were not responsible for stimulating production. They were, however, required for mineralisation.

4.6. Markers of Osteogenic Differentiation at 100 mg/l CaCl$_2$

Calcium deposition, ALP activity, collagen I formation and bone-related gene expression (osteopontin, osteocalcin, collagen I and ALP) were quantitated at distinct time points during induction in mineralised and control cultures in Alpha MEM (CaCl$_2$: 100 mg/l). The data was normalised to cell counts per microscopic field at induction.

4.6.1. Calcium deposition

Calcium deposition was shown using Alizarin red S staining (2.7.3.b. 'Mineralisation'), and quantitated by demineralising in HCl. The optical density was measured using a spectrophotometer (2.7.3.c. 'Demineralisation').
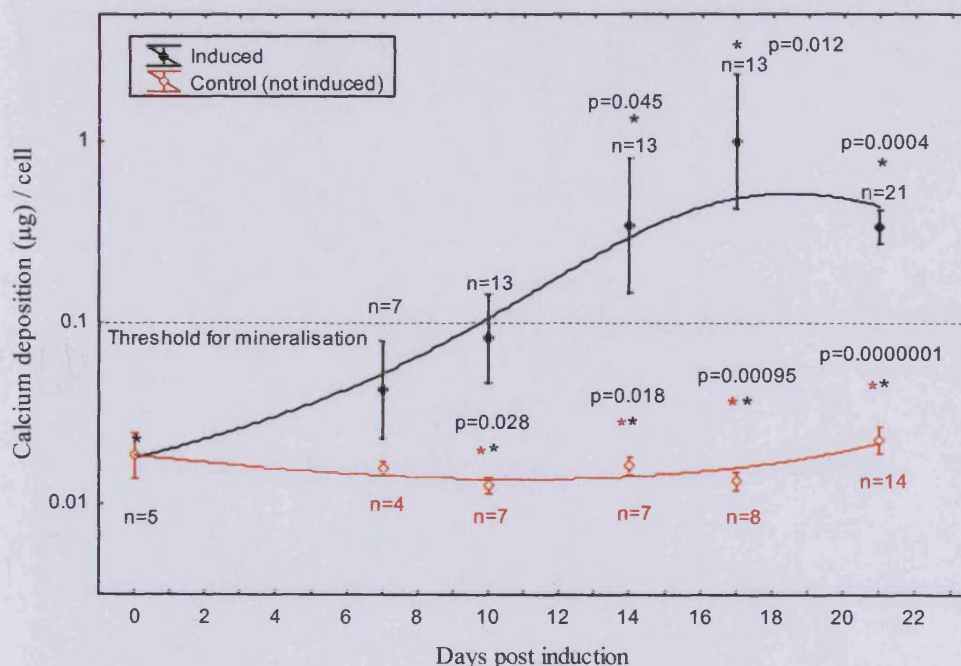
Figure 4.22. Calcium deposition per cell in osteogenically induced (black closed) and control (red open) cultures as a function of time during induction in Alpha MEM (CaCl$_2$: 100 mg/l). Mean ± SE

There was a significant increase in calcium deposition from the time of induction (day 0, n=5) to day 14 (n=13, p=0.045), day 17 (n=13; p=0.012) and day 21 (n=21; p=0.0004) in the osteogenically induced cultures in Alpha MEM. Photo 4.13 shows the increase in Alizarin Red S staining with induction time, which reaches a plateau by day 14. The control cultures showed no calcium deposition throughout the induction period with significantly more calcium in the

induced cultures at day 10 (n=13, n=7; p=0.028), 14 (n=13, n=7; p=0.018), day 17 (n=13, n=8; p=0.00095) and day 21 (n=21, n=14; p=0.000001).

Photo 4.13. Alizarin Red S staining at 0, 10, 14 and 21 days post induction

a) Calcium removed from the medium during 21 days of induction

To assess the origin of the calcium used in the mineralisation process the calcium concentration in the conditioned Alpha medium of mineralised and control cultures at each feeding was quantitated using the O'cresolphthalein complexone technique (2.7.3.a. 'Medium calcium utilization by cells'). Uninoculated flasks were incubated in parallel with Alpha MEM as a baseline medium calcium concentration.
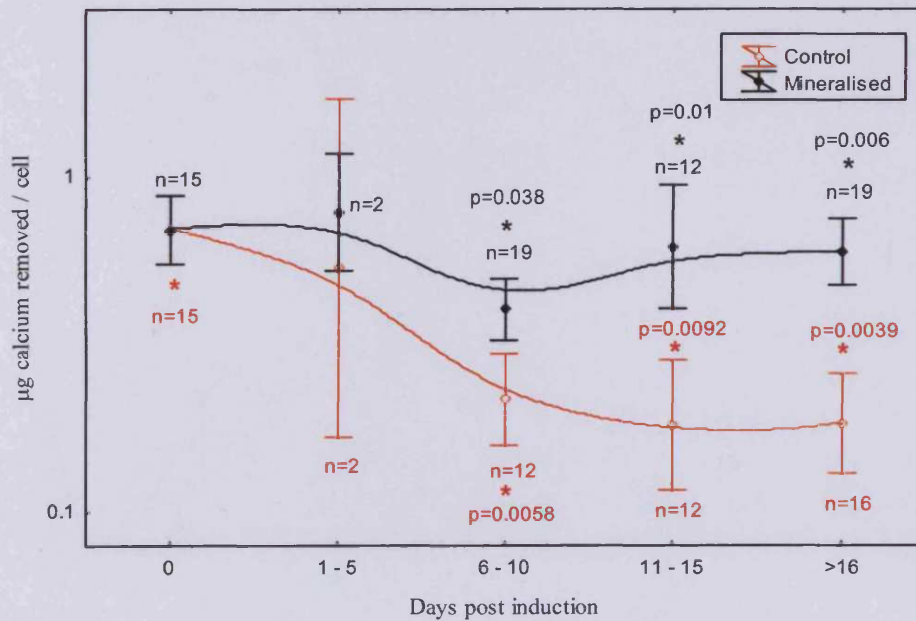
Figure 4.23. Calcium removed from conditioned Alpha MEM from mineralised (black closed) and control (red open) cultures as a function of time during induction. Mean ± SE

Significantly less calcium was removed from the medium of the control cultures (red open) as a function of time with 6 – 10 days (n=12, n=15; p=0.0058), 11 – 15 (n=12; p=0.0092) and >16 days (n=16; p=0.0039) removing significantly less calcium from the medium than at day 0. This could reflect a diminishing proliferative capacity with time (cells becoming senescent). The induced cultures were removing significantly more calcium from the medium than the control cultures by 6 – 10 days (n=19, n=12; p=0.038), 11 – 15 (n=12; p=0.01) and >16 days (n=19, n=16; p=0.006) post induction, but mineralised cultures showed no significant increase in calcium uptake post induction. In this respect, day 0 represents the maximum uptake of calcium from the medium, which is maintained in mineralised cultures during mineralisation. Balint (Balint et al 2001) osteogenically induced mouse BMdSCs at confluence and reported minimal uptake of calcium by the cells until 16 – 18 days post induction (matrix maturation and calcification). Further studies would be to analyse the calcium concentration of the conditioned medium during proliferation (days before induction) to ascertain how much calcium was utilised from the medium for growth. This could then be compared to the amount of calcium utilised for differentiation.

4.6.2. Cellular alkaline phosphatase activity

Cellular ALP activity was assayed at various time points within the induction period by measuring the change in absorbance per minute following the addition of p-Nitrophenyl phosphate (2.7.3.e. 'Alkaline phosphatase').
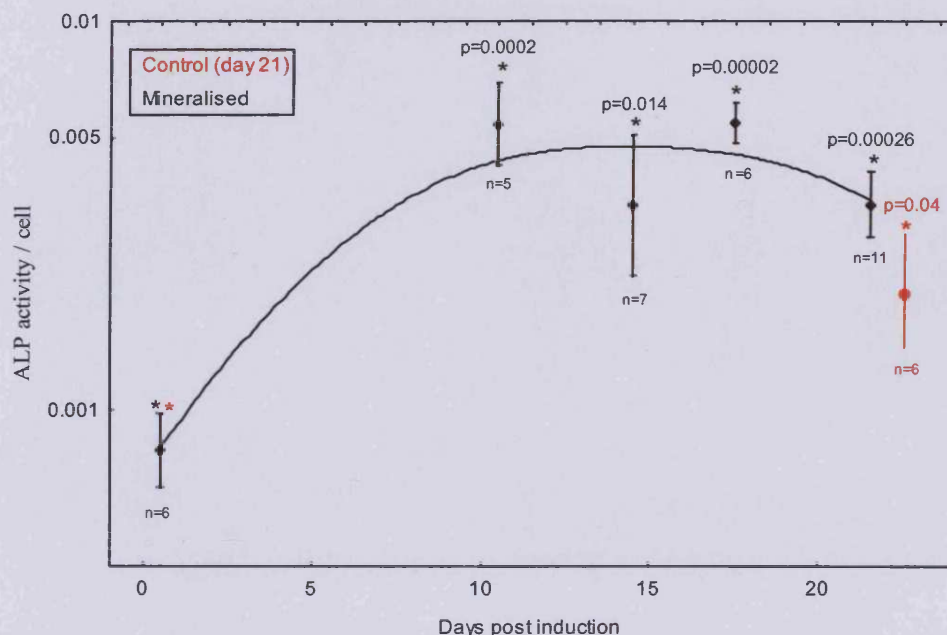
Figure 4.24. ALP activity per cell in osteogenically induced (black) and control (red) cultures as a function of time during induction in Alpha MEM (CaCl$_2$: 100 mg/l). Mean ± SE

There was a significant increase in ALP activity per cell in the osteogenically induced (mineralised) cultures from the time of induction (day 0) to day 10 (n=5, n=6; p=0.0002), day 14 (n=7; p=0.014), day 17 (n=6; p=0.00002) and day 21 (n=11; p=0.00026). The levels reached a plateau by day 10. There was a significant increase in ALP activity in the control cultures from the time of induction to day 21 (n=6, n=6; p=0.04), which may represent continuing proliferation and ECM formation within the culture. Unfortunately no control cultures were assessed for ALP activity between 0 and 21 days, and no induced cultures were assessed between 0 and 10 days post induction. No significant differences were observed between the control and mineralised cultures.

4.6.3. **Collagen I**

Collagen I was stained at various time points within the induction period using the ABC immunocytochemical technique (2.7.3.d.i 'Collagen I') and quantitated using the flask reader.
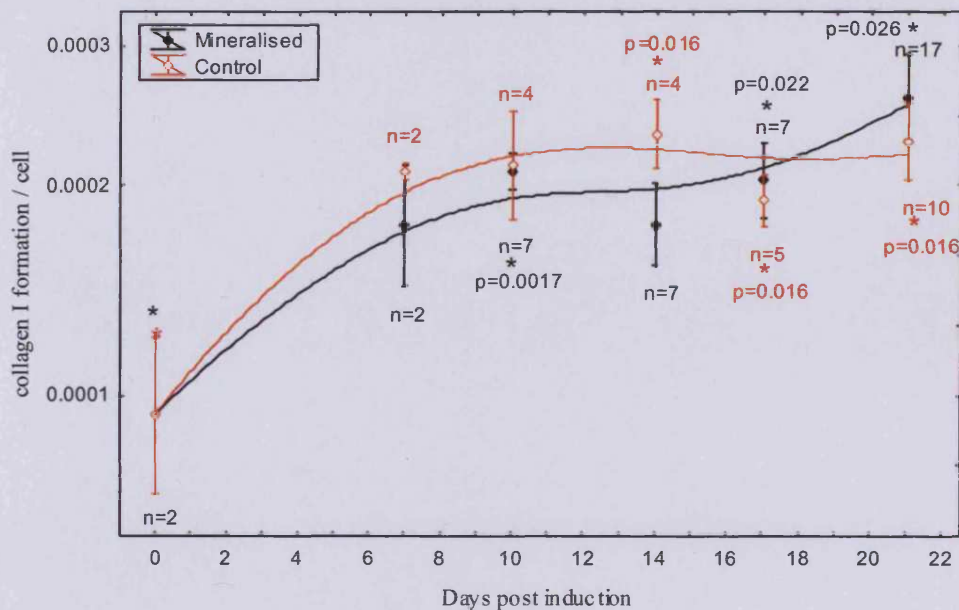
Figure 4.25. Collagen I formation per cell in osteogenically induced (black closed) and control (red open) cultures as a function of time during induction in Alpha MEM ($CaCl_2$: 100 mg/l). Mean ± SE

The pattern of collagen I staining per culture and per cell was similar. The mineralised cultures showed a significant increase in collagen I deposition per cell from day 0 (n=2) to day 10 (n=7; p=0.0017), 17 (n=7; p=0.022) and day 21 (n=17; p=0.026). The control cultures also showed a significant increase in collagen I formation from day 0 to day 14 (n=4; p=0.016), 17 (n=5; p=0.016) and 21 (n=10; p=0.016) (figure 4.25). The staining reached a plateau by day 10. The deposition of collagen I post induction follows on from that observed during proliferation (figure 3.40). Photo 4.14 shows dense comparable staining at 10, 14 and 21 days post induction in comparison to the weak staining at the start of induction.

Photo 4.14. Collagen I staining at 0, 10, 14 and 21 days post induction

4.6.4. Calcium deposition in relation to collagen I formation

Photo 4.15 shows cultures initially stained for Alizarin red S, which were demineralised and subsequently stained for collagen I formation.
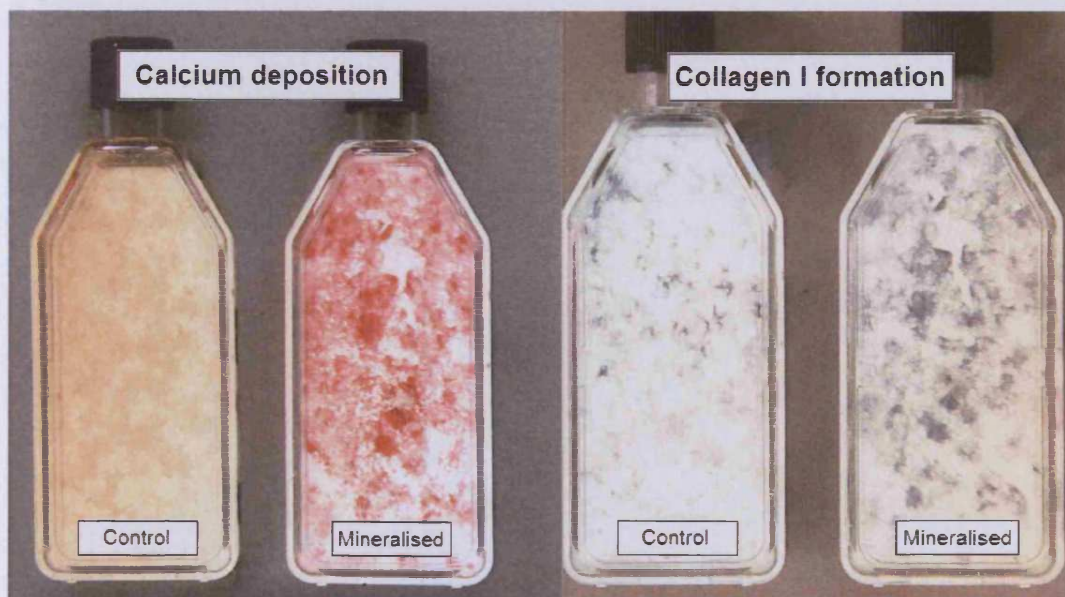
Photo 4.15. Calcium deposition and collagen I formation in control and mineralised cultures at 21 days post induction in Alpha MEM.

The pattern of Alizarin red S staining and collagen I staining were comparable, showing the interaction of hydroxyapatite crystals and ECM.

4.6.5. **Relative gene expression related to osteogenic induction**

RNA was extracted (2.9.1. 'RNA extraction') from mineralised and control BMdSC cultures at distinct time points during induction. The RNA was converted to cDNA (2.9.2. 'Reverse transcriptase reaction') and the gene expression (osteopontin, osteocalcin, ALP and collagen I) was analysed by real time PCR (2.9.4. 'Real time analysis'). The results were normalised to the housekeeping gene, S14, using the following equation: Index of gene expression = $2^{(\text{Housekeeping CT} - \text{Test CT})}$. The PCR products were confirmed by sequencing (2.9.7. 'Automated sequencing') (Appendix III). The relative gene expression of the four osteogenic-related genes is shown in figure 4.26 (the housekeeping gene is equivalent to 1).
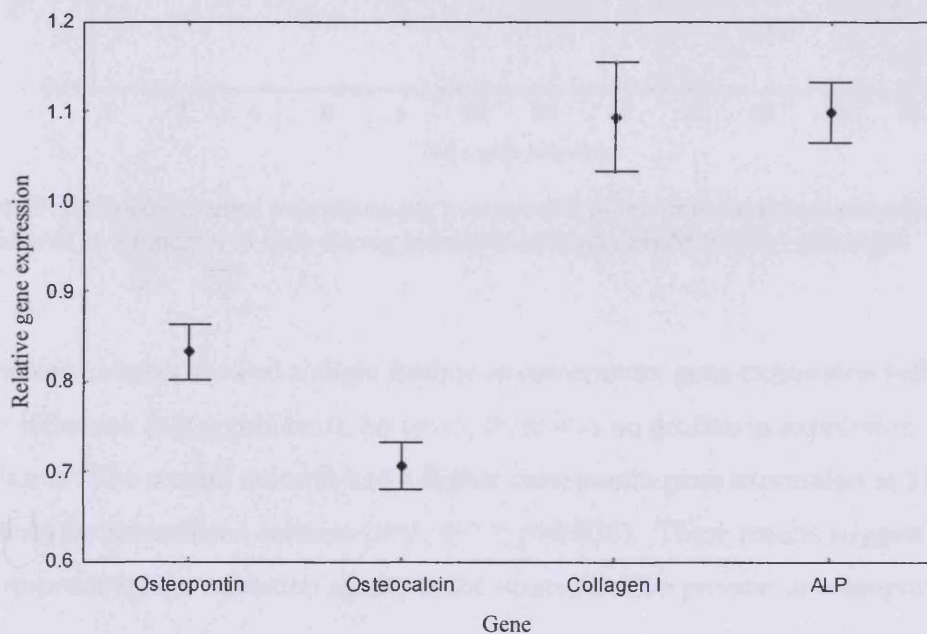
Figure 4.26. Relative gene expression in mineralised cultures at 21 days post induction in Alpha MEM (CaCl$_2$: 100 mg/l). Mean ± SE

The relative gene expression for osteocalcin (n=6) was significantly less than osteopontin (n=11; p=0.028), collagen I (n=9; p=0.02) and ALP (n=11; p=0.018) in the mineralised cultures at 21 days post induction. The relative gene expression for osteopontin was significantly less than collagen I (p=0.0077) and ALP (p=0.0051). It is possible that the same cells were responsible for producing all bone-related mRNA, but ALP and collagen I were produced at a significantly higher level, or it may reflect more cells within the culture actively producing ALP and collagen I mRNA and less cells producing osteopontin and osteocalcin mRNA.

a) **Osteopontin**
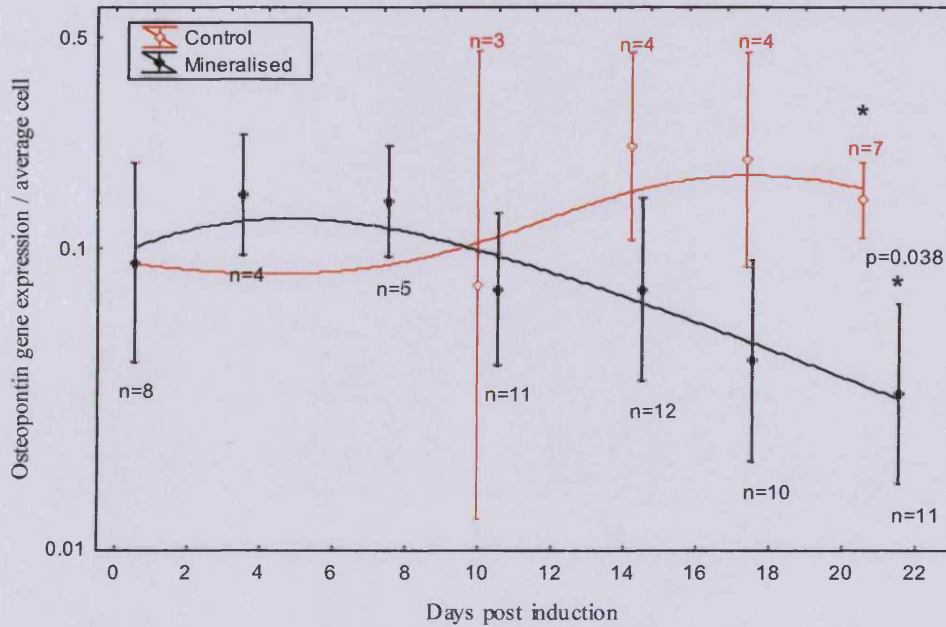
Figure 4.27. Osteopontin gene expression per average cell in mineralised (black closed) and control (red open) cultures as a function of time during induction in Alpha MEM (CaCl$_2$: 100 mg/l). Mean ± SE

The mineralised cultures showed a slight decline in osteopontin gene expression following 3 to 7 days post induction (not significant), however, there was no decline in expression within the control cultures. The control cultures had a higher osteopontin gene expression at 21 days post induction than the mineralised cultures (n=7, n=11; p=0.038). These results suggest an inhibitory response by the induction agents or the mineralisation process to osteopontin gene expression.
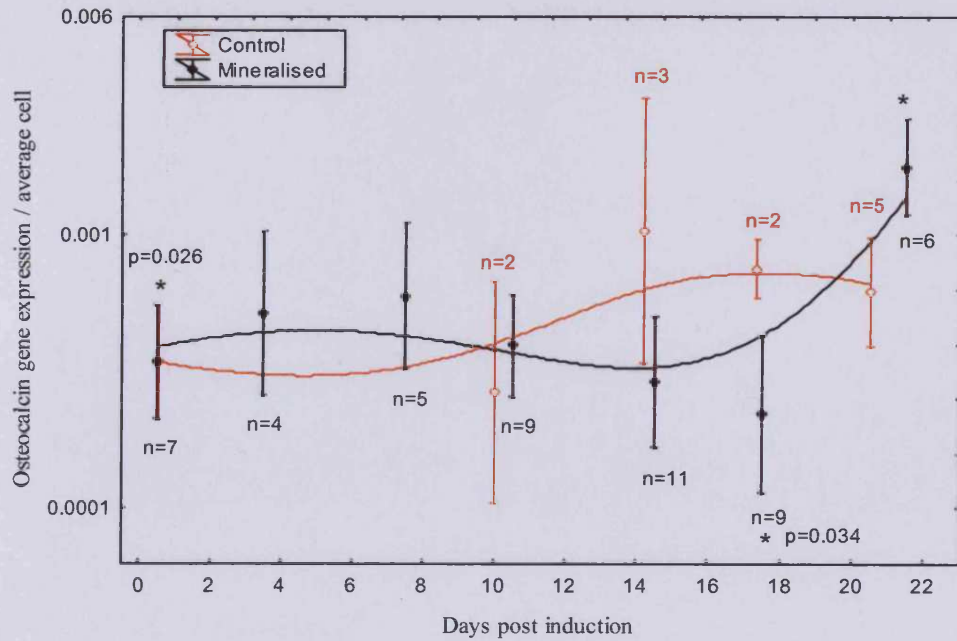
b) **Osteocalcin**

Figure 4.28. Osteocalcin gene expression per average cell in mineralised (black closed) and control (red open) cultures as a function of time during induction in Alpha MEM ($CaCl_2$: 100 mg/l). Mean ± SE

No significant differences were observed between the osteogenically induced and control cultures. There were no significant changes in osteocalcin gene expression in the mineralised cultures between 0 and 17 days, but the level of expression rapidly increased from day 17 to day 21 (n=9, n=6; p=0.034). This may have increased further if analysed for longer.

c) Collagen I
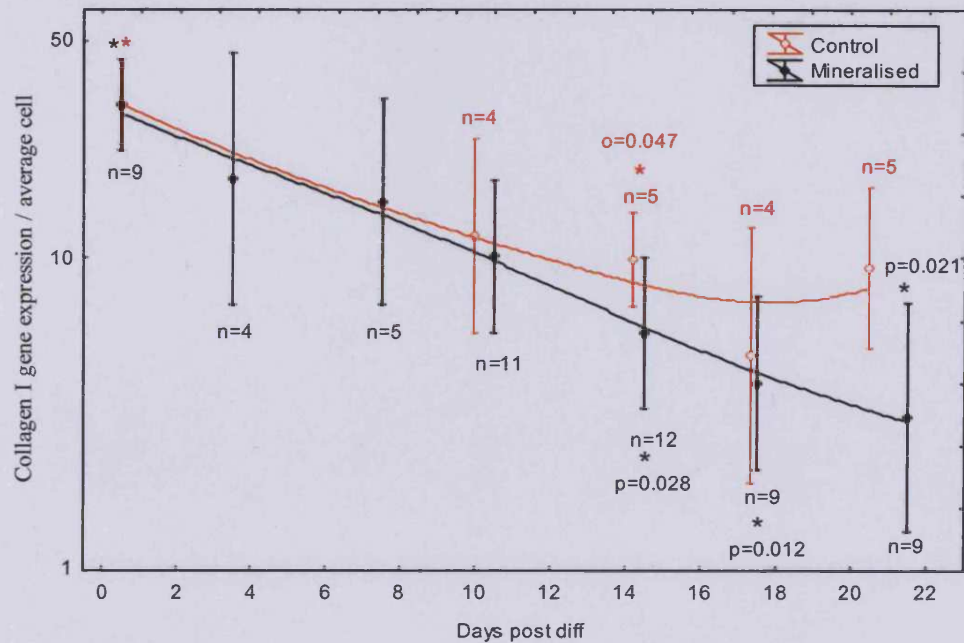
Figure 4.29. Collagen I gene expression per average cell in mineralised (black closed) and control (red open) cultures as a function of time during induction in Alpha MEM (CaCl$_2$: 100 mg/l). Mean ± SE

Collagen I gene expression was significantly down-regulated in mineralised cultures from day 0 to day 14 (n=9, n=12; p=0.028), day 17 (n=9; p=0.012) and day 21 (n=9; p=0.021), and in the control cultures from day 0 to day 14 (n=9, n=5; p=0.047) (figure 4.29). No significant differences were observed between the mineralised and control cultures. This decline in expression following induction may have indicated that collagen I gene expression was no longer required for ECM formation and correlated with a plateau in collagen I staining by day 10 (figure 4.25).

d) Alkaline phosphatase
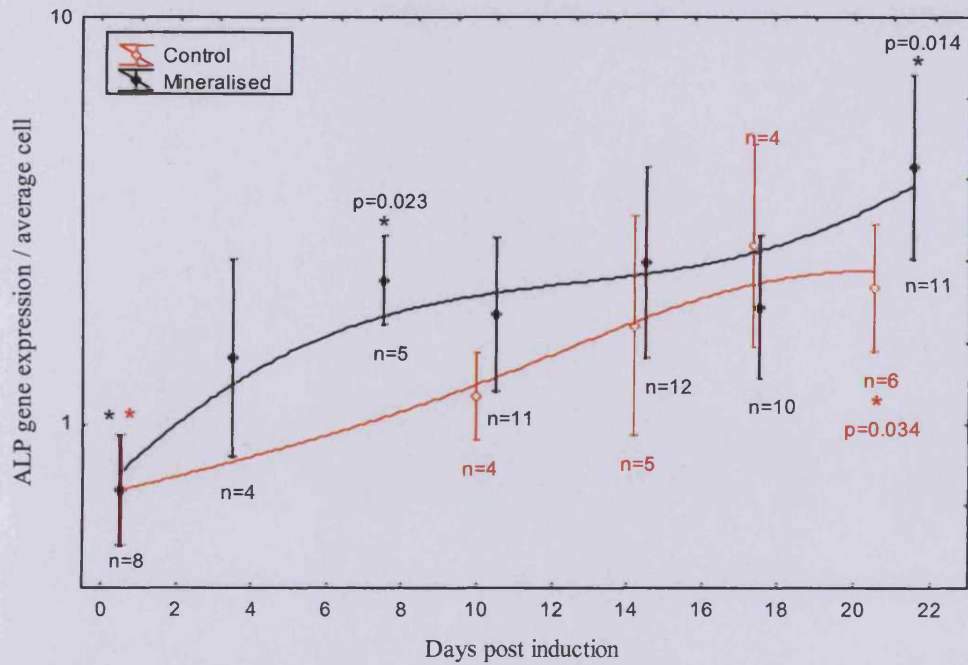
Figure 4.30. ALP gene expression per average cell in mineralised (black closed) and control (red open) cultures as a function of time during induction in Alpha MEM ($CaCl_2$: 100 mg/l). Mean $\pm$ SE

ALP gene expression was significantly upregulated in the mineralised cultures from day 0 (n=8) to day 7 (n=5; p=0.023) and day 21 (n=11; p=0.014), and in the control cultures from day 0 to day 21 (n=6; p=0.034). No significant differences were observed between the control and mineralised cultures.

4.6.6. Summary
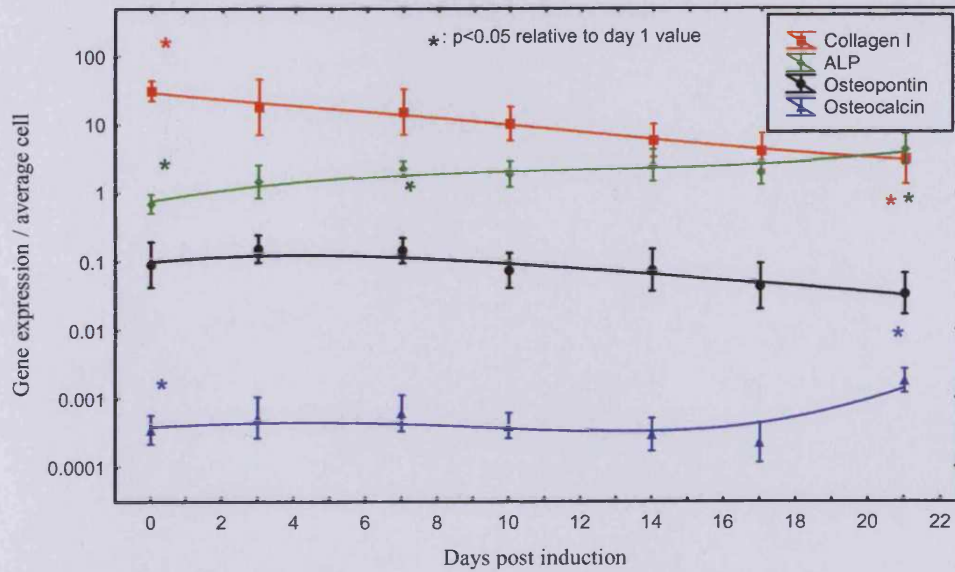
Figure 4.31. Collagen I, ALP, osteopontin and osteocalcin gene expression as a function of time post induction. Mean ± SE (*: $p<0.05$ relative to day 1 value)
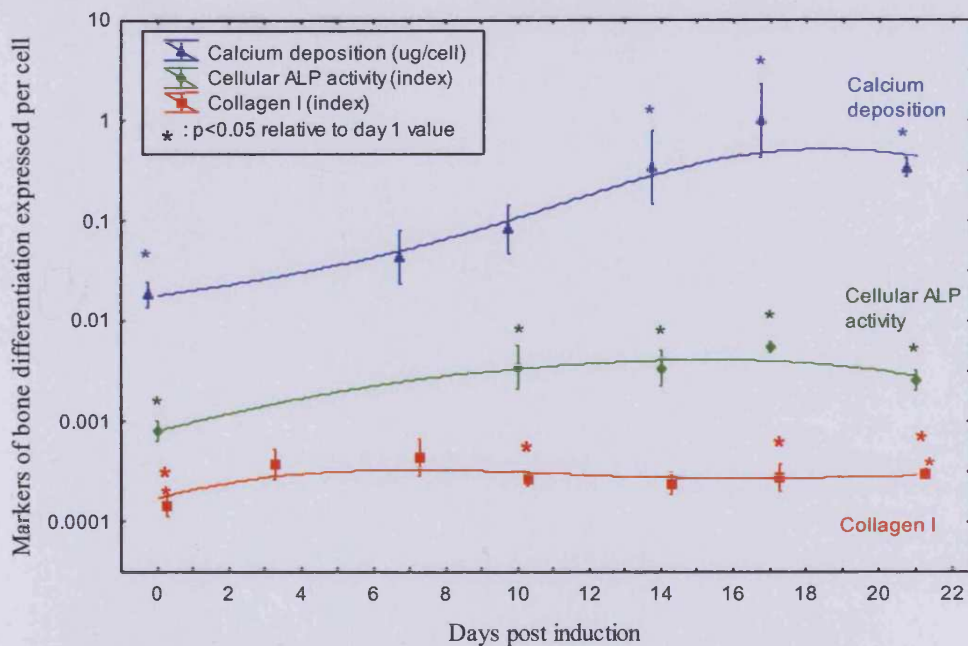
Figure 4.32. Calcium deposition, ALP activity and collagen I formation as a function of time post induction. Mean ± SE (*: $p<0.05$ relative to day 1 value)

Throughout the time course of the experiment there was a significant increase in calcium deposition, cellular ALP activity and collagen I staining in the osteogenically induced cultures. Levels plateaued at approximately day 14 for calcium deposition and day 10 for ALP activity and collagen I staining. This correlated with a down-regulation of collagen I gene expression post induction, however, ALP gene expression continued to increase, suggesting a continued need for ALP. Osteopontin gene expression within the mineralised cultures declined post

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induction (not significantly), but remained fairly constant within the control cultures, which may suggest a slight inhibitory response by the induction agents. Malaval (Malaval et al 1994) also reported a reduction in expression following the onset of mineralisation but found no significance. There was no significant change in osteocalcin gene expression from day 0 to day 17.

With the exception of mineralisation and osteopontin gene expression, there were no significant differences between the control and mineralised cultures at 100 mg/l $CaCl_2$ in Alpha MEM. This indicated that the induction agents did not significantly influence bone-related markers of differentiation *in vitro*. It may suggest that osteogenesis was occurring spontaneously in the cultures, requiring the induction agents simply to calcify.

4.7. Comparison between Alpha MEM and DMEM

BMdSC cultures were osteogenically induced in either DMEM or Alpha MEM (100 mg/l $CaCl_2$).

Table 4.3. Cultures induced at 100 mg/l $CaCl_2$ in DMEM or Alpha MEM

Culture	Passage	Medium	Origin Sex /Age	Age of LTC at subculture (wks)	Age at induction (days)	Cells per field at induction	Mineralised
N5	3	DMEM	826 M / 40	7	9	100	NO
ABC	2	DMEM	YH F / 62	5	14	120	NO
NA	3	DMEM	J6 M / 25	13	10	100	Day 10
ABR	1	DMEM	881 M / 72	5	10	100	NO
AHV	2	DMEM	888 M / 21	5	9	200	Day 7
AHV	2	ALPHA			9	200	Day 10
AHH	2	ALPHA	886 M / 39	9	6	200	Day 10
AHP	2	ALPHA		5	7	150	Day 10
AJR	3	ALPHA		6	9	200	Day 10
AJR	3	DMEM		6	9	200	Day 10
AB4	3	DMEM	880 F / 39	4	10	170	Day 10
AB4	3	ALPHA			10	170	Day 10
AB9	2	DMEM		4	13	120	NO
AB9	2	ALPHA			13	120	Day 21
AFD	4	ALPHA		7	19	200	Day 10
ABL	3	ALPHA		4	15	115	Day 17
OQ	2	DMEM	NC F / 46	5	14	120	NO
OR	2	ALPHA		5	14	100	NO

Parallel cultures induced in either Alpha MEM or DMEM were comparable for age at induction and time of mineralisation. Cultures 'AHV' and 'AJR' were induced in both Alpha MEM and DMEM at 9 days and mineralised by day 10 post induction. Culture 'AB4' was induced at 10 days in Alpha MEM and DMEM and also mineralised by 10 days post induction. Culture 'AB9' was induced in Alpha MEM and DMEM at 13 days and only the Alpha MEM culture mineralised by 21 days post induction, however, the DMEM culture may also have mineralised if incubated for longer. There was considerable variation in BMdSC growth, induction and mineralisation in cultures derived from LTC '880'.

The markers of osteogenesis in mineralised cultures, in Alpha MEM and DMEM (100 mg/l CaCl₂), were quantitated and compared at 21 days post induction.

4.7.1. Calcium deposition
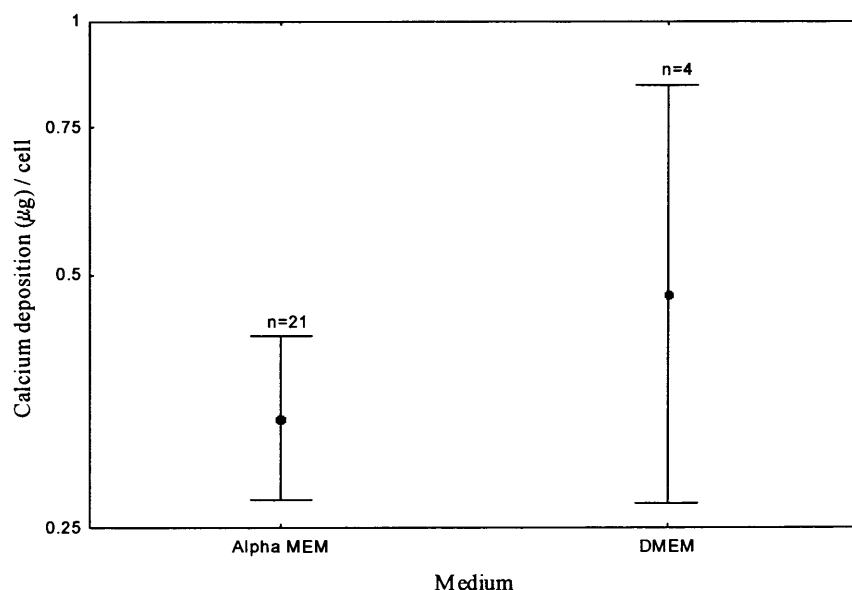
Figure 4.33. Calcium deposition per cell in mineralised cultures at 21 days post induction in Alpha MEM and DMEM (CaCl₂: 100 mg/l). Mean ± SE

No significant differences in calcium deposition per cell were observed between Alpha MEM and DMEM at 21 days post induction (figure 4.33).

4.7.2. **Alkaline phosphatase activity**
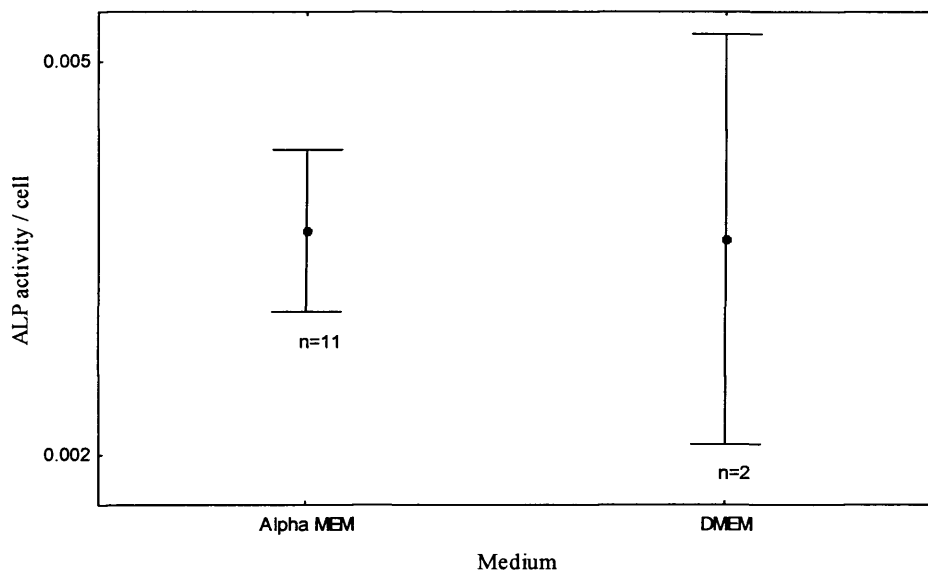
Figure 4.34. ALP activity per cell in mineralised cultures at 21 days post induction in Alpha MEM and DMEM ($CaCl_2$: 100 mg/l). Mean ± SE

No significant differences in ALP activity per cell (figure 4.34), or per culture (not shown), were observed between Alpha MEM and DMEM at 21 days post induction.

4.7.3. **Collagen I**
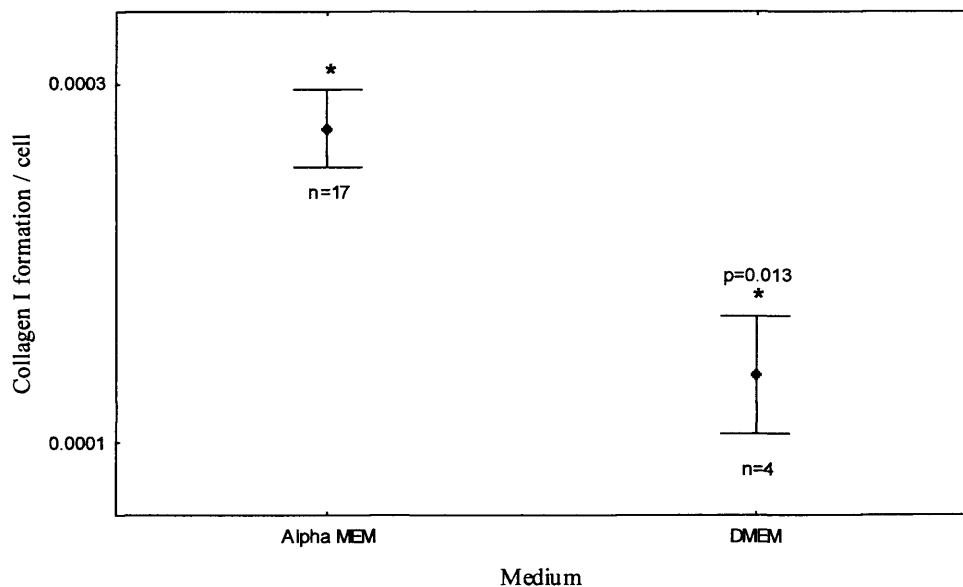
Figure 4.35. Collagen I formation per cell in mineralised cultures at 21 days post induction in Alpha MEM and DMEM ($CaCl_2$: 100 mg/l). Mean ± SE

Mineralised BMdSCs induced in Alpha MEM had significantly more collagen I deposition per cell (figure 4.35) and per culture (not shown) than DMEM (n=17, n=4; p=0.013) at 21 days post

induction. Alpha MEM contains ascorbic acid, which in addition to the ascorbic acid added at induction, acts as a cofactor in the hydroxylation of lysine and proline residues in the processing of procollagen I (Coelho & Fernandes 2000; Gundle 1998; Jaiswal et al 1997). The additional ascorbic acid in Alpha MEM in comparison to DMEM may be responsible for the increased collagen I formation by 21 days post induction.

4.7.4. Gene expression

Osteopontin, osteocalcin, collagen I and ALP gene expression were quantitated in mineralised BMdSCs induced in Alpha MEM and DMEM ($CaCl_2$: 100 mg/l).
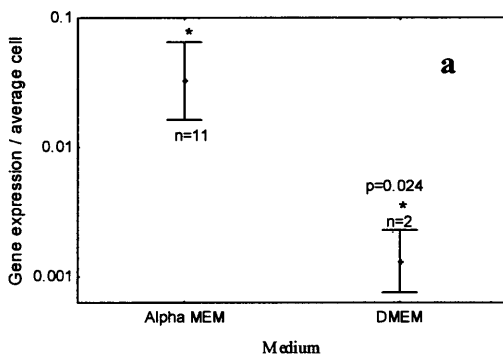
a) **Osteopontin**   b) **Osteocalcin**
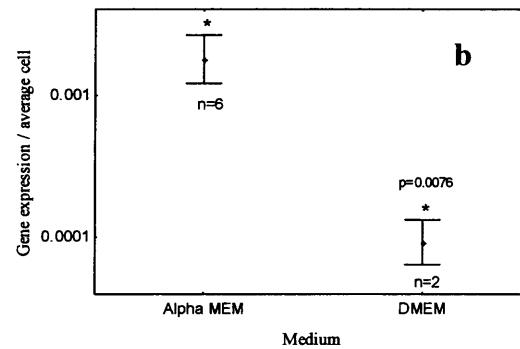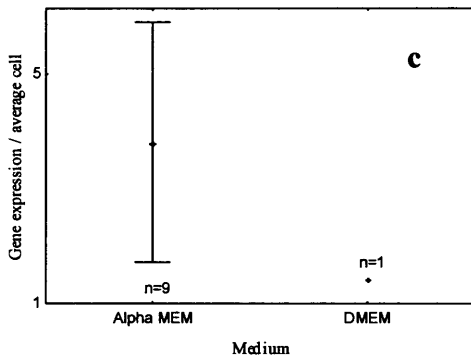
c) **Collagen I**   d) **Alkaline phosphatase**
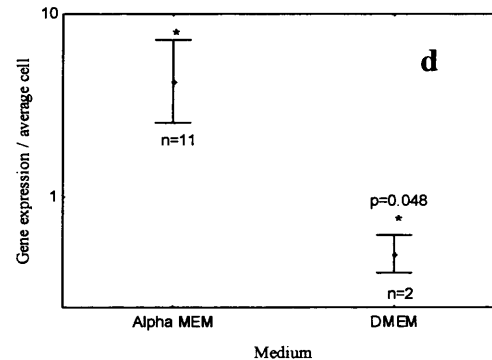
Figure 4.36. a) Osteopontin gene expression, b) osteocalcin gene expression, c) collagen I gene expression and d) ALP gene expression per average cell in mineralised cultures at 21 days post induction in Alpha MEM and DMEM ($CaCl_2$: 100 mg/l). Mean ± SE

Osteopontin (n=11, n=2; p=0.024), osteocalcin (n=6, n=2; p=0.0076) and ALP (n=11, n=2; p=0.048) gene expression were significantly higher in Alpha MEM than in DMEM.

4.7.5. Summary

Mineralised BMdSCs in Alpha MEM and DMEM were associated with comparable ALP activity and mineralisation. The significantly increased collagen I production may have resulted from increased concentrations of ascorbic acid in Alpha MEM, and may have been responsible for the increased ALP gene expression observed. However, the significantly increased ALP mRNA did not correlate with increased protein production and there was no significant increase in mineralisation. Statistical testing would likely improve with more data for DMEM.

The cultures were osteogenically induced at sub-confluence after being grown in commercial preparations of Alpha MEM (200 mg/l $CaCl_2$), therefore at induction all markers within parallel cultures should have been similar. Further studies would be to analyse the markers of gene expression before induction. At induction, the medium was changed to either Alpha MEM or DMEM with 100 mg/l $CaCl_2$. No time series experiments were carried out in DMEM. It was clear that the induction agents were not responsible for significantly increasing markers of osteogenesis. If components within Alpha MEM, that were not present in DMEM (for example, ascorbic acid), were responsible for spontaneously inducing osteogenesis, it may explain why some markers of differentiation were significantly reduced in DMEM.

4.8. Part II: Adipogenic Induction

Lipid formation was microscopically observed as multidroplet fat cells within adipogenically induced BMdSC cultures. Numerous refractile lipid globules formed within the cytoplasm of the cells surrounding the nucleus, and stained red with Oil red O (2.8.2.a. 'Oil red O staining').

4.8.1. Multidroplet cell cluster formation in relation to cell number at induction

The formation of mineral deposit in osteogenically induced BMdSC cultures was a clear morphological sign of bone formation, allowing for non-mineralised cultures to be excluded from the analysis. In a similar manner, the formation of multidroplet fat cells was a clear morphological sign of adipogenesis, however, not all adipogenically induced cultures developed them. The formation of multidroplet cell clusters (normalised to DNA content) was analysed against cell number at induction in adipogenically induced cultures (figure 4.37).
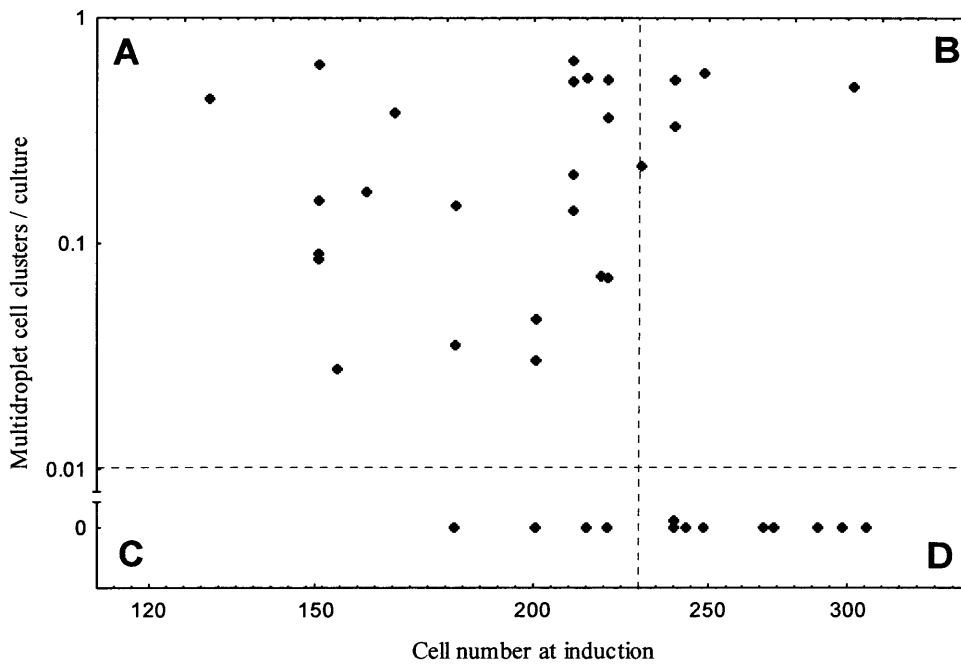
Figure 4.37. Multidroplet cell cluster formation in relation to cell number at induction.

All data plots within sections C and D (figure 4.37) were adipogenically induced cultures with no multidroplet cell cluster formation, whilst all induced cultures within sections A and B formed multidroplet cell clusters. The majority of the cultures (n=12) that did not form multidroplet cell cultures were in section D with cell counts >230 cells. The majority of cultures that formed multidroplet fat cell clusters (n=20) were in section A with cell counts between 120 and 230 cells. Therefore, cultures were significantly more likely to form multidroplet fat cell clusters if the cell counts at induction were below 230 cells (p=0.006).

Only cultures that formed multidroplet fat cell clusters were used in the quantitation of lipid-related markers (multidroplet cell cluster formation, lipid index and gene expression). No significant differences were observed between the standard and cyclic induction protocols at 21 days post induction (Appendix V), therefore, the two induction protocols were combined for analysis. Alpha MEM and DMEM were compared at 21 days post induction. The results were normalised to DNA content.

4.8.2. **Multidroplet cell cluster formation**

The multidroplet cells usually formed in colonies or clusters (photo 4.6), which could be quantitated using a low power dissecting microscope (2.8.2.a.iii. 'Analysis').
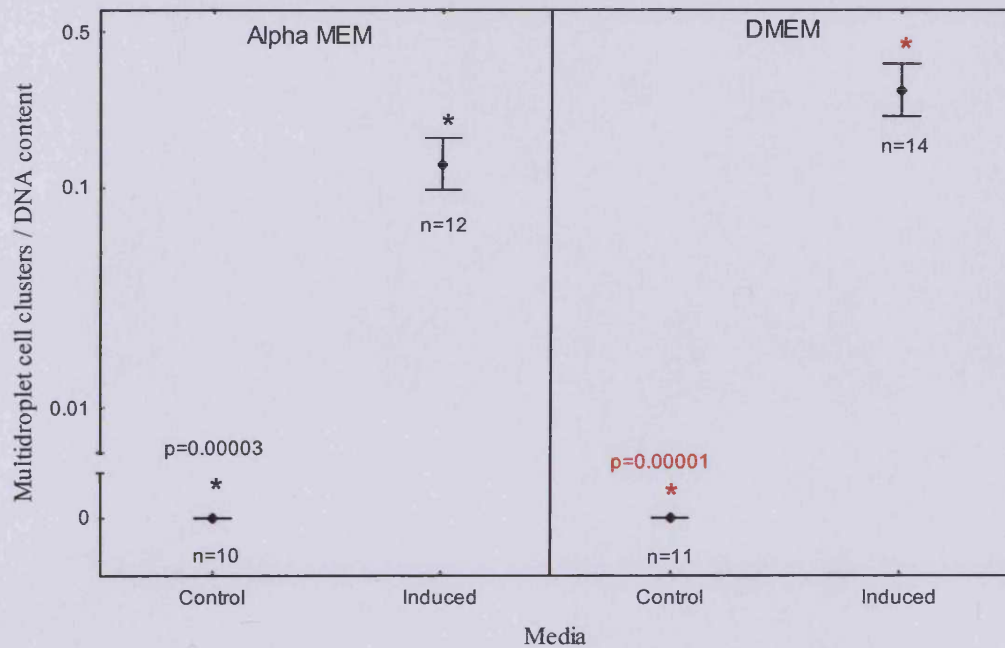
Figure 4.38. Multidroplet cell cluster formation at 21 days post induction in Alpha MEM and DMEM (CaCl$_2$: 100 mg/l), normalised to DNA content. Mean ± SE

There were significantly more multidroplet cell clusters at 21 days post induction in the adipogenically induced cultures in Alpha MEM (n=12, n=10; p=0.00003) and DMEM (n=14, n=11; p=0.00001) than the control cultures (non-parametric statistics). No statistical differences were observed between Alpha MEM and DMEM.

4.8.3. Lipid index

The lipid index was quantitated by staining with Oil red O and dissolving the stained fat in 100% isopropanol and measuring its absorbance (2.8.2.a.iv. 'Quantitation'). The lipid index was normalised to DNA content per culture.
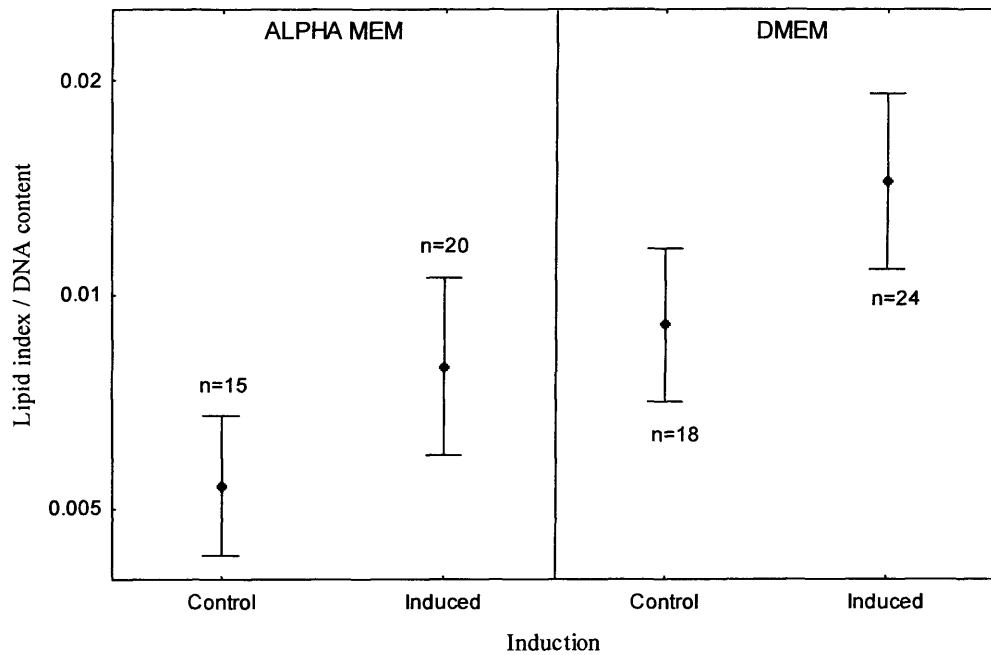
Figure 4.39. Lipid index at 21 days post induction in Alpha MEM and DMEM ($CaCl_2$: 100 mg/l), normalised to DNA content. Mean ± SE

There was an increase in the mean lipid index in the induced cultures at 21 days post induction in comparison to the control cultures, but no statistical differences were found in Alpha MEM or DMEM. The comparative results between the control and induced cultures suggested that the isopropanol extracted lipid material that was unrelated to multidroplet fat cell differentiation (background staining).

4.8.4. Gene expression related to adipogenic induction

RNA was extracted (2.9.1. 'RNA extraction) from adipogenically induced and control (not induced) BMdSC cultures at 21 days post induction. The RNA was converted to DNA (2.9.2. 'Reverse transcriptase reaction') and the gene expression was analysed by real time PCR and normalised to the housekeeping gene S14 (2.9.4. 'Real time analysis'). The PCR products were confirmed by sequencing (2.9.7. 'Automated sequencing') (Appendix IV). Gene expression was only analysed in the cultures that formed multidroplet fat cell clusters.

PPAR-γ2, LPL and aP2 gene expression was quantitated per average cell in control and adipogenically induced BMdSC cultures in Alpha MEM and DMEM at 21 days post induction.

a) **aP2**
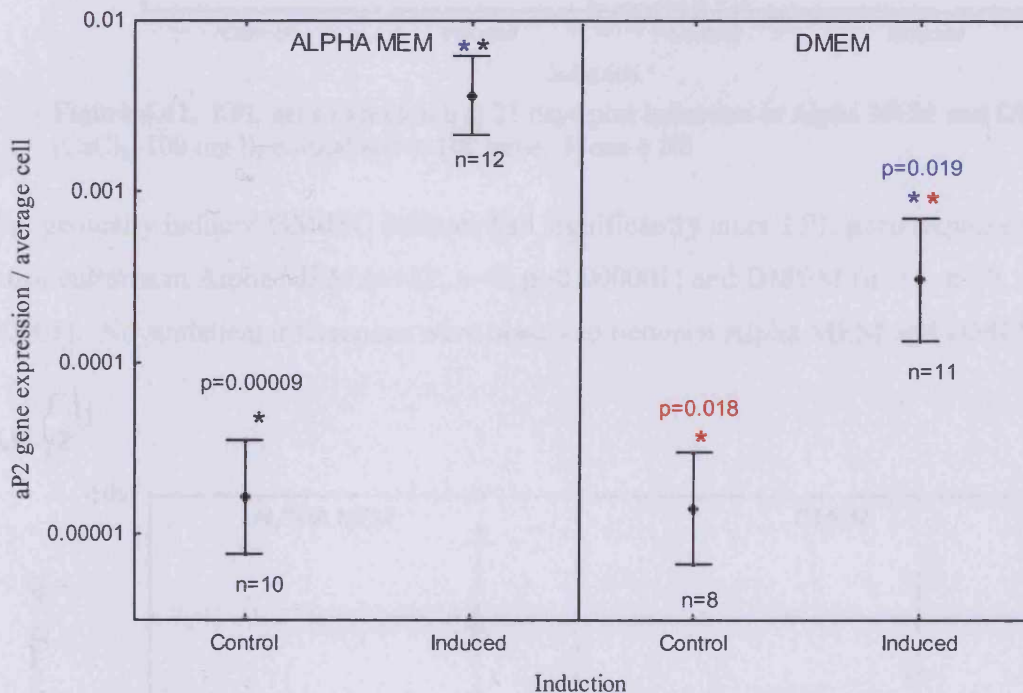
Figure 4.40. aP2 gene expression at 21 days post induction in Alpha MEM and DMEM ($CaCl_2$: 100 mg/l), normalised to HK gene. Mean ± SE

The adipogenically induced BMdSC cultures had significantly more aP2 mRNA than the control cultures in Alpha MEM (n=12, n=10; p=0.00009) and DMEM (n=11, n=8; p=0.018). The cultures induced in DMEM had significantly less aP2 mRNA than those induced in Alpha MEM (n=11, n=12; p=0.019).

c) LPL
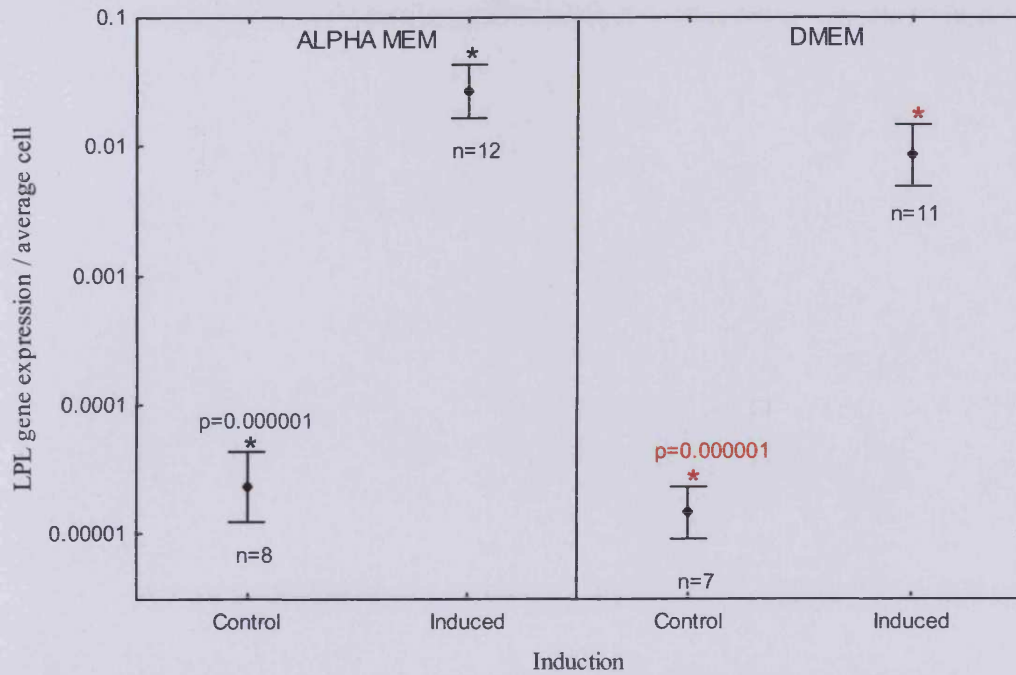
Figure 4.41. LPL gene expression at 21 days post induction in Alpha MEM and DMEM (CaCl$_2$: 100 mg/l), normalised to HK gene. Mean ± SE

The adipogenically induced BMdSC cultures had significantly more LPL gene expression than the control cultures in Alpha MEM (n=12, n=8; p=0.000001) and DMEM (n=11, n=7; p=0.000001). No statistical differences were observed between Alpha MEM and DMEM.

d) PPAR-γ2
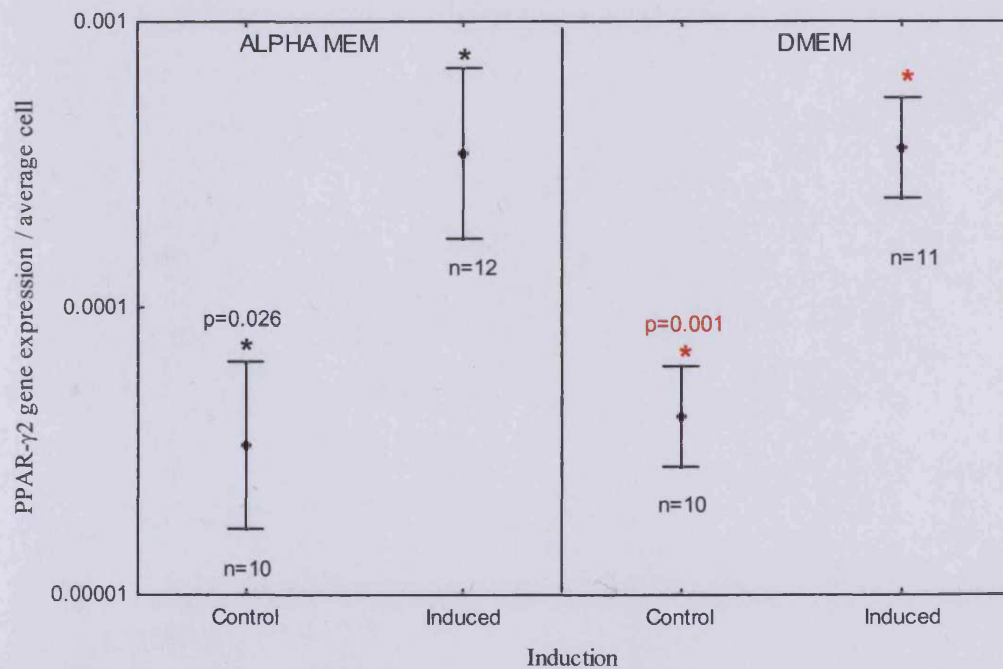
Figure 4.42. PPAR-γ2 gene expression at 21 days post induction in Alpha MEM and DMEM (CaCl$_2$: 100 mg/l), normalised to HK gene. Mean ± SE

The adipogenically induced BMdSC cultures had significantly more PPAR-γ2 gene expression than the control cultures in Alpha MEM (n=12, n=10; p=0.026) and DMEM (n=11, n=10; p=0.001). No statistical differences were observed between Alpha MEM and DMEM.

e) **Relative gene expression in adipogenically induced and control cultures**
Alpha MEM and DMEM were combined for a final comparison between aP2, LPL and PPAR-γ2 gene expression in control and adipogenically induced cultures.
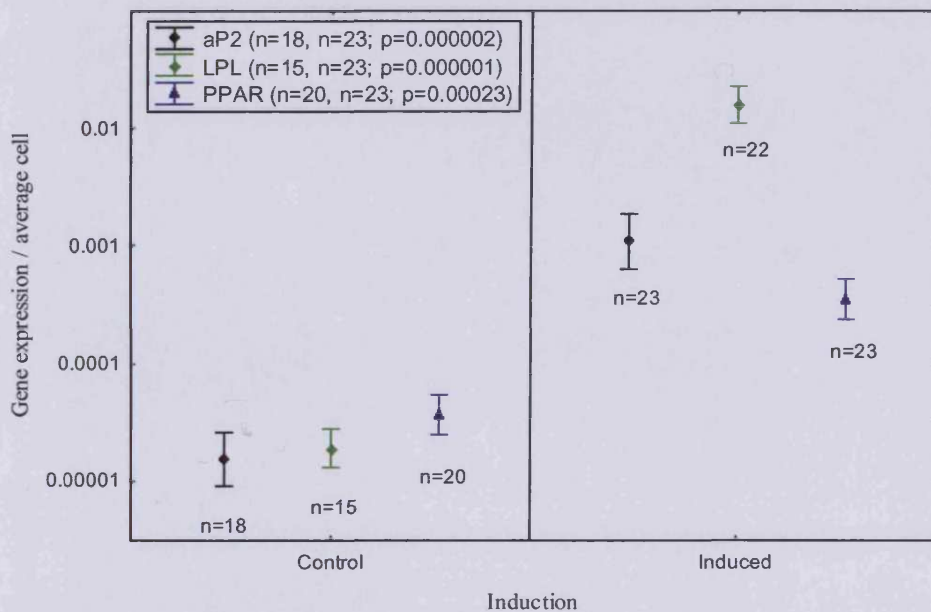
Figure 4.43. Alpha and DMEM combined. aP2, LPL and PPAR-γ2 gene expression in control and induced cultures at 21 days post induction. Mean ± SE

The expression of all three genes was significantly increased (data shown on figure 4.43) at 21 days post induction when BMdSC cultures were adipogenically induced with insulin, HC, IBMX, dexamethasone and indomethacin. This largely suggests that only the differentiating cells (multidroplet fat cells) in the induced cultures were responsible for the increased production. The relative gene expression of LPL (n=23) was significantly greater than aP2 (n=23; p=0.001) and PPAR-γ2 (n=23; p=0.00006) in normal adipogenically induced BMdSC cultures at 21 days post induction, indicating its importance in lipid accumulation.

In figure 4.37 very few adipogenically induced cultures were shown to form multidroplet cell clusters with cell counts greater than 230 cells per field, and nearly all induced cultures that did not form clusters had cell counts at induction greater than 230 cells. Figure 4.44 analyses lipid-related gene expression in relation to cell counts at induction in control and adipogenically induced cultures that did and did not form multidroplet cell clusters.
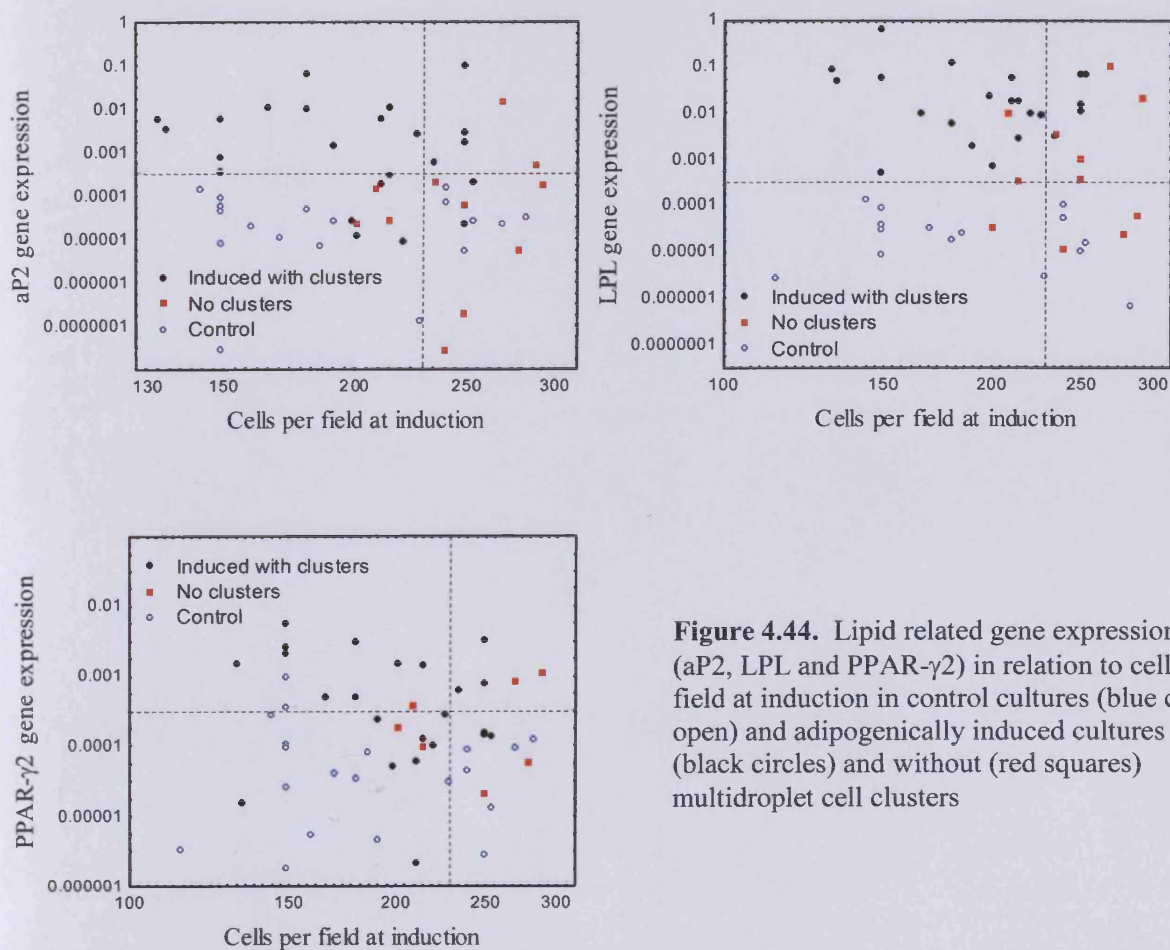
Figure 4.44. Lipid related gene expression (aP2, LPL and PPAR-γ2) in relation to cells per field at induction in control cultures (blue circle open) and adipogenically induced cultures with (black circles) and without (red squares) multidroplet cell clusters

The control cultures showed significantly lower gene expression than the induced cultures (figure 4.43). The adipogenically induced cultures that did not form multidroplet cell clusters (red squares) had higher cell counts at induction (figure 4.37), but significantly lower aP2 (n=11, n=23; p=0.003) and LPL (n=11, n=22; p=0.0004) gene expression in relation to the successfully induced cultures (figure 4.44). PPAR-γ2 gene expression was more variable with no significant differences.

4.8.5. **Summary**

The successful adipogenic induction of BMdSC cultures resulted in the upregulation of aP2, LPL and PPAR-γ2 gene expression with a significant increase the number of multidroplet cell clusters. The cultures that showed no signs of adipogenic differentiation (no multidroplet cell clusters) generally had an increased cell count at induction and a significantly lower aP2 and LPL gene expression, supporting the theory that only the differentiated cells (multidroplet cells) were responsible for lipid-specific gene expression. The increased cell count at induction in the

cultures that did not form multidroplet cell clusters indicated that more confluent cultures were inhibitory to adipogenic differentiation.

DISCUSSION

4.9.1. Quantitation

a) Quantitating markers of osteogenesis

During osteogenesis, calcium deposit (mineralisation) was quantitated by staining with Alizarin red S for 10 minutes (2.7.3.b. 'Mineralisation') and demineralising for 24 hours in HCl for spectrophotoscopic analysis (2.7.3.c. 'Demineralisation') (Stanford et al 1995). Initially the calcium concentrations within the acidified extracts were sent for atomic absorption analysis and compared to the spectrophotometer absorbance values. There was a good correlation between the two methods ($r=0.95$) enabling the calcium concentration to be determined from the optical density (2.7.3.c. 'Demineralisation'). Stanford (Stanford et al 1995) reported that 2 molecules of calcium bound to 1 molecule of Alizarin red S. The results from this investigation showed that 1 molecule of calcium bound to 1.64 molecules of Alizarin red S, which was ascertained to be 70% pure (Sigma). Quantitating collagen I was more difficult because no chemicals were found to elute the colour substrate DAB from the cultures, or digest the collagen without digesting other proteins within the ECM. Therefore, a flask reader was devised that measured the amount of light able to pass through the stain. This produced an absorbance value, which could be related back to the cellular content within the flask (2.7.3.d. 'Collagen I'). Cellular ALP activity was quantitated using a standardised method (Calzyme Laboratories) (Stinson 1993) which measured the hydrolysis of p-nitrophenyl phosphate to p-Nitrophenol. The cells were sonicated to allow the ALP to be released from the cells and the change in absorbance was measured over 5 minutes. The results were expressed as the change in absorbance per minute (2.7.3.e. Cellular alkaline phosphatase activity').

Normalising the quantitated markers of osteogenesis to the cellular material within the flask proved to be a problem because measuring protein content did not distinguish between cellular protein and ECM protein. Measuring DNA content was found to be a good method for determining cellular content but the technique was introduced too late to encompass all the results collected to make it valuable for normalising bone markers. Therefore, the quantitated markers were normalised to cell counts per microscopic field at induction. Although there was a poor correlation between cell counts at induction and DNA content at 21 days post induction, the results showed similar patterns and statistical analyses.

b).Quantitating markers of adipogenesis

During adipogenesis, the intracellular lipid droplets were stained with Oil red O (2.8.2.a. 'Oil red O staining'), which was determined to be the optimal dye to use because it selectively stained neutral lipids such as cholesterol esters and triglycerides (red), although it also stained some phospholipids (pink) (photos 4.16 & 4.17) (Filipe & Lake 1990). Sudan black strongly stains compound lipids such as phospholipids, which are present within cell membranes and was less sensitive for the resolution of smaller lipid containing structures such as intracellular droplets (Kiernan 1990). Two methods were devised to quantitate the amount of Oil red O staining. The first was subjective by placing the flask under a dissecting microscope and counting the number of multidroplet cell colonies in the flask. This method only counted visible clusters of multidroplet cells. Control cultures formed no multidroplet cell clusters. The second method used 100% isopropanol to dissolve the Oil red O stained fat for spectrophotometric quantitation. Unfortunately the results were poor with large standard error bars regardless of whether the cultures contained multidroplet cells or not, therefore, no differences were found between the control and induced cultures. This indicated that the isopropanol dissolved fat structures that were not related to multidroplet cell formation. It was also likely that the cells had a large degree of background staining, which may have been removed by washing the cultures in a very low percentage of isopropanol before using a high percentage of isopropanol to dissolve the fat for analysis.

Passaged BMdSC cultures were adipogenically induced following the establishment of the technique for measuring DNA content, therefore the quantitated markers of adipogenesis were normalised to DNA content (2.7.3.g. 'Data normalisation'). The method of normalisation (DNA or cell counts at induction) made no difference to the statistical analysis at 21 days post induction.

c) Quantitating gene expression

Quantitating gene expression was made easy by the availability of a real-time PCR machine (Roche LightCycler). Using standard PCR techniques, it was only possible to quantitate the expression of a particular gene by running the PCR product on a gel, from which the DNA could be extracted (2.9.6. 'DNA extraction'), quantitated and sequenced (2.9.7. 'Automated sequencing'). Real-time PCR allowed gene expression to be quantitated using the cycle threshold values (2.9.3.c. 'Analysis and quantitation'), which were related to the housekeeping gene using the formula $2^{(\text{Housekeeping CT} - \text{Test CT})}$. This equation assumed the reaction was

efficient with a doubling of PCR products with each cycle. The PCR products were confirmed using automated sequencing (Appendix III & IV).

4.9.2. Osteogenesis

a) Limiting the rate of mineralisation

The main problem encountered during the induction of osteogenesis was limiting the rate of mineralisation because at 200 mg/l $CaCl_2$, the cells and possibly the ECM (not bound by calcium) broke up before 21 days of induction (photo 4.8). This released both the mineral deposit from the ECM and RNA from the cells, showing a significant loss in RNA by 21 days post induction (figure 4.9). Reducing the concentration of calcium within the induction medium reduced the rate of mineralisation allowing for an adequate mineralised layer to form within 21 days of induction without any significant loss in RNA (figure 4.8). Most research teams in the literature used standard commercial medium preparations of Dulbecco's modified Eagle's medium (Bruder et al 1998; Chen et al. 2001; Frank et al 2002; Goldstein 2001; Pittenger et al 1999) or Alpha MEM (Majors et al 1997; Malaval et al 1994) for induction (200 mg/l $CaCl_2$), but no information was cited with regards to the cells breaking up during mineralisation, although Jaiswal (Jaiswal et al 1997) reported that densely seeded cultures often detached during culture. DMEM occurs more frequently in the literature, but Alpha MEM was chosen optimally in this investigation because the passaged BMdSCs grew significantly better in Alpha MEM (figure 3.11).

b) Markers of osteogenesis

The order of events important for mineralisation was not clear because insufficient non-mineralised cultures were quantitated for an induction time series of osteogenic-related markers (ALP, collagen, osteopontin and osteocalcin gene expression) in comparison to mineralised cultures, and the markers were not analysed before induction. Further studies would be to quantitate markers of osteogenesis before and after induction in both non-mineralised and mineralised cultures. Collagen I deposition was the only marker to be assessed during proliferation (before induction) and showed a gradual increase in production with an increase in cell number (figure 3.40). Post induction, collagen I gene expression was down regulated and the level of collagen I deposition plateaued by day 10 (query sufficient ECM formed for differentiation), suggesting no further need for collagen I production. Cellular ALP activity increased to approximately day 10 before reaching a plateau, with no concomitant decline in ALP gene expression (Chak et al 1995) indicating a continued basal need for ALP. There was no change in osteocalcin gene expression. Osteopontin gene expression was not determined

during proliferation, but it was likely that levels increased during proliferation in a similar manner to collagen I before being down regulated post induction. There was no down regulation of osteopontin in the control cultures, which suggested that the induction agents were responsible for directly or indirectly inhibiting osteopontin gene expression.

These results are similar to Frank (Frank et al 2002) who showed no significant change in osteocalcin gene expression (as measured by real time PCR) in osteogenically induced human BMdSCs during 20 days of induction, and no significant difference between the control and induced cultures. In contrast to our study, he also showed no significant change in ALP and collagen I gene expression through 20 days of induction and demonstrated a significant increase in osteopontin gene expression.

c) Mineralisation

Younger cultures with more cells per field at induction ($\geq$100 cells) were more likely to mineralise by 21 days post induction than older cultures with smaller cell counts at induction. There were, however, cultures with $\leq$100 cells per field at induction that mineralised within 21 days, and these cultures were shown to produce more collagen I and ALP activity per cell in comparison to the non-mineralised cultures (4.5.2.b. 'Cellular ALP' & 4.5.2.d. 'Collagen I'). It was concluded that for mineralisation to be initiated, threshold levels of collagen I and ALP activity per culture needed to be achieved. In cultures with a smaller number of cells, more of the cells needed to be actively secreting the protein, or each secreting cell needed to produce a larger amount of protein to reach the culture threshold levels. Once mineralisation had begun, ALP activity and collagen I production became less important and plateaued.

Mineralisation (calcium deposit) occurred slowly, becoming morphologically visible, on average, by day 10, the same time at which the level of collagen I staining and ALP activity plateaued. This lag phase enabled the increasing ALP activity to degrade β-glycerophosphate to inorganic phosphate for hydroxyapatite formation, and for the crystals to deposit within the ECM.

d) Necessity of induction agents

The induction agents (dexamethasone, ascorbic acid-2-phosphate and β-glycerophosphate) were important for mineralisation with no calcium deposition occurring in their absence, however, their presence did not significantly increase collagen I (protein or mRNA), ALP (activity or

mRNA), osteopontin or osteocalcin gene expression during the 21 days of induction in relation to the control cultures.

The results are not shown because very few cultures were induced and the osteogenic related markers were not quantitated, but cultures incubated with medium containing dexamethasone and ascorbic acid-2-phosphate did not mineralise. In comparison to control cultures, in which the cell layer gradually deteriorated with time (broke up or rolled up into a ball), passaged BMdSC cultures containing dexamethasone and ascorbic acid-2-phosphate were maintained for more than 50 days with no signs of deterioration. This indicated that their presence provided stability to the cell layer, presumably by promoting ECM maturation (Coelho, Cabral, & Fernandes 2000). It also suggested that ECM formation, dexamethasone and ascorbic acid-2-phosphate were insufficient for mineralisation (Herbertson & Aubin 1995; Ter Brugge & Jansen 2002). β-glycerophosphate, either alone or in combination with dexamethasone and ascorbic acid-2-phosphate, was necessary for mineralisation (Bellows, Heersche, & Aubin 1992; Coelho, Cabral, & Fernandes 2000; Gori et al 1999), although cultures induced with β-glycerophosphate alone mineralised at a later stage than with all three induction agents (either before or after 21 days post induction). Cultures induced with 5 mM β-glycerophosphate instead of 10 mM, were also seen to mineralise, particularly in association with dexamethasone and ascorbic acid-2-phosphate, but again at a later stage than using 10 mM.

In summary, dexamethasone and ascorbic acid-2-phosphate appeared to speed up the mineralisation process by promoting ECM formation, but calcium and β-glycerophosphate were necessary for mineralisation.

4.9.3. Adipogenesis

a) Morphological development of multidroplet cells

Upon adipogenic induction of BMdSCs in Alpha MEM and DMEM, changes in cellular morphology largely resembled that of the literature (Alberts et al 1998). The spindle shaped fibroblast-like cells changed to a more cuboidal shape within the first few days or first cycle of the cyclic induction protocol. By the end of the first cycle the cells had become granular with the accumulation of tiny lipid droplets (photo 4.5) (Janderova et al 2003). These droplets gradually increased in size, staining very positively red for Oil red O by 21 days post induction (photo 4.6) (Scavo et al 2004). Unfortunately due to time restrictions, no time series experiments were carried out to assess multidroplet cell formation and gene expression

throughout the 21 days of induction. In only one culture did the cells go on to become spherical adipocytes (photo 4.7), most developing clusters or individual multidroplet cells with varying sized droplets. Whether more cultures would have developed these large spherical adipocytes if induced for longer periods of time is unknown. The formation of large spherical adipocytes is more often seen within mouse LTCs, and rarely seen within human cultures, although the reasons behind this are unclear (Dexter 1979b).

Occasionally much smaller less intensely stained droplets (pink to white) were visible (photos 4.16 & 4.17), not all of which appeared to be related to the cells but lay above the cells within the ECM. These are likely to be a form of phospholipid, possibly released from the cell membranes into the cell or ECM. They were also visible within control cultures in the absence of large well stained multidroplet fat cells (photo 4.16), which indicated that they were not related to adipogenic induction. It is these smaller less well stained droplets, particularly within the control cultures, that may have interfered with lipid quantitation using 100% isopropanol.
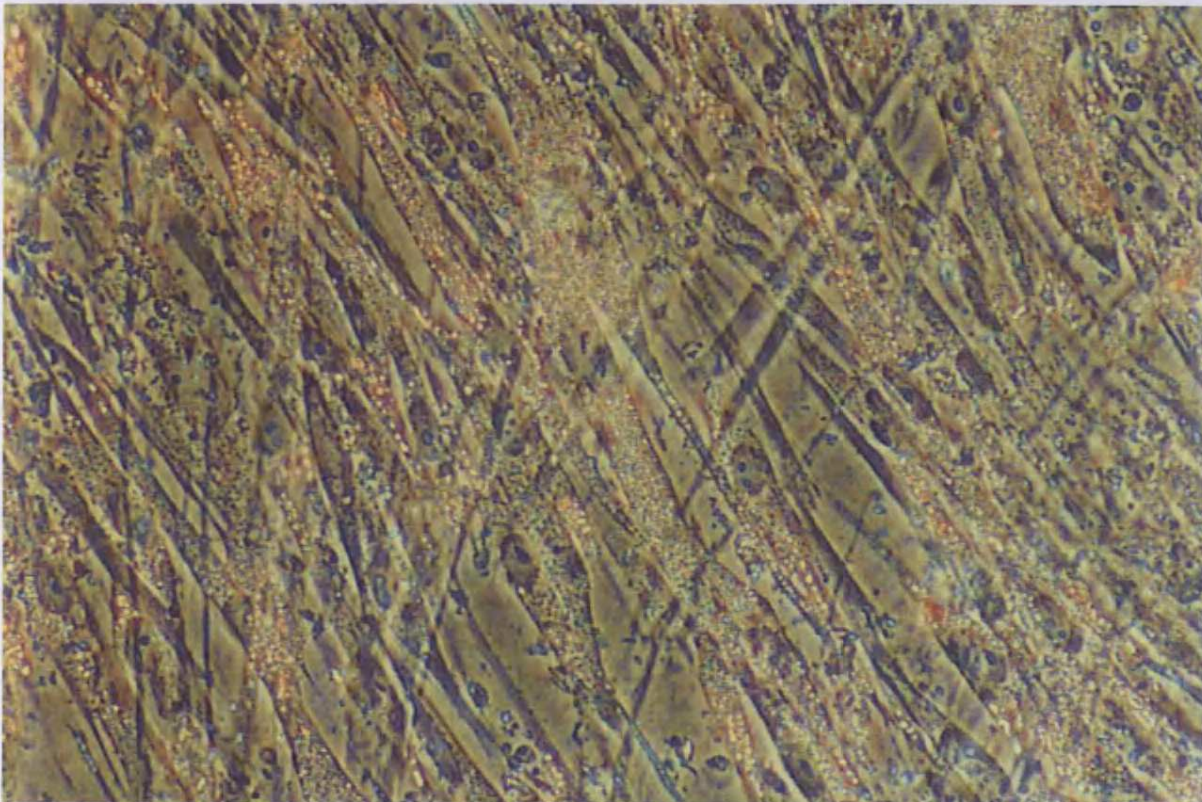
Photo 4.16. Control culture (not induced) stained with Oil red O at 21 days post induction. Tiny, weakly stained, lipid droplets scattered across flask
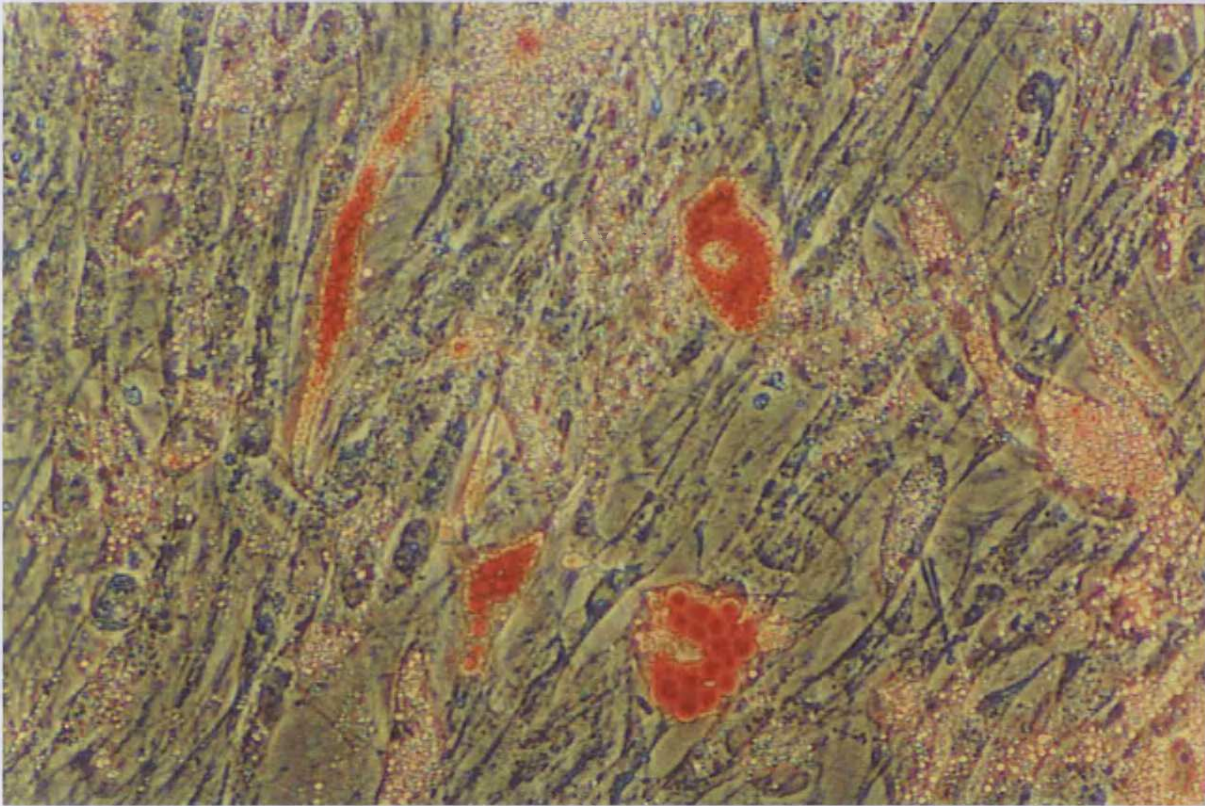
Photo 4.17. Adipogenically induced culture at 21 days post induction stained with Oil red O. Multidroplet cells with large lipid droplets and tiny, scattered, weakly stained droplets.

b) **Multidroplet cell cluster formation**

Only certain cells within the BMdSC layer were susceptible to hormonal stimulation (Janderova et al 2003) for differentiation into multidroplet fat cells, and these may already be committed to the adipogenic lineage (preadipocytes) before induction. Preadipocytes are progenitor cells or colony forming cells (CFU-Fs), therefore, these cells may be responsible for the colonies of multidroplet cells formed following induction. The capacity for preadipocytes to proliferate into multidroplet cell colonies or clusters may be restricted in more confluent cultures (figure 4.37). If preadipocytes were not present within the BMdSC layer before induction, lipid accumulation would probably take much longer, and more cells would likely become committed to the adipogenic lineage. This would require susceptible progenitor cells to commit to the adipogenic lineage post induction and continue to proliferate and differentiate into fat cell colonies. It is also possible that one susceptible cell is stimulated into differentiating by hormonal stimulation, which then produces further chemical signals that stimulate surrounding cells (paracrine interactions) to differentiate, or that the induction cocktail becomes concentrated in particular areas, although the induction cocktail was well mixed within the medium upon addition.

c) Necessity of induction agents

The presence of adipogenic induction agents were vital for the formation of multidroplet fat cell clusters, as were osteogenic induction agents (particularly β-glycerophosphate) in the formation of mineralisation. However, the presence of osteogenic induction agents did not significantly increase collagen formation, ALP, osteopontin or osteocalcin gene expression. With adipogenesis, all three fat-related genes (LPL, aP2 and PPAR-γ2) were significantly upregulated in the induced cultures by 21 days post induction in comparison to the control cultures (figure 4.43). This indicated their importance in adipogenesis. It would have been interesting to study gene expression as part of a time series to show the early upregulation of PPAR-γ2, followed by an upregulation in LPL and aP2 gene expression (Janderova et al 2003). This could then have been related to the time at which lipid accumulation was seen. Janderova reported PPAR-γ2 gene expression in undifferentiated confluent cells, which rose markedly 12 hours after induction and reached maximal levels during the second cycle of hormonal induction when lipid accumulation was observed.

The lack of multidroplet cells and significantly poorer gene expression in the control cultures indicated that cell confluence was insufficient to promote adipogenesis. Gregoire (Gregoire, Smas, & Sul 1998) indicated that growth arrest and exit from the cell cycle (required for terminal differentiation) (Janderova et al 2003; Ntambi & Young-Cheul 2000) was not initiated by cell to cell contact at confluence but required hormonal stimulation. The individual induction agents (dexamethasone, IBMX, insulin, indomethacin and hydrocortisone) were not assessed individually to determine their action upon the cell layer, but Ahdjoudj (Ahdjoudj et al 2001) reported that the reagents together had an additive effect on adipogenesis.

d) Calcium concentration

The calcium concentration for adipogenic induction was not optimised in this investigation. The cells were grown in commercial preparations of Alpha MEM ($CaCl_2$: 200 mg/l) and differentiated in the same laboratory preparations of Alpha MEM and DMEM ($CaCl_2$: 100 mg/l) that were used for osteogenesis, with good multidroplet cell formation. However, reports have shown that increasing calcium concentrations early in lipid induction inhibited adipocyte differentiation, whilst increasing them in the later stages promoted triglyceride accumulation by stimulating PPAR-γ2 expression (Shi et al 2000; Zemel et al. 2000).

e) Standard and cyclic induction protocols

This investigation found no significant differences between the standard and cyclic induction protocols (Appendix V). Lipid accumulation was observed at approximately the same time for both the standard and cyclic induction protocols (not quantitated) during the second induction phase of the cyclic protocol (8-11 days). Often the cells took on a very granular appearance towards the end of the first cycle of induction, but lipid droplets were rarely seen before 8 days. This is supported by Scavo (Scavo et al 2004), who reported no lipid droplets within the first 96 hours, and Janderova (Janderova et al 2003), who reported the most pronounced change during the second induction cycle. Janderova also reported that prolonged resting periods between each induction cycle (without dexamethasone, indomethacin and IBMX) improved the differentiation process, but this was not investigated.

4.9.4. Overlap between osteogenesis and adipogenesis

Osteogenesis and adipogenesis were very different, the obvious difference being that morphologically, osteogenesis took place within the ECM (collagen I deposition and calcium deposition) across the whole culture, whilst adipogenesis was more cellular with lipid accumulation occurring within colonies of cells. This meant that whilst adipogenically differentiated cells were easily distinguished by their morphology (lipid accumulation), it was very difficult to establish which cells within the BMdSC layer were osteogenically differentiated and responsible for mineralisation. Intracellular staining of ALP clearly showed the ALP positive cells, but were these cells also responsible for collagen I secretion, osteopontin and osteocalcin production? The relative levels of osteocalcin and osteopontin gene expression were significantly lower than ALP and collagen I (figure 4.26), which could suggest that the same cells were responsible for producing variable amounts of bone-related mRNA, or that fewer cells within the cell layer were responsible for expressing osteopontin and osteocalcin (query bone differentiating cells). Multidroplet cell cluster formation (colonies of lipid accumulating cells) was likely to be a result of proliferating preadipocytes (CFU-F) within the cell layer. In a similar manner, osteogenically differentiated cells were likely to be formed from an osteogenic progenitor (CFU-F) pre-existing within the cell layer, therefore, it was likely that osteogenesis also occurred in colonies (not obvious with ALP positive cells) (photos 4.10 & 4.11).

4.10. **Final Summary**

This investigation osteogenically and adipogenically induced BMdSC cultures and assessed methods for quantitating the markers of differentiation. The induction agents had a huge impact on adipogenesis with the formation of multidroplet cell clusters (quantitated using a low power dissecting microscope) and a significant increase in aP2, LPL and PPAR-γ2 gene expression (quantitated using real time PCR). The induction agents did not have such a big impact on the markers of osteogenesis (quantitated using the spectrophotometer or the flask reader) with the control cultures behaving similarly to the induced cultures, although β-glycerophosphate was necessary for mineralisation.

Chapter 5

Comparison between BMdSCs from Normal and Pathological Bone Marrows

Comparison between Normal and Pathological BMdSC Cultures

5.0. Aims of Chapter 5

To compare bone marrow derived stromal cell growth, osteogenesis and adipogenesis in cultures derived from normal and pathological bone marrows. To determine the presence of any abnormalities in the stromal compartment that may contribute to, or be caused by, the haematological disease. LTC cell counts, cell doubling and CFU-F colony formation (normal ranges outlined in 3.3 'Long-term culture (LTC) BMdSCs') were compared between normal and pathological groups. Markers of osteogenesis (calcium deposition, ALP, collagen I, osteopontin and osteocalcin gene expression) and adipogenesis (multidroplet cell cluster formation, aP2, LPL and PPAR-γ2 gene expression) were compared between normal and pathological groups (normal ranges outlined in 4.4. 'Part I: Osteogenic Induction' & 4.8. 'Part II: Adipogenic Induction').

INTRODUCTION

5.1. Haematological Malignancies

5.1.1. Leukaemia

Leukaemia is characterised by defective haemopoiesis and the accumulation of malignant haemopoietic cells. The increased proliferation and / or cell survival is usually associated with poor differentiation resulting in an abundance of immature cells in the bone marrow, which can filter out into the peripheral circulation (Izraeli 2004). Patients are often susceptible to infection because the abundant cells are often non-functional (Campbell 2001f).

Leukaemia arises mainly from gene mutations or deletions that control mechanisms of cell growth and development, or from translocations that create abnormal fusion genes such as bcr/abl, explained in chronic myeloid leukaemia. The causes of these genetic instabilities are unknown although many risk factors have been identified, the risks increasing with age (Campbell 2001f). Some infections, particularly DNA or RNA-containing viruses can act as co-factors to trigger neoplastic genetic mutations, for example, the Epstein-Barr virus has been associated with Hodgkin's disease and the human T-cell leukaemia / lymphoma virus has been shown to cause leukaemia or lymphomas. Environmental factors such as chemicals, particularly ones containing benzene, can be carcinogenic or mutagenic, and may cause myelodysplasia. Cigarette smoking is also a common risk factor for acute myeloid leukaemia (AML). Radiation was shown to be a prominent risk factor in survivors from the atomic bomb explosion in Japan, 1945, and a close proximity to nuclear power stations or electricity pylons

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has also been linked to an increased risk of leukaemia (Campbell 2001f; Wickremasinghe & Hoffbrand 1999).

Leukaemia can be sub-divided into acute or chronic, myeloid or lymphoid according to the haemopoietic lineage affected. Acute leukaemias are usually very aggressive and can be distinguished from chronic leukaemias by the presence of immature blast cells (>30%) of either the myeloid or lymphoid lineage (Hoffbrand, Moss, & Pettit 2001). In chronic leukaemias the cells are more mature and differentiated.

a) Acute Myeloid Leukaemia

AML, as its name indicates, affects the myeloid lineage of haemopoiesis (figure 1.9). Immature blast cells accumulate in the bone marrow, which are unable to differentiate and eventually spill out into the bloodstream. Although the myeloid lineage is primarily affected, the overproduction of cells in the bone marrow often prevents the effective proliferation and differentiation of other lineages. This leads to a progressive loss of circulating mature blood cells resulting in anaemia, bleeding from thrombocytopenia (low platelets) and an inability to fight infection (Campbell K 2001). The disease affects all age groups with an increasing incidence with age (Hoffbrand, Moss, & Pettit 2001).

Chromosomal abnormalities occur in approximately 70% of AML patients (Secker-Walker 1999). The most frequent mutation in AML is the FLT3/ITD mutation with an incidence of 13.2% to 34%, irrespective of age, classification or cytogenetics. FLT3 (fms-like tyrosine kinase) is a tyrosine kinase receptor predominantly expressed on haemopoietic progenitor cells and regulates proliferation. Mutations such as ITD (internal tandem duplication), a tandemly duplicated sequence varying in length between 12 and 204 base pairs, constitutively activates FLT3 causing the continual proliferation of haemopoietic cells. Mutations within the second tyrosine domain (incidence: 6.4% to 7.7%) can also cause constitutive activation leading to AML (Kottaridis et al. 2003). AML is divided into three different risk groups according to cytogenetics: favourable, intermediate and poor risk. Favourable risk groups are usually associated with the translocations (15;17), (8;21) and inv(16) cytogenetics, whilst poor risk groups often have complete or partial loss of genetic material such as –7 or –5, del(5q), del(3q) or complex karyotypes (Kottaridis, Gale, & Linch 2003). A balanced chromosomal translocation, such as (8;21)(q22;q22), involves the even exchange of material between two chromosomes (Wikimedia Foundation Inc. 2006). The t(8;21) is predominantly found within the M2 FAB subtype and results in the fusion of AML-1 with the ETO gene (AML1-ETO or

RUNX1-RUNX1T1). The ETO gene represses transcription through *AML1 (RUNX1)* and arrests maturation (Flandrin 2006).

Table 5.1. FAB classification of AML (Catovsky & Hoffbrand A.V 1998)

Sub-type	Disease characterisation
M0	Undifferentiated myeloblastic
M1	Myeloblastic leukaemia without maturation (high N:C ratio)
M2	Myeloblastic leukaemia with maturation (lower N:C ratio). Requires cytochemical staining for diagnosis (peroxidase or sudan black)
M3	Hypergranular promyelocytic characterised by presence of promyelocytes
M4	Myelomonocytic with granulocytic and monocytic differentiation. Requires cytochemical staining (naphthol AS-acetate esterase)
M5	Monoblastic (>80% blasts), Monocytic (<80% blasts)
M6	Erythroleukaemia (>50% erythroblasts with <30% or >30% blasts)
M7	Megakaryoblastic

Table 5.2. World Health Organisation (WHO) classification of AML (Tallman 2004; Vardiman et al. 2002)

AML with recurrent genetic abnormalities	AML with t(8;21) (q22;q22), (*AML1-ETO* or RUNX1-RUNX1T1) AML with inv(16) or t(16;16)(p13;q22) (*CBFβ/MYH11*) APL with t(15;17)(q22;q12) (*PML/RARα*) AML with 11q23 (*MLL*) abnormalities
AML with multilineage dysplasia	Following MDS or MDS/MPD Without MDS but with dysplasia in 50% of cells
AML and MDS	Alkylating agent/radiation-related type Topoisomerase II inhibitor-related type
AML (not categorised)	As FAB classification and acute basophilic leukaemia, acute panmyelosis with myelofibrosis and myeloid sarcoma

AML is classified according to the FAB (French American British) classification (table 5.1), based solely on the morphology of the leukaemic cells and basic cytochemical stains, and the WHO (World Health Organisation) classification (table 5.2), which takes into account cytogenetic abnormalities, molecular genetic changes, antigenic expression and previous haematological disorders (secondary AML), which may evolve after exposure to chemotherapy

(Tallman 2004). The WHO classification also includes a reduction in the percentage of blasts (20% threshold) sufficient to diagnose AML.

It has been reported that the bone marrow derived stromal compartment is affected in active AML with reduced numbers of stromal cells and colony formation, and often a poor supporting ability to normal haemopoietic cells (Duhrsen & Hossfeld 1996; Mayani 1996; Sparrow et al. 1997). Also, adipocyte growth is effected in 50% of AML patients (Mayani 1996). It is possible that the leukaemic cells are inhibitory to the LTC stromal compartment through the production of inhibitory chemicals, for example, Ben-Ishay and Duhrsen & Hossfeld (Ben-Ishay et al. 1984; Duhrsen & Hossfeld 1996) reported the inhibition of mouse and human CFU-F colony formation by the addition of AML cells, AML cell-conditioned medium or AML serum. However, it has also been suggested that the reduced numbers of CFU-F colonies observed may be due to the dilution of stromal cells by neoplastic cells within the MNC inoculum (Mayani 1996). The most likely cell responsible for the abnormalities observed in BMdSCs from AML patients is the macrophage (Mayani 1996), which may be derived from the malignant clone. These cells adhere alongside BMdSCs and have been shown to produce a soluble inhibitory factor (most likely tumour necrosis factor (TNF) -α) that promotes apoptosis (Sparrow et al 1997).

b) Chronic Myeloid Leukaemia

CML is a clonal myeloproliferative disorder, which accounts for about 15% of all leukaemias (Hoffbrand, Moss, & Pettit 2001). It is characterised by the presence of the Philadelphia (Ph) chromosome in over 90% of cases, which is acquired during the t(9;22)(q34;q11) translocation. The translocation involves the bcr gene on chromosome 22 and the abl gene on chromosome 9, resulting in the formation of the bcr/abl fusion gene (Bhatia et al. 1995; Mayani 1996). This gene codes for a fusion protein with tyrosine kinase activity in excess of the normal protein, which results in the excessive proliferation of malignant myeloid cells (Hoffbrand, Moss, & Pettit 2001).

CML involves two phases, a chronic phase characterised by a slow progression rate and expansion of the myeloid compartment with retention of full differentiation, and a myeloid blast crisis phase, which is a much more progressive disease in which differentiation is arrested at the precursor stage (Mayani 1996; Shet et al. 2002). The disease affects all ages, although is rare in children below 15 and more common between 40 and 60 years of age (Campbell 2001c).

Bhatia (Bhatia et al 1995) reported a poor interaction between normal haemopoietic progenitors and BMdSCs derived from CML bone marrows, although this did not appear to be related to an abnormal production of soluble cytokines because similar concentrations were found in both normal and CML BMdSC layers. The reduced interaction was more likely due to abnormalities within the cyto-adhesion receptors (5.1.5. 'Haemopoietic microenvironment') or due to macrophages derived from the bcr/abl clone (Duhrsen & Hossfeld 1996). Addition of CML macrophages to a normal BMdSC layer was reported to reduce haemopoietic progenitor growth (Mayani 1996). CML BMdSCs have shown similar proliferative capabilities to normal BMdSCs with CFU-F colony counts within the normal range, however the number of adipocyte colony forming cells were often increased. Once CML progressed to the more rapid progressive blast-like disease, results became similar to those of AML (Duhrsen & Hossfeld 1996).

c) Acute Lymphoblastic Leukaemia

ALL, as its name suggests, affects the lymphoid lineage (figure 1.9) with the accumulation of immature blast cells (arrested) in the bone marrow that eventually spill out into the bloodstream in a similar manner to AML. The increased production of blast cells affects the production of other lineages leading to a reduced number of mature blood cells within the bloodstream (Campbell 2001a). Immunologically both B-cells (75% early B-cell, 5% mature B-cell) and T-cells (20%) can be affected with the disease most commonly affecting people under the age of 25 years and above 75 years (Catovsky & Hoffbrand A.V 1998).

Clonal abnormalities are found in up to 90% of patients with ALL. Mature B-cell ALL is often characterised by t(2;8), t(8;14), t(8;22), and common B-cell ALL (c-ALL) often has 6-, t(9;22) (poor prognosis), 9- and / or t(12;21) (good prognosis). T-cell ALL is commonly associated with the translocations t(8;14), t(11;14), t(10;14) and t(1;14), and in about 40% of cases involves a breakpoint at 14q11, 7q32 or 7p15 (Secker-Walker 1999).

ALL is classified according to the FAB system (table 5.3), although this is not as clear cut as with AML. The L3 classification is immunophenotypically known as B-cell ALL, and is currently seen as the leukaemic form of Burkitt's lymphoma (Szczepanski et al. 2003).

Table 5.3. FAB classification for ALL (Hoffbrand, Moss, & Pettit 2001)

Sub-type	Disease characterisation
L1	Small blasts, uniform, monomorphic lymphocytes. High N:C ratio
L2	Larger, heterogeneous blasts, nucleolated lymphocytes. Low N:C ratio with prominent nucleoli
L3	Larger lymphocytes with basophilic, vacuolated cytoplasm and prominent nucleoli

The stromal microenvironment in ALL is generally much more disrupted than with AML or CML, the composition showing reduced fibroblast-like cells and adipocytes with an increase in endothelial cells and macrophages. This relates to a diminished proliferative capacity and reduced CFU-F colony formation in LTCs (Duhrsen & Hossfeld 1996).

d) Chronic Lymphoid Leukamias

The chronic lymphoid leukaemias involve several disorders that are characterised by the proliferation of mature lymphocytes of either B or T-cell type. Chronic lymphocytic or lymphoblastic leukaemia (CLL) is the most common, in which a relatively mature B cell is affected (Hoffbrand, Moss, & Pettit 2001). CLL is characterised by an increase in the number of poorly functioning, fragile, mature lymphocytes in the bloodstream that are not apoptosed at the end of their normal lifespan and accumulate in the blood, bone marrow, liver, spleen and lymph nodes.

About 15% of CLL cases undergo transformation to CLL / PLL (prolymphocytic leukaemia), a rapidly progressive disease characterised by an increased number of prolymphocytes, and about 10% of cases transform to Richter's syndrome, which rapidly progresses to large cell lymphoma associated with a poor prognosis. PLL appears similar to B-cell CLL but the prolymphocytes are twice the size of CLL lymphocytes with a prominent central nucleolus (Hoffbrand, Moss, & Pettit 2001). The disease usually occurs over the age of 65 with 20-30% occurring before 55 years of age, and is more common in men (Campbell 2001b).

Chronic B-cell leukaemia can be classified into B-CLL (85-90% of chronic B-cell leukaemias), hairy cell leukaemia (5-10%), hairy cell variant leukaemia (rare) and B-cell prolymphocytic leukaemia (B-PLL), which is diagnosed with the presence of more than 55% prolymphocytes (<5%). Chronic T-cell leukaemias (often associated with inv(14q) and t(14q) cytogenetics) are more difficult to classify due to their heterogeneity, but can be sub-divided by

immunophenotyping into T-cell PLL, adult T-cell leukaemia, Sezary syndrome and large granular lymphocyte leukaemia (Szczepanski, van der Velden, & van Dongen 2003).

5.1.2. Lymphoma

Lymphomas are cancers of the lymphatic tissues, which drain fluid back into the peripheral blood circulation. During infection, the lymph nodes, found at various places within the lymphatic system including armpits, groins, neck, pelvis, abdomen and chest, can become swollen due to a large increase in the number of lymphocytes. The organs (e.g. spleen, thymus, tonsils and adenoids) and lymph nodes involved usually dictate the type of lymphoma (Campbell 2001f).

There are two main groups of lymphoma: Hodgkin's and non-Hodgkin's lymphoma. Hodgkin's disease (HD) is a primary lymph node based neoplasm that occurs mainly in young people with an incidence peak in old age, and involves the bone marrow in approximately 5-15% of cases. It is largely of B-cell origin, although rare cases of T-cell have been recorded, and is characterised by the presence of low numbers of Reed-Sternberg cells amongst a varied cellular infiltrate of inflammatory cells, accessory cells (lymphocytes), plasma cells, eosinophils, neutrophils, dendritic cells and histocytes (Viswanatha & Foucar 2003). Various cytokines are usually over expressed including the circulating interleukins 1 to 9, transforming growth factor (TGF)-β and TNF, which may have implications for the stromal compartment (Linch, Goldstone, & Mason 1999). The World Health organisation divides HD into two primary groups including the lymphocyte predominant form (5%) and classic HD, which is further sub-divided into nodular sclerosis, mixed cellularity, lymphocyte rich and lymphocyte depleted (Viswanatha & Foucar 2003).

Non-Hodgkin's lymphoma (NHL) can be either B-cell (more common) or T-cell (rare in Western countries) in origin. B-cell NHL usually involves the bone marrow and can be sub-divided into many diseases including precursor B-lymphoblastic leukaemia / lymphoma, small lymphocytic leukaemia, lymphoplasmacytic lymphoma, mantle cell lymphoma, follicular lymphoma, marginal zone lymphoma, large B-cell lymphoma and Burkitt lymphoma. As a few examples, lymphoplasmacytic lymphoma commonly involves the bone marrow and causes Waldenstrom's macroglobulinaemia. Marginal zone lymphoma is a B-lymphocytic leukaemia, characterised in 70% of cases by trisomy 3 and trisomy 18. The translocation (11;18) is present in about 50% of extranodal cases. Mantle cell lymphoma is an aggressive subtype of small B-lymphocytic lymphoma, characterised by the (11;14) translocation in about 50% of cases, and

frequently involves the bone marrow (50% of cases). Follicular lymphoma involves the bone marrow in 40-70% of cases and is the most common low-grade form, often characterised by the (14;18) translocation (85% of cases). Aggressive large B-cell lymphomas involve the bone marrow in approximately 20-30% of cases (Viswanatha & Foucar 2003). The aetiology of NHL is unclear but it can arise as the result of chronic antigenic stimulation, for example, following persistent bacterial infections, or following infection by the Epstein-Barr virus, which is also implicated in HD (Linch, Goldstone, & Mason 1999).

Minimal residual disease (MRD) in the bone marrow has been implicated in the recurrence of the disease following bone marrow transplantation. It is likely that the modulation of haemopoietic proliferation and differentiation by BMdSCs, plus the latter's anti-apoptotic effect, plays an important role in the maintenance and eventual expansion of MRD (Weekes et al. 1998). The elucidation of the mechanisms responsible for maintaining malignant cells *in vivo* might allow for the production of treatments that disrupt the adhesive interactions between stromal and haemopoietic cells to trigger apoptosis (Bradstock et al. 1996).

5.1.3. Multiple Myeloma

Multiple Myeloma (MM) is a malignant proliferation of a single plasma cell clone at various bone marrow sites and is characterised by paraproteinaemia in the serum or urine (Hoffbrand, Moss, & Pettit 2001). Plasma cells are antibody-producing B-lymphocytes and their malignant proliferation results in an excess production of monoclonal antibodies termed gamma globulins or paraproteins. Normally serum immunoglobulins are polyclonal representing the combined output from different plasma cells, however, in MM, the paraprotein will often only correspond to one type of molecule (IgG, IgA, IgD or IgE) termed monoclonal gammopathy. Normal antibody production is reduced so the patient is often susceptible to infections, and because the disease occurs at multiple bone marrow sites, bone structure often breaks down with resultant hypercalcaemia, leading to dehydration and kidney damage. It is a disease of later life, which primarily affects males with a peak incidence in the 70s (Campbell 2001d).

Myeloma plasma cells preferentially accumulate in the bone marrow and it is thought that the bone marrow microenvironment supports the growth of these cells and protects them from apoptosis (Wallace et al. 2001). It has been suggested that the interaction of MM cells with the BMdSC microenvironment results in the upregulation of IL-6 (interleukin 6), a multifunctional cytokine that normally induces terminal differentiation to antibody producing cells. However, in MM, IL-6 acts as a major growth factor (Duhrsen & Hossfeld 1996). It also appears to

protect the cells from many apoptosis inducing treatments such as dexamethasone (Cheung & Van Ness 2001; Wallace et al 2001). In addition, the MM cells produce a variety of cytokines that may modulate the growth and function of stromal cells. It has been reported that stromal cells from bone marrows affected by myeloma show an increased proliferative capacity in comparison to age matched controls with similar monoclonal gammopathies, however ECM formation was reduced with poorer organisation (Duhrsen & Hossfeld 1996). This may be apparent *in vitro* during osteogenesis by collagen I production and mineralisation of the ECM. MM may also be responsible for inhibiting osteocalcin production, which suppresses bone formation in the latter stages of the disease (Duhrsen & Hossfeld 1996).

5.1.4. Myelodysplastic Syndrome (MDS)

MDS is a heterogeneous group of clonal multipotent stem cell disorders, characterised by variable cytopenias despite increased haemopoiesis and hypercellular bone marrows (Oscier 1998). MDS arises from an unknown genetic transformation within a single haemopoietic precursor cell, which gives rise to a selective growth advantage over other normal haemopoietic cells (Flores-Figueroa et al. 1999). The vast majority of cells are destroyed by apoptosis before they leave the bone marrow, which in addition to ineffective haemopoiesis, leads to a generalised pancytopenia in the blood despite having a normal to increased cellularity within the bone marrow. It has been reported that cytokines, some of which may be produced by the BMdSCs, may predispose haemopoiesis to premature apoptosis, for example increased levels of IL-1β (interleukin-1β), TNF-α and the up-regulation of Fas and Fas-ligand have all been reported in MDS marrows, which are essentially involved in apoptosis (Deeg et al. 2000; Hirayama et al. 1993). It is thought that TNF-α is largely produced by macrophages, but other cells within the stromal layer may also produce it during MDS.

Myelodysplasia has been recognised as a preleukemic stage (Flores-Figueroa et al 1999) with approximately 30% of cases transforming to AML at varying times, which is harder to treat than primary AML. About 50% die before transformation of MDS-related problems such as infection or haemorrhage (Alvi et al. 2001). The disease is more common in the elderly population, particularly in men (Hoffbrand, Moss, & Pettit 2001) and treatment is simply to relieve the symptoms of anaemia, infections and bleeding, the only cure being a stem cell transplant in younger healthier patients (Campbell 2001e).

MDS was classified by the FAB system into five categories depending on the percentage of blasts, ring sideroblasts (>15%) and monocytes in the bone marrow and peripheral blood

(Bennett et al. 1982). WHO further classified MDS into eight categories (table 5.4) and proposed a new grouping called Myelodysplastic / Myeloproliferative disorders, which includes chronic myelomonocytic leukaemia (CMML), Juvenile Myelomonocytic leukaemia (JMML, a rare chronic myelodysplastic / myeloproliferative disorder mainly effecting children under four years) and atypical cases of CML.

Table 5.4. Original WHO classification of MDS (Brunning et al. 2001; Vardiman, Harris, & Brunning 2002)

Sub-type	Disease characterisation
Refractory anaemia	Mild to moderate anaemia with macrocytosis. <1% blasts in the blood and <5% in the bone marrow. <15% ringed sideroblasts. Median survival is 50 months
Refractory anaemia with ring sideroblasts	Red cells are unable to use iron, so iron is deposited in rings. <1% blasts in the blood, <5% in the bone marrow and ≥15% ring sideroblasts. Median survival is 50 months
Refractory cytopenia with multilineage dysplasia	Cytopenias, <1% blasts in the blood. <5% blasts, <15% ringed sideroblasts in the bone marrow with dyplasia in ≥10% of the cells.
Refractory cytopenia with multilineage dysplasia and ringed sideroblasts	Cytopenias, <1% blasts in the blood. <5% blasts, ≥15% ring sideroblasts, no Auer rods in the bone marrow. Dysplasia in ≥10% of the cells
Refractory anaemia with excess blasts 1	<5% blasts in the blood, 5-9% blasts in the bone marrow with dysplasia. No Auer rods Reduced platelets, leucocytes and erythrocytes. Median survival 11 months with a 40% transformation rate.
Refractory anaemia with excess blasts 2	Cytopenias, 5-19% blasts in the blood, 10-19% blasts in bone marrow with auer rods. Patients with >30% blasts considered to have AML. Median survival 5 months.
MDS, unclassified	Cytopenias, <1% blasts in blood. <5% blasts in bone marrow, no Auer rods. Unilineage dysplasia.
MDS associated with isolated del(5q)	Anaemia with normal or increased platelet count and <5% blasts in the blood. <5% blasts in bone marrow with isolated del5(q) cytogenetics. No Auer rods. Normal to increased megakaryocytes

BMdSCs from MDS patients with good prognosis have been shown to develop normal confluent layers within Dexter-type cultures and good CFU-F colony formation, however the size of the colonies (proliferative capacity) has been reported to be reduced. BMdSCs from MDS patients with poor prognosis have shown poor stromal growth and reduced lipid accumulation (Duhrsen & Hossfeld 1996). Similar to AML and CML, the macrophage population is thought to be abnormal with the production of cytokines that are inhibitory to the stromal population. There are conflicting reports about the supporting ability of MDS BMdSCs to normal haemopoietic cells. Some studies have shown impaired haemopoiesis being sustained at lower levels for shorter periods of time compared to normal cultures (Tennant et al. 2000b), whilst others have shown normal function (Coutinho et al. 1990; Hirayama et al 1993).

5.1.5. Haemopoietic microenvironment

The haemopoietic microenvironment was defined by Mayani (Mayani 1996) as a complex network of stromal and accessory cells (e.g. macrophages), plus associated products (adhesion receptors for haemopoietic cells, extracellular matrix molecules and membrane bound or secreted cytokines), capable of regulating haemopoietic cell proliferation, differentiation and function. The close interaction between the haemopoietic cells and the stromal microenvironment largely suggests that abnormalities within the stromal microenvironment may effect the regulation of haemopoiesis and contribute to the aetiology and pathogenesis of haematological disease. In contrast, stromal abnormalities may occur as a consequence of the haemopoietic disease (Sparrow et al 1997).

In the absence of the stromal microenvironment the haemopoietic cells do not survive *in vitro* and presumably *in vivo*. Normal and leukaemic haemopoietic cells rely on membrane contact and diffusible cytokines (G-CSF, CM-CSF, M-CSF, IL-3, IL-6 and steel factor) secreted by BMdSCs for survival. In many haemopoietic malignancies, adhesion of the leukaemic haemopoietic cells to the BMdSCs confers protection from apoptosis and allows the clone to continue to proliferate (Duhrsen & Hossfeld 1996). In this respect the BMdSCs may often confer protection from certain drugs designed to induced apoptosis in leukaemic cells.

Interactions between normal myeloid progenitor cells, stromal cells and ECM mainly occur via the β_1 integrins (VLA-4 and VLA-5 on the haemopoietic cells), VCAM-1 (CD106, on the BMdSCs) and fibronectin (within the ECM) (Duhrsen & Hossfeld 1996; Makrynikola & Bradstock 1993). Antibodies to VLA-4 or VCAM-1 can completely block B-cell lymphopoiesis, and can partially block myelopoiesis *in vitro* (reduction / inhibition of

cobblestone area formation) (Obinata et al 1998) indicating a much stronger dependence on the stromal microenvironment for B-cell lymphopoiesis (Duhrsen & Hossfeld 1996; Obinata et al 1998).

The binding of VLA-4 and VLA-5 to VCAM-1 is also the primary mediator between leukaemia or lymphoma cells and their microenvironment (Weekes et al 1998), although AML subtypes with a high peripheral blast count tend to show lower VLA-4 and VLA-5 expression, and in CML this interaction is often defective with VLA-4 and VLA-5 remaining inactive. As a result, in CML, VLA-2 (CD49b) and VLA-6 are more strongly expressed than on normal myeloid precursors. Another adhesion molecule often over expressed in CML (Duhrsen & Hossfeld 1996) and ubiquitously expressed in B-ALL (Makrynikola & Bradstock 1993) is CD44 (HCAM), a lymphocyte homing receptor, which binds to hyaluronate and fibronectin and is important during haemopoiesis. Stronger expression has been linked to more mature B-cell malignancies (Makrynikola & Bradstock 1993).

RESULTS

5.2. Normal and Pathological Bone Marrow Samples

Table 5.5. Number of bone marrow samples and age ranges of each group

Pathological group	Number of donations	Age range (years)	Female	Male
Normal	51	18 – 49 Median: 38	14	37
Pathological normal*	16	1 – 78 Median: 62	6	10
AML	25	18 – 82 Median: 57	11	14
Lymphoma	15	30 – 83 Median: 60	3	12
Myeloma	12	50 – 74 Median: 58	5	7
Lymphoproliferative	6	8 – 89 Median: 60	0	6
JMML	3	2 – 8 Median: 2.5	1	2
MDS	5	62 – 75 Median: 69	4	1

* Bone marrow aspirates proven to have no diagnostic abnormality

Sixty six pathological bone marrow samples were received from patients suffering from various haematological malignancies at diagnosis (before treatment) and fifty one normal bone marrow samples were received from bone marrow donations. A further 16 pathological samples were received, which were later determined to be normal ('pathological normal') (table 5.5).
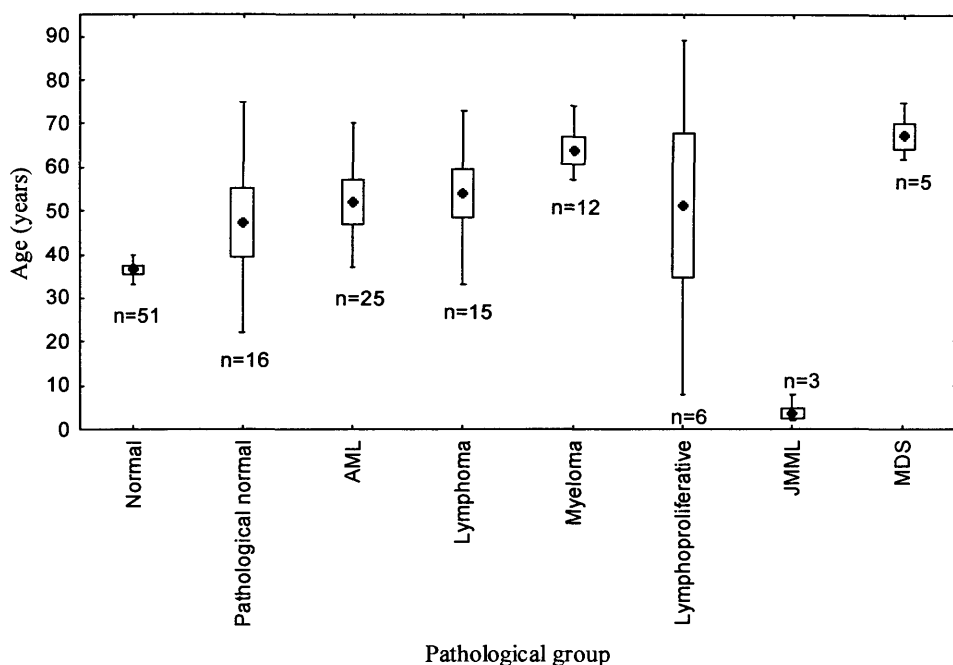
Figure 5.1. Age ranges for normal and pathological bone marrows. Mean ± SE (box) plus total range (whiskers). See table 5.5 for data

5.2.1. Age distribution

The age ranges were not always comparable when comparing the pathological groups to normal BMdSC cultures because bone marrow donors were young (<49 years) and healthy, whilst many of the haematological disorders occurred later in life. The age ranges for all the pathological groups were significantly higher than the normal group (p=<0.002) with the exception of JMML, which was significantly lower (p=0.0009). Occasionally bone marrow aspirates, taken to determine the pathology of the sample for diagnosis, were reported to be normal. These bone marrow samples formed a 'pathological normal' group with a significantly older age range (mean: 56 years, n=16; p=0.000001) than the normal group (mean: 37.6 years, n=51). The pathological normal group was more comparable to the various disease groups in terms of age (figure 5.1), and was combined with the normal group for analysis, however, JMML had no comparable normal age group.

5.2.2. Sex distribution

There were more male normal bone marrow donors (n=37) than female (n=14) (figure 3.3). More AML, lymphoma and myeloma samples were received from men than women and the lymphoproliferative group consisted entirely of males. The MDS group (n=5) included only 1 male, despite MDS being more common in elderly males.

5.3. LTC BMdSCs

5.3.1. Inoculum sizes

The inoculum sizes reflected the cellularity of bone marrows affected by malignancy. LTCs were plated at 20×10^6 cells whenever possible.
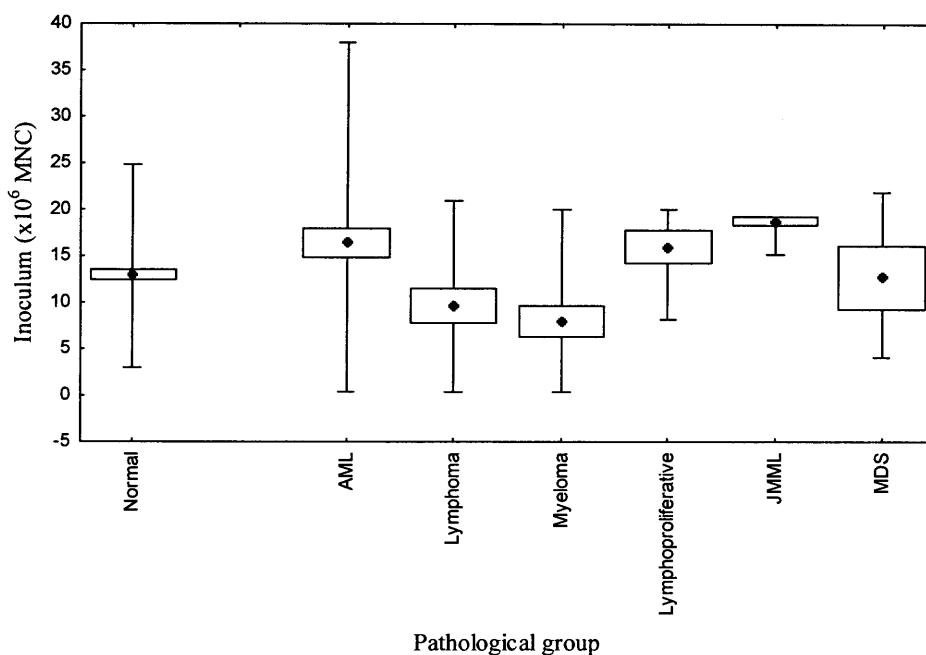
Figure 5.2. Inoculum sizes used for LTCs derived from normal and diseased bone marrows. Mean ± SE (box) and total range (whisker).

The bone marrow samples from patients with AML, lymphoproliferative disorders and JMML were often hypercellular, which is reflected by high inoculum values (figure 5.2), however, many of these cells were haemopoietic in origin and often blast-like, which became clear in culture with the presence of many tiny haemopoietic cells and very few BMdSCs (photo 5.1). Therefore, the MNC inoculum from hypercellular pathological bone marrows was likely to contain only a small proportion of BMdSCs, and an even smaller fraction of BMdSC progenitors in comparison to normal. Increasing the inoculum size increased the acidity of the cultures, which inhibited cellular growth. MDS, lymphoma and myeloma samples showed a varied cellularity in the inoculum sizes used.
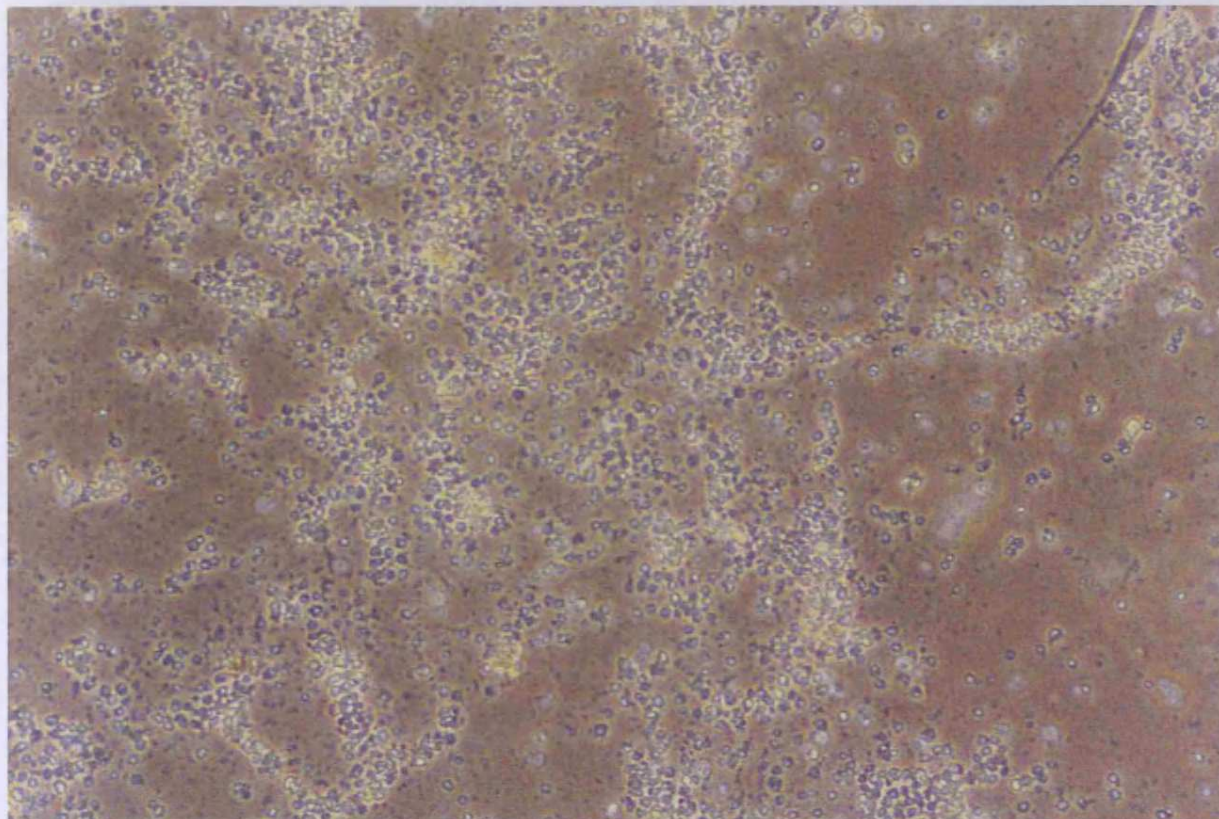
Photo 5.1. Low power magnification (x10) of an AML LTC. Multiple tiny haemopoietic cells with only one elongated BMdSC (top right).

5.3.2. **LTC BMdSC growth**

The growth of BMdSC from normal and pathological bone marrows were compared in modified McCoy's media and Alpha MEM in the presence of HC (3.3.2. 'Effect of hydrocortisone (HC) and glutamine on LTC BMdSC Growth'). All growth curves (cell counts) were plotted on the same scale to compare pathological groups.

a) Acute Myeloid Leukaemia

Twenty five AML bone marrow samples from patients aged 18 to 82 years (table 5.6), were received. All samples from patients classified as having AML were initially grouped together for analysis.

Table 5.6. AML bone marrow samples received

FAB classification	Frequency	Sex / Age	Cytogenetics
M0	1	F / 58	Inv 17, MDS transformed
M1	3	F / 56	16 +8
		F / 30	t(16;16)
		M / 72	Normal
M2	4	M / 57	t(8;21)
		F / 65	
		M / 72	Normal
		F / 62	Normal
M3	1	M / 47	Not known
M5	2	F / 20	Adverse
		M / 35	Normal
Biphenotypical	1	M / 37	Complex
Unclassified	13	M / 74	Normal
		F / 39	Normal
		M / 68	Complex (MDS Transformed)
			Not known
		M / 28	Not known
		M / 18 - 65	Not known
		F / 69 – 82	
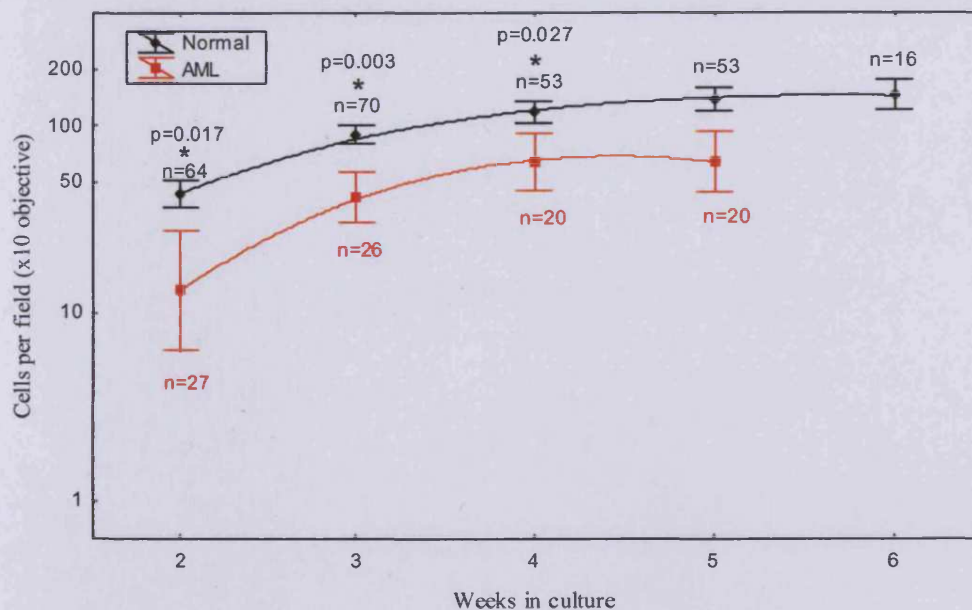
Figure 5.3. LTC BMdSC growth from normal (black circle) and AML (red square) affected bone marrows. Mean ± SE

The initial BMdSC counts for AML LTCs were significantly smaller at week 2 (n=27, n=64; p=0.017), week 3 (n=26, n=70; p=0.003) and week 4 (n=20, n=53; p=0.027) than in the normal LTCs. The ratio of adherent cells at week 2 over inoculum size was significantly reduced in comparison to normal (AML ratio: 3.66, normal ratio: 8.22; p=0.036), suggesting the presence of a smaller proportion of stromal cells in relation to malignant cells within the AML MNC inoculum. The slope of the growth curves was similar.

The AML samples were further split into different groups depending on their cytogenetics and FAB classification for comparison of LTC BMdSC growth. All AML samples with no clinical details were excluded from the analysis. Although the n numbers were small (preliminary findings only), the growth curves were similar with no significant differences observed (not shown).

b) Lymphoma

Fifteen lymphoma bone marrow samples were received, ageing from 30 to 83 (table 5.7).

Table 5.7. Lymphoma bone marrow samples received

Lymphoma classification	Frequency	Sex / Age
T-cell, stage IV NHL	1	M / 37
Follicular NHL	2	F / 52 F / 70
Marginal zone NHL	2	M / 83
Burkitt's	1	M / 58
Diffuse large B-cell	1	M / 67
Lymphoplasmacytoid	1	M / 66
Waldenstrom's macroglobulinaemia	1	M / 79
NHL (unclassified)	6	M / 33 – 73
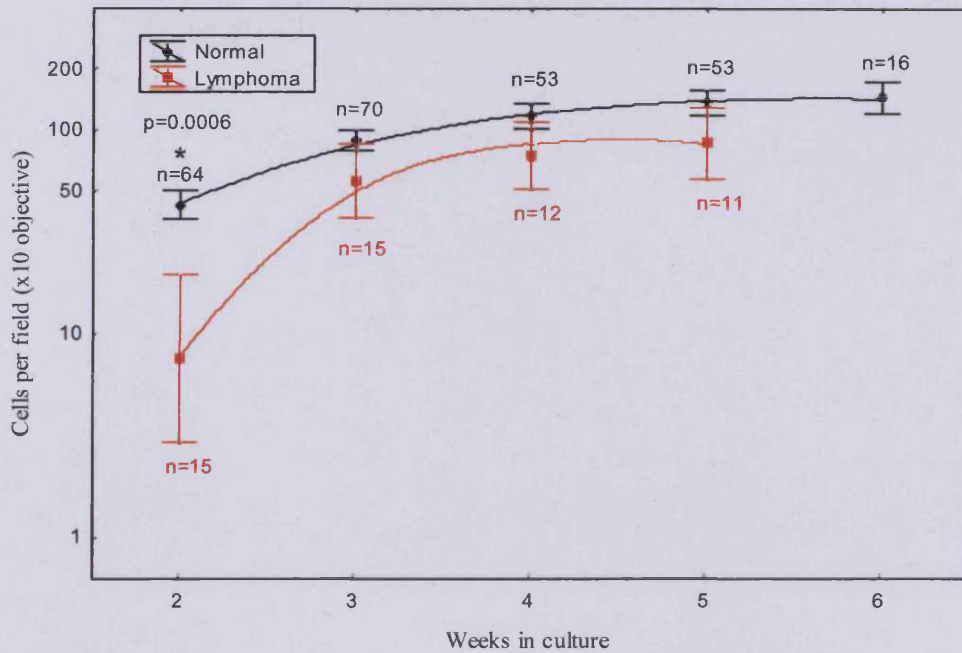
Figure 5.4. LTC BMdSC growth from normal (black circle) and lymphoma (red square) affected bone marrows. Mean ± SE

At week 2 the normal LTCs had significantly more BMdSCs per microscopic field than the lymphoma LTCs (n=15, n=64; p=0.0006). The ratio of adherent cells at week 2 over inoculum size was considerably reduced but not significant (ratio: 3.39; p=0.09), suggesting that the malignant cells were diluting out the normal adherent stromal cells to a limited extent. Figure 5.2 also shows a slight reduction in the mean inoculum values used to seed the lymphoma LTCs in relation to normal.

c) Myeloma

Twelve myeloma samples were received from patients ageing between 50 and 74 years.
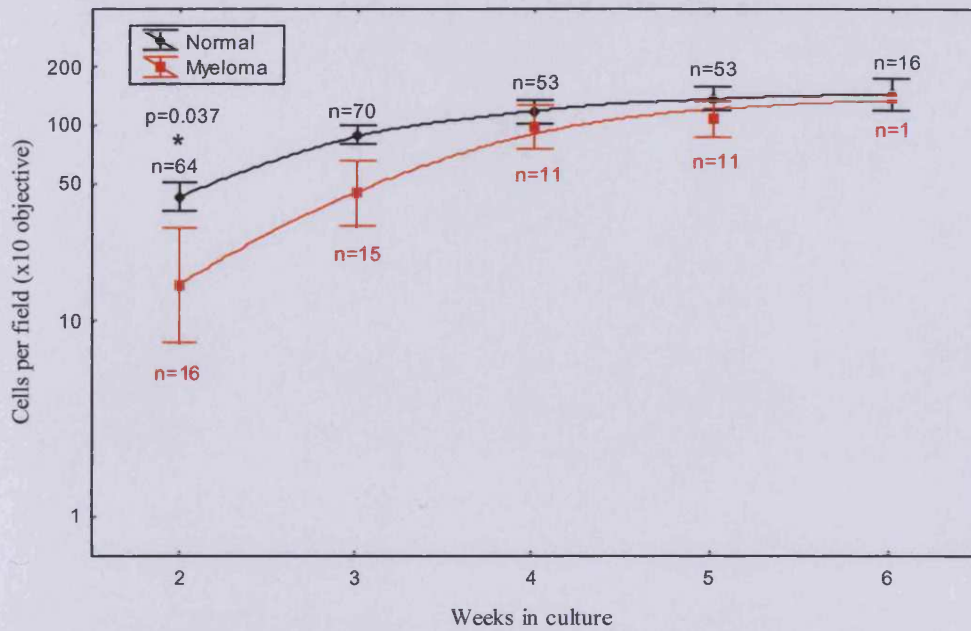
Figure 5.5. LTC BMdSC growth from normal (black circle) and myeloma (red square) affected bone marrows. Mean ± SE

At week 2 the normal LTCs had significantly more adherent BMdSCs per microscopic field than the myeloma LTCs (n=64, n=16; p=0.037), but the ratio (week 2 cell counts / inoculum: 8.82) was comparable to normal. This does not suggest a dilution effect of the stromal cells by the malignant cells within the inoculum. Figure 5.2 shows a reduction in the mean inoculum size used to seed the myeloma LTCs (not significant). The normal and myeloma BMdSC growth curves were comparable.

d) **Lymphoproliferative**

Six bone marrow samples were grouped into the lymphoproliferative group (table 5.8).

Table 5.8. Lymphoproliferative bone marrow samples

Lymphoproliferative disorder	Sex / Age
Lymphoblastic leukaemia	M / 54
	M / 60
	M / 8
CLL	M / 54
Non-CLL lymphoproliferative	M / 89
B-PLL	M / 67
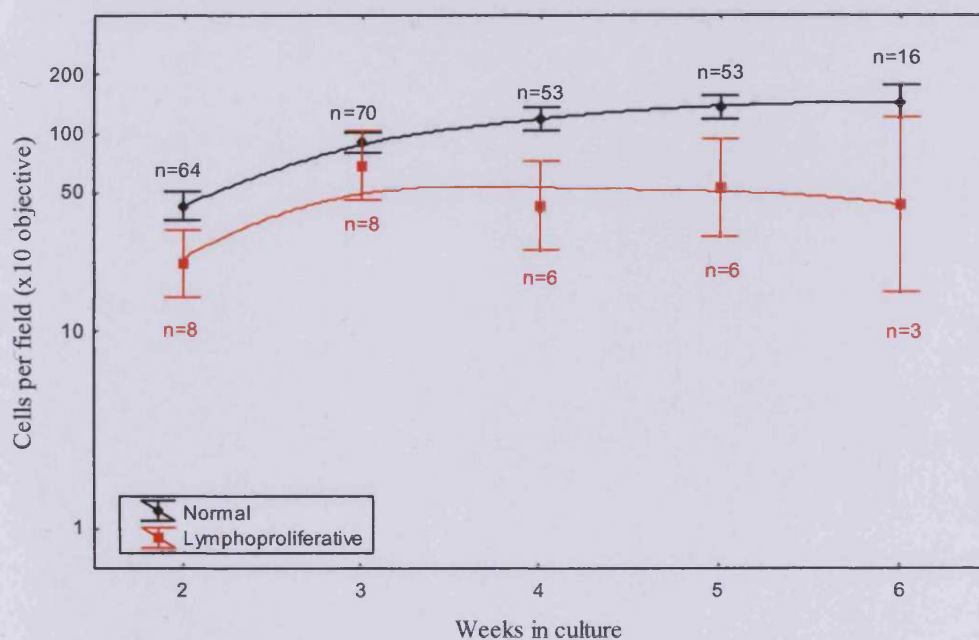
Figure 5.6. LTC BMdSC growth from normal (black circle) and lymphoproliferative (red square) bone marrows. Mean ± SE

No significant differences in LTC BMdSC growth or cell doubling (week 3 to 5) (5.3.3. LTC cell doubling) were observed between the lymphoproliferative and normal group.

e) JMML

Three JMML samples were obtained from young patients suffering from juvenile myelomonocytic leukaemia (JMML), aged 2 to 8 years old.
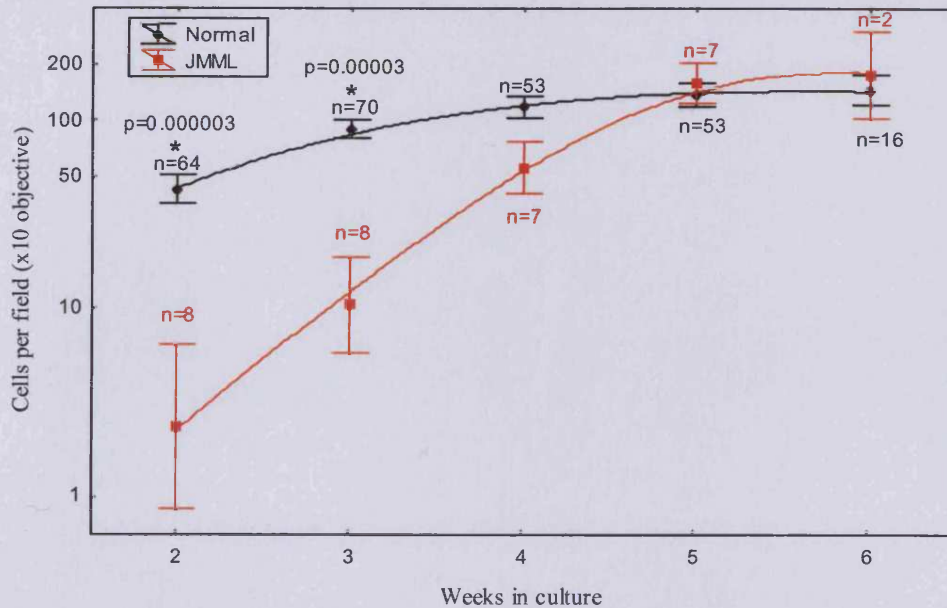
Figure 5.7. LTC BMdSC growth from normal (black circle) and JMML (red square) affected bone marrows. Mean ± SE

LTC BMdSC counts in JMML were significantly poorer at week 2 (n=8, n=64; p=0.000003) and week 3 (n=8, n=70; p=0.00003) in comparison to normal. The ratio of adherent cells at week 2 over inoculum size was significantly reduced in comparison to normal (JMML ratio: 1.46, normal ratio: 8.22; p=0.0005), suggesting a reduced proportion of stromal cells in relation to malignant cells within the MNC inoculum. However, in relation to AML (figure 5.3) and lymphoma (figure 5.4) the growth rate was rapid with a significantly higher cell doubling from week 3 to week 5 in comparison to normal (n=7, n=53; p=0.000001) (5.3.3. 'LTC cell doubling'). No normal samples were obtained within this age group to ascertain if the increased growth rate was characteristic of this age group.

f) MDS

Five MDS samples were obtained, aged 62 to 87 years (table 5.9).

Table 5.9. MDS classified bone marrow samples received

MDS classification	Sex / Age	Cytogenetics
RARS	F / 75	Normal
	F / 62	Not known
RA	F / 69	Not known
Unclassified	F / 63	Not known
	M / 75	Not known
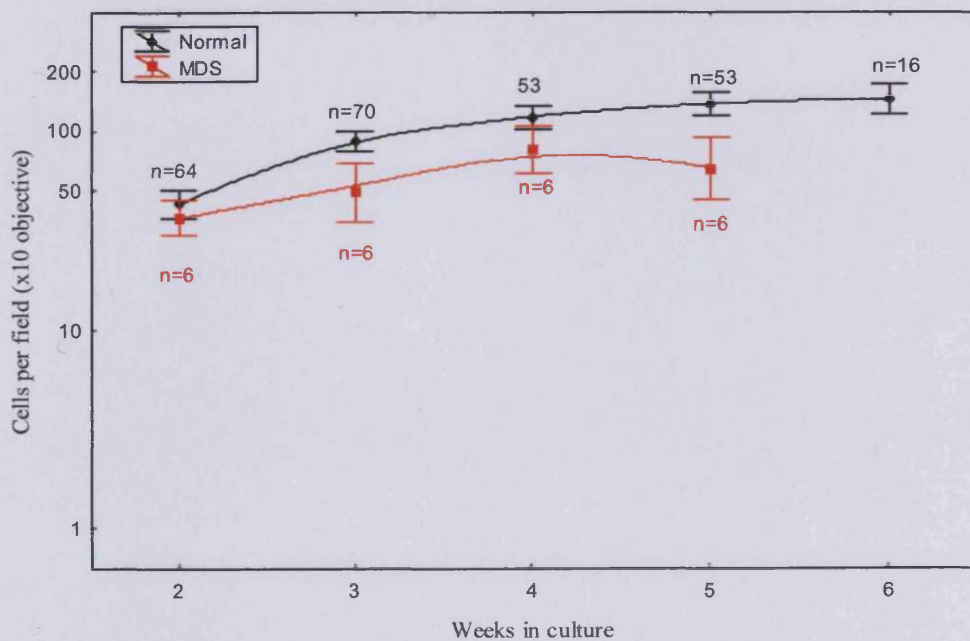
Figure 5.8. LTC BMdSC growth from normal (black circle) and MDS (red square) affected bone marrows. Mean ± SE

No significant differences in LTC BMdSC growth or cell doubling (week 3 to 5) (5.3.3. LTC cell doubling) were observed between the MDS and normal group.

g) **Summary**
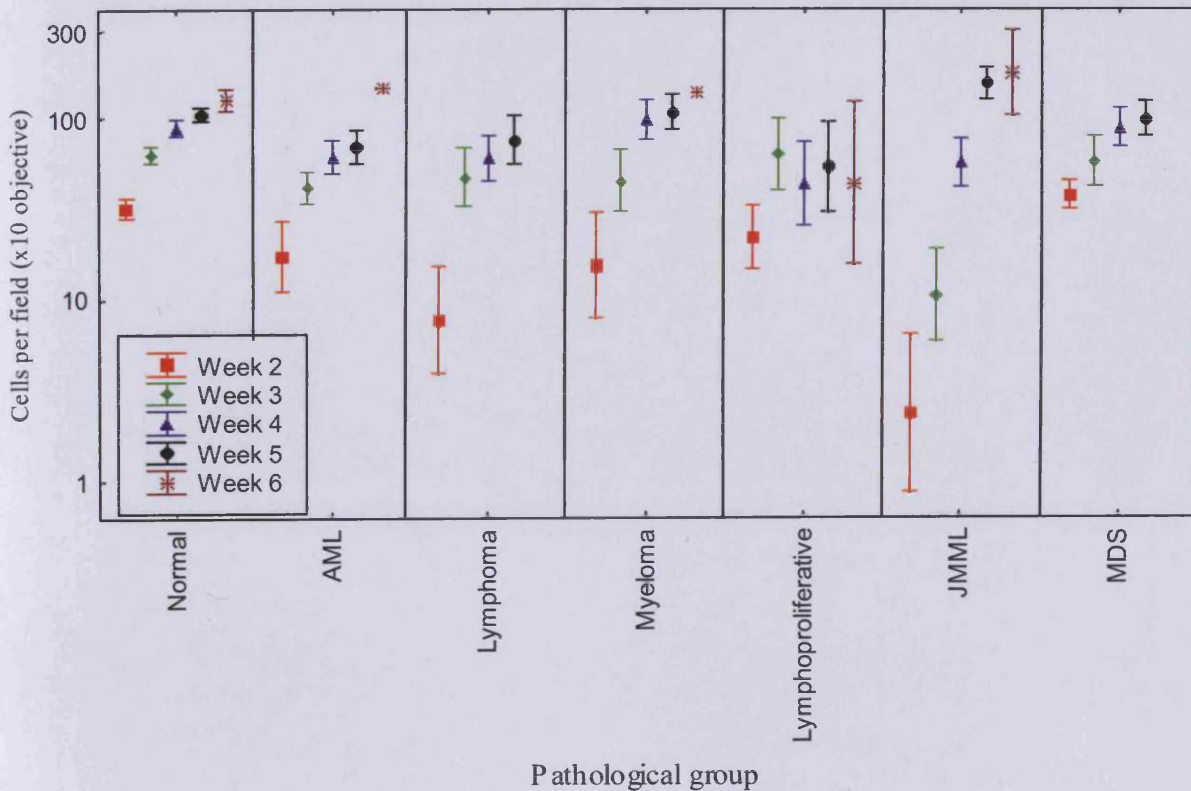
Figure 5.9. LTC BMdSC growth from normal and pathological bone marrows. Mean ± SE

The adherent cells at weeks 2 and 3 were often significantly reduced in comparison to normal when derived from hypercellular bone marrows (particularly AML and JMML), which was likely to be due to a dilution effect of the stromal population by the malignant cells within the inoculum. The growth curves were comparable to normal with the exception of JMML, which showed a significantly increased growth rate.

5.3.3. LTC cell doubling

Cell doubling was determined from weeks 3 to 5 for each pathological group in comparison to normal (figure 5.10).
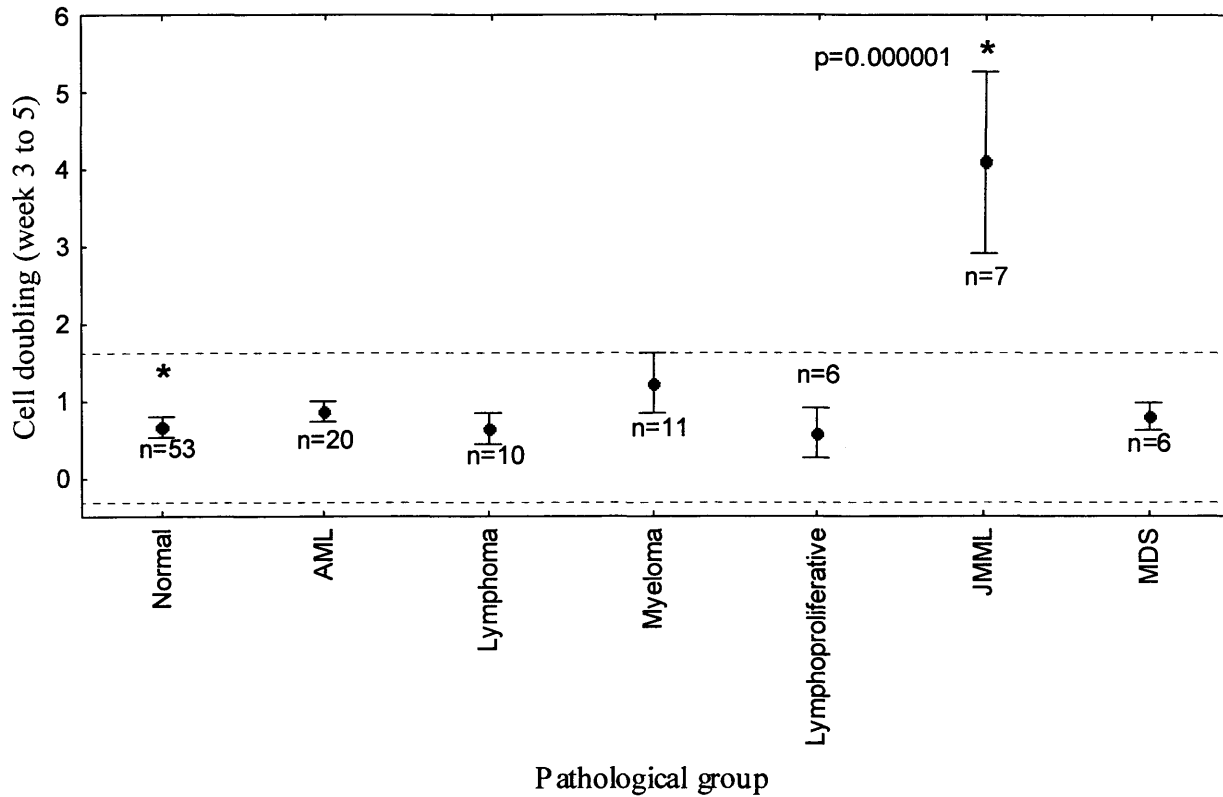
Figure 5.10. LTC cell doubling from normal and pathological bone marrows. Mean ± SE and 95% confidence interval of normal (-----)

The growth rate for normal LTC BMdSCs was comparable to AML, lymphoma, myeloma, lymphoproliferative and MDS BMdSCs, although 2 myeloma cultures (n=11) had cell doublings above the normal range (figure 5.10). With more data an increase in myeloma BMdSC proliferation may become apparent. The cell doubling was significantly increased in JMML cultures from weeks 3 to 5 as indicated previously (5.3.2.e. 'JMML').

5.3.4. **LTC CFU-F analysis**

Small (<25 cells), medium (25-100 cells) and large (>100 cells) CFU-F colonies were compared between normal and pathological groups at week 2 (figure 5.11).
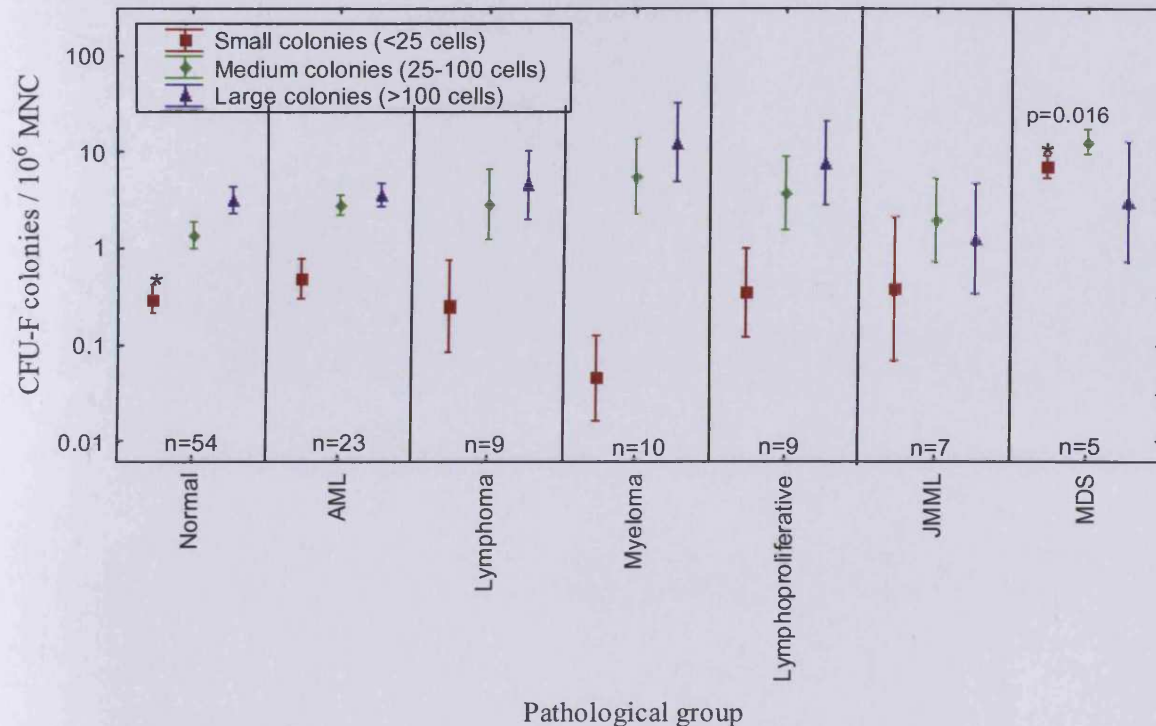
Figure 5.11. LTC CFU-F colony formation, small (<25 cells, red square), medium (25-100 cells, green diamond) and large (>100 cells, blue triangle), in normal and pathological samples. Mean ± SE

Significantly more small colonies (red square) were formed in the MDS LTCs (n=5, n=54; p=0.016) in comparison to normal. There were no significant differences in the number of large CFU-F colonies (blue triangle) between the normal and pathological groups. The MDS group was the only group analysed in which all 5 cultures contained small colonies. This could indicate the presence of an abnormal subpopulation of more committed (differentiated) progenitors, whilst the more immature progenitors, capable of forming larger colonies, were comparable to normal. Colony size has been shown to be reduced in Dexter-type LTCs in MDS patients with good prognosis, whilst still being able to form confluent cell layers (Duhrsen & Hossfeld 1996). The myeloma results showed a reduced number of small CFU-F colonies and an increased number of large CFU-F colonies (not significant). This may be related to the cultures showing an increased cell doubling (2 cultures with >95% confidence interval of normal, 5.3.3. 'LTC cell doubling'), and may suggest a stimulatory effect on the BMdSCs by abnormal cytokine production induced by the myeloma cells.

5.3.5. Summary

With the exception of JMML, no significant differences in cell growth and cell doubling were observed between normal and pathological LTC BMdSCs beyond week 3. JMML was shown to have a significantly higher growth rate, but no age-related normal marrows were received to attribute this to the age of the patient (5.9.7. 'JMML'). No significant differences in CFU-F colony formation were observed between the normal and pathological groups with the exception of MDS, which showed a significantly increased number of small colonies. With more data, it may be that the BMdSCs within the myeloma cultures start to show an increase in proliferation and CFU-F colony formation as a result of abnormal cytokine production.

5.4. Passaged BMdSC Growth

The passaged BMdSCs from pathological bone marrows behaved similarly to normal cultures (results not shown). They were sub-cultured to passage 3 and grown to sub-confluence or confluence for bone differentiation or lipid differentiation respectively at each passage. There were no significant differences in passaged BMdSC growth, cell doubling or CFU-F colony formation in Alpha MEM between normal and pathological cultures (not shown).

5.5. Passaged BMdSC Characterisation by Cell Markers

Passaged BMdSCs, derived from normal and pathological bone marrows, were characterised by cell surface markers, CD90, CD44, CD166, CD105, CD49b, CD106 and CD45 (2.5.2.b. 'BMdSC characterisation by flow cytometry'). The level of antigenic expression was determined from the G-mean ratio of the test over isotype control. The G-mean ratios from normal and pathological groups were compared to a human foetal foreskin fibroblast cell line, HFFF2 (2.2.4. Cell lines). The G-mean ratios for each antibody were shown on the same scale to indicate the varying levels of expression.

5.5.1. CD90
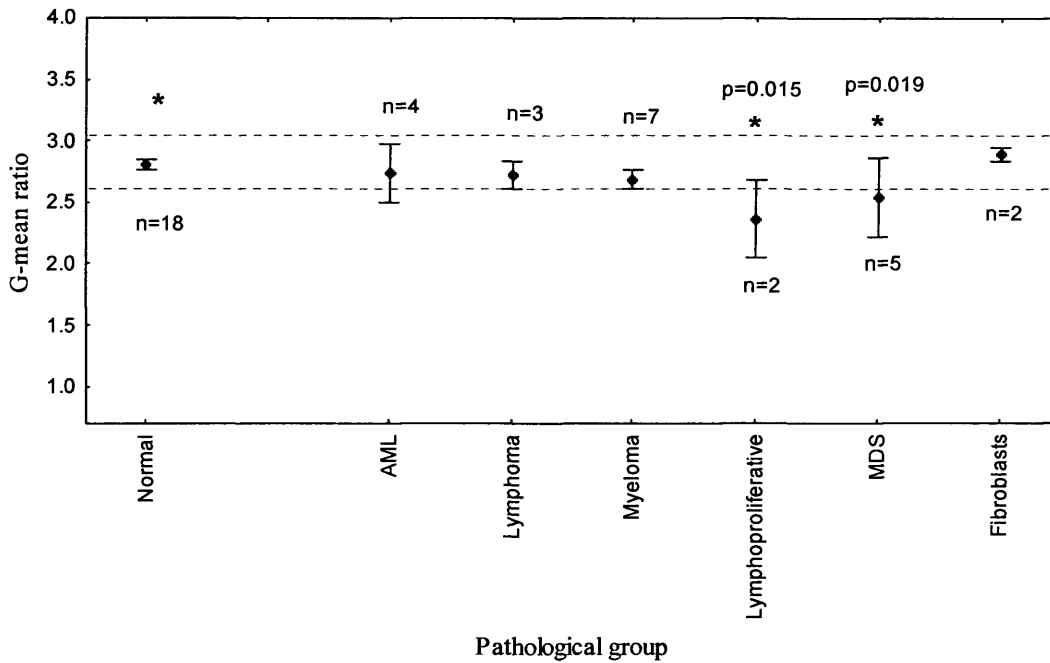
Figure 5.12. CD90-FITC antigenic expression on passaged normal and pathological BMdSCs and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The lymphoproliferative group (n=2, n=18; p=0.015) and the MDS group (n=5, n=18; p=0.019) had a significantly reduced CD90 expression in comparison to normal (figure 5.12). The mean values of the remaining pathological groups were comparable to normal. One AML culture showed expression below the normal range.

5.5.2. CD44
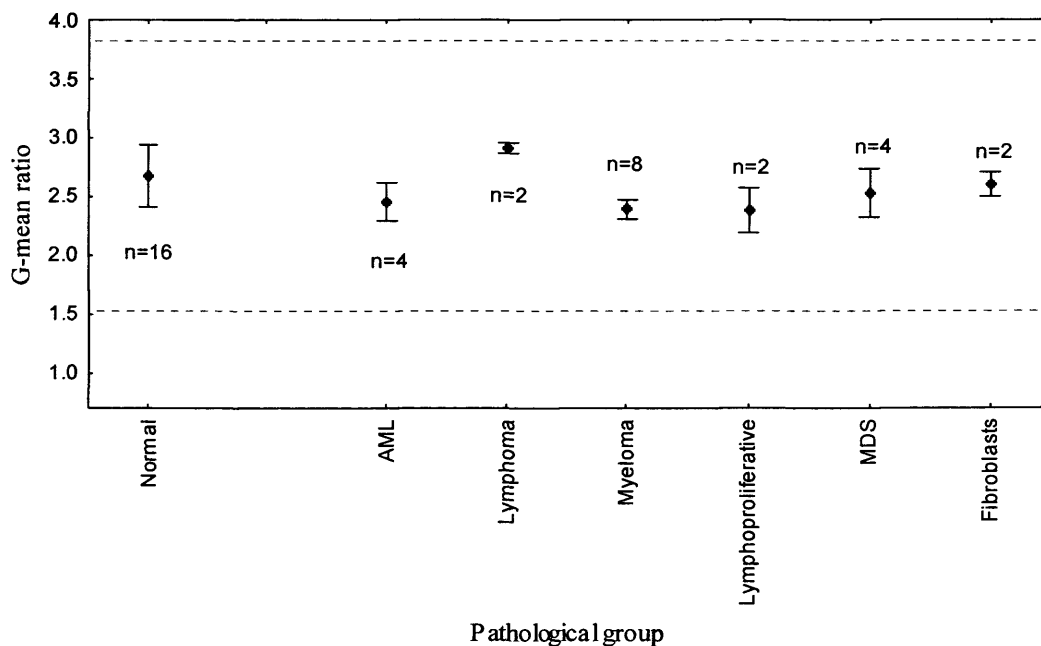
Figure 5.13. CD44-PE antigenic expression on passaged normal and pathological BMdSCs and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The level of CD44 expression was highly variable in the normal passaged BMdSC cultures. All pathological cultures were comparable to normal (figure 5.13).

5.5.3. CD166
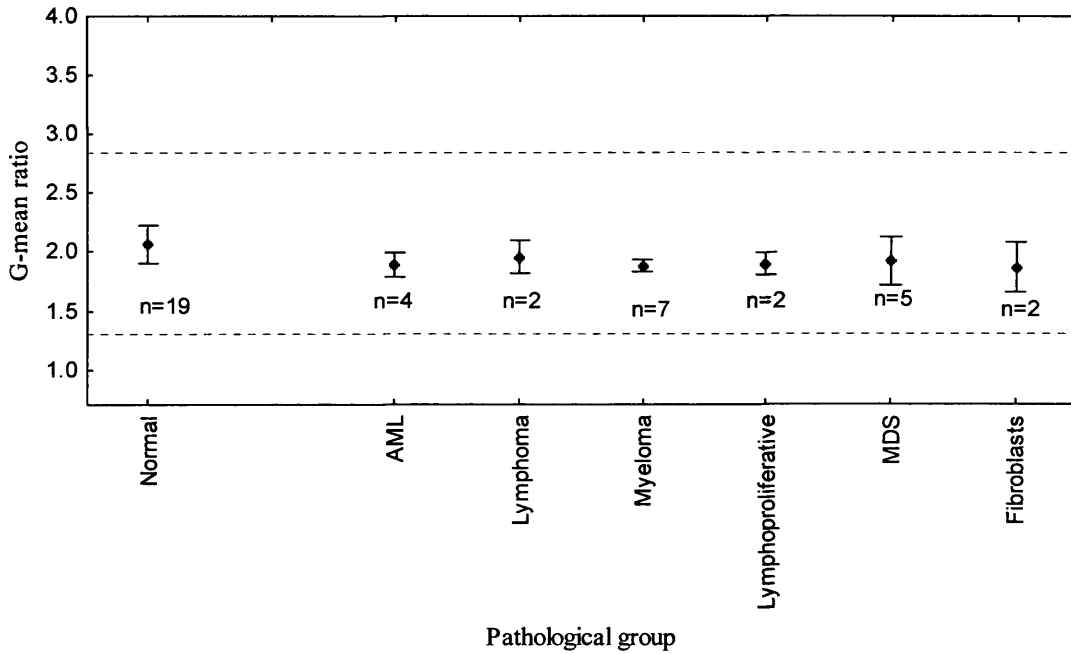
Figure 5.14. CD166-PE antigenic expression on passaged normal and pathological BMdSCs and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

There were no significant differences in CD166 expression between the normal and fibroblast cell line or pathological groups (figure 5.14).

5.5.4. CD105
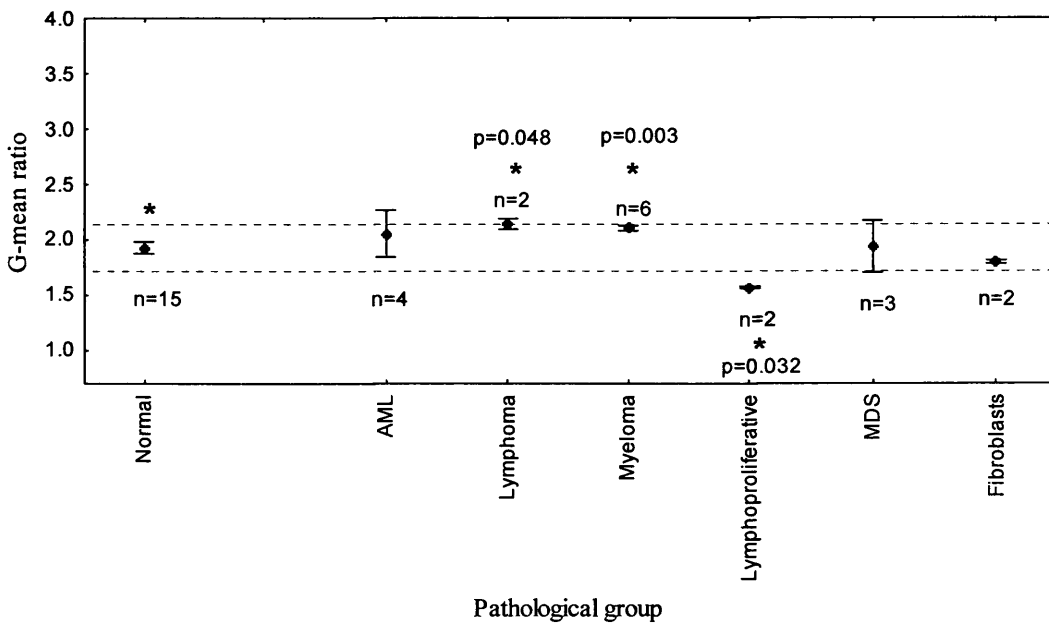
Figure 5.15. CD105-RAMPE antigenic expression on passaged normal and pathological BMdSCs and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The lymphoproliferative group had a significantly reduced CD105 expression (n=2, n=15; p=0.032) (both cultures below the normal range), whilst the lymphoma (n=2; p=0.048) and the myeloma (n=6; p=0.003) groups showed a significantly increased expression in comparison to normal (figure 5.15). The mean values for the AML and MDS groups, and the fibroblast cell line were comparable to normal. One AML culture had expression above the normal range.

5.5.5. CD49b
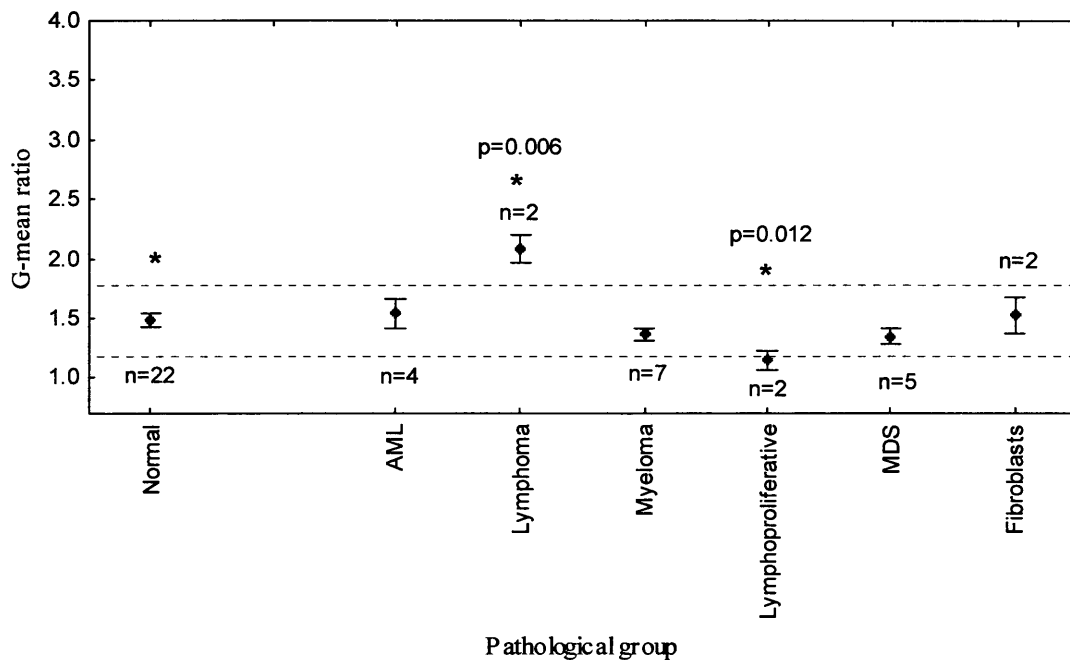
Figure 5.16. CD49b-FITC antigenic expression on passaged normal and pathological BMdSCs and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The lymphoma group had a significantly increased CD49b expression (n=2, n=22; p=0.006) (both cultures showing expression above the normal range) whilst the lymphoproliferative group showed a significantly reduced expression (n=2; p=0.012) (both cultures showing expression below the normal range) in comparison to normal (figure 5.16). The mean values of the remaining pathological groups were comparable to normal.

5.5.6. CD106
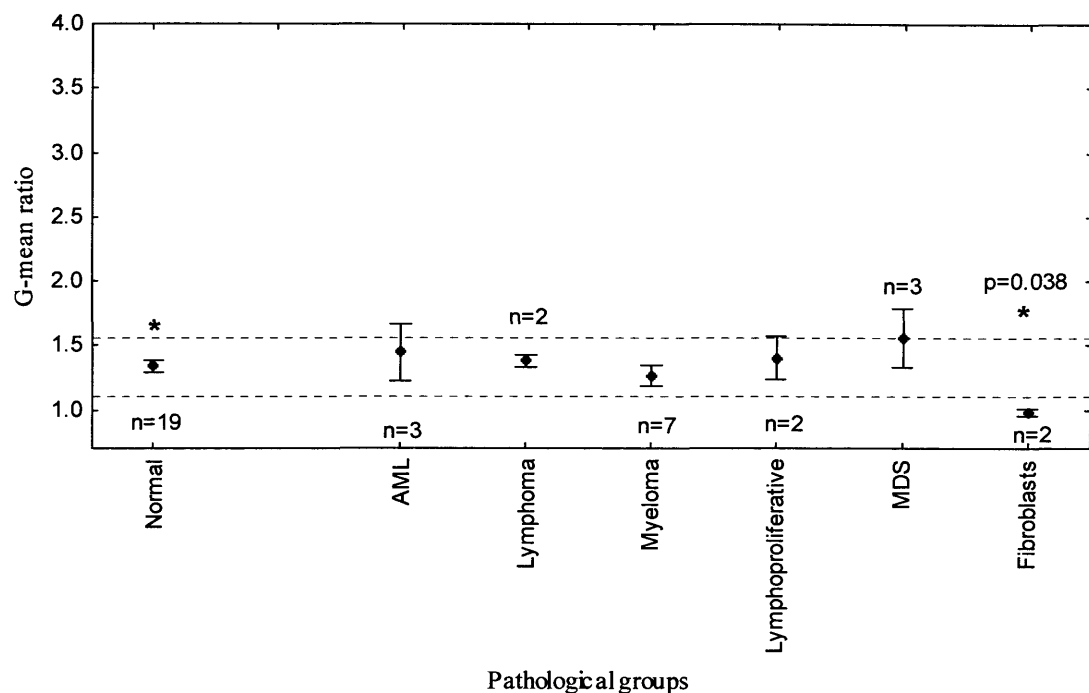
Figure 5.17. CD106-RAMPE antigenic expression on passaged normal and pathological BMdSCs and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

So far the fibroblasts have shown similar phenotypical and morphological expression to normal BMdSCs, however the level of CD106 expression was significantly reduced in comparison to normal with both fibroblast cultures showing expression below the normal range (figure 5.17). CD106 (VCAM-1, Stro-1) has been found to be more specific for BMdSCs having been used to isolate the more primitive BMdSCs from the bone marrow (Gronthos et al 1994; Stewart et al 2003), and is predominant in the interaction between stromal and haemopoietic cells (Duhrsen & Hossfeld 1996; Makrynikola & Bradstock 1993). No significant differences were observed between the normal and pathological cultures, although one out of the three MDS cultures and one out of the three AML cultures were above the normal range.

5.5.7. CD45
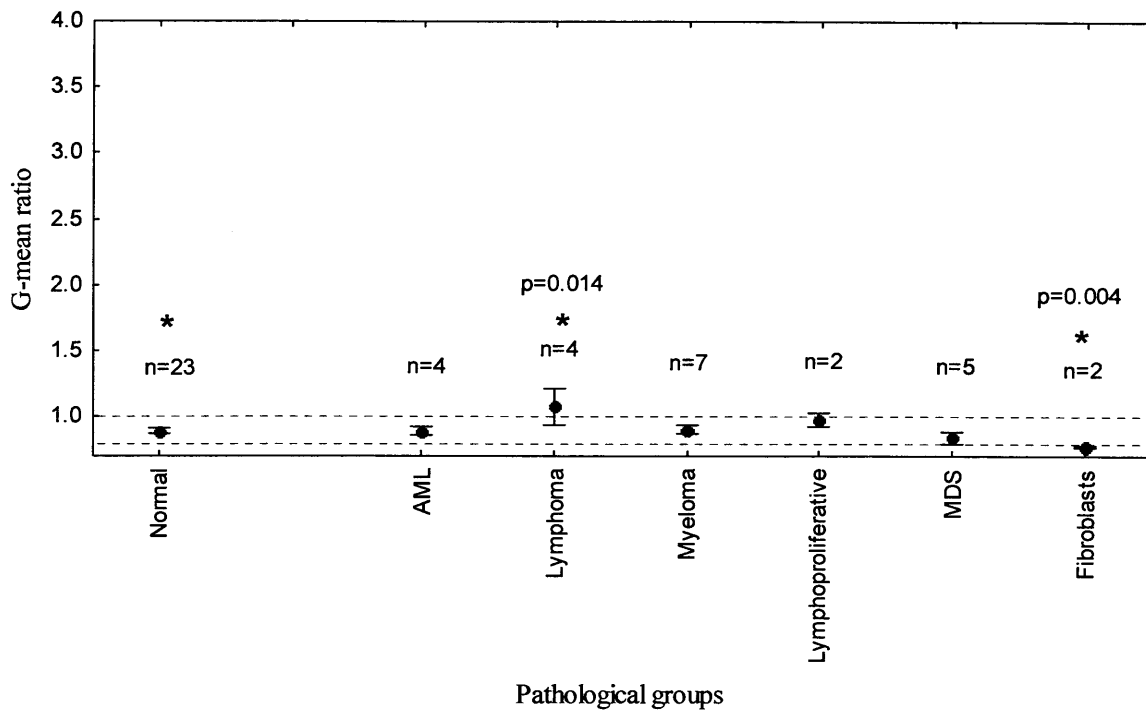
Figure 5.18. CD45-PerCP antigenic expression on passaged normal and pathological BMdSCs and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The lymphoma group showed significantly increased CD45 expression (n=4, n=23; p=0.014), whilst the fibroblasts showed significantly reduced expression (n=2, n=23; p=0.004) in comparison to normal (figure 5.18). The mean values of the remaining pathological groups were comparable to normal.

5.5.8. Summary

Table 5.10 compares the fibroblast and BMdSC antigenic expression values of normal and pathological cultures.

Table 5.10. Review of BMdSC characterisation in comparison to normal

	90	44	166	105	49b	106	45
AML	C	C	C	C	C	C	C
Lymphoma	C	C	C	SI p=0.048	SI p=0.006	C	SI p=0.014
Myeloma	C	C	C	SI p=0.003	C	C	C
Lymphoproliferative	SR p=0.015	C	C	SR p=0.032	SR p=0.012	C	C
MDS	SR p=0.019	C	C	C	C	C	C
Fibroblasts	C	C	C	C	C	SR p=0.038	SR p=0.004

C: Comparable antigenic expression (mean value within 95% confidence interval)

SR: Significantly reduced (p<0.05)

SI: Significantly increased (p<0.05)

The fibroblasts showed significantly reduced CD106 antigen expression, which has been reported to be more selective for primitive BMdSCs, and a significantly reduced non-specific binding of CD45. The remaining antigenic determinants were comparable. The results indicate that the expression profile analysed could not be used to specifically isolate or enrich BMdSC populations. The pathological groups had comparable CD106 and CD44 antigen expression, which indicates that the BMdSCs were capable of normal interaction with haemopoietic cells and ECM. They also had comparable CD166 antigen expression, which is reported to decline during osteogenesis.

5.6. **Cytogenetics**

Table 5.11. Cytogenetics of pathological cultures

Origin	Passage	Pathology	Haemopoietic cytogenetics	BMdSC Cytogenetics	Cells analysed
845	1	Pro T-cell ALL		46XY	20
AC6	2	MDS		46XX	15
ADB	2	Myeloma		46XY	10
AD6	1	AML		46XX	15
ADO	2	AML	Complex	46XY	15
846	2	AML relapse	-4 add (5)	46XX	9

Cytogenetics was only analysed in 6 pathological BMdSC cultures at passages 1 or 2, from which only 2 had haemopoietic cytogenetics available from diagnosis. The passaged BMdSCs (absent in macrophages which may carry the same cytogenetic defect if derived from the same leukaemic clone) did not have the same cytogenetic abnormalities as the pathological haemopoietic cells. Table 3.11 showed that BMdSCs did not undergo cytogenetic transformation as a function of passage.

5.7. **Osteogenic Induction**

Mineralisation (calcium deposition), cellular ALP activity, collagen I formation and gene expression (osteopontin, osteocalcin, collagen I and ALP) were compared between normal and pathological samples (table 5.12) in Alpha MEM ($\geq$100 mg/l $CaCl_2$) at 21 days post induction. The effect of the osteogenic induction agents on a human foreskin fibroblast cell line (HFFF2) and the pathological groups was compared to the effect on normal BMdSCs. The normal range for the mineralised cultures (95% confidence interval of normal) was shown by black dotted lines. The normal and 'pathological normal' groups were combined for analysis.

5.7.1. Pathological samples

Table 5.12. Bone marrow donations / aspirations per group and donor age

Pathological group	Bone marrow donations/aspirates	Age range
Normal	40	21 – 46
Pathological normal	5	33 – 62
AML	7	30 – 72
Lymphoma	3	33 & 67
MDS	2	69 & 75

a) AML

Seven AML bone marrow samples were osteogenically induced (table 5.13).

Table 5.13. AML samples osteogenically induced

FAB	Number of samples received	Sex / Age	Cytogenetics
M1	1	F / 30	t(16;16)
M2	3	M / 72 F / 36 F / 62	Normal -4 add (5) Normal
M5	1	M / 37	Normal
Biphenotypic	1	M / 37	Complex
Not known	1	M / 37	Not known

b) Lymphoma

Three lymphoma bone marrow samples were osteogenically induced (table 5.14).

Table 5.14. Lymphoma samples osteogenically induced

Classification	Frequency	Sex / Age
Not known	1	M / 33
T-cell, stage IV NHL	1	M / 37
Diffuse large B-cell	1	M / 67

c) MDS

Two bone marrow samples were osteogenically induced (table 5.15).

Table 5.15. MDS samples osteogenically induced

Classification	Sex / Age
RA	F / 69
RARS	F / 75

5.7.2. **Mineralisation**

Calcium deposit was stained with Alizarin red S at 21 days post osteogenic induction in Alpha MEM. Mineralisation was quantitated by demineralising the cultures in HCl and measuring the optical density at 420 nm (2.7.3.c. 'Demineralisation'). Not all BMdSC cultures mineralised within 21 days of osteogenic induction, therefore, markers of osteogenesis were assessed within the normal and pathological groups in both mineralised and non-mineralised cultures.
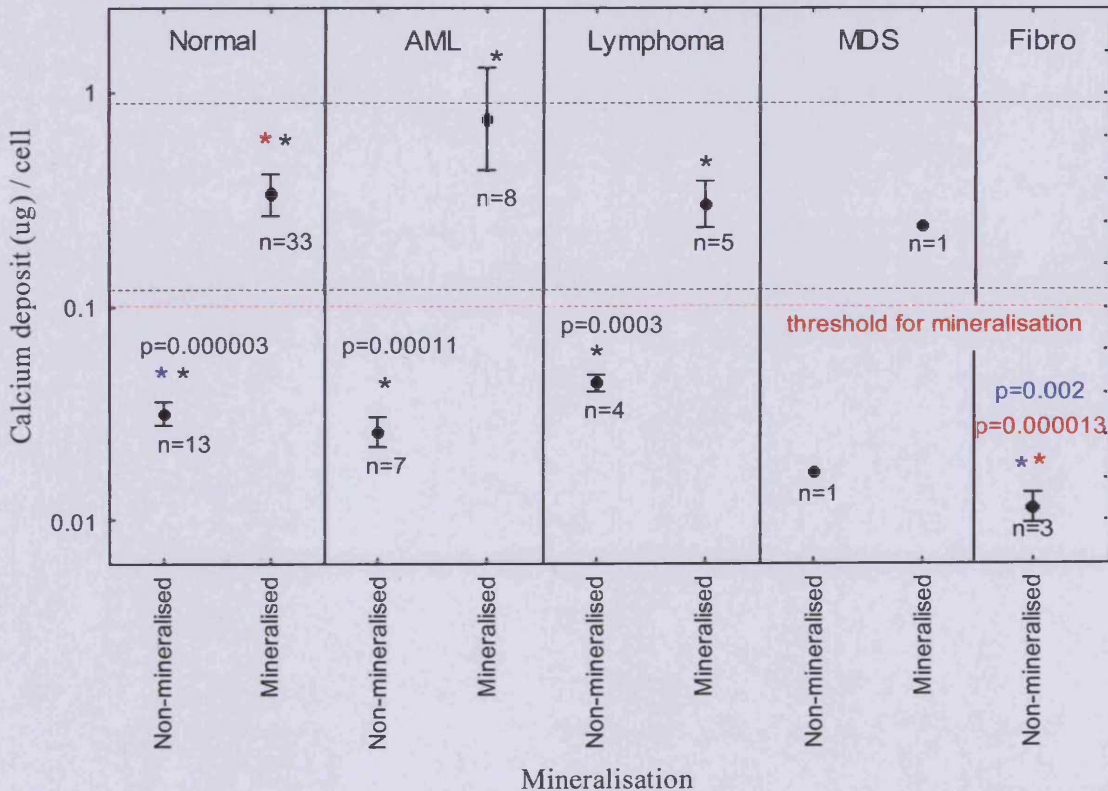
Figure 5.19. Calcium deposition per cell in mineralised and non-mineralised cultures from normal and pathological bone marrows, and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (----)

Significantly more calcium was deposited in the mineralised cultures than the non-mineralised cultures by 21 days post induction in the normal (n=33, n=13; p=0.000003), AML (n=8, n=7; p=0.00011) and lymphoma cultures (n=5, n=4; p=0.0003) (figure 5.19). Approximately 50% of cultures mineralised within each group. From the two MDS samples, one mineralised (RA) and one did not (RARS), but insufficient samples were induced to relate this to the disease. No significant differences were observed between the normal and pathological BMdSC cultures that mineralised. The fibroblast cell line (non-mineralised) showed significantly less calcium deposition than the normal non-mineralised cultures (n=3, n=13; p=0.002). This is shown in photo 5.2, which demonstrates Alizarin red S staining of a confluent osteogenically induced fibroblast culture, a control culture and a normal osteogenically induced mineralised culture at 21 days post induction. No mineralisation occurred in the absence of induction agents or BMdSCs. The induction medium showed no precipitation of calcium when incubated (dotted line on figure 4.12).

Photo 5.2. Alizarin Red S staining of an osteogenically induced fibroblast cell line, control and mineralised BMdSC culture at 21 days post induction

In chapter 4, mineralisation was equated to the time of induction and the total cell counts at induction (figure 4.10), therefore, the numbers of cells at induction were established for each mineralised and non-mineralised pathological culture (figure 5.20).
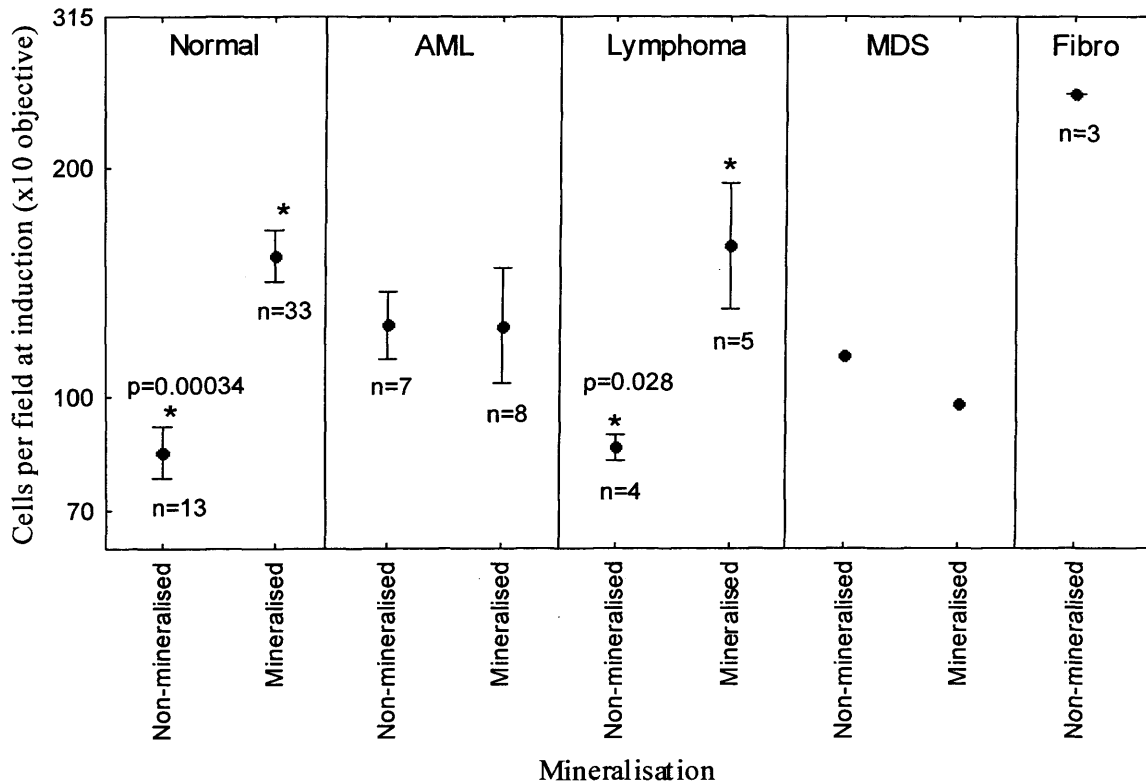
Figure 5.20. Cell counts per field at induction for normal, pathological and fibroblast cultures. Mean ± SE

The non-mineralised cultures showed significantly fewer cells per field at induction than the mineralised cultures in the normal (n=13, n=33; p=0.00034) and lymphoma groups (n=4, n=5; p=0.028) (figure 5.20). The AML cultures showed no relationship between the cell number at induction and mineralisation.

5.7.3. **Cellular ALP activity**

Cellular ALP activity was quantitated by measuring the hydrolysis of p-nitrophenyl phosphate to p-Nitrophenol (2.7.3.e. 'Cellular ALP activity').
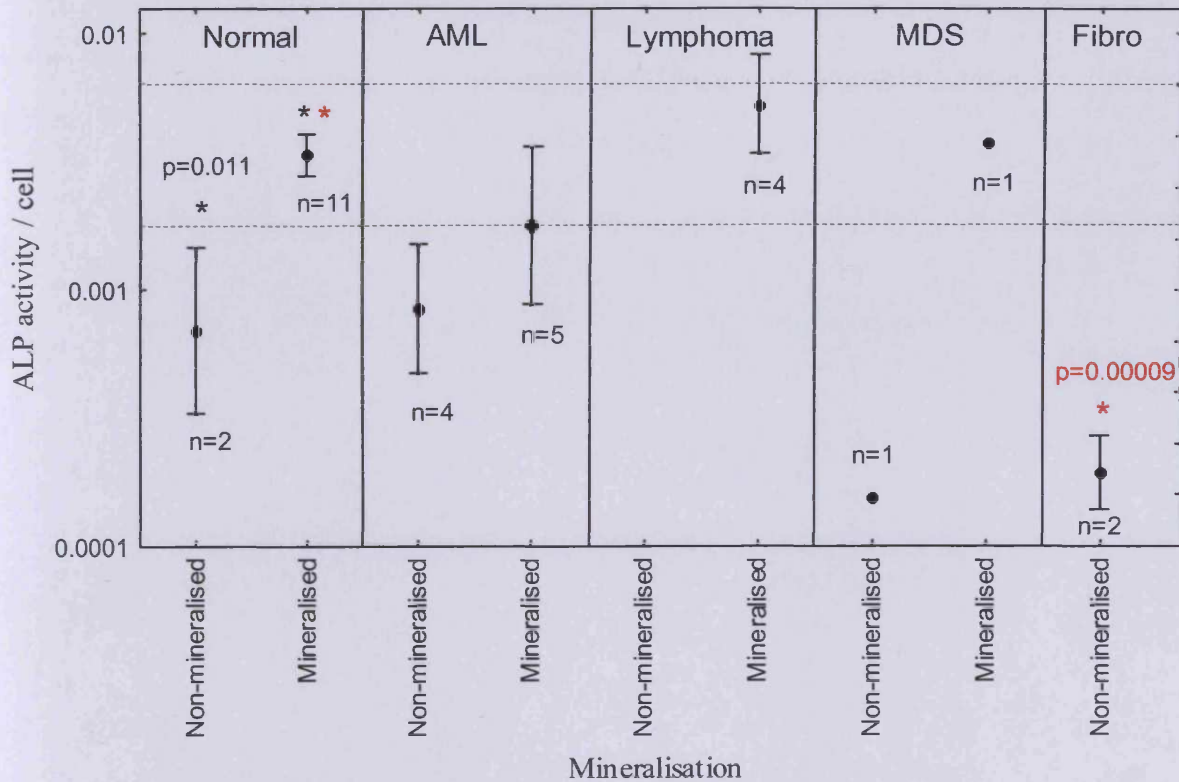
Figure 5.21. Cellular ALP activity per cell in mineralised and non-mineralised cultures from normal and pathological bone marrows and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The normal mineralised cultures were associated with significantly more ALP activity per cell (figure 5.21), and per culture (not shown), than the non-mineralised cultures (n=11, n=2; p=0.011). The AML mineralised group did not show a significant increase in ALP activity per cell or per culture at 21 days post induction. No significant differences were observed between the normal and pathological mineralised cultures. Two AML cultures showed ALP activity below the 95% confidence interval, whilst two lymphoma cultures showed ALP activity above the normal range. The fibroblasts showed reduced ALP activity in comparison to the normal non-mineralised cultures (not significant) and were associated with significantly less ALP activity per cell than the normal mineralised cultures (n=2, n=11; p=0.00009).

5.7.4. **Collagen I**

Collagen I was stained using an immunocytochemical technique involving the ABC (Avidin-Biotin Complex) reaction and quantitated using the flask reader (2.7.3.d. 'Collagen I').
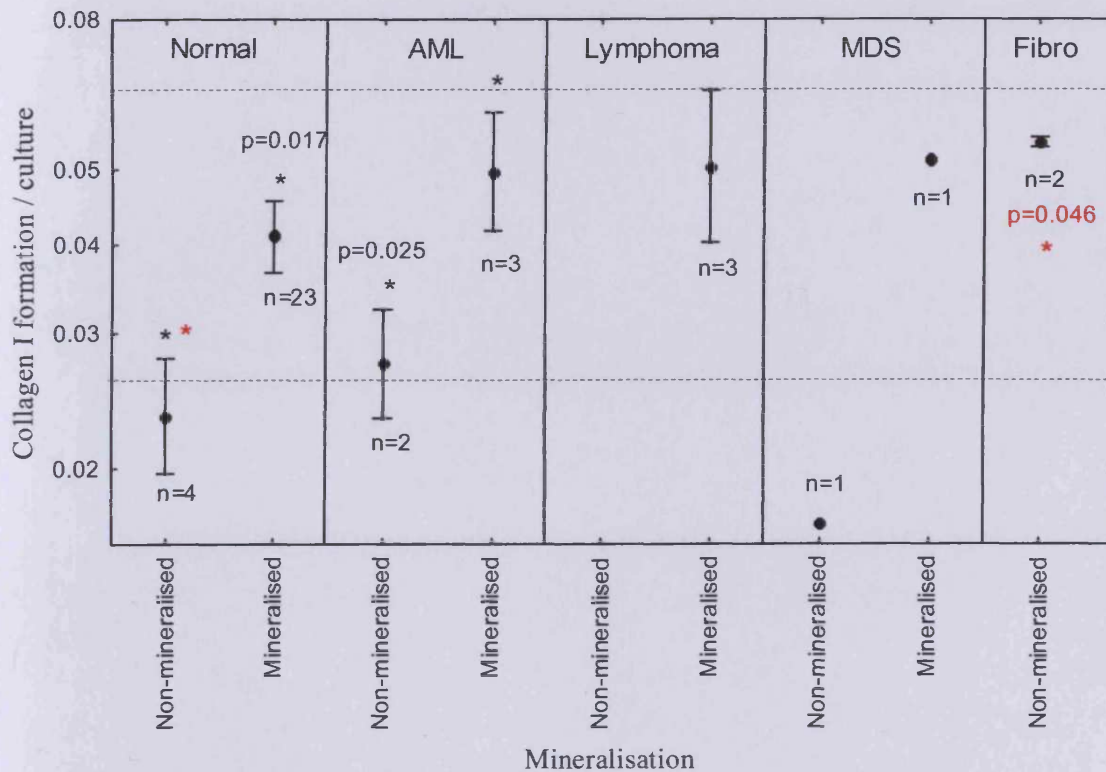
Figure 5.22. Collagen I formation per culture in mineralised and non-mineralised cultures from normal and pathological bone marrows and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The normal and AML mineralised cultures were associated with significantly more collagen I formation per culture (but not per cell, 4.5.4.d.'Collagen I') than the non-mineralised cultures (normal: n=23, n=4; p=0.017 & AML: n=3, n=2; p=0.025) (figure 5.22). No significant differences were observed between the normal and pathological mineralised or non-mineralised cultures. The non-mineralised fibroblast cell line produced significantly more collagen I than the normal non-mineralised BMdSC cultures (n=2, n=4; p=0.046).

5.7.5. Gene expression analysis

Osteogenic-related gene expression (osteopontin, osteocalcin, collagen I and alkaline phosphatase) was compared between normal and pathological, non-mineralised and mineralised, cultures at 21 days post induction in Alpha MEM ($\geq$100 mg/ml $CaCl_2$).

a) Osteopontin
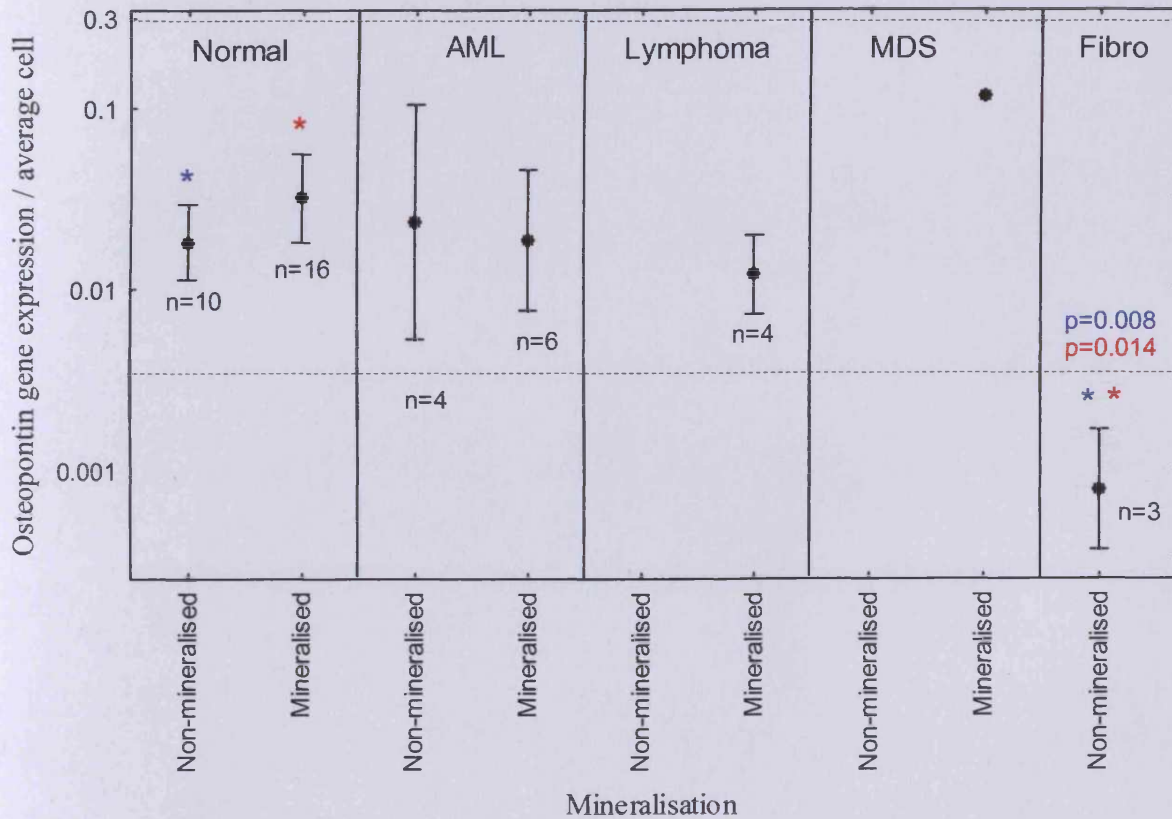
Figure 5.23. Osteopontin gene expression per average cell in mineralised and non-mineralised cultures from normal and pathological bone marrows and a fibroblast cell line. Mean $\pm$ SE and 95% confidence interval of normal (-----)

No significant differences in osteopontin gene expression at 21 days post induction were observed between the non-mineralised and mineralised cultures in normal or pathological groups (figure 5.23). There were no significant differences between normal and pathological mineralised or non-mineralised cultures. The non-mineralised fibroblast cell line had significantly less osteopontin gene expression per average cell than the normal non-mineralised cells (n=3, n=10; p=0.008).

b) Osteocalcin
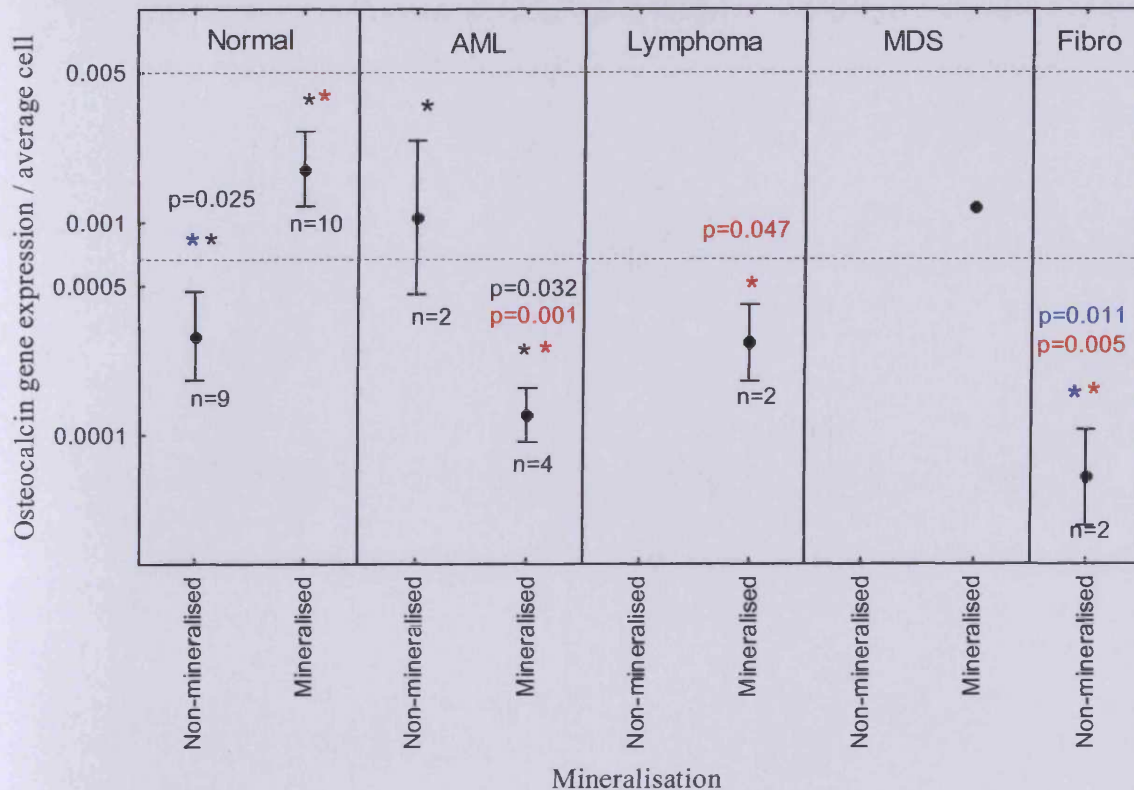
Figure 5.24. Osteocalcin gene expression per average cell in mineralised and non-mineralised cultures from normal and pathological bone marrows and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The normal mineralised cultures had significantly more osteocalcin gene expression per average cell than the non-mineralised cultures (n=10, n=9; p=0.025), but the AML mineralised cultures had significantly less osteocalcin gene expression than the non-mineralised cultures (n=4, n=2; p=0.032) (figure 5.24). No significant differences were observed between the normal and pathological non-mineralised cultures, but the normal mineralised cultures had significantly more osteocalcin gene expression per average cell than the mineralised AML cultures (n=10, n=4; p=0.001) and the mineralised lymphoma cultures (n=2; p=0.047). The non-mineralised fibroblast cell line had significantly less osteocalcin gene expression than the normal non-mineralised cultures (n=2, n=9; p=0.01).

c) Collagen I
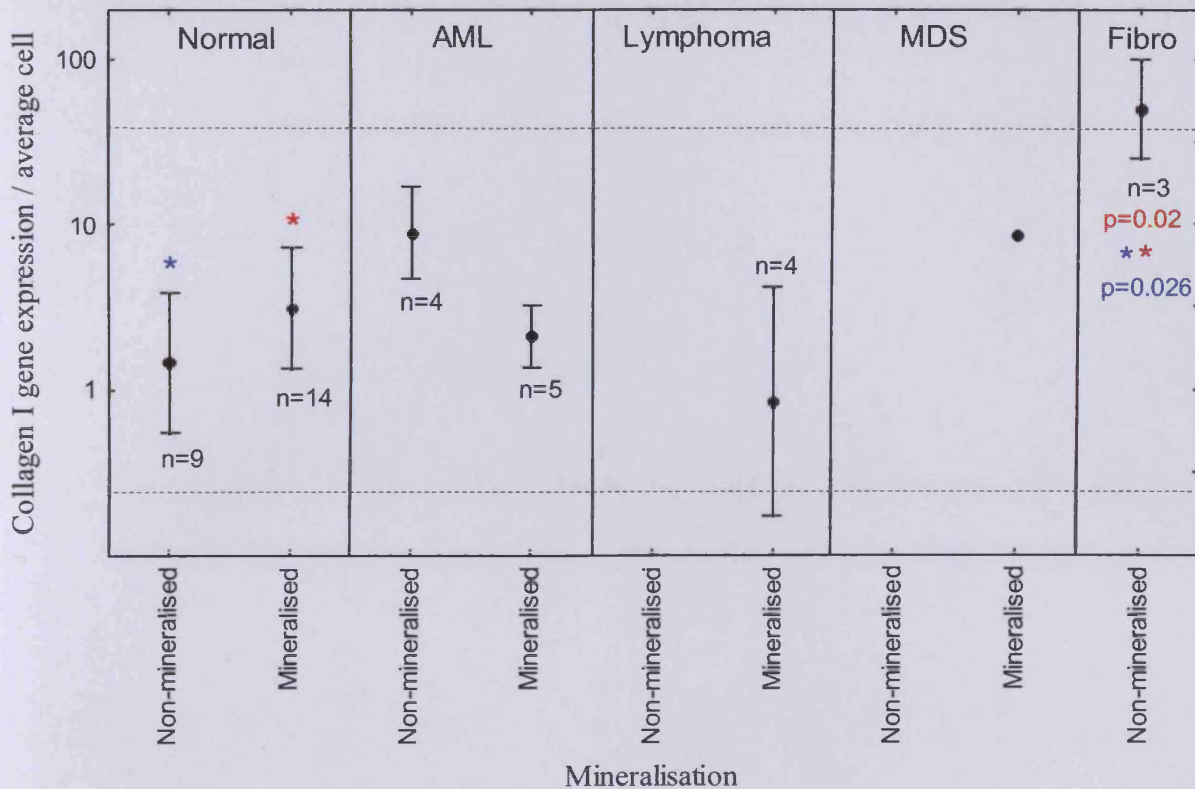
Figure 5.25. Collagen I gene expression per average cell in mineralised and non-mineralised cultures from normal and pathological bone marrows and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The normal mineralised cultures showed comparable collagen I gene expression to the normal non-mineralised cultures, whilst the AML mineralised cultures showed a reduction in collagen I gene expression in comparison to AML non-mineralised cultures (not significant) (figure 5.25). No significant differences were observed between normal and pathological mineralised or non-mineralised cultures. The non-mineralised fibroblast cell line showed significantly increased collagen I gene expression per average cell than the normal non-mineralised (n=3, n=9; p=0.026) and mineralised cultures (n=3, n=14; p=0.02).

d) Alkaline phosphatase
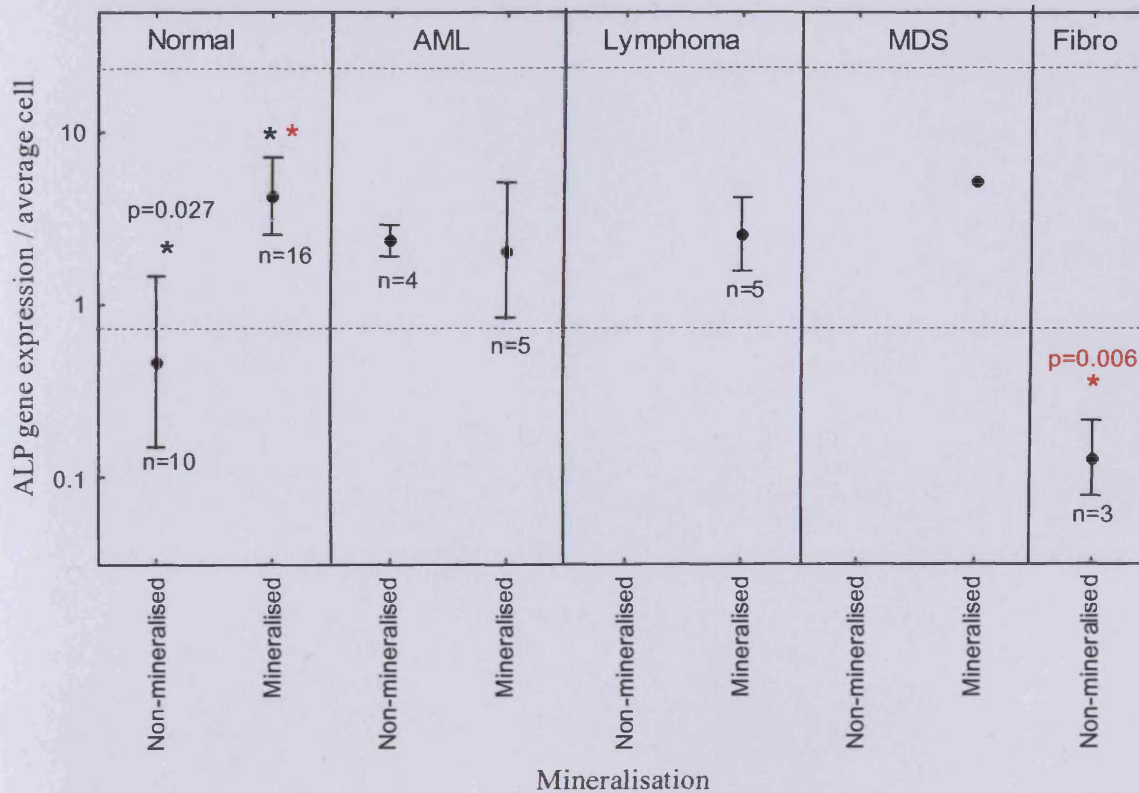
Figure 5.26. ALP gene expression per average cell in mineralised and non-mineralised cultures from normal and pathological bone marrows and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The normal mineralised cultures had significantly more ALP gene expression per average cell than the non-mineralised cultures (n=16, n=10; p=0.027), however there were no significant differences between the mineralised and non-mineralised AML cultures (figure 5.26). No significant differences were observed between the normal and pathological mineralised or non-mineralised cultures. The fibroblast cell line had reduced ALP gene expression in comparison to the normal non-mineralised cultures (not significant) and significantly less ALP gene expression per average cell than the normal mineralised cultures (n=3, n=16; p=0.006).

5.7.6. **Summary**

Table 5.16 compares the fibroblast cell line to normal non-mineralised BMdSCs, and the normal mineralised cultures to the pathological mineralised cultures.

Table 5.16. Osteogenic markers of differentiation in normal and pathological mineralised cultures

	Mineral deposit	ALP activity	Collagen I protein	Osteo-pontin	Osteo-calcin	Collagen I	ALP
AML	C	R	C	C	SR	C	C
Lymphoma	C	I	C	C	SR	C	C
MDS	C	C	C	C	C	C	C
Fibroblasts	SR	R	SI	SR	SR	SI	R

C: Comparable (all cultures within 95% confidence interval)

R: Reduced (cultures below 95% confidence interval)

I: Increased (cultures above 95% confidence interval)

SR: Significantly reduced ($p=<0.05$)

SI: Significantly increased ($p=<0.05$)

[   ]   Gene expression

The fibroblast cell line showed no signs of osteogenesis with significantly less calcium deposition, osteopontin and osteocalcin gene expression than the normal non-mineralised cultures. Collagen I provides tensile strength to tissues, therefore, a significant increase in collagen I (mRNA and protein) would be expected from a fibroblast cell line derived from skin (human foreskin), but the highly collagenous ECM did not mineralise.

The normal and AML mineralised cultures deposited significantly more calcium and collagen I than the non-mineralised cultures at 21 days post induction. Besides calcium and collagen I deposition, the pattern of osteogenesis was very different between the normal and AML cultures. The AML mineralised cultures showed reduced osteocalcin and collagen I gene expression in comparison to the non-mineralised cultures, whilst the normal mineralised cultures showed significantly increased expression. The AML mineralised cultures also showed comparable ALP (activity and mRNA) to the non-mineralised cultures in comparison to the normal mineralised cultures having significantly increased ALP (activity and mRNA). No significant differences were observed between the normal and AML non-mineralised cultures, but the AML mineralised cultures showed significantly reduced osteocalcin gene expression

and reduced ALP activity (cultures below the 95% confidence interval of normal) in comparison to the normal mineralised cultures (table 5.16). The mineralised lymphoma cultures showed significantly reduced osteocalcin gene expression and increased ALP activity (cultures above normal range) in comparison to the normal mineralised cultures (table 5.16).

5.8. Adipogenic Induction

Adipogenically induced normal and pathological cultures (table 5.17) were compared at 21 days post induction for multidroplet cell cluster formation (normalised to DNA content) and adipogenic-related gene expression (LPL, aP2 and PPAR-γ2). The normal and 'pathological normal' groups were combined for analysis, as was Alpha MEM and DMEM. The effect of the adipogenic induction agents on a human foetal foreskin fibroblast cell line (HFFF2) was compared with the effect on normal BMdSCs. The normal range for the successfully induced cultures (95% confidence interval of normal) was shown by black dotted lines.

Table 5.17. Bone marrow samples, disease classification and donor age

Pathological group	Bone marrow donations / aspirates	Age range	Disease classification
Normal	14	M / 21 - 39 F / 39 - 43	
Pathological normal	10	F / 22 - 69 M / 70 - 72	
AML	2	M / 37 M / 37	Unclassified M5, normal cytogenetics
Lymphoma	4	M / 33 M / 30 & 54 F / 54	NHL NHL Diffuse large B-cell

5.8.1. **Multidroplet cell cluster formation**

Not all adipogenically induced normal and pathological cultures formed multidroplet cell clusters (figure 4.37), therefore, markers of differentiation were compared in cultures that did and did not form clusters. Only multidroplet cell colonies visible using a dissecting light microscope were counted.
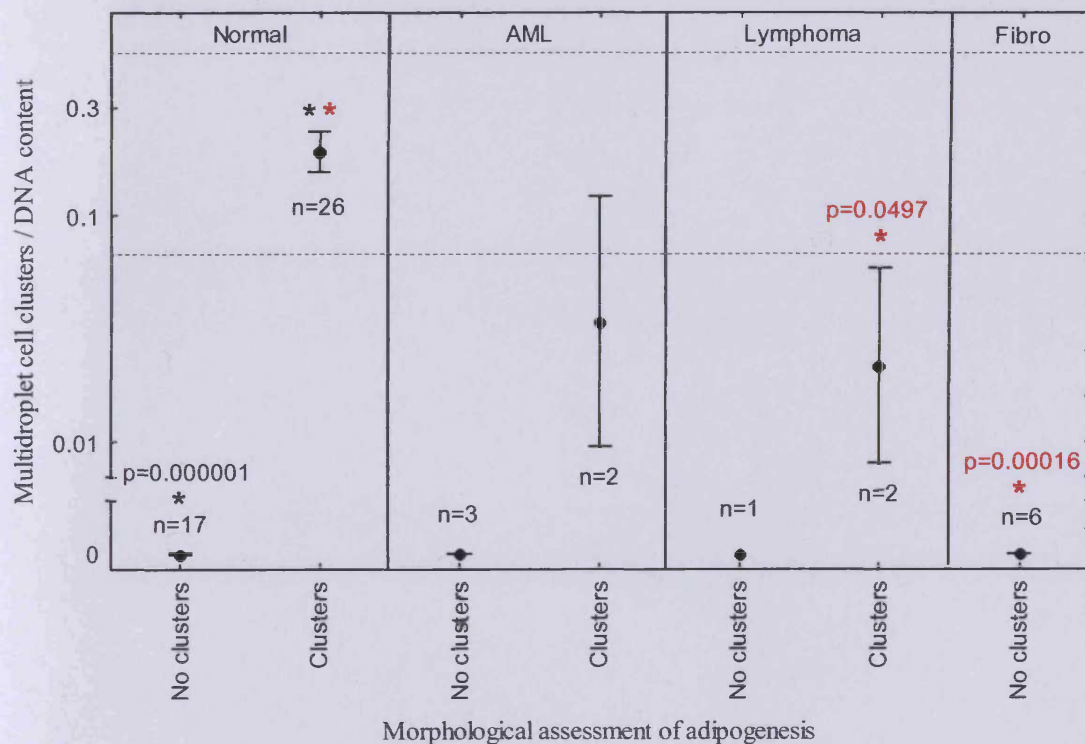
Figure 5.27. Multidroplet cell cluster formation in normal and pathological cultures, and a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

Multidroplet cell clusters were observed in approximately 50% of cultures. The successfully induced AML cultures had fewer multidroplet cell clusters (one culture below the 95% confidence interval), and the lymphoma cultures had significantly less multidroplet cell clusters, than the normal cultures showing morphological signs of adipogenic differentiation (n=2, n=26; p=0.0497) (figure 5.27). With further analysis, it is likely that the data for AML would also become significant. The fibroblasts formed no multidroplet fat cells.

Reduced numbers of cells at induction appeared to be associated with an increase in the number of multidroplet cell clusters by 21 days post induction in the normal cultures (figure 4.37), therefore, the cell counts at induction were analysed within the pathological cultures (figure 5.28).
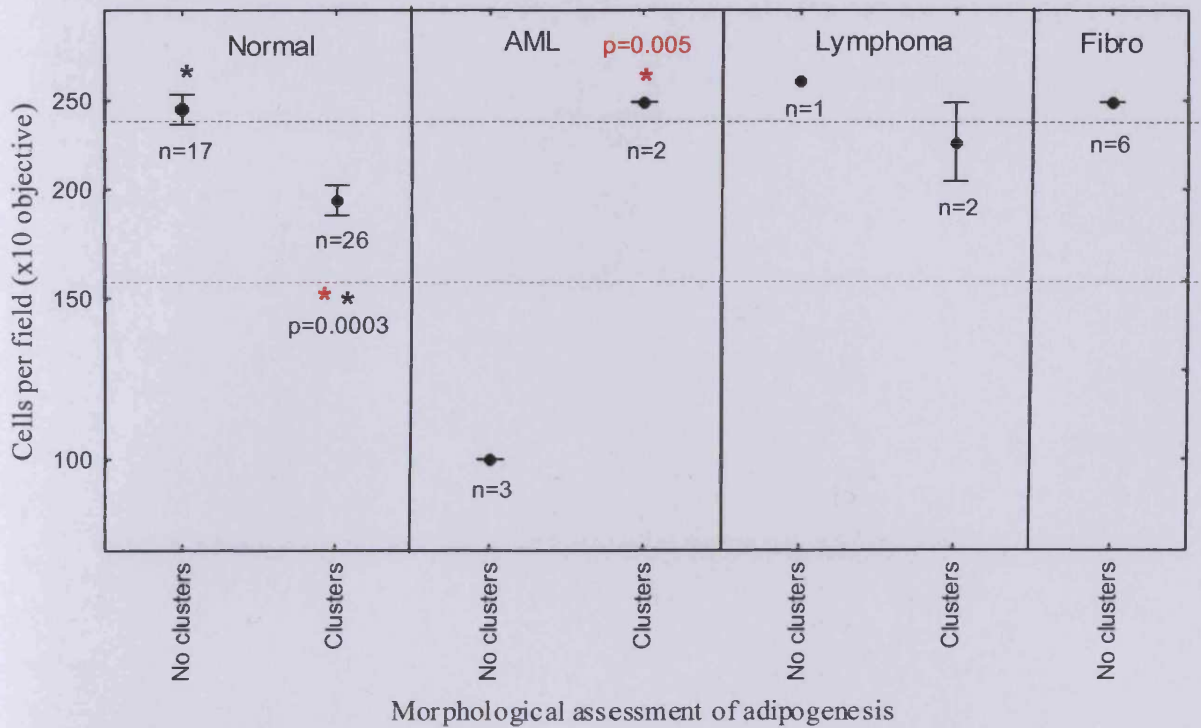
Figure 5.28. Cell counts at induction for normal and pathological cultures with and without multidroplet cell clusters. Mean ± SE and 95% confidence interval of normal (-----)

The successfully induced normal cultures had a significantly reduced number of cells per microscopic field at induction in comparison to the cultures that did not develop clusters (n=26, n=17; p=0.0003) (figure 5.28). The AML cultures (with clusters) had a significantly higher number of cells at induction than the normal cultures (with clusters) (n=2, n=26; p=0.005) with a reduced multidroplet cell cluster formation. The lymphoma cultures (with clusters) had a higher number of cells at induction than the normal cultures (with clusters) (not significant) with a significantly reduced number of multidroplet cell clusters by 21 days post induction. This relates to the theory that more confluent cultures inhibit adipogenic colony formation.

5.8.2. Gene expression analysis

Lipid-related gene expression (aP2, LPL and PPAR-γ2) (normalised to the housekeeping gene S14) was analysed at 21 days post induction in adipogenically induced normal and pathological BMdSCs, and human fibroblast cells.

a) **aP2**
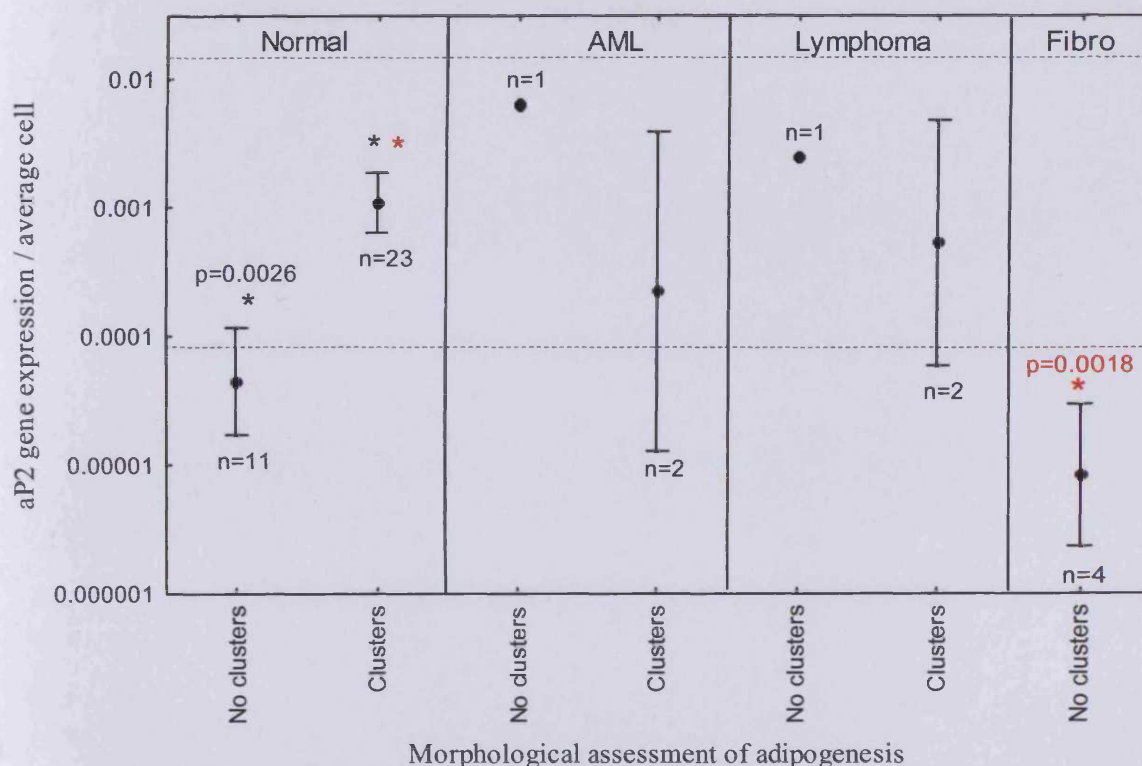
Figure 5.29. aP2 gene expression per average cell in normal and pathological groups and in a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The successfully induced normal cultures had significantly more aP2 gene expression than the cultures with no multidroplet cell clusters (n=23, n=11; p=0.0026). This pattern was not observed in the pathological groups, however, only 1 AML and 1 lymphoma culture were analysed with no multidroplet cell clusters. No significant differences were observed between the successfully induced normal and pathological groups (low sample numbers), but one out of two lymphoma cultures showed expression on the lower limit of normal, and one out of two AML cultures showed expression below the normal range. The fibroblast cell line had reduced aP2 gene expression in comparison to the normal cultures with no clusters (not significant).

b) LPL
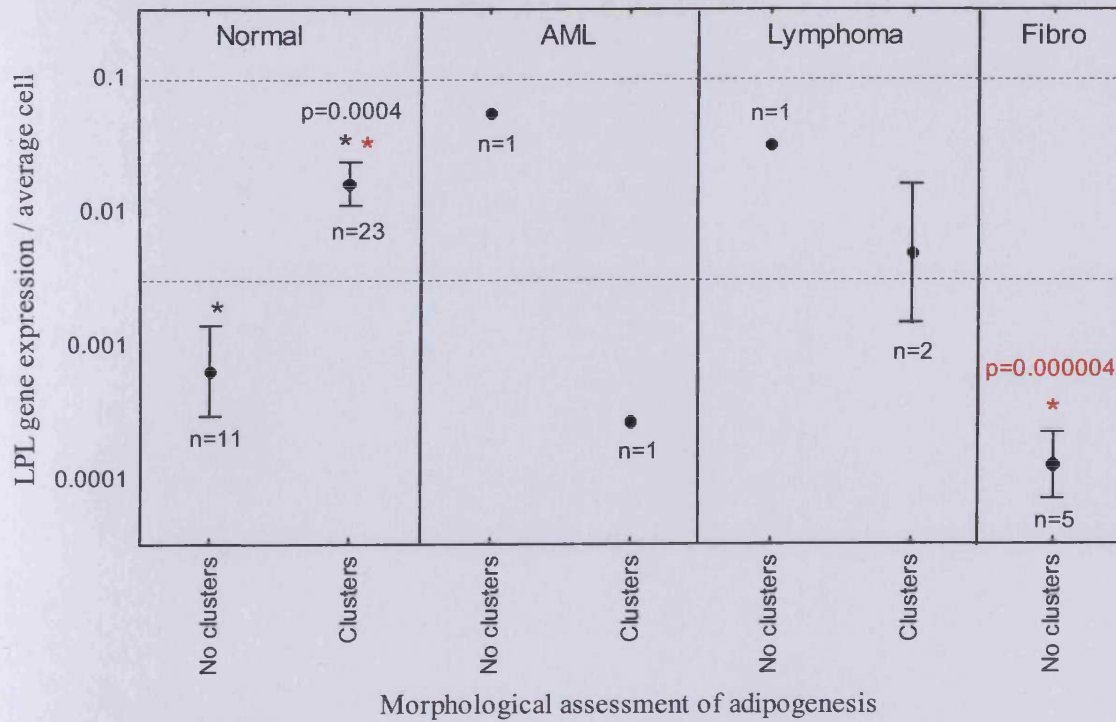
Figure 5.30. LPL gene expression per average cell in normal and pathological groups and in a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

The successfully induced normal cultures had significantly more LPL gene expression than the normal cultures with no multidroplet cell clusters (n=23, n=11; p=0.0004). This pattern was not observed in the pathological groups, although very low numbers of samples were analysed. The pathological cultures with no multidroplet cell clusters had higher LPL gene expression than their successfully induced counterparts and the normal cultures (not significant). The one successfully induced AML culture had LPL gene expression below the normal range, and one lymphoma culture was below the normal range. The fibroblast cell line had reduced LPL gene expression in comparison to the normal cultures with no clusters (not significant).

c) PPAR-γ2
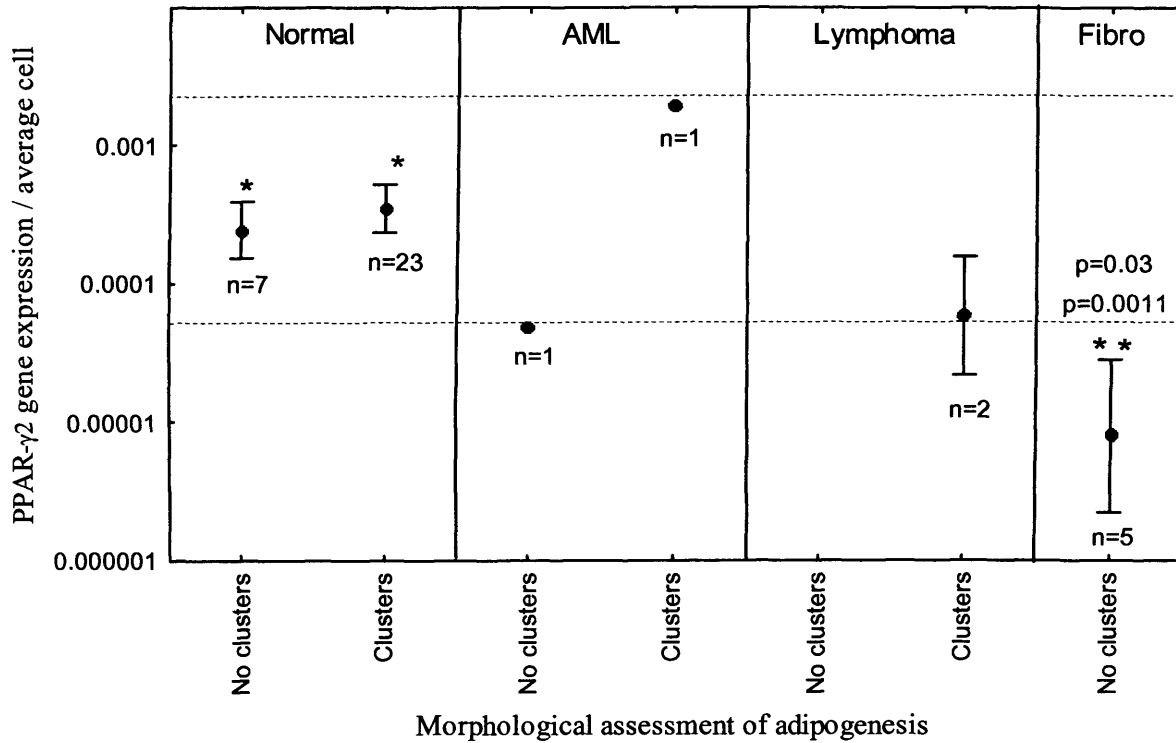
Figure 5.31. PPAR-γ2 gene expression per average cell in normal and pathological BMdSCs and in a fibroblast cell line. Mean ± SE and 95% confidence interval of normal (-----)

PPAR-γ2 gene expression was comparable between the normal cultures that did and did not develop multidroplet cell clusters, whilst the successfully induced AML cultures had a higher expression than the cultures with no clusters (opposite of aP2 and LPL gene expression). One lymphoma culture showed PPAR-γ2 gene expression below the normal range. The fibroblast cell line had significantly less PPAR-γ2 gene expression than the normal cultures with no multidroplet cell clusters (n=5, n=7; p=0.03).

5.8.3. Summary

The fibroblast cell line was compared to the normal BMdSCs with no evidence of differentiation (no clusters), whilst the successfully induced normal and pathological cultures were compared (table 5.18).

Table 5.18. Adipogenic markers of differentiation in successfully induced normal and pathological cultures, and a fibroblast cell line

	Multidroplet cell clusters	aP2	LPL	PPAR-γ2
AML	R	C	C	C
Lymphoma	SR	C	C	C
Fibroblasts	None	R	R	SR

C: Comparable (mean values within 95% confidence interval of normal)

R: Reduced (mean value below 95% confidence interval of normal)

SR: Significantly reduced ($p < 0.05$)

▭ Gene expression

Very few pathological samples were analysed, therefore, the mean values were compared to normal in table 5.18, however, in AML and lymphoma, often one culture out of two was below the normal range for aP2, LPL and PPAR-γ2 gene expression. Further data is required for good statistical analysis to determine if lipid-related gene expression was reduced in AML and lymphoma, which may then be related to a reduced multidroplet cell cluster formation. The cell counts at induction were higher in AML and Lymphoma cultures and may also be related to a reduced multidroplet cell cluster formation, or it may be that the AML and lymphoma cultures had fewer preadipocytes within the cell layer in comparison to normal. With further analysis, it may be that aP2, LPL and PPAR-γ2 gene expression is normal, but problems exist within a more complex network (e.g. protein production) (1.6.1. 'Network interactions'), reducing the cell's ability to accumulate lipid.

The fibroblast cell line showed no evidence of adipogenic differentiation with no multidroplet cell cluster formation and reduced aP2, LPL and PPAR-γ2 gene expression in comparison to normal cultures that developed no clusters.

DISCUSSION

This is a preliminary study comparing the growth, osteogenic and adipogenic differentiation of normal and pathological BMdSCs. Although the numbers of samples within each pathological group were small, there were some statistical differences suggesting the presence of some abnormalities within the stromal compartment. Additional testing needs to be carried out to explore these results further.

5.9.1. Fibroblast cell line

The fibroblasts behaved differently to the BMdSCs. They showed significantly reduced CD106 (Stro-1) expression, an antibody previously used to isolate the more primitive BMdSCs (Gronthos et al 1994; Stewart et al 2003) and a lower non-specific binding of CD45. The remaining antigenic markers analysed were comparable suggesting that they could not be used to specifically identify or enrich BMdSC populations. The fibroblast cell line showed no evidence of either osteogenesis or adipogenesis following induction with significantly reduced differentiation markers in comparison to normal.

5.9.2. Interplay between the haemopoietic and stromal cells

There is a great deal of debate as to whether stromal cells and haemopoietic cells share a common stem cell (Flores-Figueroa et al 2005; Huss & Moosmann 2002), but if they did and the malignant transformation occurred within that stem cell, both the stromal and the haemopoietic compartments would likely be effected. BMdSCs rarely demonstrate the same genotypic abnormalities as the leukaemic cells, therefore evidence suggests that the stromal compartment in leukaemia is not derived from the malignant clone (Wetzler et al. 1995), however, BMdSC function can become abnormal in the presence of leukaemic cells, leukaemic-conditioned media or serum (Ben-Ishay et al 1984). This is largely due to the presence of abnormal concentrations of cytokines, many of which become constitutively expressed in malignancy (Wetzler et al 1995). There is also evidence to support abnormal interactions between the stromal microenvironment and haemopoietic cells (Duhrsen & Hossfeld 1996). The level of abnormality within each pathological group can vary considerably and this is most likely to be due to the heterogeneous nature of many diseases. The level of involvement of the stromal compartment in the pathogenesis of haematological disease is still uncertain (Flores-Figueroa et al 2005).

Within this investigation the LTC growth of pathological BMdSCs was analysed in the presence of interacting malignant cells, which exert their own influence on the stromal

compartment by abnormal cytokine production, cellular or ECM interactions. If the stromal compartment is not affected from a hierarchical source (no malignant transformation with the stromal stem / progenitor cells) it is possible that removal of the interacting malignant cells returns the stromal compartment to within normal parameters. In other words, the passaged and LTC BMdSCs from pathological bone marrows may behave differently. In this investigation, no significant differences in BMdSC growth (cell counts, doubling and CFU-F colony formation) were found between the normal and pathological passaged cultures (results not shown), however, there were significant differences between the antigenic expression of normal and pathological BMdSCs (5.5. 'Passaged BMdSC Characterisation by Cell Markers'). It was difficult to ascertain if these differences were due to the retained up regulation or down regulation of cell surface receptors by the malignant cells within the LTC (increased / decreased susceptibility to growth factors or inhibition), or whether the BMdSCs within themselves were abnormal.

The literature mainly focuses on the effect the malignant cells have on the stromal microenvironment in a LTC setting and on the supporting capability of pathological BMdSCs for normal haemopoiesis. This study aimed to determine the presence of any abnormalities (growth or differentiation) within the BMdSC layer of pathological cultures with and without the interference from malignant cells. LTC growth was compared in the presence of malignant cells, whilst antigenic expression and differentiation were analysed in passaged stroma in the absence of malignant cells (no haemopoietic cells detected by flow cytometry). Very few contaminating haemopoietic cells (e.g. macrophages) may have been present within the early passaged cultures, but these were unlikely to significantly effect the results.

5.9.3. AML

a) **Growth**

Many studies have shown that in AML the BMdSC compartment is affected by the presence of leukaemic cells with reduced cell growth and CFU-F colony formation (reduced bone marrow CFU-F numbers in 35% of patients at diagnosis) (Duhrsen & Hossfeld 1996; Mayani 1996; Sparrow et al 1997). This study worked with a variety of AML samples (low sample numbers) with different cytogenetic abnormalities and FAB group classifications and demonstrated no significant differences in LTC BMdSC growth (figure 5.3) or CFU-F colony formation (figure 5.11), however, the numbers of cells per field were significantly reduced during the first few weeks in culture. A significantly reduced ratio of adherent cells at week 2 over inoculum size

indicated the presence of a reduced proportion of stromal cells in relation to malignant cells within the MNC inoculum (photo 5.1).

b) Osteogenesis

During osteogenesis, the pattern of AML BMdSC differentiation was different to normal. The AML mineralised cultures showed reduced osteocalcin and collagen I gene expression in comparison to the AML non-mineralised cultures, whilst the normal mineralised cultures showed significantly increased osteocalcin gene expression (figure 5.24) and comparable collagen I gene expression (figure 5.25) in comparison to the normal non-mineralised cultures. Also, whilst the normal mineralised cultures demonstrated significantly increased ALP gene expression in comparison to the non-mineralised cultures, the AML mineralised cultures showed no increase in expression (figure 5.26). The AML mineralised cultures did show a comparable significant increase in calcium deposition (figure 5.19) and collagen I formation (figure 5.22) to normal, but unlike the normal cultures, mineralisation in the AML cultures was not related to a significant increase in cell number at induction.

There were no significant differences between the normal and AML non-mineralised cultures, but the AML mineralised cultures showed a significantly reduced osteocalcin gene expression in comparison to the normal mineralised cultures. No leukaemic cells were present to inhibit osteocalcin gene expression, which has been reported to be inhibited by myeloma cells (Duhrsen & Hossfeld 1996). Gene expression is regulated by signalling cascades initiated from receptor-ligand binding, therefore, a reduction in osteocalcin gene expression may indicate a reduction in either the associated receptor or the relevant signalling molecule, which may suggest dysfunctional cytokine production. No significant differences in the antigenic expression of adhesion molecules analysed within this investigation were observed between the normal and AML BMdSCs (5.5. 'Passaged BMdSC Characterisation by Cell Markers').

c) Adipogenesis

During adipogenesis, the AML group showed increased cell numbers at induction (n=2) (figure 5.28) and reduced multidroplet cell cluster formation (figure 5.27) in comparison to normal. The increased cellularity may have partially inhibited fat colony formation in AML, although previous studies have reported a decline in adipogenesis in AML cultures (no adipocyte growth in 50% of patients) (Mayani 1996), which may indicate a smaller number of adipogenically committed progenitor cells within the BMdSC layer. PPAR-γ2 gene expression (figure 5.31) in the AML cultures was comparable to normal at 21 days post induction, whilst one AML culture

showed levels of aP2 (figure 5.29) and LPL (figure 5.30) gene expression below the normal range. Insufficient cultures were analysed to equate this reduced expression to reduced lipid accumulation.

The pattern of gene expression for the successfully induced cultures and cultures with no multidroplet cell clusters was very different between the normal and AML groups. The successfully induced normal cultures showed a significantly lower cell number at induction (figure 5.28), a significantly higher aP2 and LPL gene expression and comparable PPAR-γ2 gene expression (figures 5.29 to 5.31) in comparison to normal cultures with no clusters. The successfully induced AML cultures showed a larger cell count at induction, a lower aP2 and LPL gene expression and a higher PPAR-γ2 gene expression in comparison to AML cultures with no clusters (results not significant due to low sample numbers). With further analysis these differences may have become significant. Further studies would be to adipogenically induce AML cultures with varying levels of confluence and analyse them for multidroplet cell cluster formation and aP2, LPL and PPAR-γ2 gene expression.

5.9.4. Lymphoma

a) Growth

No significant differences in LTC BMdSC growth (figure 5.4) or CFU-F colony formation (figure 5.11) were observed between the normal and lymphoma cultures. Initially the numbers of adherent cells were significantly reduced in comparison to normal with a ratio (week 2 cell counts / inoculum) approaching significance, suggesting a dilution effect of the stromal cells by the malignant cells within the inoculum.

b) Antigenic expression

The passaged BMdSCs from the lymphoma cultures showed a significant increase in CD105, CD49b and CD45 antigenic expression in comparison to normal, but it was unclear if the malignant lymphoma cells induced the upregulation during the LTC or whether the antigens were upregulated abnormally within the BMdSCs. An increase in CD45 may suggest the presence of contaminating haemopoietic cells within the BMdSC layer (one out of two lymphoma cultures were analysed at passage 1). CD49b associates with CD29 during the interaction between haemopoietic cells and ECM, and may have been upregulated for increased interaction during the LTC. CD49b has been reported to be more strongly expressed on CML BMdSCs when the predominant form of interaction (CD106 – VLA-4) is disrupted (Duhrsen & Hossfeld 1996), however CD106 expression was comparable to normal. CD105 is reported to

be involved in cellular proliferation, differentiation, ECM formation and migration (Lastres et al 1996) but increased expression did not significantly alter BMdSC growth or collagen I formation. Further studies may be to look at the time lapse photography of lymphoma cultures in relation to cell growth, cell doubling, CFU-F colony formation and collagen I production. CD105 and CD106 are also hallmarks of activated endothelial cells, which are reported to be increased in the peripheral blood of patients with vascular injury and in cancer patients including lymphoma (Mancuso et al. 2001). It may be that endothelial cells are also increased in number within the BMdSC compartment of lymphoma patients, which would account for an increase in CD105 expression, but CD106 expression was not affected.

c) Osteogenesis

The pattern of osteogenesis in the lymphoma cultures was similar to normal. Both the normal and lymphoma mineralised cultures deposited significantly more calcium by 21 days post induction than the non-mineralised cultures, with significantly higher cell counts at induction. No non-mineralised cultures were quantitated for ALP (protein and mRNA), collagen I (protein and mRNA), osteopontin or osteocalcin gene expression. The lymphoma mineralised cultures demonstrated comparable collagen I (protein and mRNA), osteopontin and ALP gene expression to normal mineralised cultures, with two out of four lymphoma mineralised cultures demonstrating ALP activity above the normal range (results not significant). With further analysis the data may become significant. Increased ALP activity at 21 days post induction had no additive effect on hydroxyapatite formation, which is supported by Bellows (Bellows, Heersche, & Aubin 1992), who reported that ALP was required to initiate mineralisation in embryonic chick and foetal rat osteoblasts, but was not required to maintain it, suggesting that once threshold levels were reached to initiate the reaction, only basal levels were required to maintain it. This was shown in the normal cultures by a plateau in ALP activity by day 10 (figure 4.24) the time at which mineralisation was most frequently observed. The pattern of osteocalcin gene expression was comparable to mineralised AML cultures with a significant reduction in osteocalcin gene expression in comparison to normal mineralised cultures. No literature exists to support these findings.

d) Adipogenesis

The results for lymphoma adipogenesis were similar to AML with a reduction in multidroplet cell cluster formation (figure 5.27) with an increased number of cells per field at induction (figure 5.28), largely suggesting that increased cell numbers were inhibitory to multidroplet cell cluster formation. No literature was found to support these findings, although Janderova

(Janderova et al 2003) and Pittenger (Pittenger et al 1999) maintained their cultures at confluence for 2 to 3 days before induction, whilst Colter (Colter, Sekiya, & Prockop 2001) adipogenically induced human bone marrow stromal cultures at 50 to 70% confluence. Further studies would be to adipogenically induce AML and lymphoma cultures at increasing cell densities to determine if more multidroplet cell clusters formed in less confluent cultures. Few lymphoma cultures were assessed for lipid-related gene expression, with one out of two cultures showing levels of aP2 and LPL gene expression below the normal range, therefore, it is possible that reduced expression (aP2 and LPL) resulted in reduced lipid accumulation. Further samples need to be analysed to assess this theory.

5.9.5. Myeloma

a) Growth

The behaviour of myeloma LTC BMdSCs was very similar to normal with comparable growth curves (figure 5.5) and no significant differences in the number of CFU-F colonies formed (figure 5.11). There were, however, a reduced number of small colonies and an increased number of large colonies in comparison to normal (not significant), which may become significant with more data, and suggests a stimulatory effect on the more immature progenitor cells within the population. It has been reported that MM cells upregulate IL-6 production, which acts as a major growth factor on myeloma cells (autocrine stimulation), but may also have a stimulatory effect on the more primitive stromal cells. Rodriguez (Rodriguez et al. 2004) reported an almost 50% reduction in CFU-F colonies and a reduction in CD106 (VCAM-1) and Thy-1 (CD90) expression on BMdSCs derived from bone marrows deficient in IL-6. Multiple myeloma cells produce a variety of cytokines upon interaction with BMdSCs that may stimulate the growth of stromal cells, but no significant effects were observed in this investigation.

b) Antigenic expression

The passaged myeloma cultures showed no reduction or upregulation of CD90 or CD106 antigenic expression, but they did show an up regulation in CD105, which modulates cellular response to TGF-β, one of the cytokines produced by MM cells (Cook et al. 1999). As with lymphoma, an upregulation of CD105 may also be related to an increase in the number of endothelial cells within the BMdSC layer (Zhang et al. 2005).

5.9.6. Lymphoproliferative

a) Growth

The lymphoproliferative group included a wide range of diseases. The ALL and B-PLL samples consisted mainly of immature blast-like haemopoietic cells, whilst the CLL group consisted mainly of mature cells. The number of samples analysed were small, but the LTC growth curves of the blast group and the more mature group were similar to normal (results not shown). When all the results were combined no significant differences in LTC BMdSC growth (figure 5.6) or CFU-F colony formation (figure 5.11) were observed. The number of fibroblast-like cells and adipocytes have been reported to be reduced in the BMdSC layer of ALL patients with a reduced proliferative capacity (Duhrsen & Hossfeld 1996).

b) Antigenic expression

The lymphoproliferative group showed a significant reduction in CD90, CD105 and CD49b antigenic expression in relation to normal. Reports have shown that B-cell precursors in lymphoblastic leukaemia are more reliant on cellular interactions with BMdSCs for survival with antagonists to VCAM-1 (CD106) completely blocking B-cell lymphopoiesis (Obinata et al 1998), but no up regulation in any adherence receptors were noted. It is unclear why adherence receptors such as CD49b, or growth factor receptors such as CD105 would be down regulated. No literature was available to support these findings.

5.9.7. JMML

a) Growth

The JMML BMdSCs showed a significant increase in growth rate (figure 5.10) with no concomitant increase in large CFU-F colonies at week 2 (figure 5.11), suggesting that the increased growth rate was not the result of more primitive progenitors within the culture. There was a significant reduction in the number of BMdSCs per microscopic field within the first 3 weeks of culture in comparison to normal, and the ratio of adherent cells at week 2 over inoculum was significantly reduced suggesting a reduction in the proportion of stromal cells to leukaemic cells within the JMML inoculum. However, AML (figure 5.3) and lymphoma (figure 5.4) cultures also showed significantly reduced cell counts at week 2 and reduced ratios, but growth continued at a similar rate to normal with comparable CFU-F colony formation. It is possible that at week 2, having been demi-fed once, the JMML leukaemic cells were producing abnormally high concentrations of cytokines that may have been inhibitory to the stromal cells. With further demi-feeding, the inhibition may have been diluted out, allowing the BMdSCs to proliferate. In addition, the BMdSCs may have been abnormally responding to

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growth factors responsible for over-stimulating growth. With the two theories working side by side, it is possible that the inhibition was preventing the BMdSCs from abnormally responding to the growth factors within the first couple of weeks, explaining the comparable CFU-F colony formation to normal. As the inhibition was diluted out, the JMML BMdSCs may have become increasingly responsive to the growth factors, suggesting that if CFU-F analysis was carried out at week 3 or week 4, much more larger CFU-F colonies would have been apparent in comparison to normal. Further studies would be to perform a serial dilution of cell concentration for inoculation in LTCs and quantitate BMdSC growth and CFU-F colony formation weekly.

5.9.8. MDS

a) Growth

No significant differences in LTC BMdSC growth or growth rate were observed between MDS and normal cultures. The numbers of large colonies were comparable to normal, but MDS cultures showed a significantly increased number of small colonies. Reports have shown normal development of BMdSC layers and CFU-F colony formation in MDS patients with good prognosis, but the doubling rates tend to be much slower with 45% of MDS cultures failing to reach confluence by day 14 (Flores-Figueroa et al 2005), and colony sizes were reported to be reduced (Duhrsen & Hossfeld 1996). A significantly increased number of small colonies within this investigation, suggests the presence of more less proliferative progenitor cells within the BMdSC layer, however, there were more CFU-Fs within the MDS LTCs (approximately 1 per 30,000 MNC) than normal (1 per 62,000 MNC) (results not significant) (figure 5.11). Flores-Figueroa (Flores-Figueroa et al 2005) also demonstrated a higher frequency of CFU-Fs in the MNC fraction of MDS bone marrow (1 per 17,000 MNC) than normal (1 per 31,000 MNC) (results not significant). The variations in the results may reflect the heterogeneity of the different bone marrow samples and culture conditions.

b) Antigenic expression

MDS BMdSCs were found to have a significantly reduced CD90 expression in relation to normal BMdSCs, which was supported by Flores-Figueroa (Flores-Figueroa et al 2005), who showed comparable expression profiles for CD29, CD105, CD34 and CD68 between normal and MDS stroma but reduced CD90 expression.

5.10. **Final Summary**

This investigation compared the growth, cell doubling, CFU-F colony formation and differentiation (osteogenic and adipogenic) of normal and pathological cultures. Unfortunately small numbers of pathological samples were received and grown without contamination for assessment of differentiation. The preliminary results suggest the presence of some abnormalities within the BMdSC compartment, but more studies need to be carried out to explore these results further.

Chapter 6

Conclusions and Further Studies

6.1. **Final Conclusions**

6.1.1. **LTC BMdSC growth**

Following inoculation of the MNC population into tissue culture flasks (long-term primary cultures) the BMdSCs that adhered to the flask surface grew significantly better (higher cell doubling with more large CFU-F colonies) in the presence of hydrocortisone (in modified McCoy's with or without glutamine and Alpha MEM) (figures 3.5 & 3.6). These cultures were also more haemopoietically active (figure 3.8). HC appeared to maintain the more primitive cells in an undifferentiated state within the LTCs, whilst promoting proliferation.

6.1.2. **Passaged BMdSC growth**

Following subculture into passage 1, the BMdSCs grew significantly better in Alpha MEM (greater cell growth and large CFU-F colony formation) in comparison to modified McCoy's medium and DMEM (figures 3.11 & 3.13). Reducing the calcium concentration from 200 mg/l to 100 mg/l did not affect BMdSC growth in Alpha MEM (figure 3.10). HC had little effect on passaged stromal growth (figure 3.15), suggesting that HC did not have a direct effect on the BMdSCs. The optimal time for passage was before sub-confluence when cellular migration (figure 3.40) and cell doubling (figure 3.24) were maximal with little collagen I deposition (figure 3.40).

6.1.3. **BMdSC antigenic characterisation**

BMdSCs could not be specifically identified by the expression profile analysed, but they were negative for the haemopoietic markers CD34, CD45 and CD65, and the stem cell markers CD117 and CD133. They were strongly positive for CD90, CD44, CD166 and CD105, and weakly positive for CD49b and CD106 (3.5.2. 'Cell markers'). The fibroblasts showed a similar expression profile to normal BMdSCs, but they also showed a significantly reduced CD106 antigen expression, which has previously been used to isolate the more primitive BMdSCs, and a significantly reduced non-specific binding of CD45 (5.5. 'Passaged BMdSC Characterisation by Cell Markers').

6.1.4. **Osteogenesis**

Reducing the calcium concentration from 200 mg/l to 100 mg/l medium prevented the cells from breaking up with a significant loss in RNA (figure 4.9) and allowed measurable amounts of calcium to deposit (figure 4.8). The osteogenic induction agents, particularly β-glycerophosphate, were necessary for mineralisation, but other markers of differentiation

(ALP, collagen and osteocalcin gene expression) did not appear to be affected by induction. Not all osteogenically induced cultures mineralised and this was thought to be due to cultures failing to reach threshold amounts of collagen I or ALP required to initiate mineralisation (4.5.2. 'Markers of osteogenesis in mineralised and non-mineralised cultures'). Mineralisation was seen on average by day 10 post induction, at which point collagen I formation (figure 4.25) and ALP (activity and mRNA) (figures 4.24 & 4.30) plateaued. Osteocalcin gene expression did not change during 17 days of induction, but rose sharply from 17 to 21 days (figure 4.28). Osteopontin (figure 4.27) and collagen I (figure 4.29) gene expression slowly declined post induction. Levels were not assessed prior to induction.

6.1.5. Adipogenesis

The adipogenic induction agents significantly increased aP2, LPL and PPAR-γ2 gene expression in comparison to the normal control cultures (figure 4.43), and were likely to be responsible for the accumulation of lipid observed within the cytoplasm of the differentiating cells (multidroplet fat cells). The normal induced cultures that did not form multidroplet cell clusters had significantly less aP2 and LPL gene expression (figure 4.44). No lipid accumulation was seen in the absence of induction agents (figure 4.38). Multidroplet cells usually formed in clusters (colonies) suggesting the proliferation and differentiation of committed preadipocytes within the cell layer.

6.1.6. Normal and pathological BMdSCs

This preliminary comparative study suggested the presence of some abnormalities within the bone marrow stromal compartment of pathological bone marrows but additional testing needs to be carried out to explore these results further. It was unclear if the abnormalities found were a contributing factor to the haematological disease, or whether the malignant cells were responsible for the abnormality. It may be possible that the malignant cells upregulated / down regulated gene or antigenic expression during the LTC, which were retained through passaged culture.

a) Growth

Only the JMML group showed significantly different growth results to normal with a significant increase in LTC cell growth (figure 5.7) and cell doubling (week 3 to week 5) (figure 5.10), but no concomitant increase in large colonies were observed (figure 5.11), suggesting that the more primitive cells were not responsible for the increased growth. The malignant cells may have secreted a stromal inhibitory factor, which prevented the formation of large colonies

by week 2. With weekly feeding regimes, the inhibition may have been diluted out, which in addition to a possible abnormal stimulatory effect on BMdSCs, resulted in increased growth post week 2. The hypercellular pathological cultures had significantly reduced cell counts at weeks 2 and 3 in comparison to normal (5.3.2. 'LTC BMdSC growth'), which was likely to be due to a reduction in the proportion of stromal cells in relation to haemopoietic cells within the MNC inoculum. The MDS group showed a significant increase in small colonies, which may suggest the presence of an abnormal population of more committed cells.

b) Antigenic characterisation

Antigenic expression was different to normal in many pathological groups (5.5. 'Passaged BMdSC Characterisation by Cell Markers'). The lymphoma group showed an increased CD105, CD49b and CD45 expression in comparison to normal. The myeloma group showed increased CD105 expression. The lymphoproliferative group showed reduced CD90, CD105 and CD49b antigenic expression, and the MDS group showed reduced CD90 expression. These may represent abnormalities in the interaction (upregulation or down regulation of receptors) between haemopoietic cells, stromal cells and ECM during the LTC, but no haemopoietic cells were present at the time of analysis, which may suggest an abnormality within the stromal compartment, or the abnormality may have been retained from the LTC.

c) Osteogenesis

With osteogenesis, the pattern of differentiation between the cultures that did and did not mineralise was different between the normal and AML cultures (5.7. 'Osteogenic Induction'). The normal mineralised cultures had significantly more osteocalcin, collagen I and ALP gene expression than the normal non-mineralised cultures, whilst the AML mineralised cultures showed a reduction in osteocalcin and collagen I gene expression with comparable ALP gene expression in relation to the AML non-mineralised cultures. The AML and lymphoma mineralised cultures had significantly less osteocalcin gene expression (calcium binding protein) than the normal mineralised cultures at 21 days post induction, but calcium deposition was comparable.

d) Adipogenesis

The numbers of cultures adipogenically induced was poor, but the AML and lymphoma cultures had more cells per field at induction (figure 5.28) and developed fewer multidroplet cell clusters (figure 5.27) by 21 days post induction than the successfully induced normal cultures. The higher cell numbers at induction in the AML and lymphoma cultures may have been

responsible for the reduced multidroplet cell cluster formation (figure 4.37). The aP2 and LPL gene expression was either comparable or slightly reduced in the pathological cultures in comparison to normal (figures 5.29 & 5.30) but due to small sample numbers it was difficult to equate poor multidroplet cell cluster formation to aP2 or LPL gene expression. Further work needs to be carried out.

6.2. **Further Studies**

6.2.1. **BMdSC Growth**

It would be useful to carry out further studies to assess the function of HC in both the LTCs and the passaged stromal cultures. It was clear that HC stimulated the growth and CFU-F colony formation of BMdSCs in the LTC, but preliminary results showed no effect on passaged stromal growth. This needs to be further assessed in modified McCoy's medium and in Alpha MEM. The results suggested that HC had no direct effect on passaged BMdSC growth, therefore, HC may have acted through a secondary mechanism (e.g. a haemopoietic cell such as a macrophage) or through ECM interactions (3.7.6. 'Effect of hydrocortisone on BMdSC growth'). It would be a useful comparison to look at the effect of HC on LTCs demi-fed weekly and those fed twice weekly (all the medium replaced twice a week). Twice weekly feeding regimes would remove the haemopoietic cells much quicker and may effect the actions of HC. HC appeared to support the proliferation of more primitive progenitor cells whist maintaining their immaturity in culture. It would be useful to continue HC treatment in the LTC and passaged stromal cultures in modified McCoy's medium and Alpha MEM. This may prolong the life-span of the cultures by maintaining the more primitive cells in population. It may be that glucocorticoids at the relevant concentrations are essential for promoting long-term progenitor cell growth, and for promoting differentiation. HC is also used to induce adipogenesis, and dexamethasone, another glucocorticoid, is used to induce both osteogenesis and adipogenesis.

6.2.2. **BMdSC antigenic Characterisation**

The ultimate goal of working with bone marrow derived mesenchymal cells for stem cell therapy is to select out the more immature undifferentiated populations of cells for expansion (proliferation) and controlled differentiation *in vitro* and eventual implantation back into the patient. Using antigenic markers, identification of the stem cell population has been unsuccessful due to the heterogeneous nature of the cell layer and lack of specific markers (Steindler & Pincus 2002). Sekiya (Sekiya et al 2002) screened over 200 antibodies and failed to distinguish between the more proliferative progenitor cells and the more mature stromal cells

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by antigenic characterisation. Campioni (Campioni et al. 2003) immunoselected BMdSCs from the bone marrow using anti-fibroblast antibody and CD105 to eliminate haemopoietic contamination from the long-term culture. This has important implications for haematological diseases such as AML in which the leukaemic cells may interfere with the stromal colony forming activity. Campioni showed that immunomagnetic enrichment of BMdSCs restored the *in vitro* stromal compartment of AML patients.

It is important to remember that any attempt to isolate an adult stem cell may not capture the intended cell, or may alter its characteristics (antigenic or gene expression, and function) by changing its environment ('systems science') (1.6.1. 'Network interactions').

This investigation observed three sub-populations of cells in the passaged BMdSC cultures identified by R1, R2 and R3 (figure 3.30). R3 contained a heterogeneous population of cells of varying size and granularity with a very active cell cycle (3.5. 'BMdSC Characterisation'). The sub-populations R1 and R2 showed a more limited cell cycle (assuming all doublets were excluded) and may have included a more discrete population of cells (e.g. more stem cell-like) (Colter et al 2000). These may be responsible for replacing those within the R3 sub-population over time resulting in a reduction in the size of R1 and R2 during culture and as a function of passage. Further studies would be to inoculate BMdSCs at various seeding densities and compare the changes in the sub-populations R1, R2 and R3. Plating at lower seeding densities may stimulate the stem cell-like cells within R1 and R2 to proliferate, which can be assessed by cell cycle analysis using cell-cycle specific antigen K_i-67 (Colter et al 2000) and / or DRAQ5. The viable cells within R1 and R2 could then be further characterised by antigenic expression. In an extension to the study, flow sorting techniques could be optimised to enable the isolation of the R1, R2 and R3 sub-populations of cells using the FSC, SSC plot. These cells could then be inoculated into culture and quantitated for CFU-F colony formation. CFU-F assays for sub-populations R1 and R2 may identify a more primitive population of progenitors (larger colonies) than in R3.

It would also be useful to characterise BMdSCs undergoing adipogenic or osteogenic differentiation, for example, CD166 (ALCAM) is reported to decline once the cells embark on the osteogenic pathway (Barry & Murphy 2004).

6.2.3. Osteogenesis

The induction agents (dexamethasone, ascorbic acid-2-phosphate and β-glycerophosphate) and concentrations used were derived from the literature (Bruder et al 1998; Pittenger, Mosca, & McIntosh 2000) and combined for osteogenesis, but the effects of the individual agents were only briefly observed within this investigation. Preliminary observations showed that dexamethasone and ascorbic acid-2-phosphate prevented the cells from breaking up during prolonged culture in comparison to the control cultures, indicating an increased ECM formation and maturation. It would be useful to induce the BMdSC cultures in the absence of β-glycerophosphate and continue the time series beyond 21 days of induction to quantitate any differences in osteogenic-related markers between the induced and control cultures. It would also be valuable to prolong the induction period beyond 21 days of induction with all three induction agents to determine if any markers of bone differentiation were altered, for example, osteocalcin gene expression sharply rose from 17 to 21 days post induction, but no further cultures were quantitated to determine if osteocalcin continued to increase beyond 21 days.

The induction agents were not responsible for increasing the markers of bone differentiation, however, they were necessary for mineralisation, particularly β-glycerophosphate. It would be useful to compare Alizarin red S staining and calcium quantitation throughout the induction period in cultures osteogenically induced with β-glycerophosphate with or without dexamethasone and ascorbic acid-2-phosphate. This would highlight the importance of using dexamethasone and ascorbic acid-2-phosphate in the induction cocktail. It is possible that the cultures were spontaneously differentiating down the osteogenic lineage before induction, which accounts for the comparable results observed between the induced and control cultures. The induction agents may simply have been necessary to induce calcification.

It would be a useful extension to the study to assess the markers of osteogenesis before and after induction to provide more clues about the initiation of mineralisation. Osteopontin (not significant) (figure 4.27) and collagen I (figure 4.29) (significant from 0 to 14, 17 & 21 days post induction) gene expression were down-regulated post induction, therefore, quantitating expression before induction would likely show an upregulation in expression. Collagen I deposition was the only marker of osteogenesis to be quantitated during 17 days of proliferation before induction, and showed a significant increase in deposition from day 0 to day 10, 14 and 17 (figure 3.40). Osteopontin gene expression was not down regulated in the control cultures,

which suggested that the induction agents were inhibitory to osteopontin production (figure 4.27).

It would also be useful to quantitate markers of osteogenesis in mineralised and non-mineralised cultures as part of a time series before and after induction. This would likely show a significant difference between the mineralised and non-mineralised cultures in the upregulation of ALP and collagen I production. ALP and collagen I production could then be related to the time of mineralisation, for example, an increase in production may be observed sooner in cultures that mineralise earlier.

Cell confluence was shown to have an effect on mineralisation, with significantly more cultures mineralising when induced before 14 days with cell counts ≥ 100 cells. In future more robust results can be obtained by selecting more confluent cultures for osteogenic differentiation.

Mineralisation (photo 4.3) and collagen I formation (photo 4.12) occurred within the ECM overlying the cells within osteogenically induced BMdSC cultures. It was, therefore, difficult to establish which cells were responsible for secreting the relevant proteins within the cell layer. The differentiating cells in adipogenically induced BMdSC cultures were clearly identified as multidroplet fat cells, which were likely to be responsible for the significant increase in aP2, LPL and PPAR-γ2 gene expression observed post induction. The differentiating cells were not clear during osteogenesis. Intracellular ALP staining identified ALP positive cells (photos 4.10 & 4.11), but were these cells also responsible for secreting collagen I and factors important in mineralisation? Also, were the same cells responsible for producing osteopontin and osteocalcin, or was a smaller, more discrete population of cells (possibly differentiating cells) responsible for their production? Osteocalcin and osteopontin bind to hydroxyapatite within the ECM during mineralisation, therefore, immunocytochemical staining following mineralisation would likely produce results similar to that of collagen I (photo 4.12). It may therefore be useful to histochemically or immunocytochemically stain BMdSCs for ALP, collagen I, osteocalcin and osteopontin at various time points before and after induction to determine the point of production and observe which cells were responsible for their production.

6.2.4. Adipogenesis

All lipid induction agents (dexamethasone, indomethacin, hydrocortisone, IBMX and insulin) and concentrations used were derived from the literature (Digirolamo et al 1999; Pittenger, Mosca, & McIntosh 2000) and combined for inducing adipogenesis. The combined action of

all the induction agents resulted in a significant increase in lipid-related gene expression (figure 4.43) and multidroplet cell cluster formation (figure 4.38) (Ahdjoudj et al 2001). Further studies would be to look at the effects of the individual agents, or various combinations of induction agents, on the markers of adipogenic differentiation (2.8.1. 'Adipogenic induction').

Adipogenically induced cultures were more likely to form multidroplet cell clusters (colonies) when induced with 120 to 230 cells per field at induction (figure 4.37). This information will be used in future studies to select less confluent cultures to give more robust results.

No time series experiments were performed for adipogenesis due to time restrictions, but this would be very useful to relate PPAR-γ2, LPL and aP2 gene expression to lipid accumulation. The time series may show an early peak in PPAR-γ2 gene expression, followed closely by an up regulation in LPL and aP2 gene expression alongside lipid accumulation (Janderova et al 2003; Scavo et al 2004).

Only one culture formed spherical adipocytes by 21 days of induction (photo 4.7), therefore, it would be useful to prolong the induction period beyond 21 days to establish if more multidroplet fat cells developed into spherical adipocytes (photo 4.7).

BMdSC cultures within this investigation were adipogenically induced in the presence of 10% FCS and HS. It has been reported that replacing FCS with rabbit serum (RS) increases adipocytic differentiation with more lipid accumulation and lipid-specific gene expression in mouse cell lines (Diascro, Jr. et al. 1998). This is thought to be due to a higher fatty acid content in RS (enriched in linoleic acid, palmitic acid and oleic acid), which act as ligands to members of the PPAR family (Diascro, Jr. et al 1998). Future studies would be to replace FCS with RS and adipogenically induce the BMdSC cultures as normal (2.8.1. 'Adipogenic induction'), or add palmitic acid, oleic acid and linoleic acid to BMdSC cultures in the presence of FCS and the induction agents and quantitate the markers of adipogenesis over a period of time.

6.2.5. Overlap between osteogenesis and adipogenesis

There is reported to be a reciprocal relationship between osteogenic and adipogenic differentiation (Ahdjoudj et al 2001; Gori et al 1999) with the down regulation of bone-related markers in adipogenesis and vice versa. Further studies would be to assess the markers of adipogenesis (Oil red O staining and lipid-specific gene expression) in osteogenically induced

cultures and the markers of osteogenesis (calcium, ALP, collagen I, osteopontin and osteocalcin) in adipogenically induced cultures. No osteogenically induced cultures developed multidroplet fat cells during 21 days of induction.

Parallel BMdSC cultures were successfully differentiated towards fat (multidroplet cell cluster formation) or bone (mineralisation) depending on the induction protocol used (bipotential differentiative capacity of cell layer). In an extension to differentiating parallel cultures, it is possible to selectively isolate and detach an individual colony (includes the CFU-F and its progeny) using a cloning ring. This colony of cells can then be replated and osteogenically or adipogenically induced. Assuming no contaminating cells (cells not derived from the CFU-F) were present within the cloning ring and that the colony formed from one CFU-F (difficult to prove), evidence of osteogenic and adipogenic differentiation suggest the presence of a single bipotential progenitor cell (CFU-F). This investigation found there to be approximately 28 large CFU-Fs (>100 cells per colony) per 10^4 cells (1 per 357 cells inoculated) in passaged stromal cultures (table 3.5), and consistently detaching the cultures within 10 days maintained a high growth rate until passage 4 (figure 3.26). Therefore, inoculating 25cm^2 flasks with a range of seeding densities from 200 to 500 cells should in theory produce 1 large colony per flask. This colony could be easily isolated, with less chance of contamination, and replated for adipogenic or osteogenic differentiation. Further work would then be to induce differentiation into other mesenchymal, and possibly non-mesenchymal lineages.

6.2.6. Pathological BMdSCs

Many more pathological samples need to be analysed, however, the quantitation of LTC BMdSC growth, antigenic characterisation and markers of differentiation within this investigation have shown subtle differences within the stromal compartment in comparison to normal that have been analysed statistically. Further cytogenetic characterisation of normal and pathological BMdSC cultures also needs to be carried out as a function of passage for a more in depth comparison.

The capability of pathological BMdSC layers to support normal haemopoiesis was not assessed within this investigation but is of great importance in any future studies. Confluent BMdSC layers can be irradiated and seeded with normal cord blood cells to assess their ability to support normal haemopoiesis.

JMML was found to have a significantly higher cell doubling from week 3 to week 5 (figure 5.10) and a comparable CFU-F colony formation at week 2 (figure 5.11) in comparison to normal. This was theorised to be due to an early inhibition of stromal cell growth by the malignant cells, which may have been diluted out with weekly demi-feeding regimes. This theory can be assessed with serial dilutions of the inoculum until JMML stromal growth and CFU-F colony formation relate to normal. If JMML BMdSC growth and cell doubling remain high, and the CFU-F colony formation increases by week 2 in comparison to normal, it is likely that the BMdSCs were abnormally responding to growth factors, possibly secreted by the malignant cells.

This investigation showed a possible relationship between cell proliferation (cell doubling) (figure 3.24), cell migration and collagen I production (figure 3.40). Early in culture (<7 days) normal BMdSCs had a high doubling rate and cells were rapidly migrating across the flask. As cultures became more confluent with more collagen I deposition, cell doubling and cell migration declined. The link between these parameters needs to be more extensively studied in both normal and pathological cultures. Collagen I formation was only assessed in pathological cultures at 21 days post induction and cannot be related to cell doubling, and no time lapse photography was carried out on pathological samples.

With osteogenesis and adipogenesis, only a small number of AML and lymphoma cultures were studied. Further studies would be to analyse more AML and lymphoma cultures and a wider range of pathological groups for BMdSC differentiation. Preliminary results suggested that increased cell counts at induction related to reduced multidroplet cell cluster formation during adipogenesis, and unfortunately the small number of pathological samples analysed all had high cell counts at induction. Further studies would be to induce AML and lymphoma cultures at varying cell densities to determine if multidroplet cell cluster formation was reduced as a result of the high cell numbers, or due to other factors, for example, aP2 and LPL gene expression. In osteogenesis, the normal samples were shown to mineralise in cultures induced earlier at higher cell densities (≥ 100 cells), therefore, pathological cultures could be osteogenically induced at a range of seeding densities to determine if they behave in the same way. Preliminary results from this investigation have shown no relationship between cell number at induction and collagen I production, ALP gene expression and mineralisation in the AML cultures.

It would be useful to quantitate markers of osteogenesis and adipogenesis in pathological cultures during a time series from before induction and following induction (for longer than 21 days) for comparison to normal.

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Appendix

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University Hospital of Wales
Ysbyty Athrofaol Cymru

Eich cyf/Your ref
Ein cyf/Our ref
Welsh Health Telephone Network 1872
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USE OF EXCESS BIOLOGICAL MATERIAL

PATIENT INFORMATION

You are providing a blood or bone marrow sample for testing or you are donating haemopoeitic stem cells for transplantation. You are now asked to consent to excess material being used for unrelated laboratory work and / or research.

Background

Diagnostic tests and haemopoeitic stem cell donation sometimes results in excess material being available. In the case of laboratory tests this excess is discarded. In the case of stem cell transplantation it is usually given to the patient. This excess material which is left over could be very valuable for checking the quality of laboratory tests, for establishing new tests or for research. It should be clear that excess material is not deliberately taken.

A particular area of use of this material is the diagnosis of and research into leukaemia and related disorders. You are asked to consent to the excess material being used for research and development projects in leukaemia and associated areas. The material may be used fresh or be put into a 'cell bank' for use at a later date by scientists. DNA and RNA are the genetic code which control cells. This will be prepared and stored in the bank, along with the excess cells, in an anonymised way. Your sample will be given a code number so that your name will not be known.

When the scientific results are analysed we may need to correlate the findings with clinical details which include age, sex, previous medical history, and response to treatment if relevant. Strict confidentiality will be maintained at all times, by using the code number allocated to your sample of cells, DNA or RNA. Scientist will not know your identity.

As the samples are put into a 'bank' they could remain there for a long time and be used in future research many years hence. We do not know what questions will be asked in the future but the research will relate to the understanding of blood formation, leukaemic change, response to therapy or the development of new treatments. Your samples could be provided to commercial companies working in this area to develop new tests or treatments for leukaemias or other diseases. You will not personally benefit financially from this nor will the custodians of the tissue bank.

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It is unlikely that you as an individual will benefit from the research carried out because it often takes many years to move from the first scientific discovery to clinical application.

You should also understand that:

i   Allowing your sample and the coded information about you, to be used for research is entirely voluntary.

ii   You may have your sample removed from the bank at any time.

iii Declining to give your sample for research or its subsequent removal from the bank will in no way affect your relationship with your doctor or the care you receive.

If you require further information you may contact:- Professor AK Burnett, Head of Department of Haematology University of Wales College of Medicine.
Tel No: 029 20742375

USE OF EXCESS BIOLOGICAL MATERIAL

CONSENT FORM

Name of Local
Investigator:..

| Please Initial boxes |

Contact details of Local
Investigator:..

1   I have read the information sheet on the above project and have received a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done.

2.   I agree to give an excess sample material of blood or bone marrow. I understand how the material will be collected, that giving the material for research and development is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected.

3.   I understand that the excess material could be used for genetic or stem cell research. Current studies include the genetic variability in the population that may contribute to the development of leukaemia, the genetic changes that cause leukaemia and the genetic background that may modulate response to therapy and the generation of stem cells from blood or bone marrow. I appreciate that the results of these investigations are unlikely to have any implications for me personally.

4.   I give permission for the anonymised clinical information to be used for the analysis of the results of any research emulating from for the DNA and RNA collection.

5.   I agree that the excess material I have given, and the anonymised clinical information gathered about me, can be stored indefinitely and used for future research that would improve understanding of blood formation; how this goes

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wrong in leukaemia and research that could give rise to new treatments. I understand that these samples will be fully anonymised.

6.   I understand that I will be informed if any of the results of the tests done on my DNA or RNA are important for my future medical management.

7.   I understand that my sample could be provided to a commercial company working in this area to develop new tests or treatments for leukaemia or other diseases. I understand that I will not benefit financially if the research leads to the development of a new treatment or medical test.

8.   I know how to contact the local research team. In addition I can contact the following:

Prof A. Burnett, Head of Department of Haematology University of Wales College of Medicine on: 029 20 742375

....

Name of patient (BLOCK   Date   Signature
              CAPITALS)

....

Name of person taking consent   Date   Signature
(if different from researcher)

....

Name of researcher   Date   Signature

Thank you for giving excess material for research and development.

Appendix II

Primers

a) Markers of osteogenic differentiation (Sequencing appendix III)

Table 2.3. Primers used to detect specific gene expression during osteogenic differentiation

Primer	Sequence	Product length	Reference
Osteopontin	F: 5'-CATTGACTCGAACGACTCTG-3' R: 5'-ACGGCTGTCCCAATCAGAAG-3'	355 bp	Thalmeier et al. (2001)
Osteocalcin	F: 5'-ACGGCTGTCCCAATCAGAAG-3' R: 5'-ATGAGAGCCCTCACACTCCTCG-3'	451 bp	Thalmeier et al. (2001)
Collagen type I	F: 5'-CAGCCGCTTCACCTACAGC-3 R: 5'-TTTTGTATTCAATCACTGTCTTGCC-3'	83 bp	Frank et al. (2002)
Alkaline Phosphatase	F: 5'-GACCCTTGACCCCCACAAT-3 R: 5'-GCTCGTACTGCATGTCCCCT-3'	68 bp	Frank et al. (2002)
House keeping (S14)	F: 5'-GGCAGACCGAGATGAACTCT-3' R: 5'-CCAGGTCCAGGGGTCTTGGT-5'	144 bp	

   F: forward (sense) primer
   R: reverse (anti-sense) primer
Osteopontin, ALP and Collagen I required 40 cycles on the LightCycler. Osteocalcin required 50 to 60 cycles.

b) Markers for adipogenic differentiation (Sequencing appendix IV)

Table 2.4. Primers used to detect specific gene expression during adipogenic differentiation

Primer	Sequence	Product length	Reference
aP2	F: 5'-GTACCTGGAAACTTGTCTCC-3' R: 5'-GTTCAATGCGAACTTCAGTCC-3'	419 bp	Ahdjoudj et al. (2001)
LPL	F: 5'-GATCCAGCTGGACCTAACTT-3' R: 5'-AAAGGCCTGATTGGTATGGG-3'	540 bp	Ahdjoudj et al. (2001)
PPARγ2	F: 5'-GGGAGATTCTCCTATTGACCCAG-3' R: 5'-TCAAAGGAGTGGGAGTGGTCTTC-3'	168 bp	Maeda et al. (2002)

   F: Forward (sense) primer
   R: Reverse (antisense) primer
All lipid primers required 50 cycles on the LightCycler

Appendix III

Sequencing (Osteogenic differentiation)

a) Osteopontin

```
                              10        20        30        40        50
                         ..l....l....l....l....l....l....l....l....l....l....
                         TCATTGACTCGAACGACTCTgAtGAtGTAGcATGACACTGATGATTC

seq2  19-6-03.seq(1>341)   ←   TCATTGACTCGAACGACTCTGATGATGTAG-ATGACACTGATGATTC
seq4  19-6-03.seq(1>345)   ←   NCATTGACTCGAACGACTCTGATGATGTAG-ATGACACTGATGATTC
OP sense primer.seq(1>20)  →   CATTGACTCGAACGACTCTG
seq3  19-6-03.seq(1>347)   →                     CCCT-A-GA-GTAGCN-GACACTGATGATTC
seq1  19-6-03.seq(1>337)   →                       T-A-GA-GTAGNACGACACTGATGATTC


                              60        70        80        90        100
                         l....l....l....l....l....l....l....l....l....l....l.
                         TCACCAGTCTGATGAGTCTCACCATTCTGATGAATCTGATGAACTGG

seq2  19-6-03.seq(1>341)   ←   TCACCAGTCTGATGAGTCTCACCATTCTGATGAATCTGATGAACTGG
seq4  19-6-03.seq(1>345)   ←   TCACCAGTCTGATGAGTCTCACCATTCTGATGAATCTGATGAACTGG
seq3  19-6-03.seq(1>347)   →   TCACCAGTCTGATGAGTCTCACCATTCTGATGAATCTGATGAACTGG
seq1  19-6-03.seq(1>337)   →   TCACCAGTCTGATGAGTCTCACCATTCTGATGAATCTGATGAACTGG


                              110       120       130       140
                         ..l....l....l....l....l....l....l....l....l....l....
                         TCACTGATTTTCCCACGGACCTGCCAGCAACCGAAGTTTTCACTCCA

seq2  19-6-03.seq(1>341)   ←   TCACTGATTTTCCCACGGACCTGCCAGCAACCGAAGTTTTCACTCCA
seq4  19-6-03.seq(1>345)   ←   TCACTGATTTTCCCACGGACCTGCCAGCAACCGAAGTTTTCACNCCA
seq3  19-6-03.seq(1>347)   →   TCACTGATTTTCCCACGGACCTGCCAGCAACCGAAGTTTTCACTCCA
seq1  19-6-03.seq(1>337)   →   TCACTGATTTTCCCACGGACCTGCCAGCAACCGAAGTTTTCACTCCA


                              150       160       170       180       190
                         .l....l....l....l....l....l....l....l....l....l....l
                         GTTGTCCCCACAG-TAGACACATATGATGGCCGAGGTGATAGTGTGG

seq2  19-6-03.seq(1>341)   ←   GTTGTCCCCACAG-TAGACACATATGATGGCCGAGGTGATAGTGTGG
seq4  19-6-03.seq(1>345)   ←   GTTGTCCCCACAG-TAGACACATATGATGGCCGAGGTGATAGTGTGG
seq3  19-6-03.seq(1>347)   →   GTTGTCCCCACAGCTAGACACATATGATGGCCGAGGTGATAGTGTGG
seq1  19-6-03.seq(1>337)   →   GTTGTCCCCACAG-TAGACACATATGATGGCCGAGGTGATAGTGTGG


                              200       210       220       230       240
                         ..l....l....l....l....l....l....l....l....l....l....
                         TTTATGGACTGAGGTCAAAATCTAAGAAGTTTCGCAGACCTGACATC

seq2  19-6-03.seq(1>341)   ←   TTTATGGACTGAGGTCAAAATCTAAGAAGTTTCGCAGACCTGACATC
seq4  19-6-03.seq(1>345)   ←   TTTATGGACTGAGGTCAAAATCTAAGAAGTTTCGCAGACCTGACATC
seq3  19-6-03.seq(1>347)   →   TTTATGGACTGAGGTCAAAATCTAAGAAGTTTCGCAGACCTGACATC
seq1  19-6-03.seq(1>337)   →   TTTATGGACTGAGGTCAAAATCTAAGAAGTTTCGCAGACCTGACATC


                              250       260       270       280
                         ..l....l....l....l....l....l....l....l....l....l....
                         CAGTACCCTGATGCTACAGACGAGGACATCACCTCACACATGGAAAG

seq2  19-6-03.seq(1>341)   ←   CAGTACCCTGATGCTACAGACGAGGACATCACCTCACACATGGAAAG
seq4  19-6-03.seq(1>345)   ←   CAGTACCCTGATGCTACAGACGAGGACATCACCTCACACATGGAAAG
seq3  19-6-03.seq(1>347)   →   CAGTACCCTGATGCTACAGACGAGGACATCACCTCACACATGGAAAG
seq1  19-6-03.seq(1>337)   →   CAGTACCCTGATGCTACAGACGAGGACATCACCTCACACATGGAAAG


                              290       300       310       320       330
                         ..l....l....l....l....l....l....l....l....l....l....
                         C-GAGGAGTTGAATGGTGCATACAAGGCCAT-CCCCGTTGCCCAGGA

seq2  19-6-03.seq(1>341)   ←   C-GAGGAGTTGAATGGTGCATACAAGGCCAN-CCCCGTTGCCCAGGA
seq4  19-6-03.seq(1>345)   ←   C-GAGGAGTTGAATGGTGCATACAAGGCCATNCCCCGTTGCCCAGGA
seq3  19-6-03.seq(1>347)   →   CCGAGGAGTTGAATGGTGCATACAAGGCCAT-CCCCGTTGCCCAGGA
seq1  19-6-03.seq(1>337)   →   C-GAGGAGTTGAATGGTGCATACAAGGCCAT-CCCCGTTGCCCAGGA


                              340       350       360       370
                         ..l....l....l....l....l....l....l....l....l..
                         S-CTGAACGCGCCTTCTGATTGGGACAGCCGTATC

seq2  19-6-03.seq(1>341)   ←   N-CTGAACGC
seq4  19-6-03.seq(1>345)   ←   GNCTGAACGCAAAN
seq3  19-6-03.seq(1>347)   →   C-CTGAACGCGCCTTCTGATTGGGACAGCCGTATC
seq1  19-6-03.seq(1>337)   →   C-CTGAACGCGCCTTCTGATTGGGACAGCCGTAT
OP anti primer.seq(1>20)   →              CTTCTGATTGGGACAGCCGT ,
```

b) Osteocalcin

```
                      20        30        40        50        60
                      |....|....|....|....|....|....|....|....|....|....
                      ATGAGAGCCCTCACACTCCTCGCCCTATTGGCCCTGGCCGCACTTTGCAT
osteocalcin.seq(1>451)  →  ATGAGAGCCCTCACACTCCTCGCCCTATTGGCCCTGGCCGCACTTTGCAT
calcin sense.SEQ(1>22)  →  ATGAGAGCCCTCACACTCCTCG
```

```
                      70        80        90        100       110
                      |....|....|....|....|....|....|....|....|....|....
                      CGCTGGCCAGGCAGGTGCGAAGCCCAGCGGTGCAGAGTCCAGCAAAGGTG
osteocalcin.seq(1>451)  →  CGCTGGCCAGGCAGGTGCGAAGCCCAGCGGTGCAGAGTCCAGCAAAGGTG
```

```
                      120       130       140       150       160
                      |....|....|....|....|....|....|....|....|....|....
                      CAGCCTTTGTGTCCAAGCAGGAGGGCAGCGAGGTAGTGAAGAGACCCAGG
osteocalcin.seq(1>451)  →  CAGCCTTTGTGTCCAAGCAGGAGGGCAGCGAGGTAGTGAAGAGACCCAGG
```

```
                      170       180       190       200       210
                      |....|....|....|....|....|....|....|....|....|....
                      CGCTACCTGTATCAATGGCTGGGAGCCCCAGTCCCCTACCCGGATCCCCT
osteocalcin.seq(1>451)  →  CGCTACCTGTATCAATGGCTGGGAGCCCCAGTCCCCTACCCGGATCCCCT
```

```
                      220       230       240       250       260
                      |....|....|....|....|....|....|....|....|....|....
                      GGAGCCCAGGAGGGAGGTGTGTGAGCTCAATCCGGACTGTGACGAGTTGG
osteocalcin.seq(1>451)  →  GGAGCCCAGGAGGGAGGTGTGTGAGCTCAATCCGGACTGTGACGAGTTGG
```

```
                      270       280       290       300       310
                      |....|....|....|....|....|....|....|....|....|....
                      CTGACCACATCGGCTTTCAGGAGGCCTATCGGCGCTTCTACGGCCCGGT
osteocalcin.seq(1>451)  →  CTGACCACATCGGCTTTCAGGAGGCCTATCGGCGCTTCTACGGCCCGGT
calcin anti.SEQ(1>22)   ←                        TATCGGCGCTTCTACGGCCCGG
```

c) Collagen I

Sense

CAA AAC A   GTC TG TCGATG GCTGCAC GAG TCACACCG GA⁻IC CT GGGG(G)AG.GCA GTGATT GAATACAAAAA
1   9   17   25   33   41   49   57   65   73

Antisense

A A AA C   TC AG GCT CCG G TGT GACTCGT GCAGCCACC GACAGTGACGC AGTAGGTGAAGCGGC1(GA A   A   AA
1   10   19   28   37   46   55   64   73

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```
              1510      1520      1530      1540      1550      1560      1570      1580
              |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
              GKTTTTGTATTCAATCACTG-TCT-TmGCCCCAGG-CTCCGGTGTGACTCGTGCAGCCATC-GACAGt-gA-C-GCT-G
COLLAGEN 1.SEQ(1>5921)   ←   GGTTTTGTATTCAATCACTG-TCT-T-GCCCCAGG-CTCCGGTGTGACTCGTGCAGCCATC-GACAGT-GA-C-GCT-G
Col1S_7-4-04.seq(1>76)   ←   TTTTTGTATTCAATCACTGCTCTCTCGCCCCAGGACTCCGGTGTGACTCGTGCAGCCATC-GACAG---A-C-TGT-T
COL1_AS_7-8-03.SEQ(1>25) →   TTTTGTATTCAATCACTG-TCT-T-GCC
Col1A_7-4-04.seq(1>81)   →   AAAACTCAGG-CTCCGGTGTGACTCGTGCAGCCATCCGACAGTAGAGCAGCTAG
COL1_S_7-8-03.SEQ(1>19)  ←   GCT-G

              1590      1600      1610
              |....|....|....|....|....|....|
              TAGGT-GAAGCGGcTGKTRCSCWMRRMGC
COLLAGEN 1.SEQ(1>5921)   ←   TAGGT-GAAGCGGCTGTTGCCCTCGGCGC
Col1S_7-4-04.seq(1>76)   ←   TTG
COL1A_7-4-04.seq(1>81)   →   TAGGTAGAAGCGG-TCGTACGCAAAAA
COL1_S_7-8-03.SEQ(1>19)  ←   TAGGT-GAAGCGGCTG
```

d) Alkaline Phosphatase

Sense

ALPS_014_G10
ALPS

S/N G:141 A:89 T:97 C:138
KB.bcp
KB 1.1.1   Cap:14

KB_3100_POP6_BDTv3.mob
Pts 850 to 1800 Pk1 Loc:850
Version 5.1.1   HiSQV Bases: 23   Wendy Almond
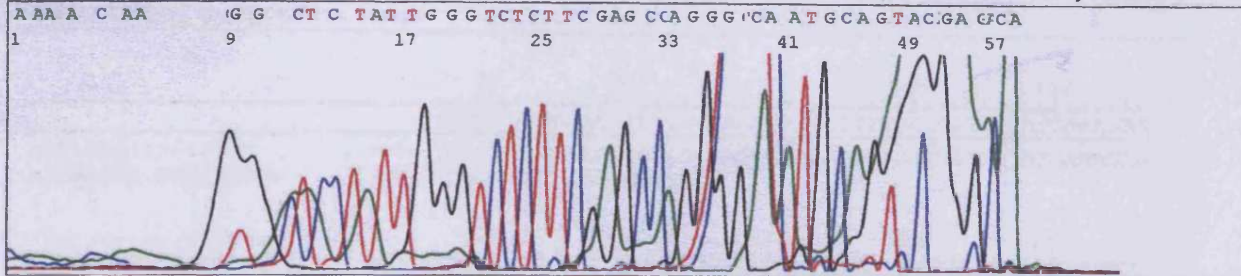
Antisense

ALPA_012_F10
ALPA

S/N G:179 A:69 T:80 C:125
KB.bcp
KB 1.1.1   Cap:12

KB_3100_POP6_BDTv3.mob
Pts 850 to 1800 Pk1 Loc:850
Version 5.1.1   HiSQV Bases: 15   Wendy Almond

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```
                    1080    1090    1100    1110    1120    1130    1140    1150
                    CTG-A-CC-CTTG-ACCCCC-ACAATGTGGACTacCTATtGGGTCTCTtCGAGCcAGGGGWCA-TGCAGtACgAGCWGAgA

Alp.seq(1>2580)       →   CTG-A-CC-CTTG-ACCCCC-ACAATGTGGACTACCTATTGGGTCTCTTCGAGCCAGGGGACA-TGCAGTACGAGCTGA-A
ALPA 7-4-04.seq(1>62) ←   TGTATCCTCTTGTACCCCCGACAATGTGGACTACCTAT-GGGTCTCT-CGATC-GTTGTTG-TT
ALPsense.SEQ(1>19)    →   G-A-CC-CTTG-ACCCCC-ACAAT
ALPS 7-4-04.seq(1>60) →              AAAACAACGGCT--CTATTGGGTCTCTTCGAGCCAGGGGT CAATGCAG-AT-AGCAGAGA
ALPantisense.SEQ(1>20) ←                                            AGGGGACA-TGCAGTACGAGC

                    1160
                    CAG
Alp.seq(1>2580)       →   CAG
ALPS 7-4-04.seq(1>60) →   CA
```

Appendix IV

Sequencing (Adipogenic differentiation)

a) aP2

```
                    70        80        90        100       110
              ....|....|....|....|....|....|....|....|....|....|....|....
              GTACCTGGAAACTTGTCTCCAGTGAAAACTTTGATGATTATATGAAAGAA
aP2.SEQ(1>619)       →   GTACCTGGAAACTTGTCTCCAGTGAAAACTTTGATGATTATATGAAAGAA
aP2sense.SEQ(1>20)  →   GTACCTGGAAACTTGTCTCC

                    120       130       140       150       160
              ....|....|....|....|....|....|....|....|....|....|....|....
              GTAGGAGTGGGCTTTGCCACCAGGAAAGTGGCTGGCATGGCCAAACCTAA
aP2.SEQ(1>619)       →   GTAGGAGTGGGCTTTGCCACCAGGAAAGTGGCTGGCATGGCCAAACCTAA

                    170       180       190       200       210
              ....|....|....|....|....|....|....|....|....|....|....|....
              CATGATCATCAGTGTGAATGGGGATGTGATCACCATTAAATCTGAAAGTA
aP2.SEQ(1>619)       →   CATGATCATCAGTGTGAATGGGGATGTGATCACCATTAAATCTGAAAGTA

                    220       230       240       250       260
              ....|....|....|....|....|....|....|....|....|....|....|....
              CCTTTAAAAATACTGAGATTTCCTTCATACTGGGCCAGGAATTTGACGAA
aP2.SEQ(1>619)       →   CCTTTAAAAATACTGAGATTTCCTTCATACTGGGCCAGGAATTTGACGAA

                    270       280       290       300       310
              ....|....|....|....|....|....|....|....|....|....|....|....
              GTCACTGCAGATGACAGGAAAGTCAAGAGCACCATAACCTTAGATGGGGG
aP2.SEQ(1>619)       →   GTCACTGCAGATGACAGGAAAGTCAAGAGCACCATAACCTTAGATGGGGG

                    320       330       340       350       360
              ....|....|....|....|....|....|....|....|....|....|....|....
              TGTCCTGGTACATGTGCAGAAATGGGATGGAAAATCAACCACCATAAAGA
aP2.SEQ(1>619)       →   TGTCCTGGTACATGTGCAGAAATGGGATGGAAAATCAACCACCATAAAGA

                    370       380       390       400       410
              ....|....|....|....|....|....|....|....|....|....|....|....
              GAAAACGAGAGGATGATAAACTGGTGGTGGAATGCGTCATGAAAGGCGTC
aP2.SEQ(1>619)       →   GAAAACGAGAGGATGATAAACTGGTGGTGGAATGCGTCATGAAAGGCGTC

                    420       430       440       450       460
              ....|....|....|....|....|....|....|....|....|....|....|....
              ACTTCCACGAGAGTTTATGAGAGAGCATAAGCCAAGGGACGTTGACCTGG
aP2.SEQ(1>619)       →   ACTTCCACGAGAGTTTATGAGAGAGCATAAGCCAAGGGACGTTGACCTGG
aP2antisense.SEQ(1>21) ←                                          GG

                    470       480
              ....|....|....|....|....|....
              ACTGAAGTTCGCATTGAACTC
aP2.SEQ(1>619)       →   ACTGAAGTTCGCATTGAACTC
aP2antisense.SEQ(1>21) ←   ACTGAAGTTCGCATTGAAC
```

b) LPL

```
                    720       730       740       750       760
                    |....|....|....|....|....|....|....|....|....|....|
                    CGATCCAGCTGGACCTAACTTTGAGTATGCAGAAGCCCCGAGTCGTCTTT
Lpl.seq(1>3549)    →  CGATCCAGCTGGACCTAACTTTGAGTATGCAGAAGCCCCGAGTCGTCTTT
LPLsense.SEQ(1>20) →  GATCCAGCTGGACCTAACTT

                    770       780       790       800       810
                    |....|....|....|....|....|....|....|....|....|....|
                    CTCCTGATGATGCAGATTTTGTAGACGTCTTACACACATTCACCAGAGGG
Lpl.seq(1>3549)    →  CTCCTGATGATGCAGATTTTGTAGACGTCTTACACACATTCACCAGAGGG

                    820       830       840       850       860
                    |....|....|....|....|....|....|....|....|....|....|
                    TCCCCTGGTCGAAGCATTGGAATCCAGAAACCAGTTGGGCATGTTGACAT
Lpl.seq(1>3549)    →  TCCCCTGGTCGAAGCATTGGAATCCAGAAACCAGTTGGGCATGTTGACAT

                    870       880       890       900       910
                    |....|....|....|....|....|....|....|....|....|....|
                    TTACCCGAATGGAGGTACTTTTCAGCCAGGATGTAACATTGGAGAAGCTA
Lpl.seq(1>3549)    →  TTACCCGAATGGAGGTACTTTTCAGCCAGGATGTAACATTGGAGAAGCTA

                    920       930       940       950       960
                    |....|....|....|....|....|....|....|....|....|....|
                    TCCGCGTGATTGCAGAGAGAGGACTTGGAGATGTGGACCAGCTAGTGAAG
Lpl.seq(1>3549)    →  TCCGCGTGATTGCAGAGAGAGGACTTGGAGATGTGGACCAGCTAGTGAAG

                    970       980       990       1000      1010
                    |....|....|....|....|....|....|....|....|....|....|
                    TGCTCCCACGAGCGCTCCATTCATCTCTTCATCGACTCTCTGTTGAATGA
Lpl.seq(1>3549)    →  TGCTCCCACGAGCGCTCCATTCATCTCTTCATCGACTCTCTGTTGAATGA

                    1020      1030      1040      1050      1060
                    |....|....|....|....|....|....|....|....|....|....|
                    AGAAAATCCAAGTAAGGCCTACAGGTGCAGTTCCAAGGAAGCCTTTGAGA
Lpl.seq(1>3549)    →  AGAAAATCCAAGTAAGGCCTACAGGTGCAGTTCCAAGGAAGCCTTTGAGA

                    1070      1080      1090      1100      1110
                    |....|....|....|....|....|....|....|....|....|....|
                    AAGGGCTCTGCTTGAGTTGTAGAAAGAACCGCTGCAACAATCTGGGCTAT
Lpl.seq(1>3549)    →  AAGGGCTCTGCTTGAGTTGTAGAAAGAACCGCTGCAACAATCTGGGCTAT

                    1120      1130      1140      1150      1160
                    |....|....|....|....|....|....|....|....|....|....|
                    GAGATCAATAAAGTCAGAGCCAAAAGAAGCAGCAAAATGTACCTGAAGAC
Lpl.seq(1>3549)    →  GAGATCAATAAAGTCAGAGCCAAAAGAAGCAGCAAAATGTACCTGAAGAC

                    1170      1180      1190      1200      1210
                    |....|....|....|....|....|....|....|....|....|....|
                    TCGTTCTCAGATGCCCTACAAAGTCTTCCATTACCAAGTAAAGATTCATT
Lpl.seq(1>3549)    →  TCGTTCTCAGATGCCCTACAAAGTCTTCCATTACCAAGTAAAGATTCATT

                    1220      1230      1240      1250      1260
                    |....|....|....|....|....|....|....|....|....|....|
                    TTTCTGGGACTGAGAGTGAAACCCATACCAATCAGGCCTTTGA
Lpl.seq(1>3549)       →  TTTCTGGGACTGAGAGTGAAACCCATACCAATCAGGCCTTTGA
LPLantisense.SEQ(1>20) ←              CCCATACCAATCAGGCCTTT
```
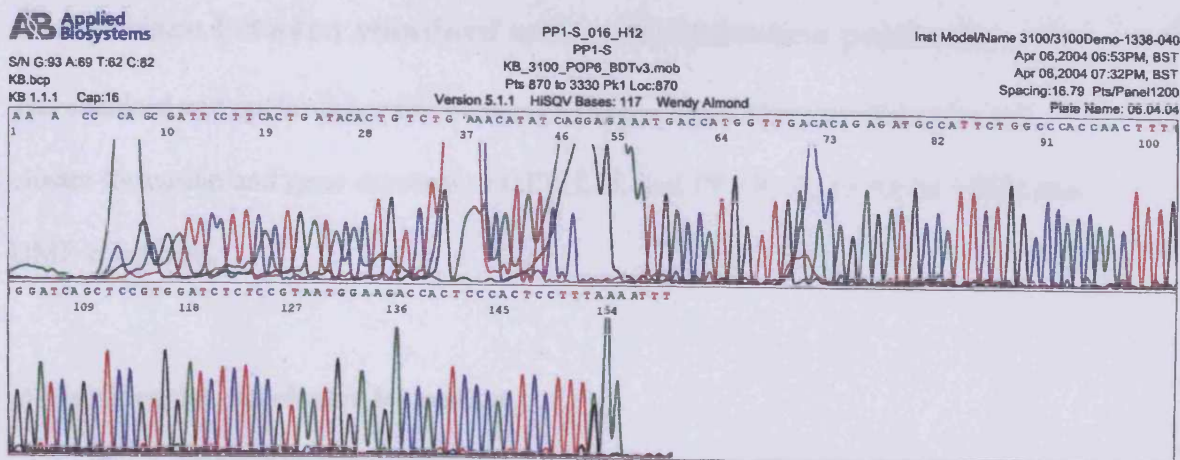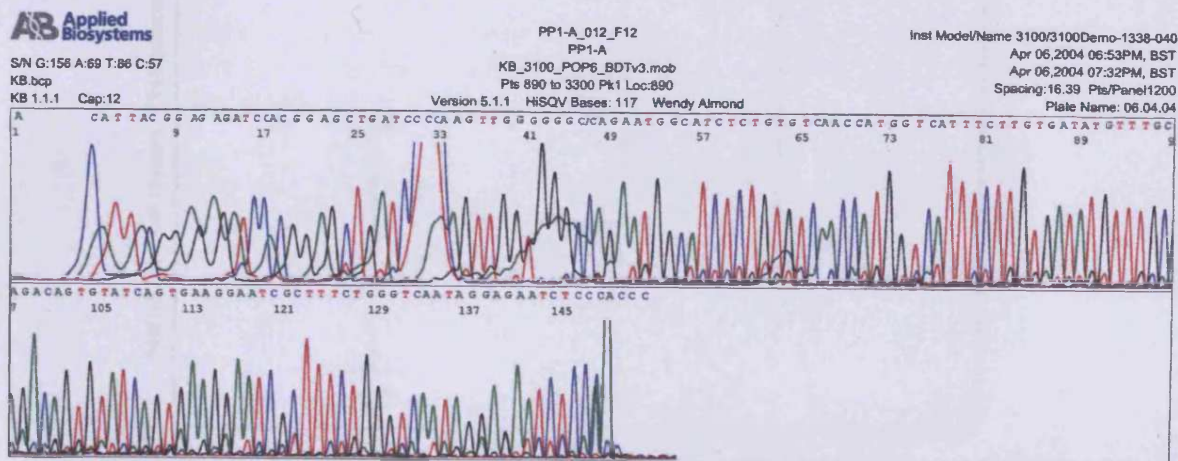
c) PPAR-γ2

Sense

Antisense

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Project: Untitled Contig 1

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PPAR2gamma.SEQ(1>1854)    →   CTCTGGGAGATTCTCCTATTGACCCAGAAAGCGGATTCCTTCACTGATACACTGTCTG-CAAACATATC-AC---AAGAA
PP1-A_7-4-04.seq(1>156)   ←   GGGTGGGAGATTCTCCTATTGACCCAGAAAGCGGATTCCTTCACTGATACACTGTCTG-CAAACATATC-AC---AAGAA
PPAR2gam sense.SEQ(1>23)  →   GGGAGATTCTCCTATTGACCCAG
PP1-S_7-4-04.seq(1>163)   →           AAACCC----AGCGGATTCCTTCACTGATACACTGTCTGTCAAACATATCGACGAGAAGAA

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PPAR2gamma.SEQ(1>1854)    →   ATGACCATGGTTGACACAGAGATGCCATTCTG-G-CCCACCAACTTTGGGATCAGCTCCGTGGATCTCTCCGTAATGGA
PP1-A_7-4-04.seq(1>156)   ←   ATGACCATGGTTGACACAGAGATGCCATTCTGTGCCCCACCAACTTGGGGATCAGCTCCGTGGATCTCTCCGTAATGT
PP1-S_7-4-04.seq(1>163)   →   ATGACCATGGTTGACACAGAGATGCCATTCTG-G-CCCACCAACTTTGGGATCAGCTCCGTGGATCTCTCCGTAATGGA
PPAR2gam anti.SEQ(1>23)   ←                                                                          GA

              310       320
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PPAR2gamma.SEQ(1>1854)    →   AGACCACTCCCACTCCTTTGATATCAA
PP1-S_7-4-04.seq(1>163)   →   AGACCACTCCCACTCCTTTAAAATTT
PPAR2gam anti.SEQ(1>23)   ←   AGACCACTCCCACTCCTTTGA
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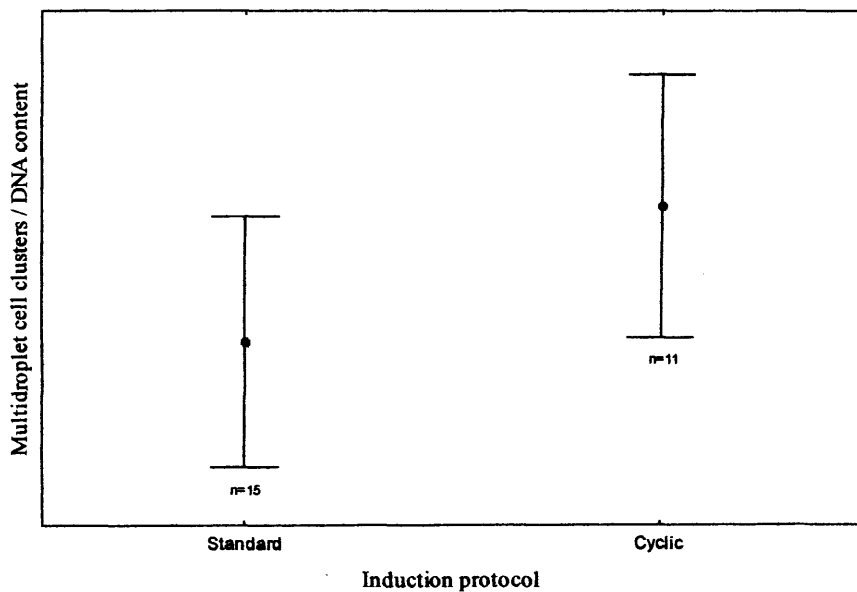
Appendix V

Comparison between standard and cyclic induction protocols

The standard and cyclic induction protocols were compared for multidroplet cell

cluster formation and gene expression (aP2, LPL and PPAR-γ2) in Alpha MEM and
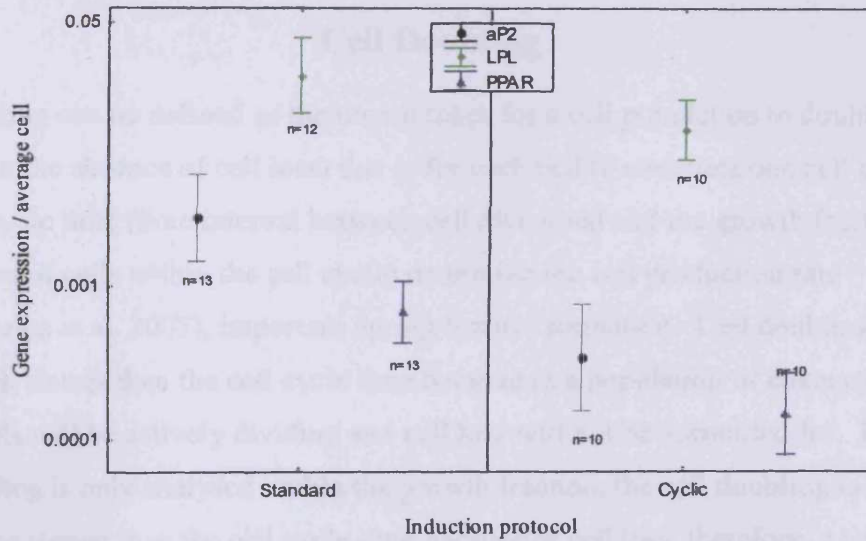
DME combined.

a) Multidroplet cell cluster formation

Multidroplet cell cluster formation / DNA content at 21 days post induction in standard and
cyclic induction protocols in Alpha MEM and DME combined. Mean ± SE

No significant differences were found between the multidroplet cell cluster formation

in adipogenically induced BMdSC cultures by cyclic or by standard induction.

b) Gene expression

Standard and cyclic induction protocols: aP2, LPL and PPAR-γ2 gene expression at 21 days post induction in Alpha MEM and DME combined (CaCl$_2$: 100 mg/l), normalised to HK gene. Mean ± SE

No significant differences were observed between the standard and cyclic induction protocols for aP2 (black round), LPL (green diamond), or PPAR-γ2 (blue triangle) gene expression.

Appendix VI

Cell Doubling

Cell doubling can be defined as the time it takes for a cell population to double in number (in the absence of cell loss) that is for each cell to complete one cell cycle. The cell cycle time (time interval between cell divisions) and the growth fraction (proportion of cells within the cell cycle) determine the cell production rate (Eidukevicius et al. 2005), important during tumour formation. Cell doubling is likely to be much slower than the cell cycle time because in a population of eukaryotic cells, not all cells will be actively dividing and cell loss will not be accounted for. Even if cell doubling is only analysed within the growth fraction, the cell doubling is still likely to be slower than the cell cycle time because of cell loss, therefore, a tumour with high cell loss can be actively dividing but look to be dormant or slow growing. During this investigation cell doubling was measured using cell counts taken twice weekly for passaged stromal cultures and weekly for long-term cultures. This did not account for the fact that not all cells were actively dividing, and did not account for cell loss through detachment and / or apoptosis, giving only an index of growth.

There are various methods for measuring cell cycle time including labelling the cells with radioactive DNA precursors (titrated thymidine) to determine the number of mitotic cells and comparing them with the total number of cells within a sample to measure the mitotic index (growth fraction) (Horst Ibelgaufts' COPE: Cytokines & cells online pathfinder encyclopaedia 2006). This investigation briefly looked at BMdSC cycle using the flow cytometer and DRAQ5 (3.5.1. 'Cell cycle analysis'), a fluorescent DNA-specific stain to measure DNA content because cells in G2/M have twice the DNA content as cells in G1 (figure 2.2). Another proliferation-related antigen that is widely used to measure the growth fraction in tumours is Ki-67, which is absent from quiescent cells (Eidukevicius et al 2005). A more recent method is the flow cytometric analysis of cells labelled with Bromodeoxyuridine (BrdUrd), which can estimate the duration of S phase and determine the growth fraction. None of these methods, however, account for cell loss within a cell population.

References

Eidukevicius, R., Characiejus, D., Janavicius, R., Kazlauskaite, N., Pasukoniene, V., Mauricas, M., & Den Otter, W. A method to estimate cell cycle time and growth fraction using bromodeoxyuridine-flow cytometry data from a single sample. BMC Cancer 5, 122. 2005.

Horst Ibelgaufts' COPE: Cytokines & cells online pathfinder encyclopaedia. Cell cycle. 2006.
http://www.copewithcytokines.de/cope.cgi?3035

Appendix VII

Publications

'Effect of calcium on bone marrow derived stromal cell growth and differentiation' at the British society of Haematology conference, 2004.

'Inhibition of bone marrow derived mesenchymal stem cell growth by glutamine in culture medium' at The first international symposium on Challenges in the era of stem cell plasticity conference, April 8 – 11, Providence, Rhode Island, 2003

'Quantitative assessment of bone marrow derived stromal cell differentiation *in vitro*' at UWCM Postgraduate day, 2003.

'The in vitro growth of bone marrow derived stromal cells' at UWCM Postgraduate day, 2002.