Isolation and Characterisation of Mesenchymal Stem Cells

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Abstract

MSCs have great therapeutic potential and are currently used in various clinical trials. However, the extremely low frequency of MSCs and the absence of a known cell-specific marker have made their purification and identification a highly challenging goal. Our hypothesis is that identification of a MSC specific cell surface marker would facilitate isolation of a pure population of MSCs, which in clinical studies may enhance the subsequent regenerative effect in comparison to a heterogeneous MSC population. Our aim therefore was to attempt to identify such MSC-specific markers using a transcriptomics approach. Individual clones were isolated after seeding bone marrow mononuclear cells from BALB/b and BALB/c mice in 5% oxygen tension. All clones had the ability to differentiate into adipocytes, chondrocytes, and osteoblasts, and to self-renew, and were therefore functionally characterised as MSCs. All murine MSC clones consistently expressed very high levels of Sca-1. Cytogenetic analysis of clones revealed an abnormal karyotype of 69 chromosomes and transplantation of cells into immuno-compromised SCID mice revealed no evidence of tumour formation after 7 months indicating that cells were not malignant. A rigorous genome-wide supervised microarray analysis revealed six genes were differentially expressed on the MSC clones in comparison to various controls - STEAP1, STEAP2, ly6f, versican, vitamin D receptor, and H2-M9. Two-dimensional hierarchical clustering of 28 different arrays revealed both STEAP genes and vitamin D receptor also had a similar expression pattern to Sca-1. Thus STEAP1 and STEAP2 were the only two cell membrane protein encoding genes identified by both analysis methods. Importantly, flow cytometry analysis revealed STEAP2 was differentially expressed in normal diploid multipotent human bone marrow stromal cells (hBMSCs) compared to fibroblasts and freshly isolated bone marrow cells. Furthermore, western blot analysis revealed STEAP1 was significantly expressed in hBMSCs, but not by human fibroblasts or human chondrocytes. STEAP1 depletion by RNA interference resulted in decreased cell adherence to tissue culture plastic. Results suggest STEAP1 and STEAP2 may be novel MSC markers in murine and in human cells. Further work is needed to elucidate their role in MSCs and to establish their usefulness as potential cell-specific markers.

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Declaration

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Dedication

In loving memory of Lalji Vishram…

...mara bapuji

Abbreviations

2D	Two dimensional
A 2-P	Ascorbic acid 2-phosphate
ABS	Adult bovine serum
AKT	V-akt murine thymoma viral oncogene homolog 1
AMC	Adipose-derived mesenchymal stem cell
B/b	BALB/b
B/c	BALB/c
BMAC	Bone marrow adherent cell
BMMNC	Bone marrow mononuclear cell
BMP2	Bone morphogenetic protein-2
BMPR1a	bone morphogenetic protein receptor, type IA
BMSC	Bone marrow stromal cell
BMT	Bone marrow transplantation
Ccl7	Chemokine (C-C motif) ligand 7
CD	Cluster of Differentiation
Cdk4	Cyclin dependent kinase 4
Cdkn2a	Cyclin-dependent kinase inhibitor 2a
CEBP	CCAAT/enhancer binding protein
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane receptor
CFU-f	Colony forming unit- fibroblast
cMET	c met proto-oncogene
CNS	Central nervous system
Crabp2	Cellular retinoic acid binding protein II
Csfr1	Colony stimulating factor receptor 1
CSPG-2	Chondroitin sulphate proteoglycan core protein-2
Cxcl1	Chemokine (C-X-C motif) ligand 1
Cxcl5	Chemokine (C-X-C motif) ligand 5
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Differentiated clone
DCAMKL1	Doublecortin-like kinase 1
DFO	Desferrioxamine
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
DU145	Human prostate cancer cell line
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

ESCs	Embryonic stem cells
FACS	Flow activated cell sorting
FCS	Fetal calf serum
FGF2	Fibroblast growth factor
FGF7	Fibroblast growth factor-7
FITC	Fluorescein Isothiocyanate
FNO	$F_{420}H_2$:NADP+ oxidoreductase
Foxa1	Forkhead box A1
Gfzf3	Glis family zinc finger 3
β-GP	β -glycero phosphate
GLUT4	Glucose transporter 4
GPI-AP	Glycosyl phosphatidylinositol-anchored protein
GVHD	Graft versus host disease
H2-M9	Histocompatability 2, m region locus 9
hBMMNC	Human bone marrow mononuclear cell
hBMSC	Human bone marrow stromal cell
HBSS	Hank's Balance Salt Solution
HGF	Hepatocyte growth factor
HIF1a	Hypoxia inducible factor-1 alpha
HIF2a	Hypoxia inducible factor-2 alpha
hMSC-TERT	Human mesenchymal stem cell- Telomerase reverse transcriptase
HS68	human newborn foreskin fibroblasts
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IBMX	3- isobutyl-1-methyl-xanthine
IGFBP6	Insulin-like growth factor binding protein 6
IGFBP6	insulin growth factor binding protein-6
IL1Ri	Interleukin 1 receptor i
IL-2Rb	Interleukin 2 receptor b
ISCT	International Society for Cellular Therapy
ITGA5	Integrin alpha 5
ITS+1	Insulin transferrin selenium
JAK1	Janus activated kinase 1
LIF	Leukaemia inhibitory factor
LNCaP	Androgen sensitive human prostate adenocarcinoma cell line
LNGFR	Low affinity growth factor receptor
M113	Melanoma cell line
Maged2	Melanoma antigen family D, 2
MALDI-TOF	Matrix-assisted laser desorption/ionization- time-of-flight
MAPC	Multipotent adult progenitor cells
MCP-3	Monocyte chemotactic protein-3

MEF	Mouse embryonic fibroblasts
α-MEM	alpha-minimal essential media
MLD	Metachromatic Leukodystrophy
MSC	Mesenchymal stem cell
Nanog	Nanog homeobox
NCAM-1	Neural cell adhesion molecule -1
NK-Cell	Natural killer- cells
NOD	Non-obese diabetic
OCT4	POU domain, class 5, transcription factor 1
OI	Osteogenesis Imperfecta
OM	Osteogenesis media
p	Passage
PE	Phycoerythrin
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDGF-BB	Platelet derived growth factor -
PPAR-γ	Peroxisome proliferator-activated receptor- γ
PTEN	Phosphatase and tensin homolog
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPLPO	Human acidic ribosomal phosphoprotein PO
RT-PCR	Reverse transcription-polymerase chain reaction
Runx2	runt-related transcription factor 2
Sca-1	Stem cell antigen -1
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOX4	SRY (sex determining region Y)-box 4
SOX9	SRY (sex determining region Y)-box 9
spl	split homolog of drosphila E
STAT5a/b	Signal transducer and activator of transcription 5a/b
STEAP1	Six-transmembrane epithelial antigen of the prostate1
STEAP2	Six-transmembrane epithelial antigen of the prostate2
TBS	Tris buffered saline
TERC	Template RNA component
Tf	Transferrin
TfR1	Transferrin receptor 1
TGF-b	Transforming growth factor- beta

TM4SF1	Transmembrane 4 superfamily member 1
UC	Undifferentiated clone
ULB	Urea Lysis Buffer
USSC	Unrestricted somatic stem cell
Vdr	Vitamin D receptor
VEGF	Vascular endothelial growth factor
α-MEM	alpha-minimal essential media
β-GP	β -glycero phosphate

Chapter 1

Introduction

1.1 Adult Stem Cells

Stem cells are defined by two basic properties: the ability to self-renew and the capacity to differentiate into one or more cell types. Adult stem cells are multipotent in their differentiation capacity and are found in many tissues of the body (Wagers et al., 2002). They are thought to serve as reservoirs of reparative cells that maintain cell turnover, and during injury or disease migrate to the site of tissue damage and participate in the repair process.

1.1.1 Epidermal stem cells

The outer layer of the epidermis is continually being shed, thus the lifetime of a skin cell is about one month depending on the region (Watt et al., 2006). Skin consists of two main parts: the outer epidermal layer and the connective tissue layer beneath. This contains the collagen-rich dermis and has an underlying fatty subcutaneous layer. The constant epidermal cell turnover can only be maintained if there is a population of self-renewing cells that can remain undifferentiated, but continuously divide via amplifying cells, which go on to terminally differentiate into distinct epidermal lineages. These lineages include the intermolecular epidermis, hair follicles, and sebaceous glands (Watt and Hogan, 2000). Epidermal stem cells are typically localised in specialised areas such as the hair follicle, at the base and or at the bulge (Panteleyev et al., 2001). They are well characterised and have been demonstrated to express high levels of β1 actin and α6 integrin, and low levels of CD71, the transferrin receptor (Jones and Watt, 1993) and more recently found to express CD200 (Watt et al., 2006). This has enabled a good understanding of the lineage pathway for each differentiated cell type.

1.1.2 Intestinal stem cells

The epithelial lining of the small intestine renews itself faster than any other tissue, with a turnover time of a week or less. The lining of the small intestine is covered with many different cell types each with a specialised function. This includes (1) absorptive cells, which are densely packed with microvilli for absorption of nutrients (2) goblet cells,

secrete mucus (3) paneth cells, which play a role in the innate immune system (4) enteroendocrine cells, which secrete serotonin and various peptide hormones (Wright, 2000). There are over 15 subtypes of paneth cells (Ganz, 2000). This constant turnover process is maintained by intestinal stem cells located in the crypts of the epithelium that descend into the underlying connective tissue. The stem cell divides to produce more intermediate cells which go through several rounds of division, with each division the cell travels towards the basal epithelium and becomes more committed to a specific lineage. Once the cell has reached the epithelium it has terminally differentiated into one of the above cell types. To date, there are no known definitive markers to identify intestinal stem cells. They are known to express Musashi (Kayahara et al., 2003), and other potential markers are BMPR1a (a phospho-PTEN), DCAMKL1, Eph receptors and integrins (Montgomery and Breault, 2008).

1.1.3 Haematopoietic stem cells

The haematopoietic stem cell (HSC) is the best characterised adult stem cell. HSCs have the potential to give rise to all the blood cells, each with very different functions from transporting oxygen around the body to the production of antibodies. In the adult, the majority of the haematopoietic process takes place in the highly dynamic environment of the bone marrow where HSCs are localised. The differentiated cells then exit into the bloodstream and are transported to where they are needed. HSCs can differentiate into myeloid lineages (macrophages, monocytes, neutrophils, basophils, eosinophils, dendritic cells, erythrocytes, megakaryocytes) and lymphoid lineages (T-cells, B-cells, NK-cells). Despite the large number of differentiated cell types, each lineage pathway has been well documented for HSCs (Orkin, 2000) (Weissman et al., 2001).

This is because HSC markers are quite well defined for each population of HSC. Human HSCs are $CD34^{+}$, $CD59^{+}$, $CD90^{+}$, $CD38^{low/-}$, $C\text{-}kit^{+}$, lin^{-}. Whilst mouse HSCs are $CD34^{low/-}$, $Sca\text{-}1^{+}$, $CD90^{low/+}$, $CD38^{+}$, $C\text{-}Kit^{+}$, lin^{-} (Wognum et al., 2003). However, it should be noted HSC populations consist of subsets with varying differentiation potentials, and this list does not cover the surface phenotype of them all.

1.1.4 Mesenchymal stem cells

The bone marrow also contains a non-haematopoietic stem cell called a mesenchymal stem cell (MSC) that can differentiate into classical mesenchymal lineages adipocytes, chondrocytes and osteoblasts, at least *in vitro* (Pittenger et al., 1999). Before the term 'MSC' was coined this cell type was thought to function predominantly as a stromal supportive cell which played an important role in the haematopoietic process (Dexter et al., 1977), (Calvi et al., 2003). Friedenstein and colleagues provided the earliest evidence for the existence of its stem cell properties by demonstrating that bone marrow derived adherent cells were capable of osteogenesis (Friedenstein, 1961). These studies have been supported by *in vivo* work (Kuznetsov et al., 1997; Lennon et al., 2001). Although its absolute *in vivo* function has not been clarified, it is logical to think that there is such a connective tissue progenitor cell involved in tissue maintenance of the skeletal system. However, the lack of a known MSC specific marker has hindered better understanding of this stem cell.

In summary, adult stem cells are vital for normal tissue maintenance and for repair after injury for the duration of an organism's life. It is not surprising that the better understood stem cells are those whose markers have been identified. This enables isolation and subsequent studies on a pure population of stem cells and yields relevant results, compared to stem cells for which no specific marker has been identified.

1.1.5 Adult stem cells vs. Embryonic stem cells

Advantages of using adult stem cells over embryonic stem cells (ESCs) for repair therapies include relatively stable differentiated phenotypes with no teratoma formation and the possibility of using autologous cells directly, thus avoiding immune response concerns. While ESCs may represent future promise for treating certain intractable diseases; for tissues where there are known adult stem cells available, the latter would seem a more promising and feasible approach. In contrast to the ESC, one type of adult stem cell currently used in clinical studies for a increasing number of diseases is the mesenchymal stem cell (MSC) (Tae et al., 2006). The multipotent nature of MSCs and their ability to proliferate significantly in culture has rendered them a potentially useful

candidate for tissue repair and gene therapy (Bianco et al., 2001; Caplan and Bruder, 2001). In addition, their potential immunosuppressive properties may increase their therapeutic application (Sotiropoulou and Papamichail, 2007).

1.1.6 Inducible pluripotent stem cells

Recent studies have reported a type of stem cell that has the pluripotency of the embryonic stem cell, but without the ethical dilemmas (Okita et al., 2007) (Nakagawa et al., 2007). So-called induced pluripotent stem cells (iPSCs), these cells are derived from non-pluripotent cells such as adult skin fibroblasts, by inducing them to express stem cell-associated genes. The Okita group cultured human skin fibroblasts and transduced them with a retroviral vector containing 4 stem cell genes- OCT3/4, SOX2, KLF4, and c-Myc (Okita et al., 2007). The subsequent cells behaved similarly to ES cells. They had a similar cell doubling time, expressed telomerase and expressed many cell surface markers and genes characteristic of ES cells. In addition, the iPSCs could differentiate into cells of the three primary germ layers (ectoderm, endoderm, and mesoderm) *in vivo* and *in vitro*. In a separate study, Yu and colleagues also reported very similar results (Yu et al., 2007). They also transduced fibroblasts with 4 stem cell genes, except this time with NANOG and LIN28 instead of KLF4 and c-myc. The resultant iPSCs exhibited similar ES cell properties to those mentioned above. In a later study Nakagawa reported that iPSCs could be formed from transduction of both mouse and human fibroblasts using only 3 genes (Nakagawa et al., 2007). This was an important study as it showed that the gene that was omitted was the oncogene c-myc which had made iPS cells prone to tumour formation (Okita et al., 2007).

This is an exciting research area, however much work needs to be done before the cells can used in a clinical setting as there are concerns over the safety of using retrovirally transduced cells.

1.2 History of MSCs

The bone marrow has long been known to be the source of haematopoietic stem cells (HSCs). In 1878 the German pathologist Clonhein was the first to suggest that non-haematopoietic cells were also present in the bone marrow (Chamberlain et al., 2007) However, it took a further 90 years before Friedenstein and colleagues demonstrated that the bone marrow contains cells which can differentiate into cells of the mesenchyme (Friedenstein et al., 1968). These investigators seeded murine whole bone marrow aspirate on plastic culture dishes. The non-adherent cells (which contain most of the haematopoietic cells) were removed after 4 hours. The remaining cells were heterogeneous in terms of morphology. However, they report the presence of spindle shaped cells that formed little clusters of 2-4 cells, which after a lag period of 2-4 days began to proliferate rapidly. The cells became more homogeneous in morphology after they had been passaged several times, i.e. the spindle shaped cells presumably out-grew the other cell types. They also found that these cells could differentiate into bone and cartilage. Furthermore, this differentiation characteristic was demonstrated both *in vitro* and *in vivo* (Friedenstein, 1961; Friedenstein et al., 1966). In the next decade these results were confirmed by others (Ashton et al., 1980; Bab et al., 1986; Castro-Malaspina et al., 1980). In addition, the latter group reported that these cells were rare, constituting 1 in 100 000 nucleated cells from the bone marrow. Subsequent studies found these cells had the ability to differentiate into the adipocytes and myoblasts (Dennis et al., 1999; Wakitani et al., 1995). Thus, these plastic adherent bone marrow cells that had the potential to differentiate into cells of the mesenchyme were called mesenchymal stem cells (MSCs). This term was used by Caplan in the 1990s (Caplan, 1991), and early studies by his group report that differentiation potential of MSCs diminished with extensive passage (Nakahara et al., 1990). However, more recent studies report that MSCs have a much broader differentiation potential (Jiang et al., 2002). Since then there has been a dramatic increase in the number of studies involving MSCs. For instance, between 1997 and 2007 there has been an over 40-fold increase in the number of pubmed entries containing ‘mesenchymal stem cell’ (Wagner and Ho, 2007).

1.3 Concept of the stem cell niche

The stem cell 'niche' is a specialised microenvironment where the stem cells reside. It is more than just the location of the stem cell as it has a multi-faceted role. It is a highly dynamic environment containing different types of cells which interact with the stem cell in many ways (Watt and Hogan, 2000), for example, the niche is important for maintaining the stem cell in its quiescent state. In response to injury or during normal tissue homeostasis the microenvironment can signal the stem cell to mobilise, self-renew and differentiate into specific cell type(s). The niche also prevents depletion of the stem cell and un-controlled proliferation; therefore, if the niche becomes dysregulated it may also cause aberrant stem cell function. The stem cell receives many different types of signals including cell-cell interactions via adhesion molecules and extracellular matrix, as well as signals via cytokine and growth factors. The physiochemical nature of the niche including the pH, calcium concentration, and oxygen tension is also important (Martinez-Agosto et al., 2007).

Throughout adult life, the *in vivo* niche maintains the adult stem cell in an undifferentiated state until required for normal tissue maintenance or repair in response to injury. It is important for scientists to understand the many properties of the niche and be able to replicate at least to some extent, the *in vivo* conditions *in vitro*. In addition, after a number of population doublings during *in vitro* culture, the stem cell undergoes an 'aging' process. The proliferation rate and the differentiation potential of the stem cell decreases and there is a morphology change. This is not ideal for regenerative medicine, where a sufficient number and quality of stem cell needs to be grown before transplantation to the patient. Current culturing conditions need to be improved so that they better mimic the *in vivo* microenvironment thus enabling stem cell properties to be better maintained.

1.3.1 The MSC niche in the bone marrow

Traditionally the main source from which MSCs are obtained is the bone marrow. The bone marrow is primarily composed of haematopoietic cells, HSCs, adipocytes,

endothelial cells, fibroblasts, osteoprogenitors, and stromal cells (Castro-Malaspina and Jhanwar, 1984; Wang and Wolf, 1990). Little is known about the *in vivo* MSC niche as there is no known marker of MSCs that can be used to identify the cell *in vivo*. However, there are a few theories. The HSC niche is slightly better understood, and since MSCs are required for the regulation of haematopoietic process (Mitsiadis et al., 2007), it is likely MSCs share a somewhat similar niche to HSCs. There are thought to be three HSC niches within the bone marrow (Dazzi et al., 2006). The first is the 'bony niche' or 'endosteal niche' located at the trabeculae, and lined with osteoblasts (Wilson et al., 2007). The second is the 'stromal niche' composed of reticular cells, fibroblasts, endothelial cells, and macrophages. The third is the 'adipocyte niche' which occupies the space within the bone marrow. MSCs are thought to be present largely in the stromal niche as they produce osteoblasts, fibroblasts, and adipocytes which interact with the HSC.

1.3.2 Tissue specific MSC niche

Another possibility to the one above is that MSCs or MSC-like cells are present in most tissue types. This is based on the fact that cells with the *in vitro* characteristics of MSCs have been isolated from many different tissues including: bone marrow, adipose tissue(De Ugarte et al., 2003), peripheral blood (Kuznetsov et al., 2001), synovial membrane (De Bari et al., 2001), human umbilical cord perivascular cells (Sarugaser et al., 2005), skeletal muscle (Williams et al., 1999), deciduous teeth (Tuan et al., 2003), trabecular bone (Noth et al., 2002) and amniotic fluid (De Coppi et al., 2007).

This could be due to the premise that there is only one source of MSCs, the bone marrow, and when mobilised MSCs exit the niche and circulate in the blood to replenish cell populations during tissue maintenance or injury. This could be achieved in two ways, (1) the MSC may differentiate into the organ-specific cells, or (2) it may secrete cytokines to facilitate tissue regeneration. In addition, the immunosuppressive properties of MSCs may also be favourable to tissue repair.

Transdifferentiation is when a cell differentiates into a lineage outside its developmental origin, for example when a MSC differentiates into a neural cell. There are some

investigators that say this could be due to cell fusion (Terada et al., 2002; Ying et al., 2002). However, recent cell tracking studies have disputed this suggestion for different cell types (Forbes et al., 2002). Since, undifferentiated MSC-like cells have been isolated from various tissues the latter possibility may be more likely.

1.3.3 The perivascular MSC niche

In more recent studies, investigators have postulated that MSCs are the bone marrow stromal supportive cells, also called marrow adventitial reticular cells or pericytes. Furthermore, they suggest the perivascular niche which is the cell lining the outer surface of blood vessels, is where the *in vivo* MSC is located. (da Silva Meirelles et al., 2008; Jones and McGonagle, 2008; Short et al., 2003). Supporting this suggestion is a study that showed MSCs sorted by STRO-1^{+}CD106^{+} expressed the pericyte specific marker α-smooth muscle actin (Shi and Gronthos, 2003). However, cells with MSC characteristics have been isolated from articular cartilage which is avascular (Barbero et al., 2003; Dowthwaite et al., 2004) which contradicts the above possibility. Therefore, more work needs to be performed in this area and significant progress can only be accomplished once a MSC specific marker is identified.

1.4 *In vitro* stem cell characteristics of MSCs

1.4.1 Self- renewal of MSCs

The self-renewal potential of a stem cell is defined as the ability to generate identical copies of themselves with the same multilineage potential as the mother cell. This can occur during the entire lifetime of an organism. The *in vitro* self-renewal ability of MSCs has been demonstrated. The cells have the ability to form CFU-f and to differentiate into multiple lineages. However, studies have reported MSCs lose their differentiation potential with passage in culture ((Gronthos et al., 2003; Muraglia et al., 2000; Pittenger et al., 1999).

1.4.2 Differentiation capacity of MSCs

Differentiation is a series of events involved in the progression of a stem/progenitor cell to a more specialised cell type, in order to perform a specific function. This process of differentiation involves co-ordinated regulation. Genes specific to the mode of differentiation are switched on and/or upregulated, whilst expression of stem cell genes and genes for other lineages is repressed. Differentiation can result in a change of cell size, morphology, metabolic activity, polarity, biomechanical properties, extracellular matrix, and surface phenotype, as well as the way a cell responds to signals (Tsonis, 2004). Therefore, it is a tightly regulated process that occurs in a stepwise manner. This way a limited number of stem cells can give rise to a highly expanded number of differentiated cells. This could explain why the fraction of undifferentiated mesenchymal stem cells is so small in the total bone marrow population. As mentioned above, many studies have reported that MSCs have the capacity to differentiate into adipocytes, chondrocytes, and osteoblasts (Tropel et al., 2004). This is the main functional criterion by which MSCs are defined *in vitro*.

1.4.2.1 Osteogenesis

The classical method of osteogenic differentiation *in vitro* involves culturing MSCs in a monolayer in the presence of ascorbic acid-2- phosphate, a source of phosphate such as β- glycerophosphate and dexamethasone. Bone morphogenetic proteins (BMPs) can also be added to induce osteogenesis. Although there are species differences, BMPs work better in murine MSCs (Edgar et al., 2007) than in human MSCs (Diefenderfer et al., 2003; Hanada et al., 1997). During culture the MSCs acquire an osteoblastic morphology as they form aggregates or nodules. There is an increase in their expression of alkaline phosphatase and the cells deposit a calcium-rich mineralised matrix which can be visualised histochemically with either Alizarin Red or a Von Kossa stain. The gene expression of MSCs differentiated into osteoblasts also changes; there is upregulation of osteoblast genes such as bone sialoprotein, osteocalcin, Runx2, and osteopontin (D'Ippolito et al., 2004).

1.4.2.2 Chondrogenesis

Chondrogenesis can be induced when MSCs are pelleted to form a micromass which is cultured in a serum-free medium in the presence of TGF-β, dexamethasone, sodium pyruvate, insulin-transferrin-selenium, and ascorbic acid-2-phosphate (Mackay et al., 1998). Under these conditions the cells lose their spindle shape, begin to aggregate and increase expression of cartilage-specific extracellular matrix components including glycosaminoglycan, aggrecan, link protein, chondroitin sulphate and type II collagen. Histochemically the presence of glycosaminoglycans and chondroitin sulphate can be demonstrated with toluidine blue and alcian blue staining respectively. A more specific method for demonstrating chondrogenesis is showing type II collagen staining via immunohistochemisty. MSCs differentiated into chondrocytes show increased gene expression levels of type II collagen, SOX9, and aggrecan (Kafienah et al., 2007; Tropel et al., 2004).

1.4.2.3 Adipogenesis

To promote adipogenesis MSCs are cultured in a monolayer in a cocktail of dexamethasone, insulin, 3- isobutyl-1-methyl-xanthine, and indomethacin. After one week there is evidence of lipid filled droplets in the cells which stain red with Oil Red O. Eventually the lipid droplets will merge and fill the cell. The cells express nuclear receptor transcription factor peroxisome-activated receptor γ-2, fatty acid binding protein aP2, and lipase (Tropel et al., 2004).

1.4.2.4 Alternative differentiation

MSCs also have the ability to differentiate into muscle cells. Rat MSCs exposed to 5-azacytidine and amphotericin B have been reported to differentiate into myoblasts that fuse into rhythmically beating myotubes (Wakitani et al., 1995). This has also been demonstrated in mouse MSCs (Phinney et al., 1999). An increasing number of

investigators have reported neural differentiation of MSCs (Azizi et al., 1998; Sanchez-Ramos et al., 2000). In addition, studies have also reported MSCs can be induced to differentiate into tendon (Young et al., 1998), ligament, endothelial cells (Davani et al., 2003) and insulin- expressing cells (D'Ippolito et al., 2004) when exposed to the appropriate environmental cue(s).

1.5 *In vivo* differentiation of MSCs

It is difficult to have a true understanding of the absolute *in vivo* function of the MSC since it is not possible to identify it *in vivo*. However, given its ability to differentiate into the mesenchymal lineages, one would conjecture that that is at least one function. It is also possible to speculate the prospective function of the MSC from *in vivo* studies.

Studies have shown that when MSCs are injected into animals, they home to various tissues and engraft and differentiate there. For instance, Gojo and colleagues demonstrated that murine MSCs differentiated into cardiomyocytes, endothelial cells, smooth muscle cells and pericytes when injected directly into a healthy adult heart (Gojo et al., 2003). They also contributed to the vasculature in the skeletal muscle and lung. In another study, human MSCs were injected into fetal lamb early in gestation via *in utero* transplantation (Liechty et al., 2000). The investigators report the MSCs differentiated into adipocytes, bone marrow stromal cells, chondrocytes, cardiomyocytes, myocytes, and thymic stroma. Jiang and colleagues have demonstrated a sub-population of murine MSC, called multipotent adult progenitor cells (MAPCs) contribute to most, if not all, somatic cell types when injected into an early blastocyst, (Jiang et al., 2002). MAPCs can engraft into various tissues and differentiate not only into mesenchymal cells but cells of mesodermal, neuroectodermal, and endodermal derived cells. On transplantation into a non-irradiated host, MAPCs engraft and differentiate to haematopoietic lineages, in addition to the epithelium of liver, lung and gut.

Rat MSCs have the ability to differentiate into bone and cartilage *in vivo* (Lennon et al., 2001). Indeed the ability of MSCs to form bone has been extensively reported (Friedenstein et al., 1966), (Kuznetsov et al., 1997). This is typically performed with ceramic implants loaded with MSCs and implanted into an animal. Allogeneic canine

MSCs have been reported to form bone when implanted into canines with a segmental defect in the femur (Bruder et al., 1998). Furthermore, the regenerated bone could be assessed by histology and radiology.

1.6 Homing ability of MSCs

MSCs have the ability to home to sites of injury. Many studies have reported the migration, engraftment and differentiation of MSCs into specific cell types at sites of injury. In a mouse model of pulmonary fibrosis where mice were exposed to bleomycin, murine MSCs migrated to the lung and adopted an epithelium-like phenotype (Ortiz et al., 2003). In addition the cells reduced inflammation and collagen deposition in the lung tissue, thus ameliorating its fibrotic effects. In another example, in rats with cerebral ischemia, culture expanded human MSCs were injected intravenously and were found to migrate to sites of brain injury (Li et al., 2002). The investigators report that there was significant recovery of function in rats treated with MSCs in comparison to controls. This was partly due to an increase in growth factors in the ischemic tissue, a decrease in apoptosis in the lesion area, and proliferation of endogenous cells in the sub-ventricular zone. Orlic and colleagues demonstrated the efficacy of MSCs to treat coronary heart disease (Orlic et al., 2001). They reported that murine bone marrow cells injected into the heart can generate *de novo* myocardium suggesting one *in vivo* function of the MSC may be in wound repair.

Despite these promising results, there are a few issues that need to be resolved. One is whether there is a difference in the level of MSCs in these injury/diseased sites compared to non-affected tissues. Another is whether the level of biological improvement is directly due to the MSC as it may be that the number of MSCs present post-transplantation is too few to account for the significant regeneration. In fact, Caplan and colleagues have postulated that the regenerative effect maybe due to the trophic properties of MSCs (Caplan and Dennis, 2006), which include secreting cytokines to assist the repair and regenerative process and their immunosuppressive properties as well.

1.7 Immunosuppressive activity of MSCs

It is not just the ability of MSCs to home, engraft and differentiate at sites of tissue damage that could contribute to tissue repair. The immunomodulatory effect of MSCs is now well documented (Aggarwal and Pittenger, 2005). MSCs secrete a wide range of bioactive molecules that are immunoregulatory and also structure regenerative microenvironments in areas of tissue injury (Caplan, 2007). Initial studies reported human MSCs suppress T-cell activation and proliferation when exposed to alloantigens and non-specific mitogens (Di Nicola et al., 2002; Krampera et al., 2003). A subsequent study reported this suppressive effect is independent of the major histocompatability complex (Le Blanc et al., 2003). Further studies have found that the immunodulatory effect is extended to impairing the maturation and function of dendritic cells (Jiang et al., 2005), as well as the proliferation and differentiation of B-cells (Corcione et al., 2006). Thus the immunosuppressive effect of MSCs appears to be wide-ranging. This is an advantageous property because it could be exploited therapeutically. For example, Osiris Therapeutics have clinical trials in place that are utilizing the immunoregulatory capacity of MSCs to treat graft-versus-host-disease (GVHD) and Crohn's disease (Taupin, 2006).

1.8 Clinical studies

The multilineage differentiation potential of MSCs, their immunosuppressive activity, and the fact that they can be easily isolated from the patient and expanded in culture makes them very attractive candidates for regenerative medicine and tissue repair. MSCs are currently being tested in clinical studies for their potential use in cell and gene therapy for a number of diseases which have no known cure.

1.8.1 Orthopaedic studies

The ability of MSCs to differentiate into bone and cartilage makes them very attractive candidates for orthopaedic medicine. In clinical studies MSCs were used to treat children with Osteogenesis Imperfecta (OI), a genetic bone disorder involving defective production of type I collagen. There is no cure for OI and the only drugs that have shown

any therapeutic potential are bisphosphonates, however the effect is only partial (Bembi et al., 1997). In a clinical trial, two infusions of cultured allogeneic MSCs were given to children who had already undergone standard bone marrow transplantation for severe OI (Horwitz et al., 1999), (Horwitz et al., 2001), (Horwitz et al., 2002). The preliminary results have been promising, as there was skeletal engraftment of the transplanted MSCs as well as osteogenic differentiation. There was also an increase in total bone mineral density, and an accelerated growth during the first 6 months post-infusion. The results suggest MSCs offer a feasible cell source for the therapy of bone disorders.

In a separate study the effectiveness of autologous MSC transplantation in the repair of articular cartilage defects in the patellae of two adults was examined (Wakitani et al., 2004). Bone marrow cultured MSCs were embedded in a collagen gel and transplanted into the articular cartilage defect. Six months post-transplantation the clinical symptoms of the disease, pain and walking ability, had improved significantly. Furthermore, this improvement was maintained five years and nine months in one case, and 4 years in the other. Results suggest MSC transplantation is a promising approach for promoting articular cartilage repair. However, the quality of the repair is still in question. For example, the investigators found via arthroscopy that the defect was repaired with fibrocartilage, which does not have the same biomechanical qualities of articular cartilage.

1.8.2 Graft-Versus-Host-Disease

The immunosuppressive property of MSCs renders them particularly suitable for clinical application in haematopoietic pathologies. Haematopoietic stem cell transplantation (HSCT) is an important medical procedure used to treat many conditions such as leukaemia, multiple myeloma, severe combined immunodeficiency, congenital neutropenia, aplastic anemia, and myelodysplastic syndrome. A severe complication of HSCT is graft-versus-host-disease (GVHD). This is unique to allogeneic transplantation; it is a form of rejection where conversely it's the donor cells that attack the recipient tissue. Immunosuppressive treatment can be given to the patient, but this can lead to deadly infections. GVHD also causes inflammation, and may cause development of

fibrosis, scar tissue and functional disability. It is mediated by donor T cells which react to the foreign peptides presented on the MHC proteins of the host cell. As mentioned above, MSCs can inhibit T-cell activity, and in addition MSCs secrete cytokines that support haematopoiesis and enhance bone marrow recovery after chemotherapy (Koc et al., 2000).

One recent example of MSC transplantation is in a trial of 46 patients with haematologic malignancies (Lazarus et al., 2005). HSCs and culture expanded MSCs obtained from HLA-matched sibling donors were infused intravenously. Haematopoietic recovery following transplantation occurred quickly for most of the patients. Furthermore, 23 out of 46 patients did not develop GVHD. The authors concluded infusion of culture-expanded MSCs with concomitant infusion of HSCs is safe and can potentially reduce severe side-effects of HSCT.

1.8.3 Genetic disorders

MSCs are also currently being used for the lysosomal disorders metachromatic leukodystrophy (MLD) and Hurler's syndrome (Koc et al., 2002a). Both are autosomal recessive diseases. MLD is caused by a deficiency of the enzyme arylsulfatase A. In the absence of this enzyme there is a build up of sulphatides in many tissues of the body. This eventually leads to the destruction of myelin (the covering around nerve fibers that acts as insulators) and thus the nervous system. As a consequence, patients show mental retardation, tetraplegia, and spasticity. Hurler's syndrome is caused by a deficiency of the enzyme alpha-L iduronidase, which leads to a buildup of mucopolysaccharides in lysosomes. This causes a multitude of symptoms: muscle diseases, cardiac failure, hydrocephalous and mental retardation which can lead to death during infancy (Peters et al., 1998).

Previous therapies to treat patients with the above diseases involved haematopoietic bone marrow transplantation (BMT) to halt liver and heart abnormalities (Field et al., 1994; Krivit et al., 1999). However, muscle alteration and degeneration still progressed and BMT was not always successful. There was a high incidence of graft failure leading to morbidity. Koc and colleagues hypothesised that MSC implantation may improve the

efficacy of BMT (Koc et al., 2002b). In addition the multilineage differentiation capacity of MSCs may be utilised for tissue repair. The premise being MSCs would migrate to, and differentiate into, different tissue types: cartilage, bone, muscle, and peripheral and central nervous system. Five patients with Hurler syndrome and six patients with MLD were infused intravenously with allogeneic cultured MSCs. The investigators reported no infusion-related toxicity. There were significant improvements in nerve conduction velocities in the patients with MLD after MSC infusion. There was also maintained or an improved bone mineral density in all patients. However, there was no clinical improvement in mental and physical change in patients. The investigators believe the results are promising and further evaluations need to be performed.

More recently, there has been a very promising case report of a female with adult form of MLD suffering from progressive neurological deterioration (Meuleman et al., 2008). A combination of non- myeloablative haematopoietic stem cell transplantation (HSCT) with (MSCs) infusion was performed with no significant toxicity and/or side-effects. Forty months post-infusion the patient displayed stabilisation in all neurological aspects of her disease

1.8.4 Gene therapy

MSCs are potentially useful for gene therapy. This research area is largely in the developmental phase with exciting possibilities. One disease for which genetically altered MSCs are being used is Cystic Fibrosis (CF) - this is the most prevalent fatal genetic disorder in the Caucasian population. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. There is no cure for CF and most patients die young, in their 20s and 30s. Wang and colleagues have reported that MSCs have the ability to differentiate into airway epithelial cells (Wang et al., 2005a). Furthermore, MSCs isolated from patients with CF are amenable to CFTR gene correction without loss of differentiation capacity. The allogeneic CFTR-corrected MSCs can contribute to apical chloride secretion in response to cAMP agonist stimulation. Thus, MSCs may provide a potential cell-based approach for treatment of CF.

1.8.5 Conclusion and future outlook for clinical studies

Taken together, these observations suggest that MSCs have great potential for use in cell and gene therapy. MSCs offer an alternative cell source for a wide range of therapies such as OI, Hurler's syndrome, CF, and MLD. Although preliminary results are promising and fuelling a growing enthusiasm for MSCs in regenerative medicine and tissue repair, caution still needs to be exercised. There are still a number of issues involving the safety and efficacy of stem cell therapy. Patients may contract severe bacterial and viral infections from donor cultured cells. There is also an issue with long term engraftment and maintenance of the differentiated phenotype. There has been extensive research on MSCs in the past decade. On this basis there is increasing pressure to start clinical trials. However, knowledge about the basic biology of MSCs is still in its infancy. In all the clinical trials undertaken, a heterogeneous population of adherent bone marrow cells termed 'MSCs' have been used. These issues need to be addressed for MSCs to be a truly effective cell source for future trials.

1.9 Isolation of mesenchymal stem cells

A main source of MSCs is the bone marrow. However, MSCs are present at a very low frequency in the bone marrow, estimated at 1 in 100 0000 nucleated cells (Castro-Malaspina et al., 1980). The combination of a lack of a known specific MSC marker and its rarity in the bone marrow has caused great difficulty in MSC research and has prevented its reproducible isolation and purification.

In general, human MSCs are isolated from a bone marrow aspirate that is taken from the superior iliac crest (Pittenger et al., 1999). Mouse and rat MSCs are usually isolated from the bone marrow harvested from the femoral and tibial marrow (Lennon et al., 2001; Peister et al., 2004) In the case of human bone marrow, the bone marrow mononuclear cell fraction is typically isolated via density gradient centrifugation (Pittenger et al., 1999). The subsequent cells are most often cultured in basal media (usually Dulbecco's with 10% serum). The non-adherent cells consisting largely of haematopoietic cells are

removed by changing the media usually after 24 - 48 hours. Hence, MSC-enriched cultures are then obtained via the Friedenstein method of simple plastic adherence. A problem with this method is contamination by a wide variety of adherent cells present in the bone marrow such as endothelial cells, lymphocytes, adipocytes, osteoblasts and smooth muscle cells. Using this conventional method, the basis for enrichment of MSCs is by expansion and passaging of adherent stromal cells. In terms of cultivation of the subsequent cells there is also huge variation. From cell density, to the length of time the adherent cells are left in culture (D'Ippolito et al., 2004), the tissue culture surface, to the addition of various serum types and concentrations (Peister et al., 2004) as well as various growth factors (Jiang et al., 2002; Muraglia et al., 2000). Some groups also culture cells in 3D scaffolds (Grayson et al., 2006). This all contributes to a myriad of cell populations with varying characteristics, all termed MSC, which is obviously sub-optimal for research purposes. Thus, a more stringent purification method is required to identify and distinguish MSCs from other cell types present in the bone marrow.

1.10 Surface markers

Many groups have attempted to identify prospective MSC markers by raising monoclonal antibodies against adherent bone marrow stromal cells. Due to variations in isolation techniques and cell culture conditions, the results of these studies are inconsistent. The antigens identified to date are expressed on a variety of cells and do not provide the specificity required for MSC purification. For example, the SH2 antibody developed by the Haynesworth group binds to the TGF-β receptor endoglin (CD105) found on many cells (Haynesworth et al., 1992). This group also identified the SH3 and SH4 antibodies which bind to distinct epitopes on CD73, an ecto-5'-nucleotidase, which is also not restricted to MSCs.

1.10.1 STRO-1

The best known antibody to date used to enrich for MSCs is STRO-1 which was first identified 17 years ago (Simmons and Torok-Storb, 1991). Surprisingly, its antigen has

still not been identified. Although STRO-1 does enrich for CFU-fs approximately 10-fold from harvested bone marrow cells (Gronthos et al., 2003) it is unlikely it is a specific marker of MSCs as it reacts with other cell types. Especially as human myofibroblasts express STRO-1 (Strakova et al., 2008). STRO-1 positive cells have the ability to differentiate *in vitro* into adipocytes, chondrocytes, osteoblasts, and HSC supporting fibroblasts, and smooth muscle cells (Dennis et al., 2002). However, it is possible the STRO-1 antibody is binding to progenitor or committed cells rather than the primitive MSCs, which would explain the multilineage potential of the cells. More over, it is possible the STRO-1 antigen is not expressed by all MSCs and a sub-set of MSC is missing.

Furthermore, STRO-1 on its own is not sufficient to enrich for MSCs, it is often used in combination with another antibody, for example, CD106/VCAM-1 (vascular cell adhesion molecule-1). The investigators work on the premise that MSCs are pericytes and localised in a perivascular niche *in vivo* (see *in vivo* niche above) (Gronthos et al., 2003). This is because many studies have reported MSCs express CD106, which is also expressed by pericytes (Bagley et al., 2006). Using a combination of the two selects for high STRO-1 expressing cells, which form CFU-f, have a high proliferation capacity, and are multipotential *in vitro*. Although potentially useful, a drawback with this strategy is that an assumption is made about the surface antigen expression of MSC, which is not known. Another limitation of the STRO-1 antibody is due to the initial problem with the approach, which is STRO-1 was raised against a heterogeneous population of bone marrow adherent cells (Simmons and Torok-Storb, 1991) rather than a pure population of clonal, tripotent MSCs.

1.10.2 Other MSC candidate markers

Other studies choose a marker by screening a long list of antibodies against the mixed population of cells they have derived. If one marker is highly expressed by their cell population, they utilize this marker to enrich for MSCs. One group selected CD271 (low-affinity nerve growth factor receptor (LNGFR)) as their marker of choice due to its high expression on an MSC containing adherent population in comparison to other marrow

cell populations (Jones et al., 2002). Another group prefer CD133 (prominin-1) which is a marker of primitive haematopoietic and neural stem cells (Martin-Rendon et al., 2007). Other investigators attempt to enrich MSCs from bone marrow with SSEA-1 (Anjos-Afonso and Bonnet, 2007) or SSEA-4 (Gang et al., 2007). Another marker used for MSC enrichment is CD200. These cells were reported to have the potential to differentiate into adipo-, chondro-, and osteogenic lineages (Wright et al., 2001). However, this marker is also expressed by endothelial cells, thymocytes, B and T lymphocytes.

According to the literature MSC containing populations are also variably positive for CD90/Thy-1, CD44, CD29, CD13, Flk-1/CD309, and CD10 (Haynesworth et al., 1992; Pittenger et al., 1999) and mouse MSCs for stem cell antigen-1 (Sca-1) (Peister et al., 2004). This marker is commonly used to isolate mouse MSCs and HSCs in combination with other markers. Unfortunately, none of these markers are exclusive to MSCs and cannot be used to identify the stem cell *in vivo*.

1.10.3 MSC negative 'markers'

MSCs are reported to be negative for the haematopoietic markers CD11b (a leucocyte antigen), glycophorin-A (a red blood cell marker), CD45 (a marker of all haematopoietic cells), and CD34 (a primitive HSC marker), although the latter two are sometimes expressed on murine MSCs (Peister et al., 2004). Both human and mouse MSC populations are almost always negative for CD31 ((PECAM), expressed on both endothelial and haematopoietic cells) and CD117 ((c-Kit) a HSC marker).

No single isolation method described above is therefore optimal, and there appears to be no clear biological difference or advantage between the cells isolated from one method and another.

1.11 MSCs, BMSCs, or MAPCs?

It is difficult to discern the value from one MSC study to another. This is because over the years there are many different methods utilised to isolate a heterogeneous population of adherent cells from different sources and showing varying capacities to differentiate

into the mesenchymal lineages - bone, fat, and cartilage. A myriad of names have been given to these cells. These include mesenchymal stem cell/marrow stromal cell (MSC), bone marrow stem cell (BMSC), adipose-derived mesenchymal cell (AMC), unrestricted somatic stem cell (USSC) (Kogler et al., 2004), marrow-isolated adult multilineage inducible cell (MIAMI) (D'Ippolito et al., 2004), and multipotent adult progenitor cell (MAPC) (Jiang et al., 2002). The latter two are claimed to be more primitive subsets of MSCs, with a greater differentiation potential. This is reported to be partially due to the unique isolation procedure the groups adopted. MIAMI cells were derived by culturing bone marrow cells on a fibronectin-coated surface in the presence of 3 % oxygen tension. Two weeks later the non-adherent cells were removed and the adherent cells re-seeded and expanded (D'Ippolito et al., 2004). MAPCs were derived by culturing CD45 and Glycophorin-A depleted bone marrow cells in the presence of EGF, PDGF-BB, and low serum concentration, on laminin coated plates. Leukaemia inhibitory factor (LIF) was also required for the growth of murine and rat MAPCs (Jiang et al., 2002).

It is possible that the differences in isolation methods and addition of various growth factors select for a distinct population of cells with varied differentiation potential. Alternatively it is possible that there is no fundamental difference between the cells derived using the various protocols, as observed differences could be due to specific responses to various growth factor treatments. It should also be noted that the results have been difficult to replicate. In a preliminary study, $CD45^-$/Glycophorin A^- bone marrow cells, CD105 positive bone marrow cells and bone marrow adherent cells were compared (Lodie et al., 2002). The investigators reported that the cells derived from each method were virtually indistinguishable in terms of differentiation potential, cell surface antigen expression, and proliferative expansion.

Recently the International Society for Cellular Therapy (ISCT) met to propose a guideline for the identification of MSCs in order to standardize the definition of a MSC (Dominici et al., 2006). There were 3 recommended criteria: (1) ability to adhere to tissue culture substrate; (2) capacity to differentiate *in vitro* into the adipogenic, chondrogenic, and osteogenic lineage; (3) a surface antigen expression of more than 95% positive for CD73, CD90, and CD105, and less than 2% for CD14, CD19, CD34, CD45, and HLA-

DR. This identification is designed to facilitate MSC research. One drawback with the standardisation is there are no criteria to demonstrate colony forming unit- fibroblast assay or self-renewal. Another is the guidelines are based on a summary of studies on mixed population of MSCs, not a clonal, i.e., single cell derived, and thus pure population. It is possible a pure population of MSCs may have a distinct phenotype to that stated. Although these guidelines may be useful in practice, they are not sufficient. Since, differentiated cells such as human fibroblasts HS68 have a similar surface phenotype to that described above (Wagner et al., 2005). A better method to set criteria is to isolate a homogeneous population of MSCs, identify their characteristics, and then set appropriate guidelines based on those cells. Also there are no guidelines on cell isolation or cultivation. Therefore cells can be isolated using a wide range of procedures as described above (1.10-1.12) which may exclude or select for a distinct population of MSCs.

1.12 Identification of MSCs

1.12.1. Gene profiling

A problem with the studies described in (1.10) is that they were performed on heterogeneous populations of cells, resulting in phenotyping of such cells rather than pure MSCs. Even if a pure population of MSCs is used the monoclonal antibody may bind to an antigen that is highly expressed by the cells but not necessarily specific to MSCs. Thus these approaches are not optimal for the purpose of identifying a cell-specific marker.

We believe a more useful approach is to apply transcriptomics to characterise the surface phenotype of a pure MSC population in an endeavour to discover MSC candidate markers. A transcriptomic approach is suitable as it is hypothesis independent and has a high level of sensitivity which may enable detection of novel MSC surface markers as well as important cell regulators.

Previous studies have attempted to catalogue a MSC-specific transcriptome, but the results were limited as they were again performed on a heterogeneous population of cells (Jia et al., 2002; Song et al., 2006), (Wagner et al., 2006), (Wieczorek et al., 2003). Tremain and colleagues published the gene expression profile of a single human clonal

MSC population where 2,353 genes were identified (Tremain et al., 2001). The results were limited as the differentiation potential of the clone was not investigated and therefore the stem cell status of the clone not determined. Also, only a single clone from one donor was examined. The gene expression profile of a control population, for example fibroblasts was not analysed in parallel, thus preventing the identification of potential MSC-specific genes. However, the results demonstrate that gene expression profiling is a very powerful technique and when used with a more biologically relevant starting material and with relevant controls, may shed light on the identity and basic biology of MSCs.

1.12.1. Microarray vs. Proteomics

One of the major drawbacks of proteomics is the limited sensitivity of the technique, at least at present. It favours the identification of the most abundant proteins over the less abundant (Park et al., 2007). Therefore, important proteins that are present at a low frequency are often missed. Also hydrophobic protein lysates from cellular compartments are a problem for 2-dimensional electrophoresis (Wagner et al., 2006).

For example, Roubelakis and colleagues compared the proteome of amniotic fluid MSCs, and bone marrow MSCs, and identified 261 and 175 proteins respectively (Roubelakis et al., 2007) using MALDI-TOF technology. From this number 116 were present on both samples. Although this seems a significant number this was for the total number of proteins of the cell. Since there are approximately 2000 polypeptides in a mammalian cell the above number is only 13% of the total (Duncan and McConkey, 1982).

One method to characterise the surface antigen profile of MSCs is to isolate and analyse crude cell membrane fractions. Using a more refined methodology consisting of a combination of liquid chromatography and tandem mass spectrometry Foster and colleagues resolved to identify changes in membrane protein markers before and after osteoblast differentiation in MSCs (Foster et al., 2005). They identified over 400 unique proteins. Although a significant number of surface proteins were identified, the significance of the result was limited because the proteomic study was performed on an hMSC-TERT cell line.

One great advantage of microarrays is the much greater sensitivity compared to mass-spectrometry-based proteomics. With trancriptomics, the expression of all the genes in the cell can be identified. Furthermore, it is much easier, quicker and reproducible than proteomic analysis.

The absence of a MSC-specific marker is a fundamental problem in MSC biology for many reasons. It prevents their identification *in vivo*. It also prevents knowledge of their anatomical location and distribution. Due to the lack of a single definitive marker it is difficult to identify and hence purify MSCs from other cell types. This is extremely important for research, as the use of a mixed population of cells makes it difficult to attribute the reported results solely to the MSC. As discussed earlier (1.9) at best mixed populations containing MSCs are currently being used in clinical studies. It is possible that a pure population may enhance the results. Therefore, it is paramount that a MSC specific marker be identified if the full therapeutic potential of this stem cell is to be realised.

1.13 Cell culture and oxygen tension

In 1907, Ross Granville Harrison was one of the first investigators to grow cells in culture, which consisted of tissue fragments of frogs (Csete, 2005). The science of tissue culture for *in vitro* studies has undergone a revolution since then. Whilst many efforts have been made to mimic various aspects of the *in vivo* microenvironment of cells such as tissue culture surfaces, a myriad of media and serum types, growth factors, 3D-scaffolds, and co-culture systems, little attention has been paid to the atmosphere in which the cells are cultured. The air is largely composed of nitrogen (78%), oxygen (21%), argon (1%), and trace amounts of carbon dioxide (0.03%), and other gases. 5% carbon dioxide is used in *in vitro* tissue culture to maintain the appropriate pH and it also reflects levels *in vivo*. However, the oxygen tension found in mammalian tissue is considerably less than 20%. The highest oxygen concentration present *in vivo* is 10-13% in arteries, lung and liver (Grant and Smith, 1963; Kofoed et al., 1985). Whilst in avascular tissue such as cartilage it can be as low as 1% oxygen (Brighton and

Heppenstall, 1971). Therefore the oxygen tension (20%) cells are exposed to *in vitro* is not normoxic, even though in conventional tissue culture this is the name given to it.

1.13.1 Effect of oxygen tension on stem cells

Until recently (D'Ippolito et al., 2004; Grayson et al., 2006; Lennon et al., 2001), the effect of cultivating MSCs at a more physiologically relevant oxygen tension has been largely ignored. The majority of *in vitro* studies on MSCs have been performed at 20% oxygen tension. Observations from previous studies on MSCs and other stem cells suggest that cultivation of MSCs in a reduced oxygen environment could enhance their proliferation, self-renewal and differentiation capacity.

In the bone marrow MSCs exist in a relatively low oxygen environment (4-7%) (Kofoed et al., 1985). Zwartouw and Westwood were among the first to document that exposure to low oxygen tension enhances the proliferation of some cells (Zwartouw and Westwood, 1958). Experimental evidence from more recent studies suggest that cultivation of stem cells at a reduced oxygen tension (5%) enhances proliferation in comparison to normoxic controls (20%) for rat CNS-derived multipotent stem cells (Studer et al., 2000) and rat marrow-derived mesenchymal cells (Lennon et al., 2001). Studies by Parinello and colleagues suggest that reduced oxygen tension may delay or halt proliferative senescence because there is less DNA damage under these conditions (Parrinello et al., 2003). This is because there are less reactive oxygen species (ROS).

Cultivation of stem cells in a low oxygen environment also reduces apoptosis for many cell types including fetal rat derived CNS stem cells (Studer et al., 2000), murine muscle satellite cells (Csete, 2005) and CD34+ marrow progenitor cells (Mostafa et al., 2000). Mostafa suggests that this anti-apoptotic effect could be due to a reduction in reactive oxygen species under hypoxic conditions. Lowering the oxygen tension in culture also affects the differentiation patterns of stem cells. The Csete group examined the effect of oxygen tension on myogenic and adipogenic differentiation ability of skeletal muscle satellite cells (Csete, 2005). They reported that hypoxia induced myogenesis, whereas normoxia led to adipogenesis. This suggests that oxygen tension is an important regulator of differentiation. It is also known that oxygen tension is a critical signal between ‘stemness’ and differentiation, as haematopoietic-committed precursors are located near

blood vessels where the oxygen concentration is higher, and more primitive precursors inhabit areas of lower oxygen tension (Mason et al., 1989)

Lennon and colleagues also found that maintaining rat MSCs in reduced oxygen enhanced their *in vivo* and *in vitro* osteogenic and chondrogenic potential as well as their ability to form colonies relative to control cultures at 20% oxygen (Lennon et al., 2001). Chondrocytes also react to changes in oxygen tension. De-differentiated cells re-differentiate in hypoxic conditions (Murphy and Polak, 2004). The reason for the enhanced effect of low oxygen on stem cells *in vitro* is, perhaps at least two-fold. The first is that it better approximates the *in vivo* microenvironment of the cell, the second is that there are less reactive oxygen species present. High concentrations of oxygen lead to the generation of reactive oxygen species, which are very small molecules that are highly reactive due to the presence of unpaired electrons.

In summary, reports from the above studies suggest that low oxygen tension plays an important role in many aspects of stem cell biology including proliferation, apoptosis, maintenance of stemness, and differentiation. Therefore, we hypothesise that cultivation of MSCs in a low oxygen environment which better approximates their *in vivo* niche may enhance their stem cell properties and this in turn may enable derivation of more physiologically relevant stem cell populations.

1.14 Aims of present study

The major aim of this thesis was to identify candidate MSC-specific cell surface marker(s). We believe identification of a MSC specific marker would have many important advantages. Firstly it would allow identification of the *in vivo* localisation of the MSC, therefore its niche. It would also allow subsequent studies on a pure population of MSCs and thus enable a better understanding of its true function. The molecular signature of the MSC could be characterised and enable identification of its regulatory and self-renewal genes. Identification of a MSC specific marker would also allow isolation of a pure MSC population which could subsequently be used in clinical studies

and may enhance the regenerative effect compared to the standard heterogeneous population of MSCs currently used.

A microarray approach was adopted to attempt this aim. The rationale was to identify MSC specific genes by comparing the gene expression profile of murine bone marrow derived MSCs clonal cells to that of relevant control cell populations. The subsequent MSC specific genes would then be validated and investigated further in normal human bone marrow derived MSCs.

In addition, the effect of oxygen tension on MSC biology was to be investigated. This is because previous studies have reported low oxygen tension enhances the CFU-f ability, proliferation, and differentiation capacity of MSCs (D'Ippolito et al., 2004; Grayson et al., 2006; Lennon et al., 2001).

Chapter 2

Materials and Methods

2.1 Reagents

2.1.1 . Tissue and cell culture reagents

All general laboratory reagents and chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Materials were purchased from the following sources: Dulbecco's modified Eagle's medium (DMEM) from PAA (USA), fetal calf serum (FCS) from Biosera (USA). DMEM + Glutamax, α- minimum essential media (α-MEM) + Glutamax, adult bovine serum (ABS) from Invitrogen (USA). FCS and ABS were aliquoted and stored at -20ºC until required. Hank's Balance Salt Solution (HBSS), trypsin/EDTA, penicillin/streptomycin from Biowhittaker (Wokingham, UK). 70μm cell strainers and 27 gauge needles from BD Biosciences (Europe), disposable scalpels from Swann-Morton (Sheffield, England). 2X chamber slides from Nalge Nunc (Thermo Fisher Scientific, Europe).

Reagents used in differentiation assays were purchased from: TGF-β3 from (Insight Biotechnology, UK), BMP-2 from Peprotech (England). All tissue culture plastics were purchased from Becton-Dickinson (Oxford, UK). Oil Red O solution was from Raymond A Lamb Ltd., (East Sussex, UK), the type II collagen antibody (Ab2031) from Chemicon (USA), and Alizarin Red S solution from (BDH, Poole, UK).

BALB/b and BALB/c mice strains were purchased from Harlan UK Ltd. (Oxon, UK). Neonatal human dermal fibroblasts (NHDFs), and human bone marrow mononuclear cells (hBMMNCs) from donor 1 were from Cambrex Bioscience (Walkersville, USA) and donor 4 from Lonza Wokingham, Ltd (Berkshire, UK). A sample of bone aspirate was obtained (with informed patient consent and local ethics committee approval) from Hammersmith Hospital (London) and another from Stanmore Hospital (Greater London). Human articular chondrocytes were a kind donation from a member of the laboratory (Dr Jérome Lafont), derived as previously described in (Lafont et al., 2007). The chondrocytes were derived from the articular cartilage in the femoral condyle of a 50 year old female patient. The tissue was obtained with informed patient consent and ethical approval.

Cells were cultured in a Galaxy triple gas incubator (CRS Biotech, Ayrshire, Scotland, UK). All cells were cultured in 5% oxygen unless stated otherwise.

2.1.2. SDS PAGE and western blotting reagents

Materials were purchased from the following sources: NuPage 4-12% gradient gels from Invitrogen (Paisley,UK). XCell SureLock Mini-Cell electrophoresis apparatus from Novex (San Diego, USA). Electrophoresis apparatus from Cambridge Electrophoresis (Cambridge, UK). Trans-blot chamber from Biorad (Hercules, USA). Polyscreen® PVDF Transfer Membrane from Millipore (Bedford, MA, USA), ECL western blotting detection reagents from Amersham (Little, Chalfont, UK). FUJI Medical X-Ray film, Super RX form Fujifilm (Bedford, UK). Rainbow Molecular weight markers form GE Healthcare (Europe). HIF-1α antibody was from Novus Biological ((NB100-134) USA), HIF-1β antibody from BD Biosciences (USA), Telomerase from Abcam ((ab23699) Cambridge, UK), STEAP1 from Santa-Cruz ((sc-10262), USA). Anti-horseradish peroxidase secondary antibody was from DakoCytomation (Denmark).

2.1.3 Molecular biology reagents

QIAamp® RNA Blood Minikit from Qiagen Ltd. (Crawley, West Sussex, UK) 1 x Opti-mem reduced serum media, Lipofectamine 2000 (Invitrogen, Paisley, UK). RNAse-Free DNAse Set from Qiagen (Crawley, England). Promega kit Reverse Transcription from Promega (Southampton, UK). Pre-developed primer/probe sets for the following genes were purchased from Applied Biosystems (Foster City, CA, USA) for mouse: Ly6a/Sca-1, STEAP1, STEAP2, TM4SF1, Versican, PPARγ, and for human: RPLPO, STEAP1, STEAP2. TaqMan 2X PCR master mix from Applied Biosystems.

2.2 Solutions

2.2.1 General buffers

A 10X sterile phosphate buffered saline (PBS) solution (43mM Na_2HPO_4, 14mM KH_2CO_3, 14mM KCL and 1.37M NaCl at pH 7.3) was obtained from VWR (Lutterworth, UK). A 1X PBS solution was generated by diluting the 10X stock with ultrapure ddH_20.

Tris buffered saline (TBS) pH 7.6	20mM Tris-HCL 137 mM NaCl

2.2.2. Protein extraction buffers

Radio-immunoprecipitation assay (RIPA)	100mM Trizma base pH7.4 300mM NaCl 20mM NaF 2mM EDTA 0.2mM β-glycerophosphate 2% (w/v) Na deoxycholate, 0.5% (w/v) SDS 1% (v/v) NP-40

Prior to use:
10µl/ml Protease inhibitor cocktail was added to the RIPA and ULB buffer

Urea Lysis Buffer (ULB)	8M Urea in ddH2O 10% (v/v) glycerol 1% (w/v) SDS 5mM DTT 10mM Tris HCl pH6.8

2.2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffers

4 x SDS-PAGE sample buffer	0.4M Tris/Hcl pH 6.8 40% (v/v) glycerol, 0.4% (w/v) SDS 2% (v/v) β-mercaptoethanol 0.02% bromophenol blue
Bradford reagent	0.1% (w/v) Coomassie Brilliant Blue G 0.05% (v/v) Ethanol 8.5% (v/v) H_3PO_4
Running buffer (National Diagnostics, Atlanta, USA)	25 mM Tris 192 mM glycine 0.1% (w/v) SDS

2.2.4. Western blot buffers

Western blot transfer buffer	25 mM Tris 192 mM glycine 20% (v/v) methanol
2-mercaptoethanol stripping buffer	62.5 mM Tris-HCl pH 6.7 2% (w/v) SDS 100 mM 2-mercaptoethanol

2.2.5. Flow cytometry buffers

FACS wash buffer	10% (10X PBS) 1% (w/v) FBS 0.1% (w/v) NaN_3
FACS fixing buffer	1X PBS 0.5% (v/v) paraformaldehyde

2.3 Tissue culture

2.3.1. Isolation and expansion of murine clones

Murine clones were derived from the bone marrow of 5-week old male mice (BALB/b and BALB/c strains). Mice were sacrificed by CO_2 asphyxiation for 5 minutes. The tibiae and femora were aseptically removed and the ends cut to expose the interior of the marrow shaft. The bone marrow cells were collected by flushing the shaft with Hank's Balance Salt Solution (HBSS) using a syringe with a 27 gauge needle attached. The cell suspension was passed through a 70µm cell strainer and then centrifuged at 1500 rpm for 5mins. After discarding the supernatant, the red blood cells (rbc) were lysed by mixing the pellet in 25ml rbc lysis buffer for 7-10 min. An equal volume of HBSS was added and the cell suspension was passed through a cell strainer into a 50ml tube and centrifuged as above. The supernatant was discarded and the pellet was re-suspended in 50 ml complete media (DMEM + Glutamax, 10% adult bovine serum albumin, and 1% penicillin/streptomycin) (hereby called complete media) and centrifuged as before. The

bone marrow-derived mononuclear cells (BMMNCs) were seeded at a 'low' density (10 x 10^6 cells in a 15cm dish) and cultured at both 20% oxygen, and in a 5% oxygen, 5% CO_2, triple gas incubator. The non-adherent cells were re-plated 24 hours later and fresh media added to the adherent cultures.

Cells were cultured for approximately one month, and 50% of the medium was replaced each week with fresh, pre-equilibrated medium. Cells failed to adhere and survive in the low density cultures exposed to 20% oxygen. However, in the 5% oxygen cultures, after approximately 4 weeks there was the appearance of individual colonies. Great care was taken to isolate (with cloning rings) only distinct individual colonies. Initially each individual colony was passaged into a 3.5cm dish, and then a 15cm dish. At this stage cells from each clone were counted and subsequently cultured at a seeding density of 1 x 10^3 cells per cm^2. Cultures were passaged at approximately 70% confluency.

2.3.1.1 Trypsinisation of clones

Cells were trypsinised by washing twice with 1X PBS and then incubating with pre-warmed trypsin/EDTA for 1 min at 37^{o}C. The dishes were gently shaken to detach the cells, which lifted off the tissue culture substrate easily. The trypsin was neutralised by adding an equal volume of complete medium. Cell suspension was then centrifuged at 1500 rpm and the supernatant discarded. Cells were then re-suspended in an appropriate volume of complete media (typically 1-2ml for a 70% confluent 15 cm dish) and counted using a haemocytometer.

2.3.2. Isolation of murine BMMNCs and bone marrow adherent cells (BMACs)

Primary BMACs were cultured by seeding freshly obtained BMMNCs from 9 BALB/b and BALB/c mice (each strain separately) at a high density (30 x 10^6) in two 10cm dishes. Non-adherent cells were re-plated the next day. Cells were grown to ~80% confluence (2 weeks).

2.3.3. Isolation of mouse embryonic fibroblasts (MEFs)

MEFs were generated from embryos obtained from 2 BALB/b and 2 BALB/c mice (Abbondanzo et al., 1993). Briefly, pregnant mice at day 13 gestation were sacrificed by CO_2 asphyxiation for 5 mins. The peritoneal cavity was opened and the embryos removed from the uteri (approximately 4-6 embryos per mouse). The embryo was separated from its placenta and its surrounding membranes and the head and the liver removed using forceps and scalpel. The resulting embryos were finally minced by passing through a 2ml syringe with media (DMEM /10% FBS and 1% P/S) into a 20 ml universal. In the tissue culture hood a more homogeneous suspension was prepared by passing the minced embryos through a 70 μm cell strainer. The resulting cell suspension was seeded into 4 x 15cm dishes and incubated at 20% oxygen tension. After 3-4 days when the cells were confluent they were trypsinised and re-plated to expand their numbers.

2.3.4 Culture of human bone marrow stromal cells (hBMSCs)

A commercially obtained frozen vial of human bone marrow mononuclear cells (hBMMNCs) was thawed in a 37ºC water bath and transferred into a 15 ml tube containing 12 ml pre-warmed complete media. This was centrifuged at 1500 rpm, the supernatant was removed and cells re-suspended in complete media and counted using a haemocytometer. 10 $x10^6$ to 30 $x10^6$ cells seeded into a 15 cm dish ('low' density) in order to attempt to derive clones. The non-adherent cells were removed after 24 hours and fresh medium added. In most cases there were very few adherent cells in the low density cultures and these did not survive. This method was repeated several times, with some modifications such as leaving the non-adherent cells for 2-3 days, with the hypothesis that they may be required to aid the growth of the adherent cells. Once there was the appearance of adherent cell clusters, these were isolated using cloning rings and transferred to 3.5 cm dishes. There was a morphology change in these cells from a spindle shape to a more, flattened spread-out shape. These cells did not survive. Therefore we proposed to derive human bone marrow stromal cells (hBMSCs) which would contain a population of MSCs, but were not clonally derived, and hence not necessarily homogeneous. Commercially obtained hBMMNCs were thawed and 10 $x10^6$

- 30 x10^6 cells were seeded in a 3.5 cm dish. The non-adherent cells were removed the next day. Medium was changed twice a week. After approximately 3 days there was the presence of spindle shape cells. Cells were passaged when approximately 70% confluent and expanded further in culture until there was a sufficient number for freezing. This method was repeated with a fresh human bone marrow aspirate that had been ficoll-separated that we received from a local hospital. Human BMSCs isolated from the first donor were called hBMSC1, and this naming method was used for subsequent donors. hBMSC1 was derived from ficoll-separated hBMMNCs purchased from Cambrex, (obtained from a healthy 25 year old male). hBMSC2 was cultured from ficoll-separated hBMMNCs obtained from a 60 year old female (through Hammersmith hospital). hBMSC3 was cultured from a fresh bone marrow aspirate (through Stanmore hospital), from an 11 year old male. hBMSC4 was derived from ficoll separated BMMNCs obtained from Lonza (Wokingham Ltd) (donor, 25 year old male).

2.3.5 Culture of neonatal human dermal fibroblasts (NHDFs)

NHDFs were thawed as described above and seeded at 2000 cells /cm^2 and grown to ~80% confluence in 15 cm dishes containing 20 ml DMEM supplemented with 10% (v/v) FCS and 1% (v/v) Pen/Strep. Culture medium was replaced twice a week.

2.4 Adipogenic differentiation assay

For adipogenic differentiation, cells were seeded at 6000/cm^2 in 6 well plates in the presence of DMEM, 10% FBS and 1% P/S, with 10μg/ml insulin, 2 nM dexamethasone, 5 μM 3- isobutyl-1-methyl-xanthine (IBMX), 30 μM indomethacin at the three different oxygen tensions. The medium was changed twice every week for 2 weeks. The cells were fixed in 10% formaldehyde for 4 min and then covered with 1X PBS, and stored at 4^oC until stained with Oil Red O. Wells were rinsed in 60% isopropyl alcohol, and incubated in Oil Red O solution for 10 mins before rinsing again in 60% isopropyl alcohol, followed by water.

2.5 Osteogenic differentiation assay

For osteogenic differentiation, cells were seeded at 1 x 10^3 cells/cm^2 and primed in a minimal media of α-MEM with 5% FBS and 5μg/ml A2-P for 3-4 days. Cells were then re-seeded into 6 well plates at a cell density of 6 x 10^3 cells/cm^2 in the presence or absence of osteogenic media (α-MEM + Glutamax/10% FBS, 1% P/S, 50μg/ml A2-P and 10mM β-GP) and or 10ng/ml BMP2, and cultured at 20%, 5% and 1% oxygen tensions. In addition, human cells were cultured with 10 nM dexamethasone. Osteogenic medium was replaced with fresh medium every 3-4 days. Immature osteoblast differentiation was visualised after one week by alkaline phosphatase staining (Sigma), following the manufacturer's instructions. Mineralization was assessed after 4-5 weeks by alizarin red staining of calcified deposits.

2.5.1 Alizarin Red S Staining

A 40 mM solution of Alizarin Red S was made in ddH_20. Fixed cells in 6 well plates were rinsed twice with ddH_20. One ml of Alizarin Red S solution was added to each well and incubated for 15 min with gentle stirring. Alizarin Red S solution was drained and wells briefly rinsed in ddH_20 and overturned on tissue paper to dry at room temperature.

2.6 Chondrogenic differentiation assay

To investigate the chondrogenic capacity of the clones a monolayer system was adopted. Cells were seeded at a cell density of 6 x 10^3 cells/cm^2 in a 2 well chamberslide and stimulated for 4-5 days with DMEM-HG, 1% FBS, 1% insulin/transferin/selenium + linoleic acid (ITS+1), 1mM sodium pyruvate, with or without 0.1 mM ascorbic acid 2-phosphate, and 5ng/ml TGF-β3 at 20%, 5%, and 1% oxygen tension. Cells were incubated until the formation of chondrogenic nodules (4-5 days at 1% and 5% oxygen, and 1 week at 20% oxygen). Cells were then washed twice in PBS and air-dried. Chondrogenic differentiation was assessed by type II collagen immunostaining.

2.6.1 Type II collagen immunostaining

Type II collagen staining was performed by the Histology department, in Charing Cross hospital. Chamber slides were incubated for 1 hour at 60ºC to melt the glue that adhered the plastic to the slides and then fixed for 10 min in acetone. To prevent endogenous peroxidase activity, they were blocked in a solution of 30% hydrogen peroxide in 300 ml of methanol for 10 min and then rinsed in running tap water for 2 min. Slides were then treated with 200 µl of swine serum (Serotec, Oxford, UK) blocking solution containing 0.1% sodium azide for 10 min to minimise non-specific background staining. 200 µl of the primary type II collagen antibody (used at a dilution of 1:200) was applied to each slide and incubated for 1 hour and then washed twice in TBS. Slides were then incubated with 200 µl of the secondary antibody DAKO biotinylated swine-anti-rabbit IgG antibody (DAKO- Denmark) for 35 min and washed twice in TBS again. During the incubation a 1:1000 dilution of Vectastain Elite avidin-biotin complex (ABC) was prepared (Vectar Labs, Petersborough, UK). 200 µl of ABC was added to each section and the slides incubated for 35 min. The ABC binds to the biotinylated secondary antibody. In order to visualise the antibody a chromagen was applied to the slides. Vector laboratories peroxidase substrate DAB SK-4100 was used as per manufacturer's instructions. Into every 5 ml of distilled water 2 drops of pH 7.5 buffer, 4 drops of diaminobenzidine (DAB) and 2 drops of hydrogen peroxide were added. 200 µl of this solution was applied to each section for 5 min, and the reaction was stopped by immersing the slides under running tap water for 2 min. The slides were placed into pre-filtered Surgipath Harris's hemotoxylin (Surgipath-Cambridgeshire, UK) for 1 min, removed and washed in running tap water. The slides were immersed in 300 ml0.3% acid alcohol (200ml of 70% IMS and 6 ml of concentrated hydrochloric acid) for approximately 10 seconds each, differentiating the haematoxylin counterstain. Having been placed back into running tap water the slides were left to 'blue' for 10 min. The slides were placed into 300 ml 70% alcohol for 2 min, followed by 2 min in 300 ml of 90% alcohol. To completely dehydrate the cells 3 changes of 99% IMS for 2 min each were performed followed by 5x 2 min changes of xylene in order to clean the slides and remove all traces of alcohol. The sections were mounted in PERTEX mountant

(CellPath-Powys, UK), covered with coverslips, and left to dry overnight and then examined under the light microscope.

2.7 Colony forming unit-fibroblast (CFU-f) assay

Murine clones were seeded (in triplicate) at densities ranging from 100-1000 cells in a 10cm dish and incubated at three different oxygen tensions, 20%, 5%, and 1%. Media was changed once a week. The appearance of colonies was typically observed by 10-14 days. Cells were rinsed with PBS, air-dried and stained with 10% giemsa stain solution (BDH, Poole, UK) for 20 min with gentle agitation. The stain was rinsed with water and then fixed with 70% ethanol. Most colonies had more than 50 cells, and only these were counted. The number of stained colonies divided by the number of cells seeded allowed calculation of the percentage of CFU-f.

2.8 Determination of population doublings of clonally-derived cultures

BALB/b1 clone 37, BALB/b3 clone 33, and BALB/c clone 35 were seeded at a cell density of 500-1000/cm^2 and passaged every 3-5 days when the cells were approximately 70% confluent. Cells were counted at each passage using a haemocytometer. In order to examine the effect of oxygen tension on the population doubling rate of each clone the same method was used, except cells were incubated at three different oxygen tensions, 20%, 5%, and 1%. The population doubling was calculated after each passage by using the formula: log N / log 2, where N is the number of cells after 3-5 days in culture (counted after trypsinisation) divided by the number of initial cells seeded. This was calculated to determine the total population doublings over time.

2.9 Biochemical Techniques

2.9.1. Preparation of whole cell protein extracts for immunoblotting

Cells were trypsinised as discussed above and centrifuged at 1500rpm at 4ºC. The supernatant was removed and cell pellet washed once with 10 ml ice-cold PBS and

centrifuged as stated above. The supernatant was then discarded and cells lysed in 100 µl 1X RIPA buffer containing protease inhibitors and stored at -20 ºC until further use.

Alternatively for HIF-1α analysis, 0.2 x 10^6 cells were seeded in a 3.5 cm tissue culture dish and cultured in 2.5 ml complete media at each oxygen tension (20%, 5%, and 1% oxygen). One day later, 250 µl desferrioxamine (DFO) (from a 10 X 10mM solution) was added to one 20% oxygen dish and returned to the 20% oxygen incubator. Cells were cultured for another day and then rapidly washed in 1X in ice-cold PBS and immediately lysed in 150 µl urea lysis buffer containing protease inhibitors and stored at -20ºC until further use.

2.9.2. Bradford assay for protein quantitation

Protein concentration was estimated using the Bradford assay. Frozen cell lysates were thawed on ice. 10 µl of lysate was mixed with 990 µl of Bradford reagent in a cuvette. The blank cuvette contained 10 µl of the appropriate buffer (RIPA or urea lysis buffer). Absorbance at 595 nm (A_{595}) was measured in a spectrometer. Protein concentrations were estimated by comparison of the A_{595} obtained with a standard curve generated using known concentrations of bovine serum albumin (BSA).

2.9.3. SDS- PAGE

Proteins were separated by a discontinuous stacking/separation gel electrophoresis system. Gels were cast in 18.5 cm x 20 cm glass plates with 1.5 mm spacers. The resolving gel comprised 380 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 10% (w/v) acrylamide for a 10% gel and 12% (w/v) acrylamide for a 12% gel. A 10% gel was made for Telomerase and HIF1-α analysis and a 12% gel for STEAP1. Whilst the gel was polymerising it was coated with a thin layer of H_2O-saturated isobutanol to ensure no bubbles formed across the top of the gel. Once the resolving gel had set the H_2O-saturated isobutanol was poured off and the top of the gel rinsed with dH_2O prior to pouring the stacking gel. This comprised 125mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 5% (w/v) acrylamide. Once polymerised the gels were place in the electrophoresis apparatus (Cambridge Electrophoresis, Cambridge, UK) in running buffer.

50 µg of the protein lysate per sample were denatured prior to loading by addition of 4X gel sample buffer and incubating for 5 min at 95ºC. Samples were then briefly pulsed in the centrifuge to bring any condensed sample to the bottom of the eppendorf. Protein samples were loaded into the well of the stacking gel. 10 µl of molecular weight markers were run in parallel to the samples to allow size determination of proteins. Gels were run at 100V (constant voltage) for approximately 2-3 hours depending on the separation required. Alternatively, when better separation of proteins was required a 4-12% gradient gel was used using a NuPAGE mini-gel system. The gel was composed of a 4% acrylamide stacking gel and a 4-12% acrylamide gradient separating gel. Samples were loaded in the wells of the gradient gel and run as above using 1X NuPAGE MOPS-SDS-running buffer, in the XCell SureLock Mini-Cell electrophoresis apparatus which is designed for mini-gels.

2.9.2 Western blotting

Once electrophoresis was complete, the gel was removed from the electrophoresis apparatus. The stacking part and dye front were cut off, and that remaining briefly soaked in transfer buffer. The PVDF membrane was pre-hydrated for 1 min in 100% methanol and then washed in transfer buffer. The gel and PVDF membrane were sandwiched between two transfer buffer soaked Whatman filter papers and placed in a Trans-blot chamber filled with cold (4ºC) transfer buffer. Transfer of proteins was carried out at 100V (constant voltage) for 90 min. The transfer buffer in the Tran-blot chamber was kept at 4ºC by a circulating cooling pump. Membranes were blocked in 4% (w/v) Marvel (low fat dry milk) and 1% (w/v) bovine serum albumin in 1 X PBS + 0.1% Tween-20 (PBS-T) for 1 hour on a gentle shaker. Next, membranes were incubated in the same buffer overnight with the appropriate antibody HIF-1α (dilution 1:250), Telomerase (dilution 1:1000), and STEAP1 (dilution 1:500) on a rotator. The next day the membranes were washed 3 times for 10 min in 1 X PBS and then incubated for 1 hour in the appropriate secondary horseradish peroxidase-conjugated antibody (dilution 1:2000). This was in 5% (w/v) Marvel milk in 1X PBS solution, performed with gentle shaking. Next, membranes were washed three times in 1X PBS and then incubated 1 min in ECL

solution. Protein bands were visualised by autoradiography using Fuji X-Ray film. STEAP 1 antibody from Santa-Cruz (sc-10262) detected human STEAP 1 but not murine. Two other STEAP1 antibodies (Santa-cruz (sc-25514) and Abcam (ab3679) were tested on human and murine cells, but the antibodies were not specific. The SDS-PAGE and western blotting for the HIF-1α protein analysis was performed by Miss Sonia Talma, a research assistant in the laboratory.

2.10 Fluorescence activated cell sorting analysis

Cells cultured in complete medium were trypsinised and counted when ~70% confluent (2.3.1.1). After washing once with FACS buffer, 1 x 10^5 cells were transferred into 5 ml FACs tubes and pelleted by centrifugation for 5 min at 1500 rpm. The cells were re-suspended in 200µl FACS buffer and 3.5 µl of FITC or PE-labelled antibody was added. The cells were incubated at 4ºC for 30 min before pelleting as above. The cells were then fixed with FACS fixing buffer and stored in the fridge until analysis. Cells labelled with primary antibodies that were not conjugated with a fluorochrome were labelled for a second time as above with the appropriate secondary fluorochrome-conjugated antibody (mouse: FITC-conjugated anti-rat (553896) BD Pharmingen, human: PE-conjugated anti-mouse (M31504) (Caltag Laboratories, San Francisco, USA)). Mouse cells were analysed with FACScan cytometer using CellQuest software. This was then replaced with BD FACSCantoII, which was used to analyse the human cells with BD FACSDiva™ software. At least 10,000 events were collected and CellQuest and FLOWJO software used to create the histograms for the mouse cells and human cells respectively. Table 2.1 lists the mouse antibodies used, and table 2.2 the human antibodies used. STEAP2 antibody from Novus Biological detected human STEAP2, but not murine. ProsSci (Poway, CA, USA (4307)) STEAP2 antibody was tested on human and murine cells, but it was not specific.

Table 2.1 Murine antibodies used with FACS analysis

Antibody	Label	Catalogue Number	Source
Sca-1	PE	553108	BD Pharmingen, San Diego, CA, USA
SSEA-1	PE	FAB2155P	R&D Systems
CXCR-4	FITC	551967	BD Pharmingen, San Diego, CA, USA
MHC-I	PE	557000	BD Pharmingen, San Diego, CA, USA
MHC-II	PE	CBL1324P	Chemicon, UK
CD3	PE	555275	BD Pharmingen, San Diego, CA, USA
CD11b	PE	557397	BD Pharmingen, San Diego, CA, USA
CD29	-	558741	BD Pharmingen, San Diego, CA, USA
CD31	FITC	CBL1337F	Chemicon, UK
CD34	PE	551387	BD Pharmingen, San Diego, CA, USA
CD44	PE	553134	BD Pharmingen, San Diego, CA, USA
CD45	PE	553081	BD Pharmingen, San Diego, CA, USA
CD49e	PE	557447	BD Pharmingen, San Diego, CA, USA
CD73	PE	550741	BD Pharmingen, San Diego, CA, USA
CD80	PE	553769	BD Pharmingen, San Diego, CA, USA
CD90	PE	553014	BD Pharmingen, San Diego, CA, USA
CD105	FITC	FAB1320F	R&D Systems
CD106	FITC	553332	BD Pharmingen, San Diego, CA, USA
CD117	PE	CBL1359P	Chemicon, UK
CD166	PE	MAB1172	R&D Systems

Table 2.2 Human antibodies used with FACS analysis

Antibody	Label	Catalogue No.	Source
STEAP2	-	BC100-2517	Novus Biological
STRO-1	-		Developmental Studies Hybridoma Bank, University of Iowa, IW
SSEA-1	PE	FAB2155P	BD Pharmingen, San Diego, CA, USA
CXCR4	PE	555974	BD Pharmingen, San Diego, CA, USA
HLA-ABC	FITC	MAB1275F	Chemicon, UK
HLA-DR	FITC	555811	BD Pharmingen, San Diego, CA, USA
CD11b	PE	555388	BD Pharmingen, San Diego, CA, USA
CD13	PE	555394	BD Pharmingen, San Diego, CA, USA
CD29	PE	556049	BD Pharmingen, San Diego, CA, USA
CD34	FITC	555821	BD Pharmingen, San Diego, CA, USA
CD45	FITC	555482	BD Pharmingen, San Diego, CA, USA
CD49e	PE	555617	BD Pharmingen, San Diego, CA, USA
CD73	PE	550257	BD Pharmingen, San Diego, CA, USA
CD90	PE	555596	BD Pharmingen, San Diego, CA, USA
CD105	FITC	CBL418F	BD Pharmingen, San Diego, CA, USA
CD106	PE	555647	BD Pharmingen, San Diego, CA, USA
CD122	PE	554525	BD Pharmingen, San Diego, CA, USA
CD133	-	AB5558	Abcam, Cambridge, UK
CD166	PE	559263	BD Pharmingen, San Diego, CA, USA

2.11 Telomere length assay

Cells were cultured until ~70% confluence then trypsinised and centrifuged at 1500rpm for 5 min. Cells were then washed in PBS, pelleted and frozen at -20ºC. Genomic DNA was isolated using DNA isolation Kit (Qiagen) and 1µg of DNA was measured using spectrometer. Telomere Length was measured by Telo TAGGG Telomere Length Assay kit (Roche Applied Science) according to manufacturer's instructions.

2.12 Karyotype analysis

Cells in metaphase were karyotyped by TDL Cytogenetics (London, UK). Cells in culture were incubated for 20 min in 10 µg/ml colcemid, and then removed by trypsinisation and placed in a tube containing hypotonic solution (1:12 FBS/ultrapure water) and mixed by inversion. The tube was incubated at 37°C for 25 min. The cell suspension was pre-fixed with 5 drops of fresh cool fixative (3:1 methanol/glacial acetic acid), centrifuged for 10 min at 1500rpm, and the supernatant discarded and the cell pellet loosened by flicking and re-fixed. This step was repeated once more. The slide was then made. The supernatant was tipped off and the cells re-suspended in a few drops of fixative. 40 µl of suspension was applied to the top of the wet washed slide and the excess water drained off. The cells were then allowed to dry onto the slide with the aid of gentle warming from an anglepoise lamp with gentle blowing. This allowed the metaphase chromosomes to burst out of their nuclear envelope and spread sufficiently to allow analysis. Slides were then assessed with low power phase-contrast microscopy and cell suspension concentration adjusted to allow best spreading. G-banding was then performed. Slides were aged by drying in an oven (92°C for 40 min or 63°C overnight) and then rinsed in HBSS for 5 min. Slides were then treated with Trypsin (1 ml 10x Trypsin in 49 ml HBSS). This is a critical step, and the timing is variable and determined for each batch experimentally. The slides were rinsed in HBSS containing serum to stop trypsin activity and stained in 3:1 Leishman/Giemsa (Merck) (2.5 ml in 47.5 ml pH 6.8 buffer). Slides were then rinsed in purified water, gently blotted dry, and mounted with coverslips. For analysis, slides were analysed by conventional bright field microscopy. They were scanned for metaphases at low power (~100x total magnification) and examined with an

oil immersion high power objective (~1000x total magnification).The number of murine chromosomes in each cell was then counted, typically 20 or more cells per sample were examined and any obviously abnormal chromosomes, such as double-minute ones noted. A typical metaphase was digitised and stored using a Cytovision image analysing system.

2.13 Microarray analysis

Clonally-derived cells and MEFs were seeded at 1000cells/cm^2 and cultured at 5% oxygen tension (for 3-7 days). BMACs were cultured as described in section 2.3.2 and lysed when ~80% confluence. Cells were lysed when 70% confluent as described in section 2.3.1.1. Care was taken to perform the trypsinisation quickly and in cold conditions, the cells were centrifuged at 4ºC at 1500 rpm, and cell pellet washed in 10 ml ice cold 1 X PBS and centrifuged again. The supernatant was removed by aspiration and the cell pellet lysed in 350µl RLT buffer (with β-Mercaptoethanol) (Qiagen RNeasy Kit), and frozen at -80°C. Four clones and two MEF, BMAC, and BMMNC cultures per mouse strain were lysed. Total RNA was extracted from cells using Qiagen RNeasy Kit. RNA was measured (A_{260}/A_{280}) using a Nanodrop ND-1000 spectrophotometer, before assessing RNA integrity on the Agilent Bioanalyser RNA 6000 Nano system (Agilent Technologies, Berkshire, UK).

Ten µg of total RNA from each sample was used to synthesise double-stranded cDNA using GeneChip one-cycle target labelling and control reagents (Affymetrix, Santa Clara, CA) following manufacturer's instructions. *In vitro* transcription was then performed to produce biotin-labelled cRNA. Ten µg of the labelled cRNA was fragmented and hybridised to the GeneChip Mouse Genome 430 2.0 Array (Affymetrix). The chip contains over 45,000 probe sets representing over 34,000 well substantiated mouse genes. The cRNA was fragmented, hybridisation cocktails prepared and denatured at 99ºC. The hybridisation cocktails were put on the array and hybridised for 16 hrs. Hybridisation and scanning were carried out using Affymetrix 450 fluidics and scanning using the Affymetrix 3000 7G scanner. Data analysis was performed using the Rosetta Resolver® gene expression data analysis system (version 7).

2.14 RNA extraction, reverse transcription and Real Time PCR

Frozen samples were thawed on ice. RNA was then extracted and prepared using the RNeasy Mini Kit. Complementary DNA (cDNA) was generated from 1 μg of total RNA using a reverse transcription kit (Promega, USA), and following the manufacturer's instructions. 2% of the reversed transcribed solution was used for each real-time PCR reaction (done in triplicates) using the following gene-specific Taq-Man primer/probes for mouse: Ly6a/Sca-1, STEAP1, STEAP2, TM4SF1, Versican, PPARγ and the normalising reference 18S, and for human: RPLPO, STEAP1, and STEAP2. After an initial denaturation at 95 ºC for 10 min, a 2-step cycle procedure was used (denaturation at 95 ºC for 2 sec, annealing and extension at 60 ºC for 30 sec) for 40 cycles on a CORBETT Research Real-Time thermocycler. Relative gene expression levels were calculated using the delta-delta threshold cycle (ΔΔCt) method using Rotor-Gene 6000 series software (version 1.7).

2.15 Transplantation of clonally-derived cells

To determine the ability of the clonal cells to induce tumour growth *in vivo*, 1 x 10^6 cells in sterile 1X PBS were injected into 6-8 week old, male BALB/c SCID mice. Mice analysis of murine tissues were either injected intra-muscularly in the right quadriceps and sub-cutaneously in the back, or intravenously. (This procedure was kindly performed by Dr. Gabriel Criado). As a control, BALB/b MEFs (which have a normal karyotype) were likewise injected in separate animals. Mice injected with BALB/b clones and BALB/c clones were sacrificed after 4.5 months and 7 months respectively. There was no macroscopic sign of tumour formation. Lungs, heart, liver, kidneys, and the right and left quadricep were removed and fixed in neutral phosphate buffered formalin for histological analysis.

2.16 Histological analysis of murine tissues

All sections were stained with haematoxylin and eosin, and masson trichrome and the lungs and the muscle for Alizarin Red S and Alcian Blue to detect whether the clonal cells had differentiated. This was performed by the Histology department, in Charing Cross hospital.

2.16.1 Masson Trichrome staining

For masson trichrome staining formalin embedded sections were cut at 4μM using Microm HM325 microtome. Sections were baked for approximately 1 hour before de-waxing in 2 changes of xylene, 2 minutes each. Next sections were hydrated in 3 changes of absolute alcohol, 2 minutes each and then washed in distilled water. Sections were then immersed in 5% Chromic Acid for 1 hour at room temperature followed by washing in distilled water. After immersion in Celestine Blue for 5 minutes, sections were washed in distilled water followed by immersion in haematoxylin for 5 minutes. Sections were then washed in running tap water and immersed in 0.5% Acid Alcohol (70% Alcohol, 30% distilled water, plus 0.5% concentrated hydrochloric acid) for 20 seconds followed by a washing step in running tap water for 5 minutes. Sections immersed in 1% Chromazone Red in 1% Acetic Acid for 5 minutes and washed in distilled water. After immersion in 1% Phosphomolybdic Acid for 5-10 minutes, controlled microscopically, they were washed in distilled water followed by immersion in 1% Light Green in 1% Acetic Acid for 3 minutes. Next they were washed in distilled water, blot dried, then dehydrated in 3 changes of absolute alcohol, 2 minutes each.

Sections cleared in xylene, 2 changes, 2 minutes each and mounted using Pertex mountant and an appropriate cover slip.

2.16.2 Haematoxylin and Eosin staining

Formalin fixed embedded sections were cut at 4μM and baked and de-waxed as described above (2.16.1). Sections were then re-hydrated in 3 changes of absolute alcohol, 2 minutes each and washed in running tap water. Sections were then immersed in Haematoxylin solution (Surgipath) for 8 minutes, followed by washing in running tap

water. After immersion in 0.5% Acid alcohol (70% Alcohol, 30% distilled water, plus 0.5% concentrated hydrochloric acid) for 20 seconds, they were washed in running tap water. Sections were then immersed in 'bluing solution' ammoniated water ~0.1% concentrated ammonia for 2 minutes followed by washing in running tap water. Next, sections were blot dried, then dehydrated in 3 changes of absolute alcohol, 2 minutes each. Sections were then cleared in xylene and mounted as described above (2.16.1).

2.17 RNA interference experiments

Human bone marrow stromal cells (hBMSCs) were seeded at a cell density of 1,300 cells/cm^2 (approximately 50% confluence) in 6 well plates and cultured in complete medium at 5% oxygen. On day two cells were transfected with small interfering RNA (siRNA) oligos directed against luciferase (Dharmacon, Lafayette, CO, USA), a scrambled sequence, and two different STEAP1 (called STEAP1.1 and STEAP1.2) and STEAP2 (called STEAP2.1 and STEAP2.2) (MWG Biotech, Ebersberg, Germany) transcripts (Table 2.3) or with lipofectamine alone.

Prior to transfection the appropriate amounts of OptiMEM were incubated at 37ºC. Medium was removed from the cells and 3 ml of OptiMEM added to each well and cells returned to the incubator. Next the siRNAs were prepared. An appropriate volume of lipofectamine 2000 was incubated at room temperature with OptiMEM I for 5 min to give a final 10 nM concentration. A calculated volume of siRNA was also added to OptiMEM I to give a 10nM final concentration, and this mixture was incubated for 5 min at room temperature. After incubation these two mixtures were combined and mixed by pipetting 2-3 times and incubated for a further 20 min at room temperature to allow for liposome formation. The mixture of lipid and siRNA was carefully added dropwise to each well to give a final volume of 3.5 ml per well, and plates swirled very gently to mix. After 4 hours the medium was removed and 2.5 ml of complete medium was added to each well. Cells were incubated at 5% oxygen for a further 2 days. Cells were then washed once with 1X PBS and lysed in 350µl RLT buffer (for real-time PCR assays) and or lysed in 100µl RIPA (for western blot analysis) and stored at -80ºC.

Chapter 3

Isolation and Verification of Stem Cell Status of Clones

3.1 Introduction

As discussed in chapter 1, most groups work with a heterogeneous population of cells they call mesenchymal stem cells (MSCs). It is then difficult to know whether the experimental results are directly due to different populations of cells or specifically to a single population of MSCs. In the present study we developed a protocol to isolate clones to ensure a pure population of cells were being studied.

MSCs cultured under standard conditions tend to decrease their proliferative rate, and lose their differentiation capacity (Muraglia et al., 2000). A large number of parameters need to be considered when culturing MSCs. MSCs are typically cultured at 20% oxygen, however, in the bone marrow the average oxygen tension is approximately 4-7% as measured by the Kofoed group (Kofoed et al., 1985). A study by Lennon and colleagues reported that rat MSCs cultivated in a reduced oxygen atmosphere exhibited increased proliferation rates, higher colony formation, as well as enhanced *in vivo* osteo/chondro-differentiation potential relative to culture at 20% oxygen (Lennon et al., 2001). This suggests that low oxygen tension plays an important role in the maintenance of the stem cell phenotype. Therefore, to examine whether 5% oxygen was preferential to the traditional 20% we performed experiments at both oxygen environments. We endeavoured to produce a detailed characterisation of the clonally-derived MSC populations in terms of cell morphology, growth rate, differentiation capacity, and self-renewal.

3.2 Isolation of clones

Bone marrow was isolated from the long bones of BALB/b mice and depleted of red blood cells. The subsequent bone marrow mononuclear cells (BMMNCs) were cultured at a low cell density of 56,000 cells/cm^2 at 20% oxygen tension as well as 5% (figure 3.1). There were very few adherent cells in cultures that had been exposed to 20% oxygen tension and they did not survive. In contrast, after one month there was the appearance of individual colonies in the cultures that had been exposed to 5% oxygen tension (figure 3.1). These colonies were subsequently isolated and expanded in culture at

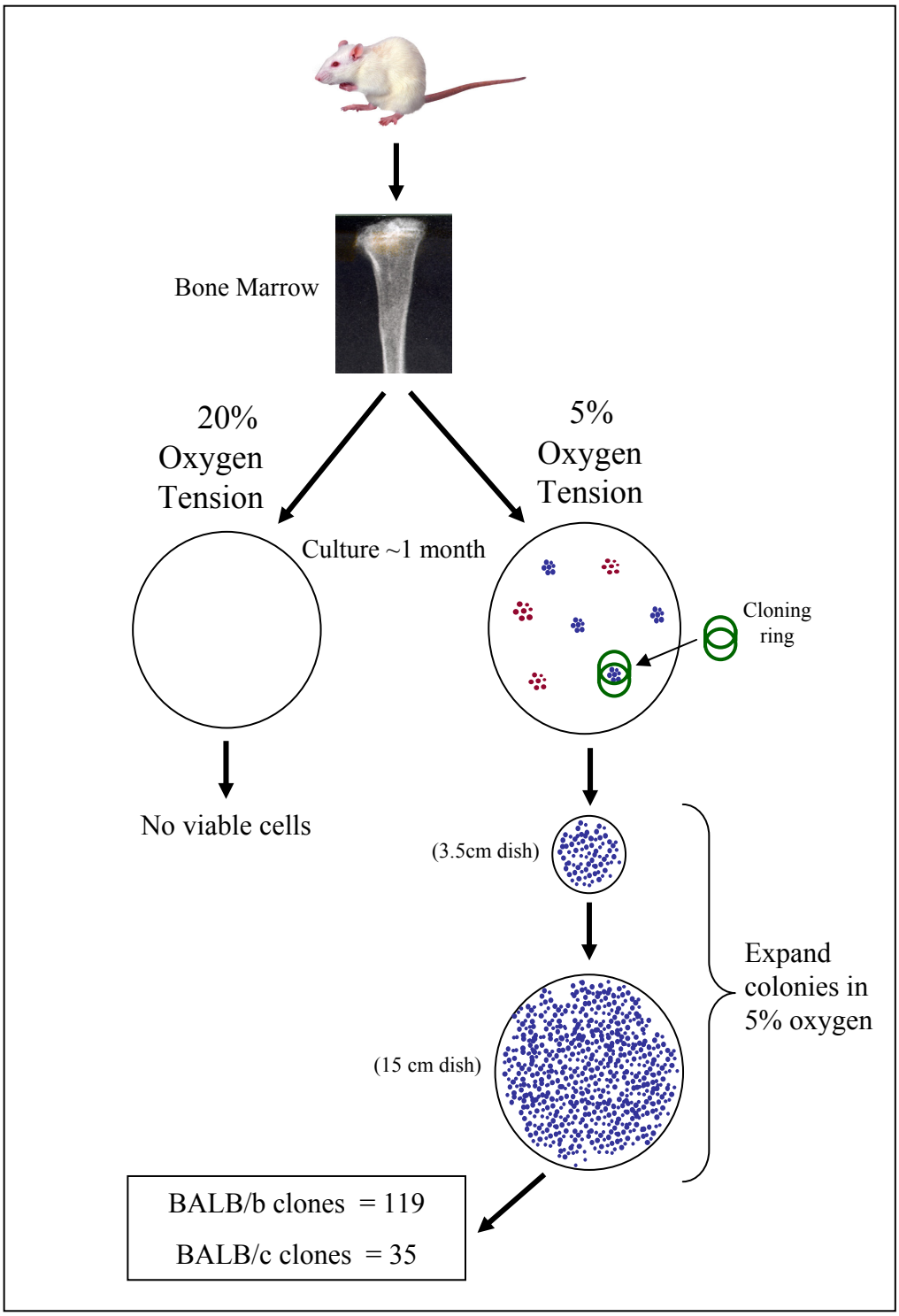


Figure 3.1 Diagram of clonal isolation procedure. Bone marrow mononuclear cells isolated from the long bones of 3 BALB/b mice were seeded at a low density and cultured at 20% and 5% oxygen tension. After one month in culture there were no viable cells in the 20% cultures. In the 5% oxygen cultures individual colonies appeared that were isolated using cloning rings and re-seeded into 3.5cm dishes and then 15cm dishes. Cells were counted when 70% confluent and frozen. This method was repeated to isolate clones from two further BALB/b isolations and a BALB/c (each isolation from 3 mice). In total 119 BALB/b clones were isolated from 3 separate isolations and 35 BALB/c clones.

5% oxygen. During expansion cells were passaged upon reaching approximately 70-80 % confluence to prevent loss of multipotency which can occur at confluency. Clones were derived from the first isolation (3 mice combined per isolation) were called BALB/b1. Using this method we isolated clones from two further BALB/b isolations (referred to as BALB/b2 and BALB/b3) and 35 from BALB/c mice. A total of 119 BALB/b clones (tables 3.1-3.3) and 35 BALB/c clones were obtained (table 3.4).

3.3 Cell doubling time of clonal cells

The doubling time calculated for the clones averaged 1.9 ± 0.2 days for BALB/b1, 1.4 ± 0.3 days for BALB/b2, 1.7 ± 0.6 days for BALB/b3, and 2.9 ± 1.7 days for BALB/c (mean ± SD) (tables 3.1-3.4). The doubling time included an initial lag phase before colonies are formed as reported by previous investigators (Colter et al., 2001). All clonally derived cells had a characteristic spindle shape and were relatively small (figures 3.2-3.3).

3.4 Determining the multipotency of clonal cells

MSCs are typically characterised by their ability to differentiate into three specific lineages - adipocytes, chondrocytes, and osteoblasts. Thus, this differentiation potential of the clones was examined to further confirm their stem cell status. Strain-matched mouse embryonic fibroblasts (MEF) were used as an additional control in differentiation experiments.

3.4.1 Adipogenic differentiation

For adipocyte differentiation cells were cultured for 1-2 weeks in the presence or absence of adipogenic media. This media contained the classic hormonal cocktail of dexamethasone, 3- isobutyl-1-methyl-xanthine, indomethacin, and insulin. Media was changed twice a week. By day 4, cells containing lipid-rich vacuoles which showed intense Oil Red O staining were detectable in the BALB/b and BALB/c clonal cultures that had been exposed to the differentiation media at 5% oxygen tension. Another week was usually necessary for all the clonal cells to commit to this lineage (figures 3.4-3.5). In the 20% oxygen cultures it usually took 3 weeks for all the cells to differentiate. There

Table 3.1 BALB/b1 clones. Bone marrow mononuclear cells from 3 BALB/b mice were seeded at low density (56,000 cells/cm^2) in 5% oxygen. After one month individual colonies appeared which were isolated and expanded further in culture. Clonal cells were counted and the population doublings and cell doubling time calculated and then clones were frozen. This was the first BALB/b isolation hence clones referred to as BALB/b1 clones.

BALB/b1 (Clone)	Cell Count ($1x10^6$)	Population Doublings	Doubling Time (Days)
C1	3.65	22	1.6
C2	2.07	21	1.7
C3	2.02	21	1.7
C4	1.06	20	1.7
C5	3.8	22	1.6
C6	2.47	21	1.7
C7	0.66	19	1.9
C8	0.545	19	2.1
C9	1.45	20	1.8
C10	1.54	21	2.2
C11	2.22	21	1.8
C12	4.23	22	1.5
C13	4.79	22	1.5
C14	2.84	21	1.6
C15	3.33	22	1.6
C16	2.3	21	1.8
C17	2.47	21	1.6
C18	1.81	21	1.7
C19	2.47	21	2.3
C20	2.1	21	2.0
C21	2.21	21	1.8
C22	1.7	21	2.1
C23	2.6	21	2.0
C24	1.6	21	2.1
C25	1.9	21	2.1
C26	3.17	21	2.1
C27	2.85	21	2.1
C28	2.17	21	2.2
C29	2.41	21	2.1
C30	0.56	19	1.9
C31	0.28	18	1.9
C32	0.94	20	1.8
C33	0.365	18	1.9
C34	1.23	20	1.8
C35	1.7	21	1.8
C36	0.3	18	2.5
C37	0.9	20	2.17
Average	2.0	20.7	1.9

Table 3.2 BALB/b2 clones. Bone marrow mononuclear cells from 3 BALB/b mice were seeded at low density (56,000 cells/cm^2) in 5% oxygen. After one month individual colonies appeared which were isolated and expanded further in culture. Clonal cells were counted and the population doublings and cell doubling time calculated and then clones were frozen. This was the second BALB/b isolation hence clones referred to as BALB/b2 clones.

BALB/b2 (Clone)	Cell Count ($1x10^6$)	Population Doublings	Doubling Time (Days)
C1	1.9	21	1.1
C2	1.9	21	1.1
C3	2	21	1.1
C4	2.2	21	1.1
C5	1.8	21	1.3
C6	3.76	22	1.2
C7	3.41	22	1.2
C8	1.18	20	1.3
C9	3.3	22	1.2
C10	2.53	21	1.2
C11	3.65	22	1.2
C12	3.43	22	1.2
C13	3.69	22	1.2
C14	4.07	22	1.2
C15	2.09	21	1.2
C16	3.3	22	1.2
C17	1.81	21	1.3
C18	2.58	21	1.2
C19	1.84	21	1.2
C20	2.58	21	1.3
C21	1.78	21	1.3
C22	2.52	21	1.3
C23	1.5	21	1.4
C24	2.33	21	1.4
C25	1.76	21	1.4
C26	1.45	20	1.4
C27	1.55	21	1.4
C28	2.13	21	1.4
C29	2.63	21	1.5
C30	1.29	20	1.6
C31	1.31	20	1.6
C32	1.94	21	1.5
C33	1.54	21	1.6
C34	1.92	21	1.7
C35	1.7	21	1.7
C36	0.83	20	1.8
C37	2.08	21	1.7
C38	1.56	21	1.7
C39	3.17	22	1.6
C40	2.74	21	1.7
C41	3.08	22	1.7
C42	2.92	21	1.8
C43	1.19	20	1.9
C44	1.55	21	1.9
C45	1.62	21	1.9
Average	2.2	21	1.4

Table 3.3 BALB/b3 clones. Bone marrow mononuclear cells from 3 BALB/b mice were seeded at low density (56,000 cells/cm^2) in 5% oxygen. After one month individual colonies appeared which were isolated and expanded further in culture. Clonal cells were counted and the population doublings and cell doubling time calculated and then clones were frozen. This was the third BALB/b isolation hence clones referred to as BALB/b3 clones.

BALB/b3 (Clone)	Cell Count ($1x10^6$)	Population Doublings	Doubling Time (Days)
C1	2.09	21	1.0
C2	2.6	21	1.0
C3	3.3	22	1.0
C4	1.09	20	1.1
C5	1.04	20	1.1
C6	0.72	19	1.1
C7	1.28	20	1.1
C8	0.82	20	1.1
C9	3.26	22	1.0
C10	2.4	21	1.0
C11	0.49	19	1.2
C12	0.61	19	1.1
C13	0.22	18	1.2
C14	0.29	18	1.2
C15	0.75	20	1.1
C16	0.33	18	1.2
C17	0.18	17	1.3
C18	0.458	19	1.6
C19	0.44	19	1.7
C20	0.52	19	1.6
C21	3	22	2.0
C22	0.82	20	2.2
C23	1.4	20	2.1
C24	2	21	2.1
C25	1.47	20	2.1
C26	0.64	19	2.3
C27	1.67	21	2.1
C28	0.69	19	2.3
C29	0.77	20	2.3
C30	0.9	20	2.1
C31	1.21	20	2.1
C32	0.27	18	2.3
C33	0.225	18	2.3
C34	0.54	19	2.2
C35	0.85	20	2.6
C36	0.46	19	2.8
C37	5.44	22	2.3
Average	1.2	19.7	1.7

Table 3.4 BALB/c clones. Bone marrow mononuclear cells from 3 BALB/c mice were seeded at low density (56,000 cells/cm^2) in 5% oxygen. After one month individual colonies appeared which were isolated and expanded further in culture. Clonal cells were counted and the population doublings and cell doubling time calculated and then clones were frozen.

BALB/c (Clone)	Cell Count ($1x10^6$)	Population Doublings	Doubling Time (Days)
C1	0.6	19	2.8
C2	0.72	19	2.8
C3	0.92	20	2.7
C4	1.2	20	2.7
C5	0.8	20	2.8
C6	0.9	20	2.7
C7	0.71	19	2.8
C8	2.23	21	2.6
C9	1	20	2.7
C10	0.74	19	2.8
C11	0.35	18	3.0
C12	0.47	19	2.9
C13	0.42	19	2.9
C14	0.91	20	2.9
C15	1.07	20	2.8
C16	0.43	19	3.0
C17	0.43	19	3.0
C18	0.41	19	3.1
C19	1.54	21	2.8
C20	1.28	20	2.9
C21	1.21	20	2.9
C22	1.51	21	2.8
C23	0.94	20	2.9
C24	0.81	20	3.0
C25	0.25	18	3.2
C26	1.32	20	2.9
C27	0.95	20	2.9
C28	0.55	19	3.0
C29	0.54	19	3.0
C30	0.8	20	3.0
C31	0.46	19	3.2
C32	0.3	18	3.4
C33	0.86	20	3.1
C34	1.24	20	3.0
C35	1.67	21	3.0
Average	0.87	19.6	2.9

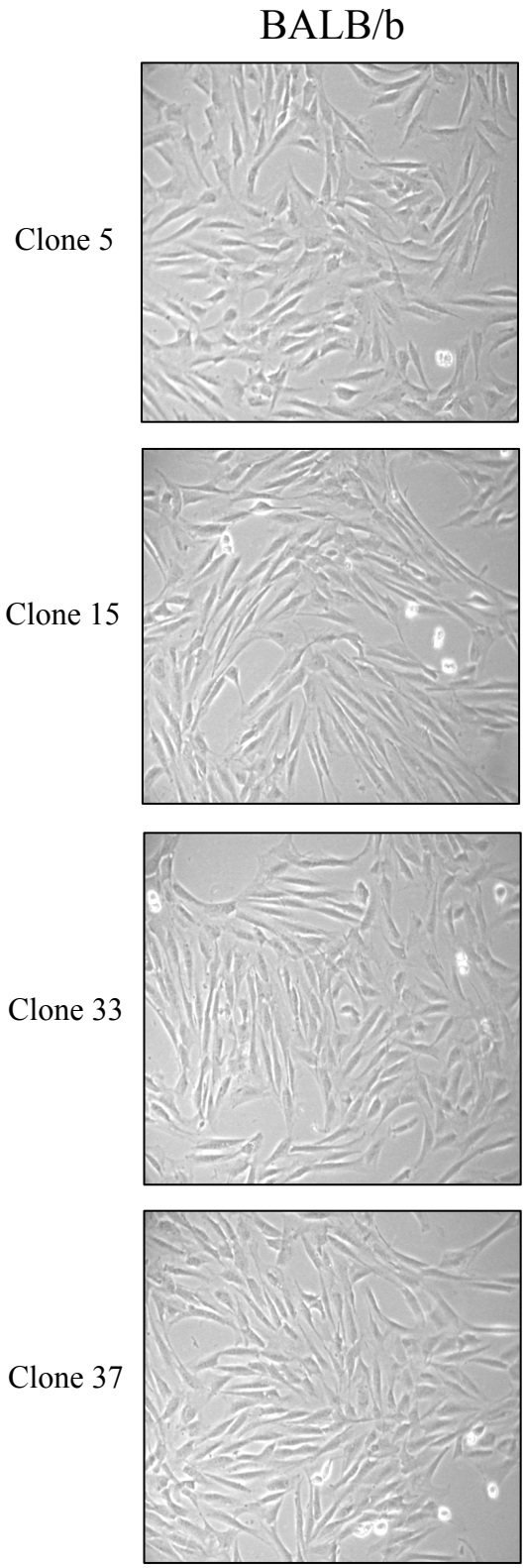


Figure 3.2 Morphology of BALB/b Clones. BALB/b clones were cultured in complete medium at 5% oxygen tension and passaged every 3-5 days when ~70% confluent. Photos of clones at passage 5-7.

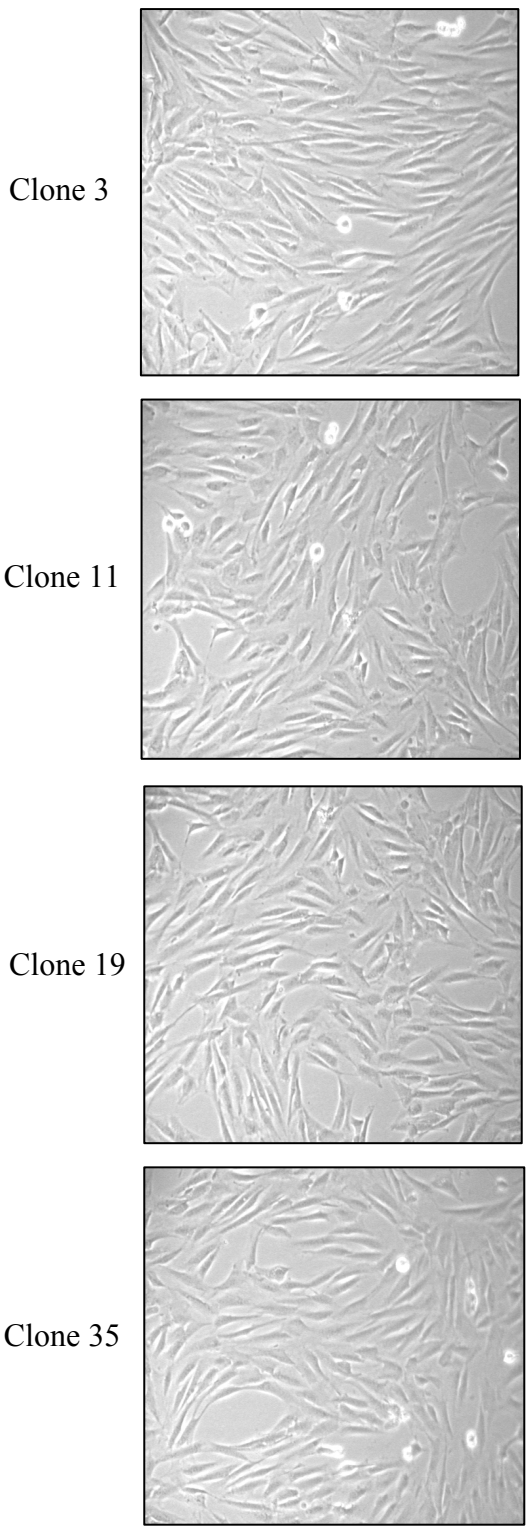


Figure 3.3 Morphology of BALB/c Clones. BALB/c clones were cultured in complete medium at 5% oxygen tension and passaged every 3-5 days when ~70% confluent. Photos of clones at passage 5-7.

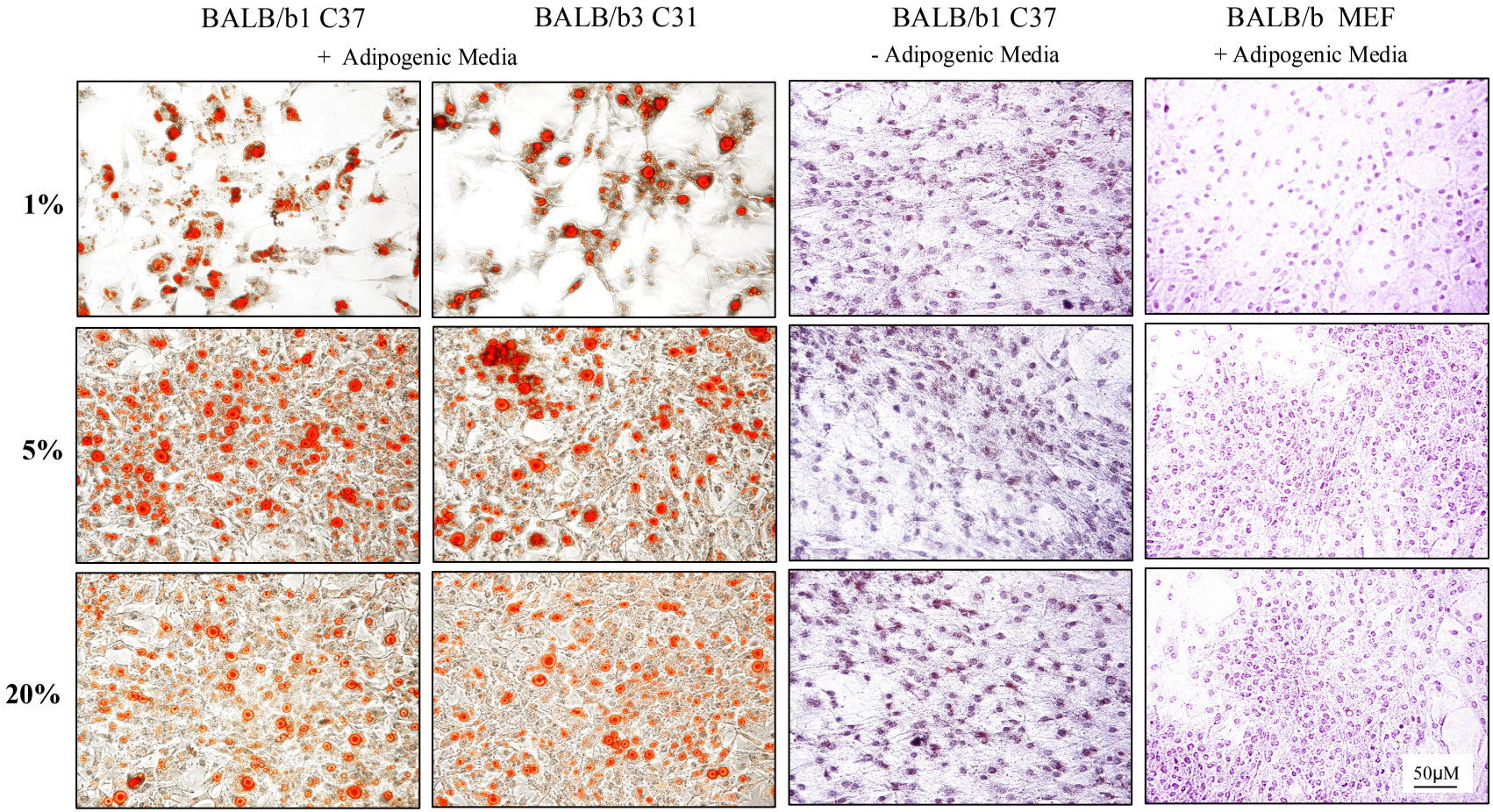


Figure 3.4 Adipogenic differentiation of BALB/b clones. Clonal cells and mouse embryonic fibroblasts (MEFs) were seeded at 6000cells/cm^2 in the presence or absence of adipogenic media (dexamethasone, 3- isobutyl-1-methyl-xanthine , indomethacin, and insulin) and cultured at 20%, 5%, and 1% oxygen tension. Clonal cells containing lipid droplets were seen 4 days after induction in the 5% oxygen cultures and by 2 weeks lipid-rich droplets were present in the majority of cells and stained with Oil Red O. The same process took 3 weeks in the 20% oxygen cultures. Cells in the 1% cultures began to slough off after 10 days. Control cells: clonal cells cultured in the absence of adipogenic media and MEFs cultured in its presence, were stained with Haematoxylin and Eosin in order to to visualise the undifferentiated cells. Photos are taken at 2 week time point for 5% and 1% oxygen cultures and 3 weeks for the 20%.

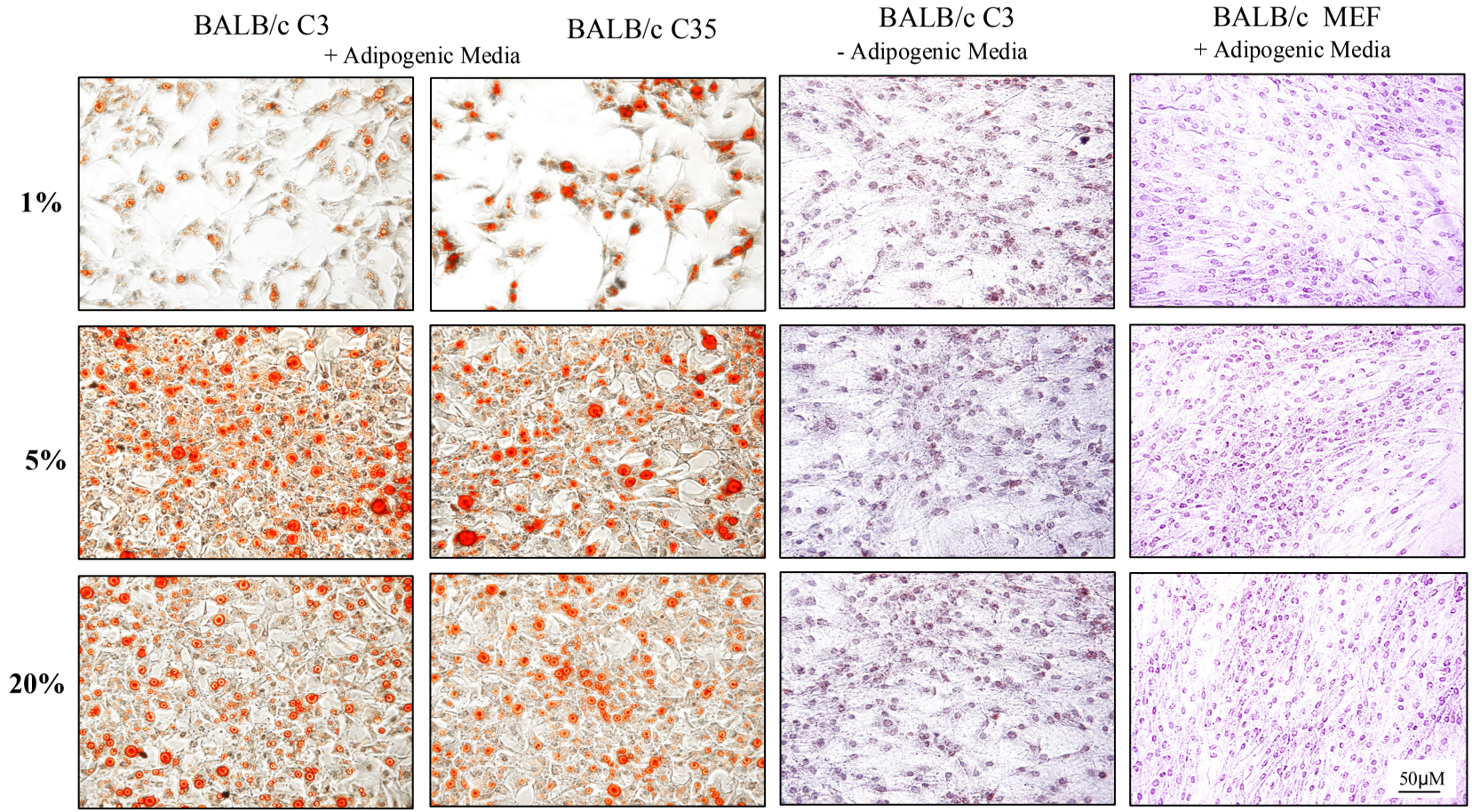


Figure 3.5 Adipogenic differentiation of BALB/c clones. Clonal cells and mouse embryonic fibroblasts (MEFs) were seeded at 6000cells/cm^2 in the presence or absence of adipogenic media (dexamethasone, 3- isobutyl-1-methyl-xanthine , indomethacin, and insulin) and cultured at 20%, 5%, and 1% oxygen tension. Clonal cells containing lipid droplets were seen 4 days after induction in the 5% oxygen cultures and by 2 weeks lipid-rich droplets were present in the majority of cells and stained with Oil Red O. The same process took 3 weeks in the 20% oxygen cultures. Cells in the 1% cultures began to slough off after 10 days. The same process took 3 weeks in the 20% oxygen cultures. Cells in the 1% cultures began to slough off after 10 days. Control cells: clonal cells cultured in the absence of adipogenic media and MEFs cultured in its presence, were stained with Haematoxylin and Eosin in order to to visualise the undifferentiated cells. Photos are taken at 2 week time point for 5% and 1% oxygen cultures and 3 weeks for the 20%.

was differentiation evident in cultures exposed to 1% oxygen tension; however after 10 days cells began to slough off the tissue culture dish. Thus adipogenic differentiation was optimal at 5% oxygen tension. Control cells cultured in the absence of adipogenic media or fibroblasts cultured in its presence displayed no signs of adipogenic differentiation, but were viable.

3.4.2 Chondrogenic differentiation

To determine whether the clonal cells had the ability to differentiate into chondrocytes we seeded the cells at a density of 5000 cells/cm^2 and treated them with media containing TGF-β3 at the three different oxygen tensions, 20%, 5%, and 1%. There was a greater number of chondrogenic nodules formed in the cultures exposed to both 5% and 1% oxygen tension and these appeared after 4-5 days. In comparison it took one week for the nodules to form at 20% oxygen tension. Staining for type II collagen which is a chondrocyte marker was also more intense in the low oxygen cultures for both mouse strains (figures 3.6-3.7). There was no type II collagen staining in clonal cultures which had been cultured in the absence of chondrogenic media or in the fibroblasts which had been cultured in its presence. In fact, there was no sign of cell aggregation in these cultures- the cells remained as a simple monolayer.

3.4.3 Osteogenic differentiation

To promote osteogenic differentiation cells were first primed in a minimal media (α-MEM media 5% FBS and 5 μg/ml ascorbic acid 2-phopshate (A2-P)) for 3-4 days. Cells were then re-seeded into 6 well plates and cultured in the presence of osteogenic media (OM) which consisted of α- MEM media 10% FBS, and 50 μg/ml ascorbic acid 2-phopshate and 10 mM β- glycerol phosphate (β-GP). Ascorbate is required for osteogenesis and β-GP is the source of phosphate required for mineralization. Cells were also cultured in OM supplemented with 1 or 10 ng/ml of bone morphogenetic protein-2 (BMP-2), a potent stimulator of osteogenesis in murine cells (Rawadi et al., 2003). Media was changed twice a week. After 1 week, cells cultured in the presence of 10 ng/ml BMP-2 (at all oxygen tensions) stained positive for alkaline phosphatase. This is an enzyme that has a role in the mineralization of bone and is an indication of immature

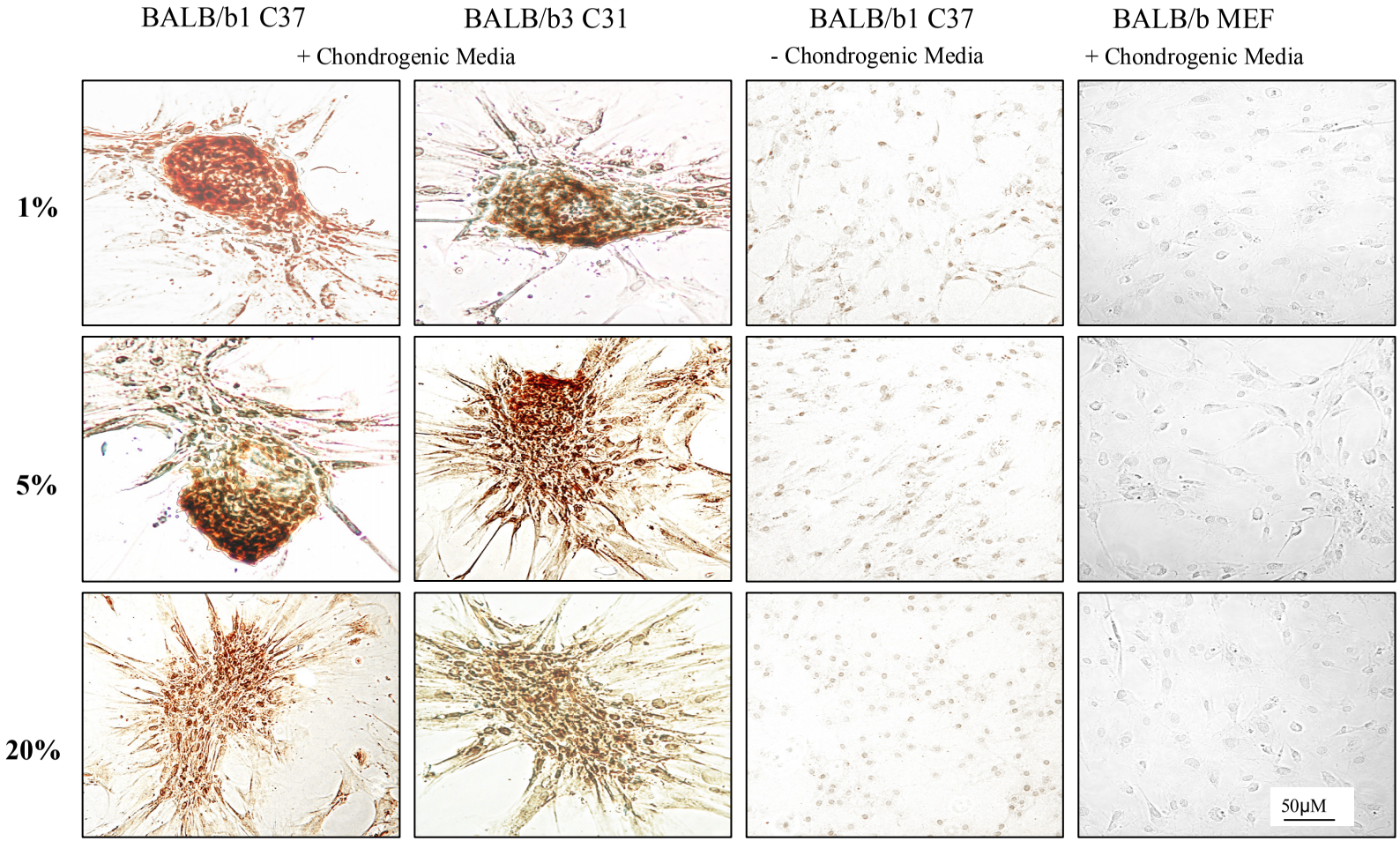


Figure 3.6 Chondrogenic differentiation of BALB/b clones. Clonal cells and mouse embryonic fibroblasts (MEFs) were seeded at 5000cells/cm^2 and cultured in the presence or absence of chondrogenic media (TGF-β3) at 20%, 5%, and 1% oxygen tension. It took 4-5 days for chondrogenic nodules to appear in the 5% and 1% oxygen cultures and 1 week in 20% oxygen culture. The presence of type II collagen in the chondrogenic nodules were visualised by immunostaining using a monoclonal antibody.

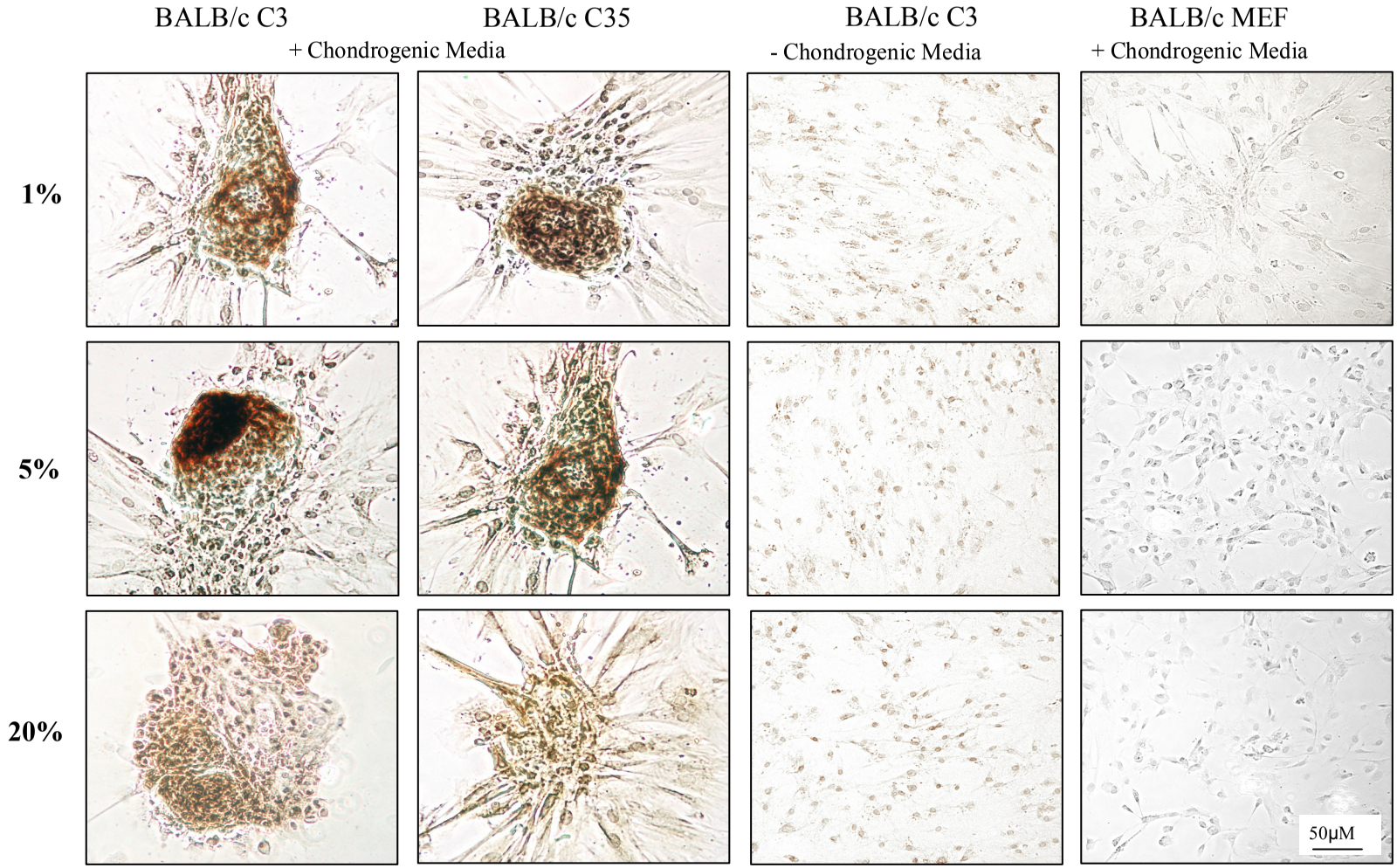


Figure 3.7 Chondrogenic differentiation of BALB/c clones. Clonal cells and mouse embryonic fibroblasts (MEFs) were seeded at 5000cells/cm^2 and cultured in the presence or absence of chondrogenic media (TGF-β3) at 20%, 5%, and 1% oxygen tension. It took 4-5 days for chondrogenic nodules to appear in the 5% and 1% oxygen cultures and 1 week in 20% oxygen culture. The presence of type II collagen in the chondrogenic nodules were visualised by immunostaining using a monoclonal antibody.

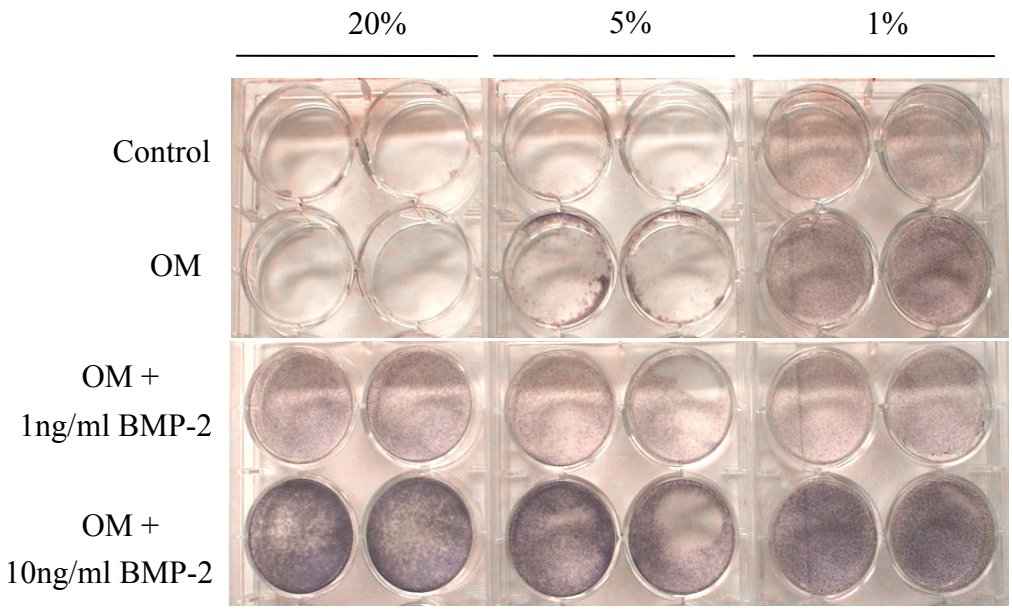


Figure 3.8 Alkaline Phosphatase Staining of BALB/b1 Clone 37. Clonal cells were first primed in a minimal media (α-MEM media 5% FBS and ascorbic acid 2-phosphate (A2-P)) for 3-4 days. Cells were re-seeded and cultured for 1 week in osteogenic media (OM) containing A2-P and β-glycerol phosphate. In addition 1ng/ml or 10ng/ml of BMP-2 was added as indicated. Cells were cultured at 20%, 5%, and 1% oxygen tension. Differentiation of the clonal cells into immature osteoblasts was visualised by alkaline phosphatase staining. Photos are indicative of alkaline phosphatase staining of BALB/b and BALB/c clones.

osteoblast formation (figure 3.8). Therefore, in subsequent experiments cells were cultured in OM supplemented with 10 ng/ml BMP-2 to induce osteogenesis. After 4 weeks of osteogenic induction, clonal cells exposed to 5% and 1% oxygen tension formed aggregates or nodules which stained positive for alizarin red (figures 3.9-3.10). This is a dye that stains the calcium phosphate in the mineralised matrix. Cultures exposed to 20% oxygen tension were viable but did not stain positive. Clonal cells cultured in the absence of osteogenic media and strain-matched fibroblasts cultured in its presence did not show any positive Alizarin Red staining. It is important to emphasise that all BALB/b clones (10) and BALB/c clones (7) examined had the ability to differentiate into all three lineages (table 3.5).

3.5 Colony forming ability of clones.

Expanded clones were tested for their colony forming ability via a colony forming unit – fibroblast (CFU-f) assay. The percentage of cells capable of forming CFU-f from early passage (p6-7) and late passage (p52-3) clones was assessed. 1000 cells were seeded in a 10 cm dish and cultured at 20%, 5%, and 1% oxygen tension (figure 3.11(A)). Individual colonies with more than 100 cells were counted. The majority of colonies at this time point had more than 100 cells as shown in figure 3.11 (A). Figure 3.11(B) shows that the clones had a CFU-f of 65 – 75%. One reason the percentage CFU-f is less than 100% is because not all the cells adhere to the tissue culture substrate. There was no heterogeneity in the morphology of the clones at 5% oxygen tension; they were all spindle shaped cells. At the low seeding densities used for these CFU-f assays, cells cultured at 20% and 1% oxygen tension did not form colonies, and there was a morphology change in these cells from a spindle shape to a broader, flatter cell.

3.6 Clonal cells have the ability to self-renew

All clones retained their ability to differentiate into the three lineages after extensive passaging (p42) (figure 3.12) and to form colonies with a CFU-f of 65 -75% at a high passage (p52-53) (figure 3.11). Thus, the clones demonstrated self-renewal by their ability to go through numerous cycles of cell division whilst maintaining their stem cell

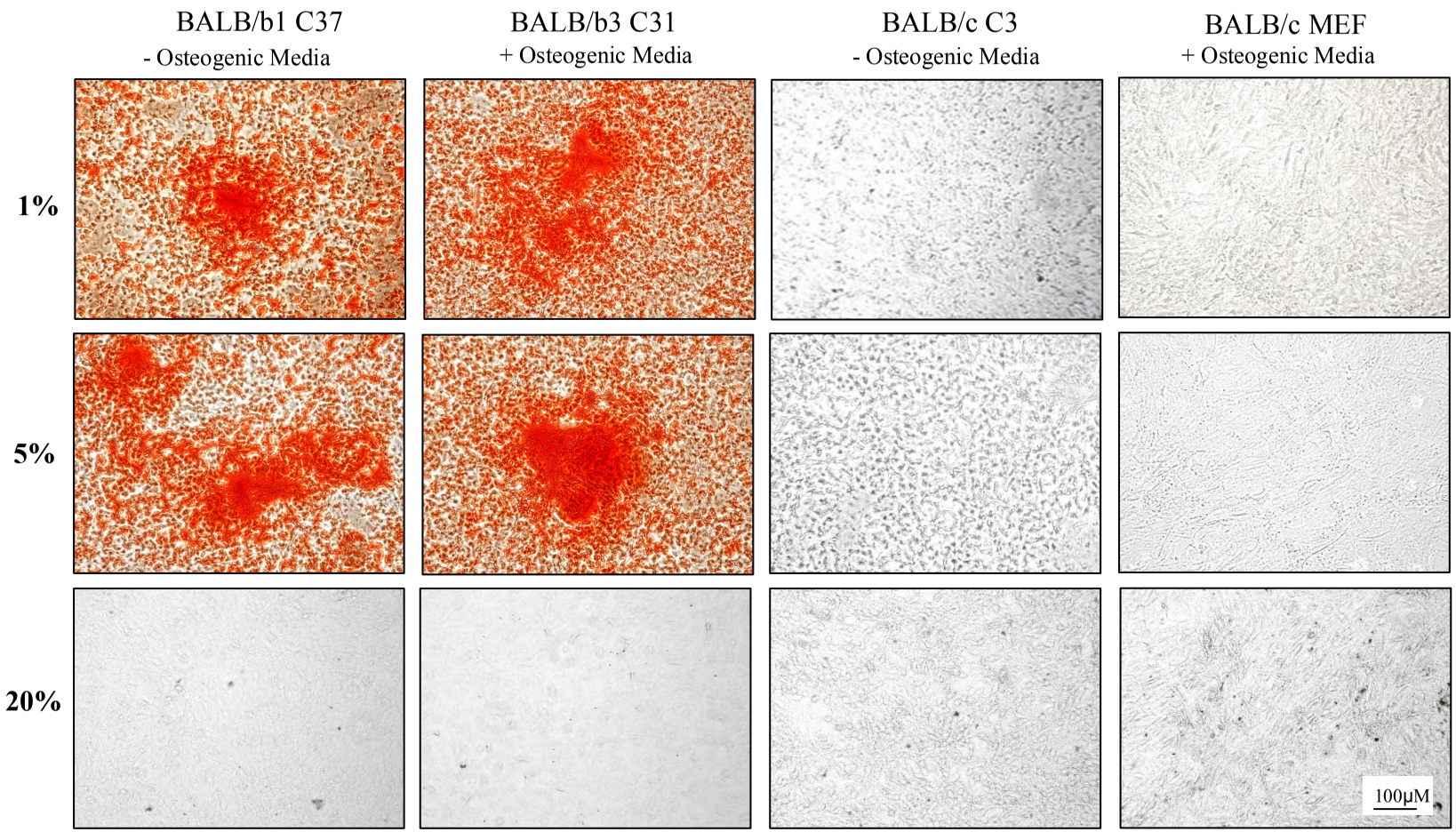


Figure 3.9 Osteogenic differentiation of BALB/b clones. Clonal cells and mouse embryonic fibroblasts (MEFs) were primed in minimal media (α-MEM media 5% FBS and ascorbic acid 2-phophate (A2-P)) for 3-4 days. Cells were re-seeded at 5000cells/cm^2 and cultured in the presence or absence of osteogenic media (A2-P, β-glycerol phosphate, and 10 ng/ml BMP-2) at 20%, 5%, and 1% oxygen tension for 4 weeks. Mineralised matrix production was visualised by alizarin red staining.

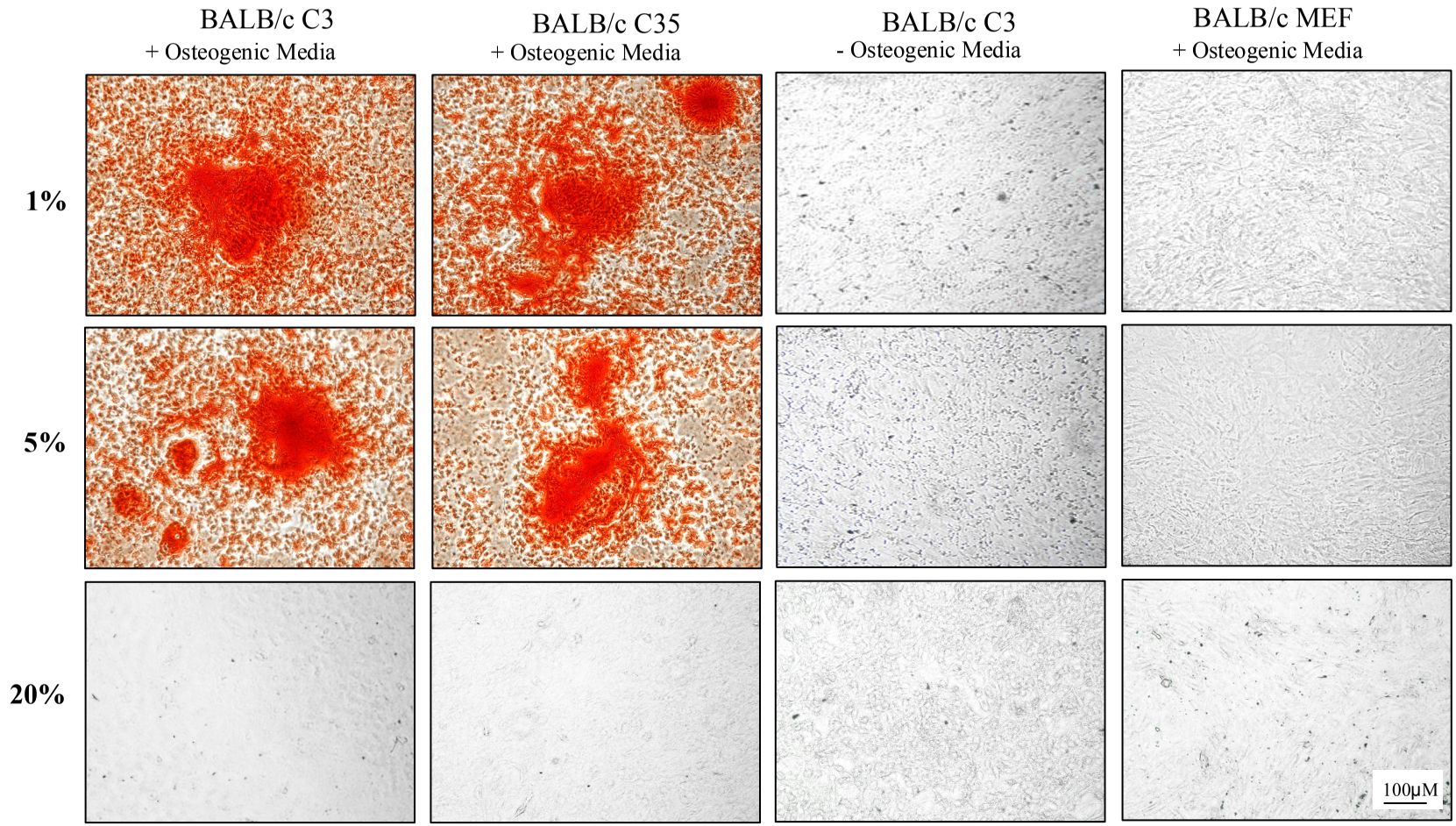
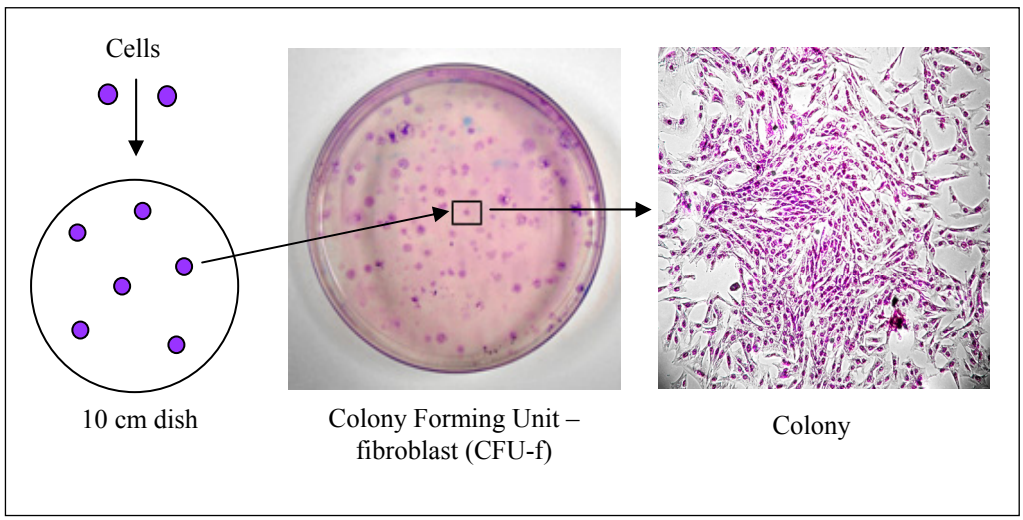


Figure 3.10 Osteogenic differentiation of BALB/c clones. Clonal cells and mouse embryonic fibroblasts (MEFs) were primed in minimal media (α-MEM media 5% FBS and ascorbic acid 2-phophate (A2-P)) for 3-4 days. Cells were re-seeded at 5000cells/cm^2 and cultured in the presence or absence of osteogenic media (A2-P, β-glycerol phosphate, and 10 ng/ml BMP-2) at 20%, 5%, and 1% oxygen tension for 4 weeks. Mineralised matrix production was visualised by alizarin red staining.

Table 3.5 Clones are multipotent. All BALB/b and BALB/c clones analysed had the ability to differentiate into adipocytes, chondrocytes and osteoblasts when cultured under the appropriate conditions.

Clone	Adipogenesis	Chondrogenesis	Osteogenesis
BALB/b1 C5	✓	✓	✓
BALB/b1 C15	✓	✓	✓
BALB/b1 C20	✓	✓	✓
BALB/b1 C32	✓	✓	✓
BALB/b1 C36	✓	✓	✓
BALB/b1 C37	✓	✓	✓
BALB/b2 C7	✓	✓	✓
BALB/b3 C3	✓	✓	✓
BALB/b3 C29	✓	✓	✓
BALB/b3 C33	✓	✓	✓
BALB/c C3	✓	✓	✓
BALB/c C4	✓	✓	✓
BALB/c C11	✓	✓	✓
BALB/c C19	✓	✓	✓
BALB/c C25	✓	✓	✓
BALB/c C29	✓	✓	✓
BALB/c C35	✓	✓	✓

A



B

Clone	Passage	CFU-f (5% O_2)
BALB/b1 C37	p6	72
BALB/b1 C37	p52	67
BALB/b3 C33	p7	70
BALB/b3 C33	p53	75
BALB/c C35	p6	65
BALB/c C35	p52	68

Figure 3.11 Clones have the Ability to Form Colonies. Cells were seeded at a low density (1000cell/ 10cm dish) and after two weeks colonies were stained with the giemsa stain (**A**). The number of colonies was counted and the CFU-f calculated (**B**). CFU-f: colony forming unit-fibroblast.

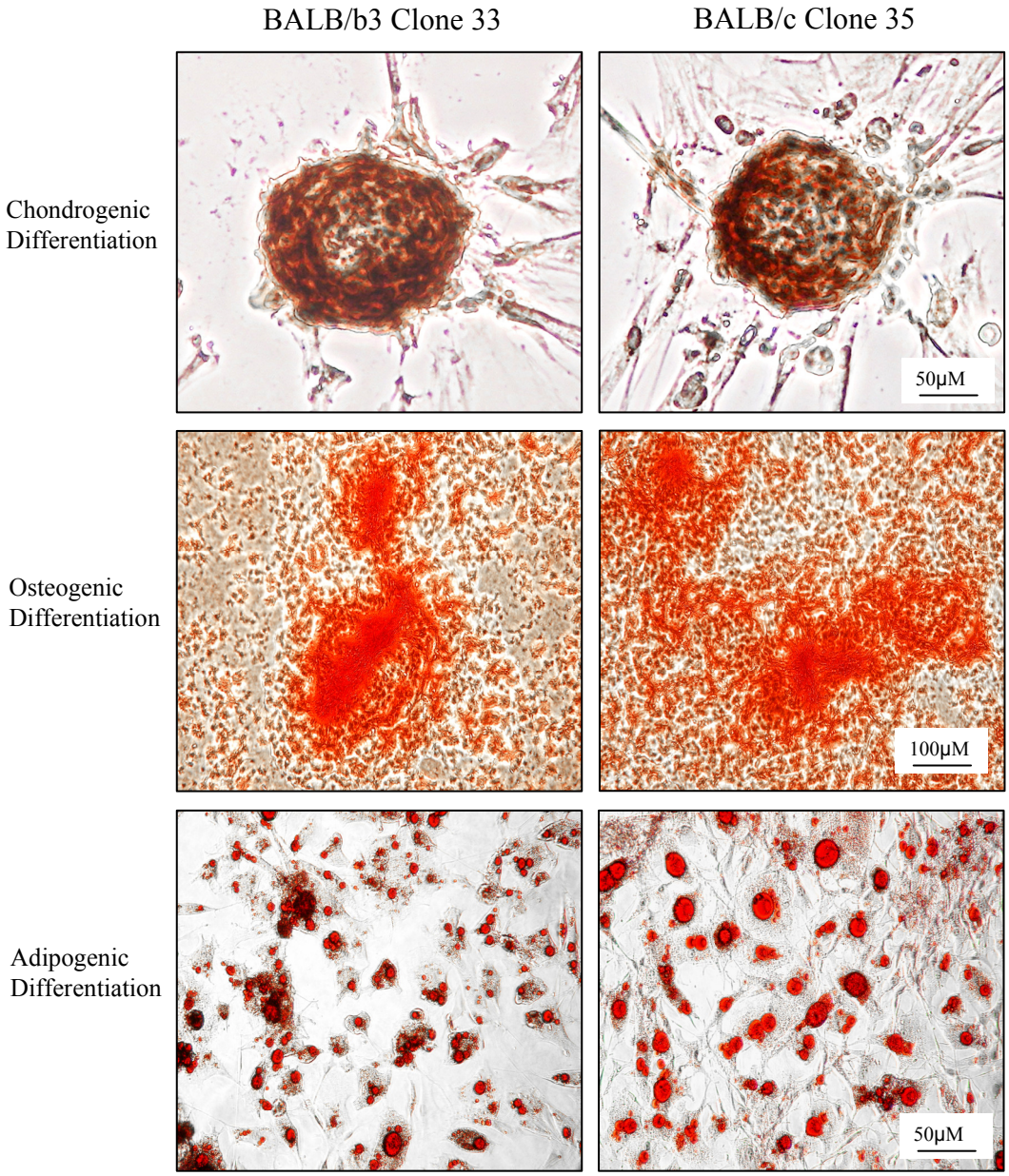


Figure 3.12 Clones Maintain Differentiation Capacity with Passage. Clonal cells were cultured in complete medium at 5% oxygen tension and passaged every 3-5 days when ~70% confluent, until passage 42. The differentiation ability of the clonal cells was assessed as indicated in figures 3.4-10.

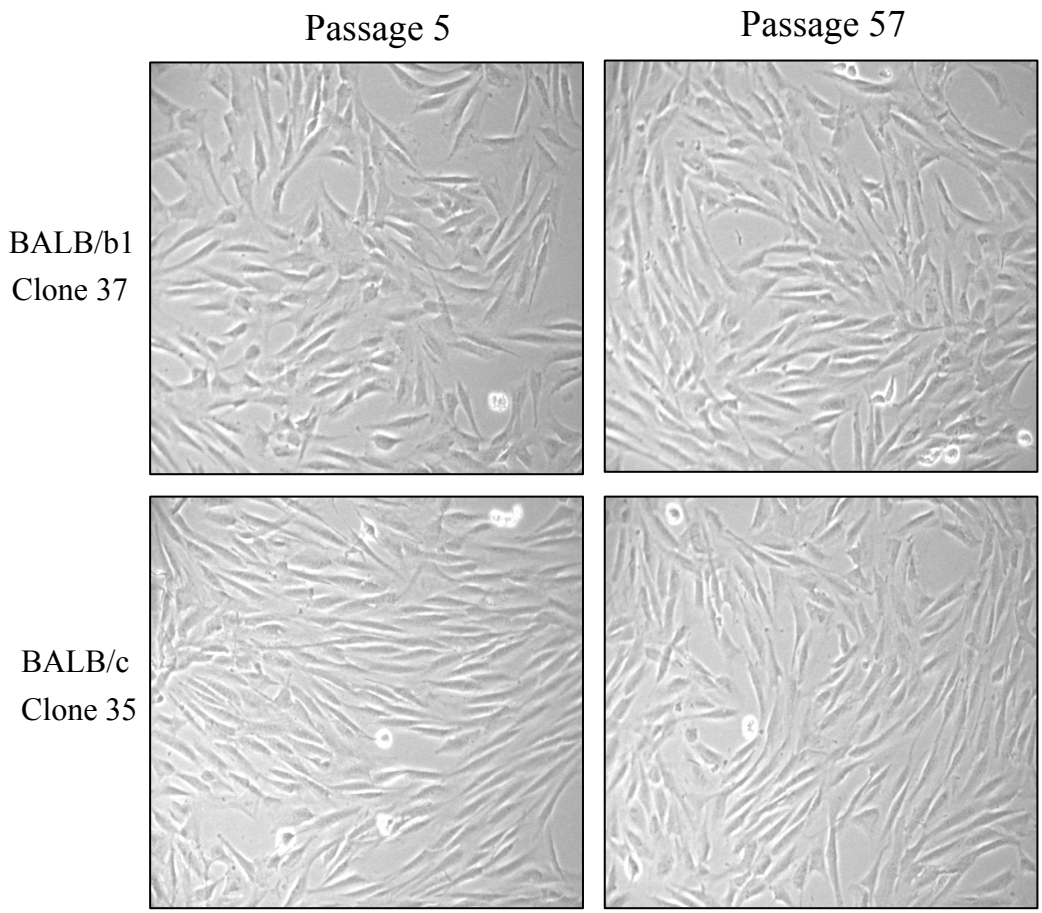


Figure 3.13 Clones Maintain Morphology with Passage. BALB/b and BALB/c clones were cultured in complete medium at 5% oxygen tension and passaged every 3-4 days when ~70% confluent.

status. In addition clonal cells cultured at 5% oxygen showed no changes in their morphology with time. (figure 3.13).

3.7 Discussion

Mesenchymal stem cells (MSCs) are currently being used in clinical studies to treat a myriad of diseases such as Osteogenesis Imperfecta (Horwitz et al., 1999), coronary heart disease (Orlic et al., 2001), Crohn's disease (Garcia-Olmo et al., 2003; Garcia-Olmo et al., 2005), and metachromatic leukodystrophy (Koc et al., 2002a) among others (Barry, 2003). Results have been promising, however, given the increasing therapeutic use of MSCs, surprisingly little is known about their basic biology. In fact, a specific surface marker that can be used to identify and purify them from other cells types has yet to be identified. The lack of a specific marker means a mixed population of cells, termed 'MSC,' are transplanted in the patient. Obviously it is not ideal to transplant such an undetermined population of cells, and it may be that a purer population may enhance the regenerative effect.

Protocols for isolating MSCs vary considerably due to the absence of a known MSC marker. The rarity of MSCs in the bone marrow further complicates matters (estimated 0.001-0.01% MSCs in total BMMNC population) (Pittenger et al., 1999). Murine MSCs are sometimes isolated by simply culturing the whole bone marrow or by centrifuging cut long bones to obtain all marrow cells (Peister et al., 2004), which can result in contamination by other adherent cells. In both cases the resulting 'MSCs' are obtained by culturing the adherent cells. Variations in cell culture protocols such as tissue culture surface (D'Ippolito et al., 2004), seeding densities (Colter et al., 2001), media (Peister et al., 2004), serum, and the addition of various growth factors (Jiang et al., 2002; Muraglia et al., 2000) further complicates matters. These procedures result in cultures with varying characteristics, but all termed MSCs. However, it is crucial to obtain a pure population of cells if we are to further our understanding of fundamental MSC biology. Thus, a consistent unbiased purification method is required for the routine isolation of these cells.

A major difficulty in culturing murine MSCs is haematopoietic contamination. One group tried a slightly extreme approach by culturing murine MSCs with a cytotoxin potassium thiocyanate to poison macrophages and other haematopoietic cell types- no assessment was made regarding the multipotentiality of the resulting cells (Modderman et al., 1994). Another group attempted to isolate MCSs from the bone marrow by negative depletion based on known haematopoietic markers (Baddoo et al., 2003). The problem with this approach is that it is biased, and since it is possible that MSCs also express these markers, the true MSC population may be missed. Wang and Wolf demonstrated that when murine bone marrow cells are seeded at a low density colonies arise that appear to be free from haematopoietic contamination. However, the method they used was impractical as it generated only 27 spindle shape colonies of 5 or more cells from a total of 200 culture dishes (Wang and Wolf, 1990). They cultured cells at the standard 20% oxygen tension. We hypothesized that culturing marrow-derived cells at the more physiologic 5% oxygen may prove to be more efficient. Indeed, observations from previous studies on MSCs have suggested that cultivation of MSCs in a reduced oxygen environment could enhance its colony forming ability, proliferation, self-renewal, and differentiation capacity (Grayson et al., 2006; Lennon et al., 2001)

Two important aspects of our study are (i) the need to obtain pure populations of stem cells and (ii) their cultivation under physiologically relevant conditions, specifically reduced oxygen tension. Murine bone marrow was isolated by flushing the marrow from the long bones which is a more sterile removal of the bone marrow. To better approximate the *in vivo* stem cell environment the cultures were exposed to a physiologically relevant oxygen tension of 5% as well as the standard 20%. This is an important aspect of our work. We found cell survival at 20% oxygen in low density cultures was very poor. In fact, in low density cultures, clones could only be derived in reduced oxygen conditions. This suggests that adherence and/or initial growth of clonal cells is enhanced at 5% oxygen tension.

Using this simple but effective protocol clones were obtained from mouse strains, BALB/b and BALB/c. Despite the initial lag phase required for clonal growth (Peister et

al., 2004), the cell doubling time of the clones is quite short ranging from 1.4 ± 0.3 days to 2.9 ± 1.7 days (mean ± SD). Murine MSCs isolated by the Baddoo group had a cell doubling time of 4 to 7 days (Baddoo et al., 2003). FGF2 was required to enhance cell proliferation; however, exposure to FGF2 inhibited the differentiation ability of the cells. No growth factor was added to the clonal cells in our study. They were simply exposed to an oxygen environment which better approximates their *in vivo* environment.

Clonal cells were first isolated from BALB/b mice due to initial studies by colleagues in our institute on MSCs from this strain and our preliminary studies on these cells highlighted their ease of use i.e. their colony forming ability and capacity to differentiate. Clonal cells were also isolated from the BALB/c strain due to their use in previous MSC studies (Peister et al., 2004) (Zhou et al., 2006). We did attempt to derive clonal cells from other common mouse strains, FVB/N and C57Bl/6. Unfortunately, clonal cells from FVB/N mice became infected and we were not successful deriving clonal cells from C57Bl/6 mice. Further attempts were not successful for the FVB/N strain. It is possible that the culture conditions used were not optimal for colony formation from these strains. Indeed, a previous study has reported that MSCs from different mouse strains vary in the requirements for optimal media conditions, which include α-MEM, DMEM, RPMI-1640, and IMDM (Peister et al., 2004).

Mets and Verdonk were the first to report the presence of two cell types in human bone marrow stromal cell cultures (Mets and Verdonk, 1981). Type I cells were spindle-shaped and rapidly dividing. Type II cells were flatter and broader and divided more slowly. They found the majority of cells in primary culture are type I. However, with passage the number of type II cells increases. Their interpretation was that type I cells are progenitor cells which give rise to type II terminally differentiated non-dividing cells, by asymmetric cell division. Further studies from various groups have found that the 'quality' of the MSC can be correlated to the cell morphology (Bruder et al., 1998; Sekiya et al., 2002). In addition the spindle shape cells are thought to expand rapidly, as they do not synthesize the large amounts of extracellular matrix that the broader, flatter cells do (Phinney et al., 1999).

The clonal cells that were isolated in this study were very similar in phenotype, they were relatively small and spindle shaped. In contrast, most groups report a variation in the size and morphology of clones (Digirolamo et al., 1999; Muraglia et al., 2000; Pittenger et al., 1999). It should be noted that these studies isolated colonies from a mixed population of bone marrow derived adherent cells cultured at 20% oxygen.

The differences between our clonal cells and the clonal cells in the above studies are twofold. Firstly, our cells were derived in a 5% oxygen environment which is more physiologically relevant (Kofoed et al., 1985). Cultures exposed to supra-physiological (20%) oxygen tensions may be incurring cell damage, e.g. due to reactive oxygen species (Csete, 2005). The higher oxygen concentration may also be inducing differentiation of the stem/progenitor cells. These stem cells are receiving signals they would not normally in the *in vivo* niche. Secondly, we obtained clonal cells directly from freshly isolated BMMNCs that were seeded at a low density which selects for cells with a high CFU-f ability, typically stem cells - this is an important aspect of our approach. Other studies seed BMMNCs at a high initial density, which also tends to encourage growth of other adherent cell types. Such adherent heterogeneous population therefore contain stem cells, progenitor cells, and committed cells. Typically, after subsequent passaging, colonies are then derived from these cultures and it is not surprising that they are heterogeneous in terms of morphology and differentiation. Finally, culturing cells at 20% is not conducive to maintenance of the stem cell phenotype. Many studies have now reported that cultivation of cells at low oxygen maintains cell 'stemness' (D'Ippolito et al., 2006). In our method we are not only selecting for stem cells, we are culturing them in a low oxygen environment which maintains their stem cell phenotype.

The Post group have recently reported the presence of 2 independent pre-osteoblastic and pre-adipocytic clonal populations in murine MSCs (Post et al., 2008). Both cell types' exhibit different morphologies, the pre-adipocytic had a round oval shape and the pre-osteoblastic a fusiform, fibroblast-like morphology. In addition to the spindle shaped cells, clonal cultures also contained a number of very small cells. These have previously

been reported by Colter and colleagues in their human MSC cultures (Colter et al., 2001). They found these cells exhibited a high self-renewal ability and greater multidifferentiation potential.

The results of the present study suggest that low oxygen has maintained morphology of clonal cells. At 5% oxygen the cell morphology is the same at high passage (after over 200 population doublings) as it was at low passage. The finding was further highlighted in the CFU-f assay where cells were seeded at a low density. At 5% oxygen cells formed colonies and were spindle shaped. However, at 20% and 1% oxygen tension the cells became broader, flatter and failed to form colonies. Thus, it seems the morphology of the MSC is an important indicator of the quality of the MSC. Many studies have reported that low oxygen increases CFU-f ability of stem cells (Fehrer et al., 2007; Grayson et al., 2006; Lennon et al., 2001). Thus, oxygen tension is an extremely important signal and should be given a greater degree of consideration when cultivating stem cells.

Each colony in a CFU-f assay is assumed to arise from a single cell since the cells are seeded at a low cell density and the subsequent colonies arise in distinct locations in the dish. Thus it follows that the total number of colonies obtained reflects the number of MSCs in the BMMNCs. In the BALB/b1 isolation thirty-seven individual colonies were obtained from 20 x 10^6 BMMNCs. This gives a MSC percentage of 0.0002 % in the BMMNCs. This figure is most likely an underestimation for two reasons: the first is there were still some red blood cells present after the cells had been processed with a red blood cell lysis buffer, and may have been included in the cell count of BMMNCs. The second is that not all the colonies were isolated since those partially merged were omitted, as were smaller sized colonies. Therefore the MSC percentage is underestimated. However, clones in this study were isolated from murine BMMNCs whilst the percentage expression that the Pittenger group found (0.001-0.01%) was obtained from human BMMNCs (Pittenger et al., 1999). It is possible that there is a species difference in the quantity of MSCs present in the bone marrow. Also in this study the MSCs are derived from immature mice, whereas the MSCs were derived from adults by the Pittenger group.

A defining property of mesenchymal stem cells is their ability to differentiate into the adipogenic, chondrogenic and osteogenic lineages. All the clones examined displayed a capacity to differentiate into these lineages when given the appropriate environmental cues (figures 3.4-3.10 and table 3.5). Adipogenesis was demonstrated by the formation of abundant lipid-rich vacuoles in cells. The formation of chondrogenic nodules, that contained type II collagen confirmed chondrogenesis. Mature osteoblast formation was visualised by the presence of a mineralised matrix which stains with Alizarin red. It is important to note that a low concentration of chemicals was used for adipogenesis in comparison to previous studies. Also chondrogenesis was induced by the addition of just 5 ng/ml TGF- β3 and without dexamethasone which is commonly added. Osteogenesis was also induced in the absence of dexamethasone which is widely used. Instead the cytokine BMP-2 was added.

In previous studies the control used for differentiation experiments was 'MSCs' cultured in the absence of the differentiation media. It is then difficult to tell whether the differentiation has physiological meaning or has occurred due to the addition of particularly strong pharmacological stimuli, which could induce differentiation in many different cell types. Thus in the present study strain-matched mouse embryonic fibroblasts (MEFs) were also used. The MEFs displayed no signs of differentiation when cultured in induction media, indicating that the differentiation capacity was specific to the clones.

Oxygen tension had a significant effect upon chondrogenesis. Low oxygen levels enhanced the number of chondrogenic nodules formed as well as the intensity of type II collagen staining in comparison to 20% cultures (figures 3.6-7). A further study in which 1 ng/ml TGF-β3 was added resulted in no or few nodule formation in the 20% cultures. However, it only took slightly longer (5-7 days) for the cultures at 5% and 1% to form nodules with 1ng/ml TGF-β3 compared to 10ng/ml. This corresponds with the recent results of Kanichai and colleagues who found that hypoxia (2% oxygen) promotes chondrogenesis in rat mesenchymal stem cells possibly through an AKT and HIF-1α mechanism (Kanichai et al., 2008).

We also found that the clonal cells expressed HIF-1α, at both 5% and 1% oxygen tension in comparison to 20% where there was negligible expression (figure 3.14), which is consistent with the above findings. Recent studies in our laboratory have demonstrated that HIF-2α is important for hypoxic induction of cartilage matrix genes in human articular chondrocytes (Lafont et al., 2007). In future studies it will be interesting to see which isoform, HIF-1α or HIF-2α or both are important for chondrogenic differentiation from MSCs.

Low oxygen tension (5%) also enhanced adipogenesis. A greater percentage of cells contained lipid-rich droplets compared to 20% cultures. However, in the lower 1% oxygen tension, although differentiation occurred, a confounding factor was that many cells sloughed off the tissue culture substrate. Hence 5% oxygen tension was optimal for adipogenesis from MSC clones.

Osteogenesis was enhanced by low oxygen tension. All clones displayed the ability to differentiate into immature osteoblasts, as all the clones that were tested stained positive for alkaline phosphatase (figure 3.8) (Jaiswal et al., 1997). This was evident at all oxygen tensions. The deposition of a mineralised matrix is a sign of a mature osteoblast. All clones formed a mineralised matrix in the presence of osteogenic media containing 10 ng/ml of BMP-2 at 5% and 1% oxygen tension. Strikingly, there was no staining evident in cells cultured at 20% oxygen tension although the cells were viable. We used alizarin red staining to demonstrate mature osteoblast formation as it stains the calcium matrix deposited by mature osteoblasts. Some groups show alkaline phosphatase staining, which is a non-specific stain and, at best, an indicator of immature osteoblast staining.

An interesting point to note is that osteogenic differentiation only occurred when the cells were in 3 ml of differentiation media and not 2 ml. This could be due to the fact that bone nodules are only thought to form at a very narrow pH range (7.2- 7.4). A decrease in pH was found to abolish mineralised bone nodule formation in rat osteoblasts (Brandao-Burch et al., 2005). We found the increased culture media volume better buffered the pH and promoted mineralised matrix formation by the clonal cells. When 2 mls of differentiation media was used the culture media became yellow indicating increased

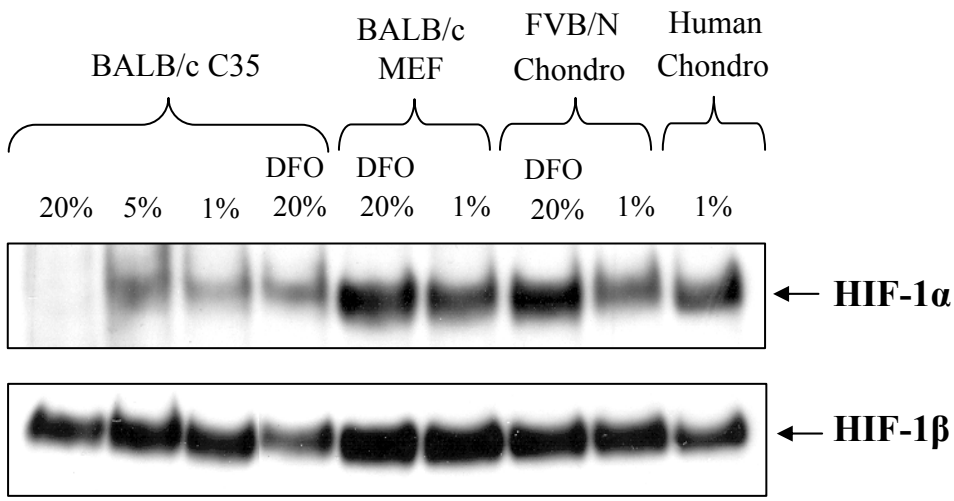


Figure 3.14 MSC clones exposed to Low Oxygen Tension express HIF-1α. Cells were seeded at a density of 0.2 x 10^6 cells in a 3.5 cm tissue culture dish and cultured at the oxygen tension (20%, 5%, and 1% oxygen) indicated. One day later, 250 µl desferrioxamine (DFO) was added to one 20% oxygen dish and returned to the 20% incubator. Cells were cultured for another 24 hours and then lysed. HIF1-α protein expression was examined by western blot analysis. FVB/N chondro (chondrocytes) were derived from the hip bones of 6 week old FVB/N mice. Human chondrocytes from the articular cartilage in the femoral condyle Chodrocytes, (Chondro). All cells at passage 2-4.

acidity. In summary the enhanced osteogenesis at low oxygen tension is consistent with the findings by Lennon and colleagues that found osteogenic differentiation of rat MSCs is enhanced at 5% oxygen tension compared to 20% (Lennon et al., 2001). However, 3% oxygen tension has been reported to inhibit osteoblast differentiation of human marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al., 2006). This could be due to a difference in species, however, it is perhaps more likely to be due to different *in vitro* culture conditions. The rat MSCs from the Lennon group and the clonal cells in this study are cultured in basal medium with 10% serum. In contrast, the MIAMI cells are cultured at 3% oxygen in the presence of EGF, PDDGF-BB, 2% FBS, and on fibronectin coated plates. Apart from hypoxia, the presence, let alone concentration of these growth factors in the *in vivo* MSC niche are unknown. Interestingly, in a previous paper the group reported that MIAMI cells did differentiate into the osteogenic lineage when cultured at 3% oxygen (D'Ippolito et al., 2004).

Our studies have found low oxygen tension enhances the differentiation potential of clonally-derived MSCs. There are some studies which report opposing results however. Adipose-derived murine MSCs show enhanced proliferation, but reduced chondrogenesis and osteogenesis when cultured in 2% oxygen tension (Malladi et al., 2006a). The same group do report in a later study that priming adipose-derived murine MSCs in 2% oxygen tension enhanced early chondrogenesis, but diminished early osteogenesis (Xu et al., 2007). The inhibiting effect of low oxygen on the differentiation potential of the adipose-derived MSCs may be explained by a few reasons. As mentioned above adipose-derived cells are exposed to a greater oxygen tension *in vivo* compared to bone marrow MSCs, as it is a vascularised tissue. This could be why the cells did not respond to the reduced oxygen atmosphere. In addition, there may be functional differences between MSC populations isolated from different tissues. The Wagner group found that MSCs isolated from the bone marrow were superior to adipose derived MSCs in terms of adhesion of haematopoietic precursor cells and their maintenance in an undifferentiated state (Wagner et al., 2007). Also, they found bone marrow MSCs are superior in their haematopoietic supportive function. Studies with cells isolated from the bone marrow exhibit enhanced colony-forming capabilities, proliferation, and differentiation in response to low oxygen

tension (Fehrer et al., 2007; Kanichai et al., 2008; Lennon et al., 2001). Indeed, recent studies by the Grayson group on human MSCs grown in 3D scaffolds at 2% oxygen tension found that cells subsequently exhibited enhanced colony-forming ability, better maintained stem cell property, and enhanced adipogenesis and osteogenesis (Grayson et al., 2007; Grayson et al., 2006). Our results are more consistent with those from the Lennon group which found that 5% oxygen enhanced most properties of the rat MSC including colony forming ability, *in vivo* and *in vitro* osteogenesis, and *in vivo* chondrogenesis. In addition we found low oxygen enhances initial colony formation, *in vitro* adipogenesis and chondrogenesis.

Another functional criterion by which a stem cell is defined is its ability to self renew. The stem cell property of the HSCs can be demonstrated by their ability to re-constitute the haematopoietic system after bone marrow transplantation. This is not possible for MSCs. The best way this can be demonstrated *in vitro* is by their ability to maintain their differentiation potential and to form colonies from a single cell after extensive proliferation. The clones derived in this study had the ability to self-renew, as witnessed by the fact that they retained their ability to form colonies at a very high passage without loss of multi-lineage differentiation capability. The spindle shaped morphology of the clone was maintained which is consistent with the literature in being high in self-renewal, i.e. colony forming ability and differentiation (Sekiya et al., 2002). The CFU-f assay is reported to be a good predictor of the life span of the cells (Digirolamo et al., 1999), with high CFU-f efficiency indicating the greatest replicative potential. This seems to be true for the clones as they were cultured to passage 58 with no sign of replicative senescence. Thus the clonal cells exhibited both of the functional characteristics used to define the MSC; they were tri-potent and had the continued capacity to self-renew.

It is important to note that of the seventeen clones tested all were tri-potent. In addition, the differentiation was very comprehensive. As evidenced by the data in figures 3.4-3.10, almost every cell differentiated into the specific lineage, as they all stained with the appropriate stain. Also there were no spindle shaped cells characteristic of MSCs present; there is a morphology change in all cultures to the differentiated phenotype. There was

also no heterogeneity in the differentiation potential of the clones and this was not lost over time, in contrast to previous studies (Colter et al., 2001; Digirolamo et al., 1999; Kuznetsov et al., 1997; Muraglia et al., 2000; Pittenger et al., 1999). Pittenger and colleagues found that only one third of their clonal cells were tri-potent. This could be because the clones were isolated from previously passaged cultures, and were continuously exposed to 20% oxygen. As discussed above such cultures could contain committed cells, or MSCs that had lost their multilineage differentiation potential with culture at such supra-physiological levels of oxygen. Extensive studies by the Muraglia group also reported similar results (Muraglia et al., 2000). The investigators report that 17% of clones were tri-potent whilst the remaining were bi-potent or uni-potent. However, 34% of the clones were tri-potent when they had been cultured in the presence of FGF2, prior to the differentiation assays. This growth factor is known to preferentially select for a certain subset of cells, one with a higher proliferation and osteogenic potential (Martin et al., 1997). Indeed the investigators report all their clones had this differentiation potential, if not the adipogenic and the chondrogenic potential. One disadvantage of adding growth factors is that it is biased to selective enrichment of subsets of cells, which may not necessarily contain the stem cell of interest.

It has almost become an accepted fact that bone marrow derived clones are heterogeneous in terms of morphology and differentiation potential. The Tuan group discusses the heterogeneity of clones at length, hypothesising that 'MSC' population contain not just the putative tripotent MSC but also subpopulations that are bi- and uni-lineage (Baksh et al., 2004). Another way to look at this is that the cell population termed MSC is usually heterogeneous because it contains other committed cell types such as immature osteoblasts, adipocytes etc, which can only differentiate into one lineage. A recent report has documented the presence of two clonal MSC lines (mMSC1 and mMSC2) derived from murine bone marrow (Post et al., 2008). Both cell lines exhibit different morphologies as mentioned previously. Interestingly, mMSC1 could only differentiate into adipocytes and mMSC2 into osteoblasts. This supports the hypothesis that MSC populations can contain sub-population of cells that are committed to one lineage. It is important to note that the committed progenitor cells did not have the

characteristic spindle shape of our multipotent clones. Yet another possibility is that there is something missing from the cell culture conditions that maintain the 'stemness' of the stem cell. In its absence the cells lose their ability to maintain their multi-lineage differentiation potential and become more committed progenitor cells. We propose that reduced oxygen tension is such a parameter.

One likely reason our clones are so convincingly tri-potent may be due to the fact that they are single-cell derived from primary bone marrow cultures – isolated and cultured at a more physiological oxygen tension which helps maintain the 'stemness' of the cells. Indeed we found differentiation and morphology of the clones was very similar, and both were retained with extensive passaging.

Oxygen tension has an effect on many aspects of stem cell biology, such as proliferation, differentiation, self-renewal, cell death, and migration. The oxygen concentration in arterial blood, lungs and liver is 10-13% (Grant and Smith, 1963; Kofoed et al., 1985). It can be as low as 1% in avascular tissue such as cartilage. The oxygen tension in the bone marrow is 4-7% (Kofoed et al., 1985). In conventional cell culture 20% oxygen tension is used, which is supra-physiological tension for the cells. Cells have evolved ways to defend themselves from the toxic effects of free radicals derived from high oxygen concentrations. These include the enzymes glutathione peroxidase, catalases, and superoxide dismutase, as well as antioxidants ascorbic acid (vitamin C), uric acid, and vitamin E (Frank and Massaro, 1980; Frank et al., 1980). However, these mechanisms may not be effective when the oxygen tension is as high as 20%, especially under chronic exposure. Thus, it is possible that such prolonged exposure to an unusually high oxygen tension may hinder many cell processes and this is why we see greater proliferation, differentiation, and colony formation when the cells are exposed to a more physiological oxygen tension (5%).

There are differences between this study and previous ones which make direct comparisons difficult. This is the first study that has isolated a large number of individual murine clones and functionally defined them as MSCs. Another particular aspect of the present study is that we took great care to isolate clones from primary (not passaged)

bone marrow cultures, and in 5% oxygen tension. The effect of oxygen tension on tri-potent differentiation of the clones was examined under 3 different oxygen conditions - hypoxic conditions (1%); low, but more physiological levels (5%); and under standard *in vitro* conditions (20%). From these studies we found that cultivation of these cells at 5% oxygen is optimal. The clones were very similar in morphology, size, growth rate, and differentiation capacity, and importantly these qualities were maintained with extensive subculture. We believe that these results originate from the fact that we isolated a pure population of cells from the bone marrow using an unbiased purification method and cells were cultured at a physiological oxygen tension (5%) which helped maintain their stem cell properties. The subject of the next chapter will be the characterization of the clonal cells and investigation of the effect of oxygen tension on their growth rate.

Chapter 4

Characterisation of MSC Clones

4.1 Introduction

The purpose of the work detailed in this chapter was to investigate the *in vitro* characteristics of clonal cells described in chapter 3. Their mesenchymal stem cell status has been functionally determined by their ability to differentiate into the three classical mesenchymal lineages (adipocytic, chondrocytic and osteoblastic) and by their capacity to self-renew. The surface antigen profile of the clones was extensively examined using flow cytometry. In the previous chapter we reported that low oxygen tension (5%) promoted colony formation and differentiation. We hypothesised it may also enhance growth rate. Therefore the growth rate of cells was examined at three different oxygen tensions. Stem cells are known to have the capacity to maintain their telomere lengths with extensive passaging and to express telomerase. Both of these were examined, as was the genetic stability of the clones, and their ability to form tumours was assessed following local and systemic administration.

4.2 Surface phenotype analysis of BALB/b clones

The surface antigen profile of 6 BALB/b clones was examined by flow cytometry using the markers listed in Table 4.1 and compared to strain matched mouse embryonic fibroblast (MEF) and bone marrow mononuclear cells (BMMNCs). All the clones and the MEFs were positive for CD49e and to a lesser extent to CD29, CD44, and CD80 and MHC-Class I (figure 4.1, table 4.2). An important point to note here (as demonstrated by the FACS histograms) is that the clones were homogeneous as they stained uniformly for the surface epitopes, 100% of the population is present in one tight peak in the histogram, and they are not distributed throughout the histogram in sub-populations. The BALB/b clones are also positive for CD34, the haematopoietic marker, and indeed murine MSCs have previously been reported to express CD34 (Peister et al., 2004). In agreement with the literature the clones were negative for CD117 (c-Kit) and MHC Class II (Peister et al., 2004). Previous studies have reported that murine MSCs do not express the haematopoietic marker CD45. However, two of the clones expressed very low levels of this marker. Clones were also negative for CXCR-4 (Wynn et al., 2004). There were two

Table 4.1 Surface antigens examined by flow cytometry for BALB/b and BALB/c clones.

Antibody	Antigen
Sca-1	Stem cell antigen-1 / Ly6a
SSEA-1	Stage specific embryonic antigen 1
CXCR-4	Chemokine (C-X-C motif) receptor 4 (Fusin)
MHC-I	Major histocompatability class -I
MHC-II	Major histocompatability class -II
CD3	Thymocyte subset
CD11b	Leucocyte antigen
CD29	Integrin β1
CD31	Platelet endothelial cell adhesion molecule (PECAM)
CD34	Cluster of differentiation 34
CD44	Hyaluronan receptor
CD45	Leukocyte common antigen, Ly5
CD49e	Integrin α5
CD73	Ecto-5′-nucleotidase
CD80	Tetraspanin
CD90	Thy-1
CD105	Endoglin/ TGF-βR
CD106	Vascular cell adhesion molecule -1 (VCAM-1)
CD117	C-kit receptor (KIT)
CD166	Activated leukocyte cell adhesion molecule (ALCAM)

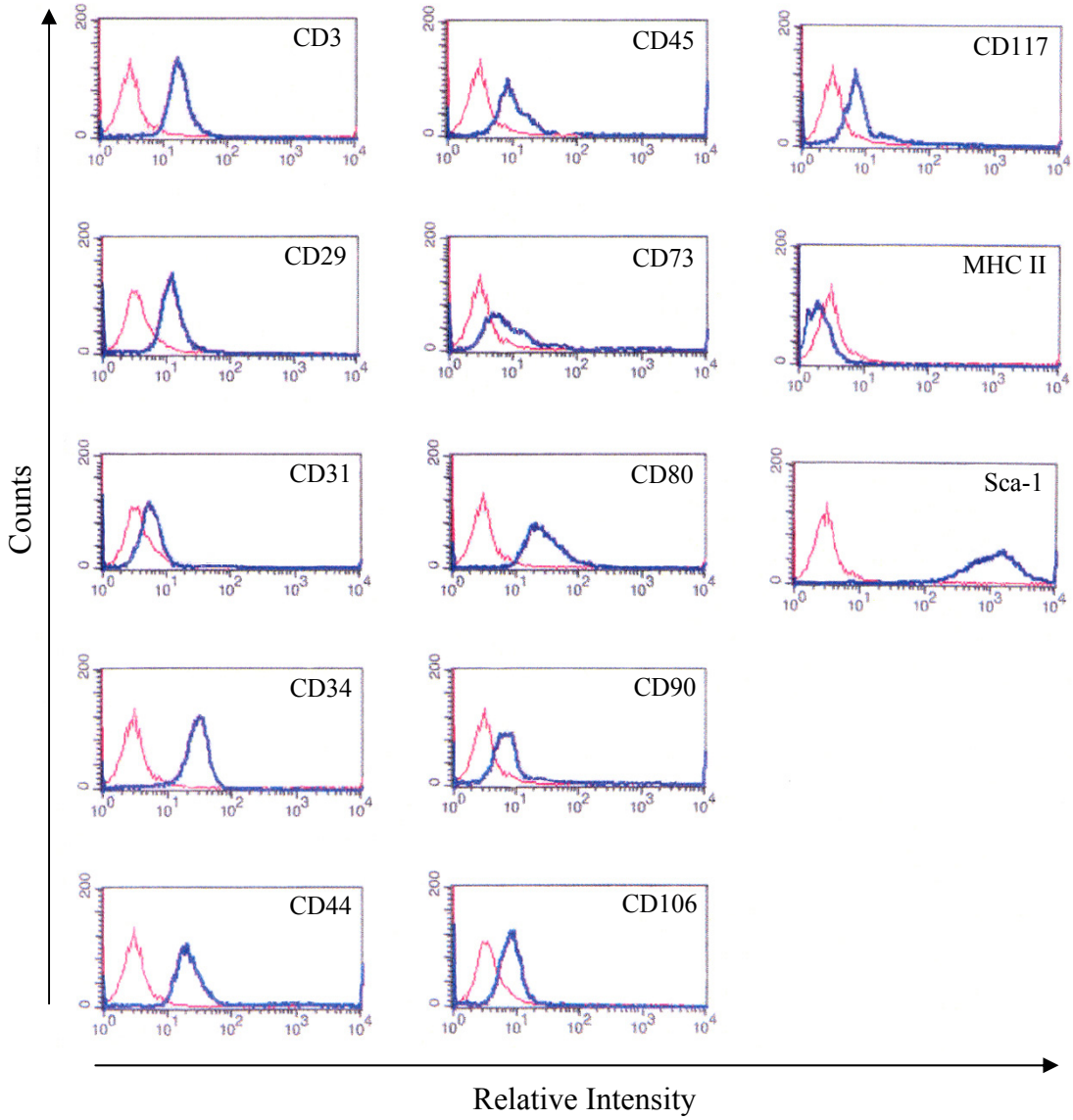


Figure 4.1 Surface antigen profile of BALB/b1 clone 37. Flow cytometric analysis of the surface proteins expressed by BALB/b1 clone 37 was performed by labelling cells with fluorochrome-conjugated antibody specific for each marker. Labelled cells were analysed by FACS using Cell Quest software. Results are representative of the surface antigen profile of other BALB/b clones. Red lines and blue lines in each histogram represent cells labelled with isotype matched control and specific monoclonal antibody, respectively.

Table 4.2 Flow cytometry characterisation of BALB/b clonal cells. BALB/b MSC clonal cells were harvested at 70% confluence and labelled with PE- or FITC- antibodies or isotype matched controls and analysed by flow cytometry at passage 3-6. The surface antigen profile of strain matched mouse embryonic fibroblasts (MEFs) and freshly isolated bone marrow mononuclear cells (BMMNCs) was also examined. Symbols refer to intensity of expression. Samples were scored as '-' if the population was in the first decade (same as the isotype control), '±' if it was between the first and second decade, '+' if it was in the second decade, '++' if it was in the third decade and '+++' if it was in the fourth decade, relative to the isotype control. ***** denotes clone at passage 53.

Antigen	UC5	UC15	UC20	UC32	UC36	UC37	UC37*	MEF	BMMNC
Sca-1	++	++	+++	+++	+++	+++	+++	+	-
SSEA-1	-	-	-	-	-	-	-	-	-
CXCR-4	-	-	-	-	-	-	-	-	-
MHC-I	+	-	±	±	+	+	+	+	-
MHC-II	-	-	-	-	-	-	-	-	-
CD3	±	-	-	+	±	+	-	-	-
CD11b	-	-	+	-	-	-	-	-	++
CD29	+	+	+	-	±	+	+	±	-
CD31	-	-	-	-	±	-	-	-	-
CD34	-	±	±	±	-	±	+	±	-
CD44	+	++	+	+	+	+	+	+	++
CD45	-	-	-	-	-	±	±	-	++
CD49e	++	++	++	++	++	++	++	++	±
CD73	-	+	-	-	-	+	+	±	±
CD80	+	+	+	+	+	+	+	+	-
CD90	-	-	-	-	-	-	-	+++	-
CD105	-	-	-	-	-	-	-	±	-
CD106	-	-	±	-	-	-	-	±	-
CD117	-	-	-	-	-	-	-	-	-
CD166	-	-	-	-	-	-	-	-	-

significant differences between the clones and the MEFs. All the clones were consistently very positive for the stem cell antigen -1 (Sca-1), which the fibroblasts expressed at a low level. Another difference was the clones were negative for the fibroblast marker CD90, which the MEFs expressed at a high intensity. The results demonstrate that the clones had a distinct phenotype to that of the MEFs. The surface antigen profile of clone 37 was also examined after extensive passaging (passage 53). The clone retained its surface antigen profile. The BMMNCs were positive for CD49e and CD73, to a lesser extent CD11b, CD44, CD45.

4.3 Surface phenotype analysis of BALB/c clones

The surface antigen profile of six BALB/c clones was also analysed by flow cytometry (figure 4.2, table 4.3). The clones displayed a remarkably similar surface antigen profile to the BALB/b clones (table 4.2). The clones were highly positive for CD49e and to a lesser extent for CD29, CD34, CD44, and CD80, which corresponded with the surface phenotype of the BALB/b clones. The 6 clones displayed variable expression of CD3, CD45, CD73, and CD106. Only two of the clones expressed fibroblast marker CD90 at a very low level, whereas the MEFs expressed it at a very high intensity, which is consistent with the result from the BALB/b MEFs. Like the BALB/b clones, the BALB/c clones were also negative for CD31, a marker for endothelial cells and they also lacked expression of MHC Class II. Again, a significant difference between the clones and the MEFs was that the clones expressed very high levels of Sca-1. BMMNCs were positive for CD45, CD49e, CD73, and slightly positive for the haematopoietic markers CD11b and CD117. The surface antigen profile of clone 35 was also examined after extensive passaging (passage 53). The clone retained its surface antigen profile during this period.

4.4 Growth rate of clones

To determine the growth capacity of the clones their population doubling rate was followed over time, for over 200 days. Cells were seeded at a density of 500-1000 cells/ cm^2 and passaged every 3-5 days when they were ~70% confluent thus ensuring cells were not contact-inhibited. Figure 4.3 shows the population doubling rate for BALB/b3

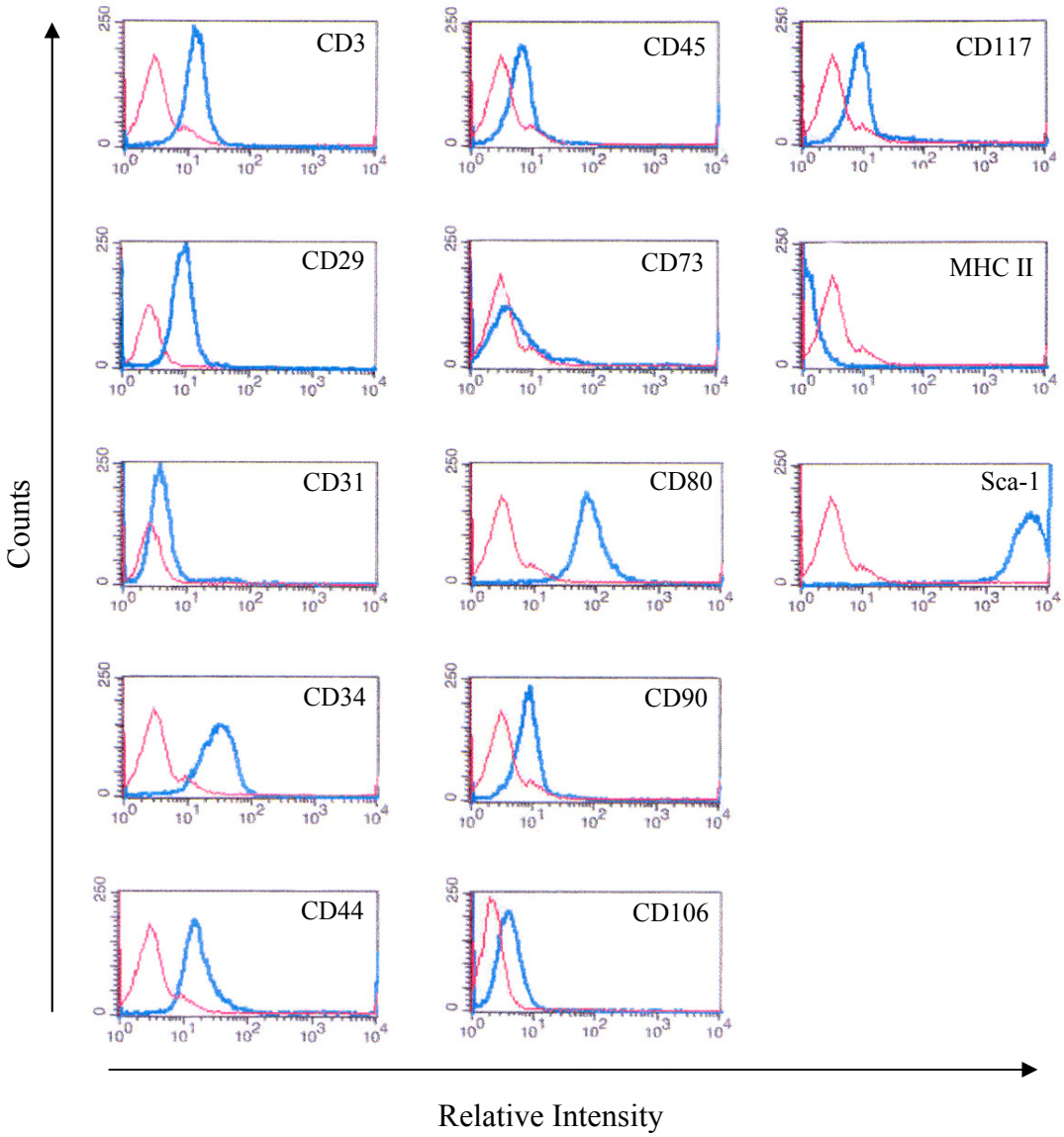


Figure 4.2 Surface antigen profile of BALB/c clone 35. Flow cytometric analysis of the surface proteins expressed by BALB/c clone 35 was performed by labelling cells with fluorochrome-conjugated antibody specific for each marker. Labelled cells were analysed by FACS using Cell Quest software. Results are representative of the surface antigen profile of other BALB/b clones. Red lines and blue lines in each histogram represent cells labelled with isotype matched control and specific monoclonal antibody, respectively.

Table 4.3 Flow cytometry characterisation of BALB/c clonal cells. BALB/c MSC clonal cells were harvested at 70% confluence and labelled with PE- or FITC- antibodies or isotype matched controls and analysed by flow cytometry at passage 3-6. The surface antigen profile of strain matched mouse embryonic fibroblasts (MEFs) and freshly isolated bone marrow mononuclear cells (BMMNCs) was also examined. Symbols refer to intensity of expression. Samples were scored as '-' if the population was in the first decade (same as the isotype control), '±' if it was between the first and second decade, '+' if it was in the second decade, '++' if it was in the third decade and '+++' if it was in the fourth decade, relative to the isotype control. ***** denotes clone at passage 53.

Antigen	UC3	UC4	UC11	UC19	UC25	UC35	UC35*	MEF	BMMNC
Sca-1	+++	+++	+++	+++	+++	+++	++	+	+
SSEA-1	-	-	-	-	-	-	-	-	-
CXCR-4	-	-	-	-	-	-	-	-	-
MHC-I	±	+	+	±	+	+	+	±	±
MHC-II	-	-	-	-	-	-	-	-	±
CD3	-	+	±	-	±	+	-	-	-
CD11b	-	-	-	-	-	-	-	-	++
CD29	±	+	±	±	+	±	+	±	-
CD31	-	-	-	-	-	-	-	-	-
CD34	+	+	±	+	+	±	+	-	-
CD44	+	+	±	±	+	+	+	±	++
CD45	±	+	±	-	-	±	±	-	+
CD49e	++	++	++	++	++	++	++	+	+
CD73	±	-	±	-	-	+	-	±	+
CD80	+	++	+	+	+	+	+	±	-
CD90	-	+	±	-	-	-	-	+++	-
CD105	-	-	-	-	-	-	-	±	-
CD106	±	±	-	-	-	-	-	±	-
CD117	-	+	+	-	-	-	-	-	+
CD166	-	-	-	-	-	-	-	-	-

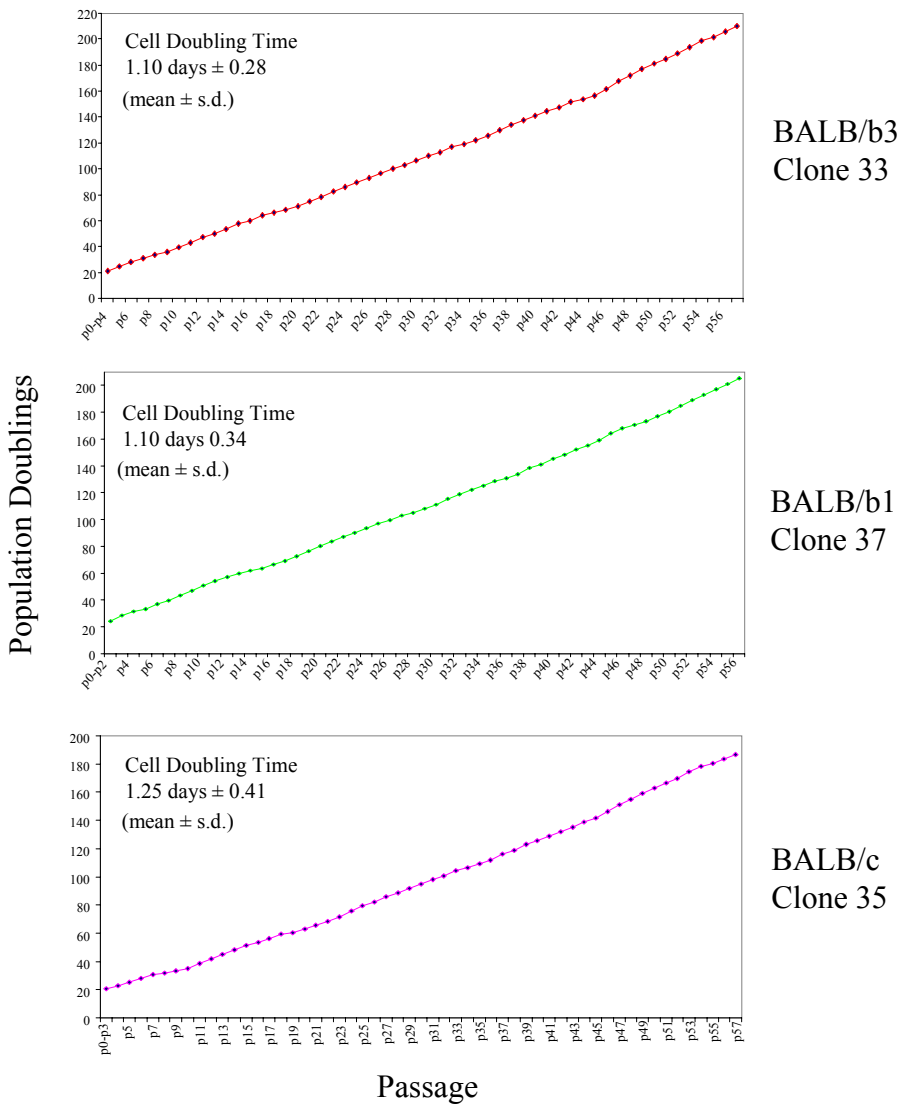


Figure 4.3 BALB/b and BALB/c clones showed extensive proliferation capacity. The growth rate of BALB/b1 clone 37, BALB/b3 Clone 33, and BALB/c Clone 35 was followed for over 200 days in 5% oxygen tension. Cells were seeded at a density of 500-1000 cells/cm^2 and passaged every 3-5 days when they were 70% confluent. Cells were counted at each passage to calculate population doubling time (standard deviation, s.d.).

clone 33, BALB/b1 clone 37, and BALB/c clone 35 cultured at 5% oxygen tension. Beginning at passage 0 to passage 58, the growth rate is constant of BALB/b3 clone 33. The same trend applies for the BALB/b1 Clone 37 and the BALB/c clone C35. Cell doubling time averaged 1.10 ± 0.28 days for BALB/b3 clone 33, 1.10 ± 0.34 days for BALB/b1 clone 37, and 1.25 ± 0.41 days BALB/c clone 35 (mean ± SD). The clones have been expanded far beyond the 50 cell doublings - the Hayflick limit (Hayflick and Moorhead, 1961). These results demonstrate the enhanced growth rate of the clones compared to differentiated cells, and since there was no evidence of cell death, the data is indicative of cell proliferation.

4.5 Effect of oxygen tension on the growth rate of the clones

To explore further the effect of oxygen tension on the clones, their growth rate was examined at 3 different oxygen tensions 20%, 5%, and 1%. The population doubling time for BALB/b3 clone 33 cultured at 5% oxygen tension was consistently shorter than that cultured at 20% oxygen tension, with 1% being intermediate (figure 4.4). Cell doubling time in 5% oxygen averaged 1.2 days, 1.7 days in 20% oxygen, and 1.6 days in 1% oxygen. The trend was similar for the BALB/c clone C35 in that the population doubling time was shortest when cultured at 5% oxygen tension compared to 1% oxygen with 20% being intermediate for this clone (figure 4.5). The cell doubling time in 5% oxygen averaged 1.3 days, 1.5 days in 20%, and 1.6 days in 1% oxygen. Taken together the results suggest that low oxygen tension (specifically 5%) enhances the proliferative capacity of the MSC clones.

4.6 MSC clones express telomerase and maintain telomere length

Given the rapid expansion demonstrated by the clonal cells without evidence of senescence, their telomere length and telomerase expression were next examined. Clones

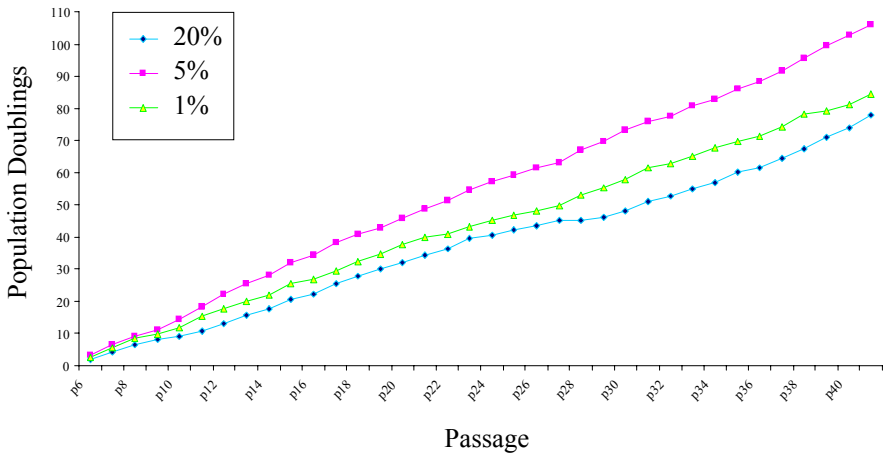


Figure 4.4 Effect of oxygen tension on the growth rate of BALB/b3 clone 33. Cells were seeded at a cell density of 500-1000 cells/cm^2 and cultured at 20%, 5%, and 1% oxygen tension. Cells were passaged every 3-4 days when they were 70% confluent. Cells were counted at each passage with a hemocytometer.

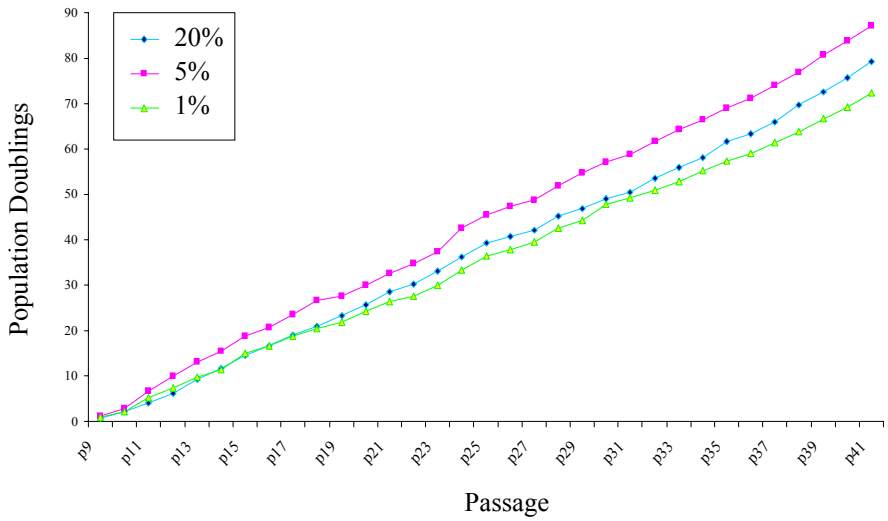


Figure 4.5 Effect of oxygen tension on the growth rate of BALB/c clone 35. Cells were seeded at a cell density of 500-1000 cells/cm^2 and cultured at 20%, 5%, and 1% oxygen tension. Cells were passaged every 3-4 days when they were 70% confluent. Cells were counted at each passage with a hemocytometer.

from both strains at low passage (p9-11) and high passage (p38-46) expressed significant levels of telomerase compared to the strain-matched fibroblasts (figure 4.6).

Telomerase is known to prolong the replicative potential and thus life span of cells by adding telomeric repeats to telomeres. Figure 4.7 shows that the telomere length of the clones was constant at >21 kbp from first passage clones to low passage clones (p9-11) to extensively passaged clones (p58-59) In comparison passaged neonatal human dermal fibroblasts had distinctly shorter telomeres.

4.7 Clones are cytogenetically abnormal

Recent studies have linked high expansion kinetics of MSCs to an abnormal karyotype (Miura et al., 2006). Karyotypic analysis of clones in this study did indeed reveal chromosomal aneuploidy, with all clones displaying an average of 69 chromosomes with the addition of 1 or 2 mini-chromosomes (figure 4.8).

A number of BALB/b clones from 3 separate isolations (BALB/b1, BALB/b2, BALB/b3) were karyotyped, and all revealed the same chromosomal aneuploidy (table 4.4). It is particularly interesting that this karyotype manifested as early as passage 1 and remained the same after extensive passaging to passage 53. Thus after being cultured for over half a year and reaching over 180 population doublings the karyotype remained constant. Cytogenetic analysis of two of the BALB/c clones also revealed the same chromosomal abnormality suggesting the transformation was not a strain specific event. The BALB/b MEFs had a normal karyotype of 40 chromosomes suggesting that chromosomal aneuploidy of the clones could be due to the selection procedure adopted for the derivation of clones. It is also possible, although less likely that the cells had the abnormal karyotype *in vivo*.

BMMNCs from FVB/N mice were seeded at a high cell density to investigate if this prevented transformation of the cells. Cells were cultured for a week until they were ~80% confluent. Karyotype analysis of these cells revealed they were tetraploid, with 1 mini chromosome (figure 4.9). This suggests cell transformation occurred very early in culture, as with the clonal cells, and was irrespective of seeding density. In order to determine whether low oxygen tension affected transformation, BALB/c BMMNCs were

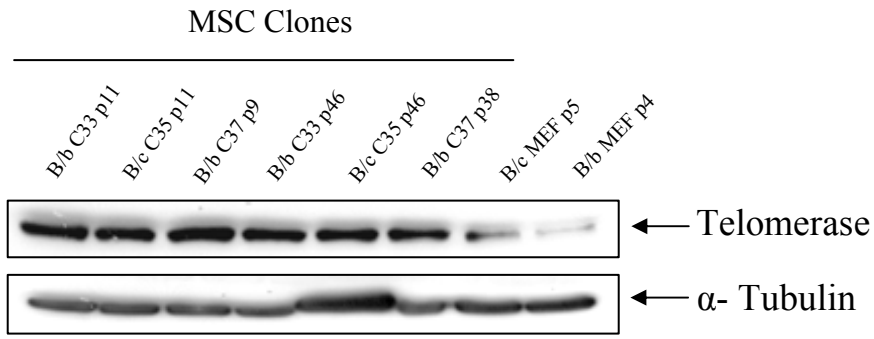


Figure 4.6 Clones Express High Levels of Telomerase. Western blot analysis of two BALB/b and a BALB/c clone showed high telomerase levels were maintained with continuous passaging (passage 9-11 to 38- 46) compared to mouse embryonic fibroblasts (MEFs). BALB/b (B/b), BALB/c (B/c).

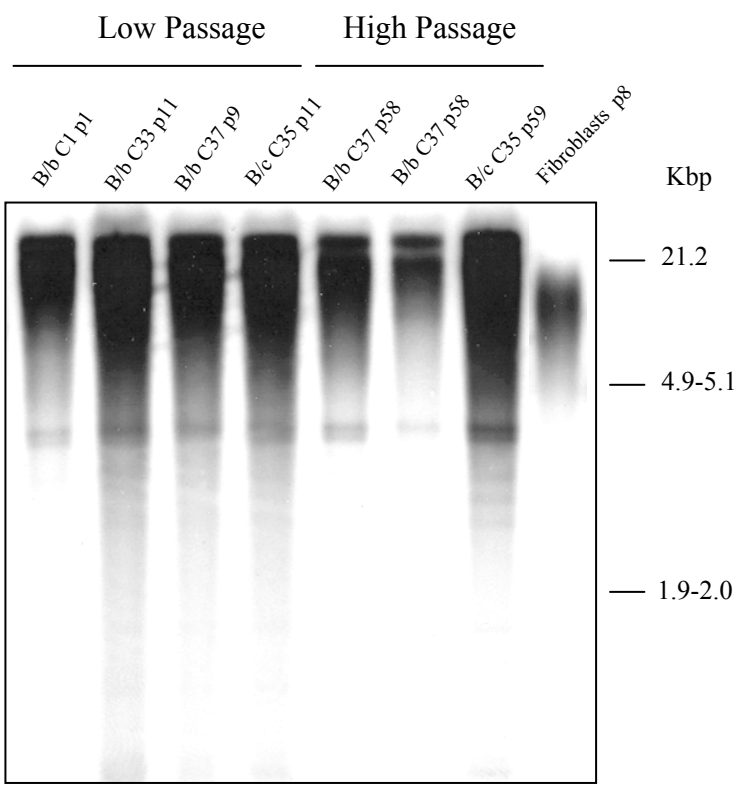


Figure 4.7 Passaged Clones Maintain Telomere Length. Telomere length of the clones did not change significantly from low passage clones (p1-11) to high passage (p58-59). Fibroblast, normal neonatal human dermal fibroblasts. BALB/b (B/b), BALB/c (B/c), Kilobase pairs (Kbp).

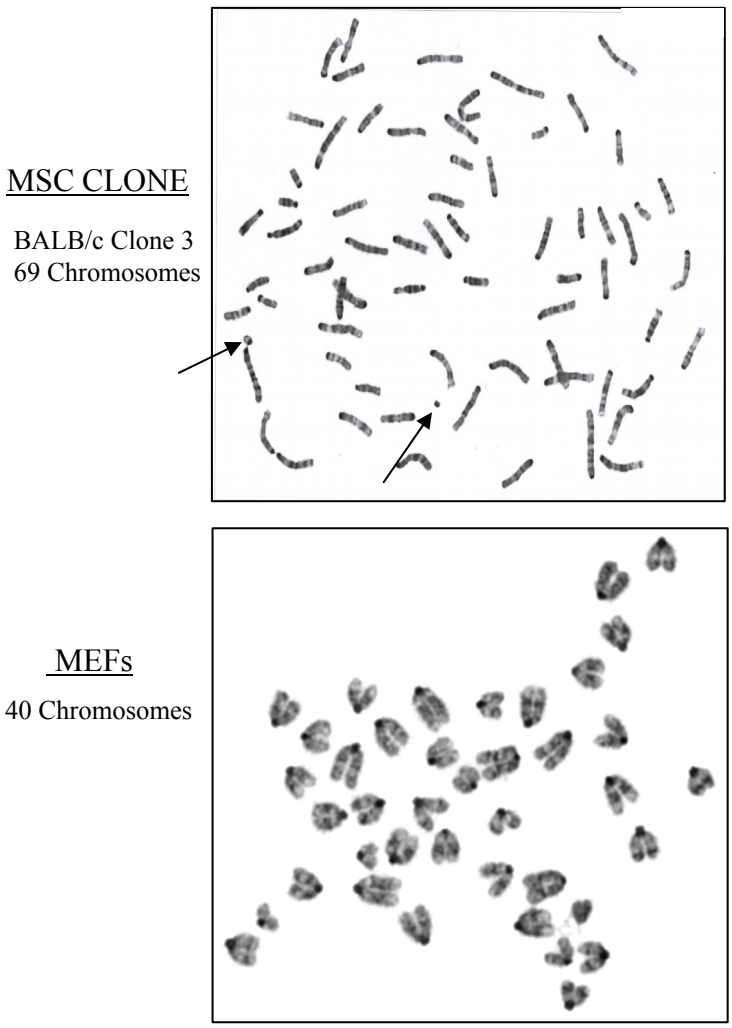


Figure 4.8 Clones have an abnormal karyotyope. Representative metaphase spread from MSC clonal cells showing 67 chromosones plus two mini-chromosomes (marked by arrows) (A). BALB/b MEFs had a normal karyotype of 40 chromosomes (B) (20 metaphases were analysed per sample). Mouse embryonic fibroblasts (MEFs).

Table 4.4 Clones have an abnormal karyotype. The karyotype of a number of BALB/b and BALB/c clones was analysed and their chromosome number summarised. All clones from p1 to p51 displayed similar numerical chromosomal aberrations of approximately 69 with the addition of 1-2 mini-chromosomes. Twenty metaphases of passage 1 freshly isolated FVB/N bone marrow adherent cells (BMACs) was also analysed and found to be tetraploid. MEFs had a normal karyotype of 40 chromosomes. Abbreviations: B/b, BALB/b (1-3 denotes 3 separate isolations); MEFs, Mouse embryonic fibroblasts; pd, population doublings.

Strain	Cells	Passage	Population Doublings	Chromosome Number	Mini-Chromosome
FVB/N	BMAC	1	6	82	2
BALB/b	MEF	1	8	40	0
BALB/b1	Clone 2	1	18	69-71	2
BALB/b1	Clone 1	3	20	68-71	1
BALB/c	Clone 3	4	23	68-69	2
BALB/b 2	Clone 3	4	24	~68	2
BALB/b 2	Clone 2	4	25	~69	2
BALB/b 2	Clone 1	5	27	~69	2
BALB/b 2	Clone 4	6	28	69	1
BALB/c	Clone 4	7	31	67	2
BALB/b 3	Clone 31	24	89	~69	2
BALB/b 3	Clone 31	51	180	~69	1

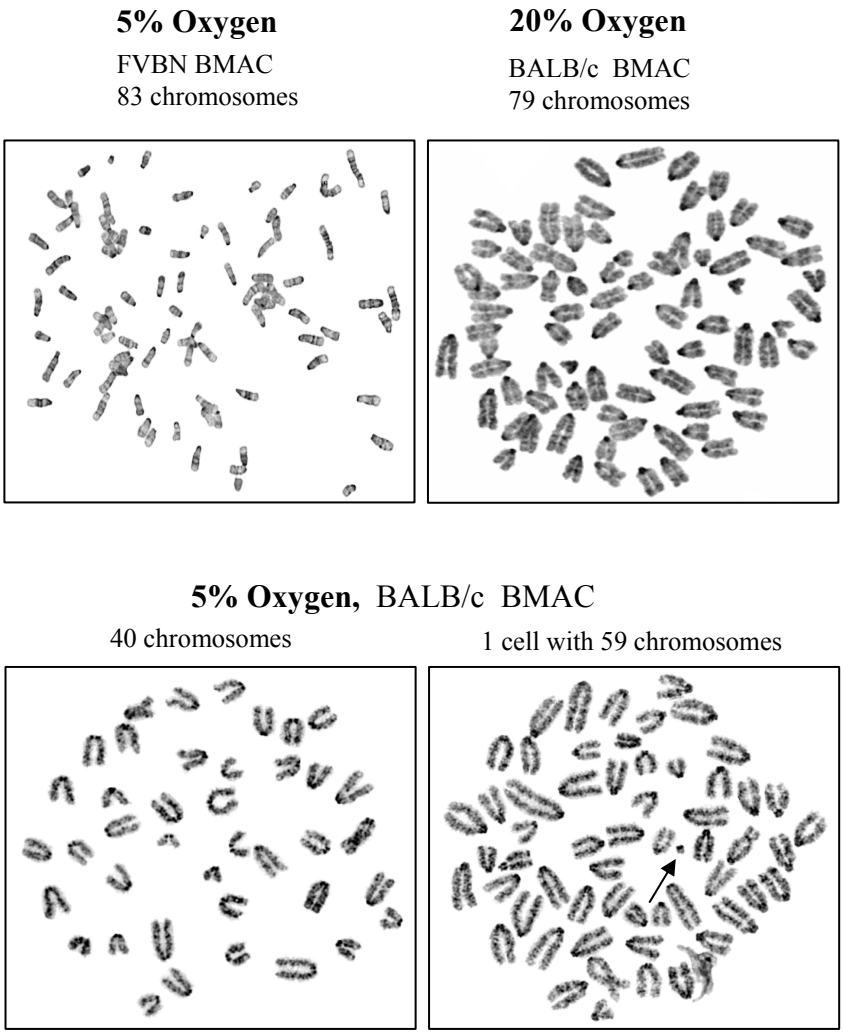


Figure 4.9 Bone Marrow Adherent Cells display Chromosomal Aneuploidy. Bone marrow mononuclear cells from BALB/c or FVB/N mice were seeded at a high density and cultured at 20% or 5% oxygen tension as indicated. After one week cells were re- seeded into a flask and cytogenetically analysed (passage 1). Representative metaphase spread from each sample is shown, FVB/N bone marrow adherent cells (BMACs) were tetraploid, BALB/c BMACs cultured at 20% were tetraploid, BALB/c BMACs cultured at 5% oxygen tension were normal except one cell contained 59 chromosomes plus one mini-chromosome (marked by arrow) (20 metaphases were analysed per sample).

seeded at a high density and cultured at both 20% and 5% oxygen tension. Cells cultured at 20% oxygen tension were found to be tetraploid whereas cells exposed to 5% oxygen were normal, with the exception of one cell that contained 59 chromosomes with one mini-chromosome, suggesting that the transformation event was beginning, but was at least delayed in reduced oxygen levels.

4.6 Clonal cells are not malignant

Since the clonal cells have an abnormal karyotype, they were transplanted into immunodeficient mice to determine whether they were actually malignant. Four different BALB/c clones were injected sub-cutaneously and intra-muscularly (i.m.) (n=2) or intra-venously (i.v) (n=2). MEFs, which have a normal karyotype were used as a control, and injected in a similar way. Previous groups have found tumour formation by 4 weeks after i.m. injection, usually at the site of the injection, and in the lungs by 8 weeks after i.v injection of cytogenetically abnormal MSCs into immuno-compromised mice (Miura et al., 2006; Tolar et al., 2007; Wang et al., 2005b; Zhou et al., 2006). In contrast to this, we found no sign of tumour growth macroscopically or microscopically after 4½ and 7 months after the mice were injected with BALB/c and BALB/b clones respectively. Histological evaluation showed all tissues were healthy. Figure 4.10 shows that the heart, lung, kidney, and prostate of SCID mouse injected with BALB/b1 clone C37 that was sacrificed at 7 months is normal with no signs of sarcoma formation.

Normal BALB/c mice were injected with 4 BALB/c clones as well as normal MEFs and were sacrificed at 4½ months. Results were the same as above there was no sign of tumour formation and or differentiation.

4.7 Despite an abnormal karyotype, clones display regulated differentiation

Transformed cells have been reported to exhibit dysfunctional patterns of differentiation

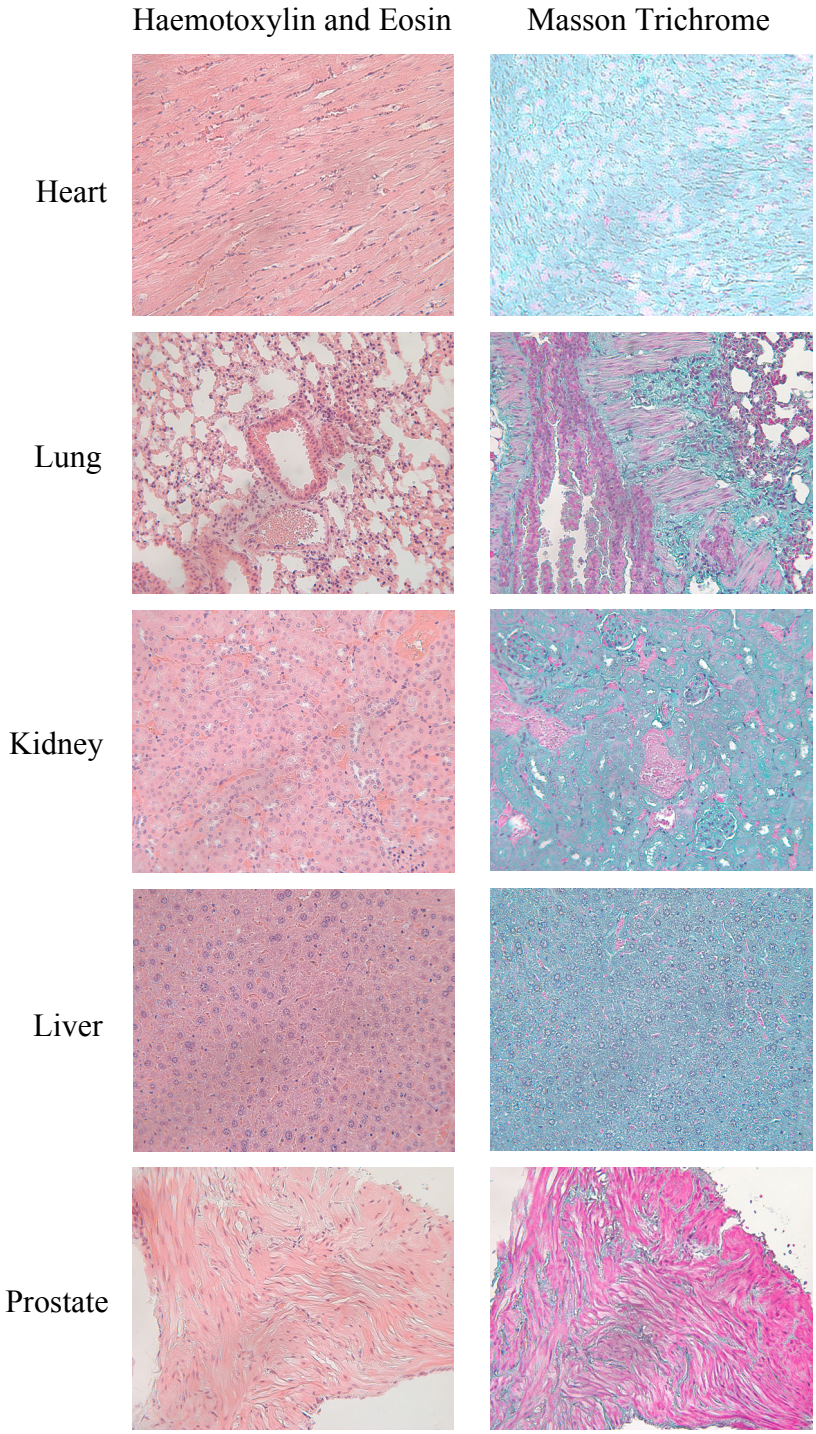


Figure 4.10 Clonal cells are not malignant. BALB/b clonal cells C37 (1 x 10^6) were injected sub-cutaneously and intra-muscularly, or intra-venously through the tail vein, into severe combined immuno-deficiency mice (SCID). Mice were sacrificed 7 month post injection. There was no sign of tumour formation macroscopically or microscopically. Photos are representative of all SCID mice injected with all BALB/b and BALB/c clones. Sections were stained with haemotoxylin and eosin, and masson trichrome. Magnification 25X.

(Bjerkvig et al., 2005). However, in contrast to this, the differentiation of clonal cells in our study was a highly regulated process. Table 4.5 shows the average gene expression intensities (arbitrary units) of Sca-1 and the adipocyte-specific transcription factor PPAR-γ during adipogenic differentiation of the clones. The undifferentiated clones from both strains express relatively high levels of Sca-1 in comparison to control cells, differentiated clonal cells, MEFs, bone marrow adherent cells (BMACs), BMMNCs. Adipocyte differentiated clones however, display high levels of PPAR-γ in comparison to the same undifferentiated clones and the controls. The results are particularly significant as they are the average of 4 MSC clones and 2 control samples.

The same trend is also found at the protein level, via flow cytometry (table 4.6). The surface antigen profile of the BALB/b1 clone 37 was examined in the presence of adipogenic media at day 1, 4, and 2 weeks when all the cells had differentiated. Interestingly, after 1 day in differentiation media Sca-1 expression decreased and after complete differentiation its expression was very low. This supports its role as a stem cell antigen as its expression was hugely down-regulated upon differentiation.

Microarray data comparing undifferentiated to adipocyte differentiated clones also revealed adipocyte genes to be highly upregulated in the differentiated clones. For example, fat specific gene 27 was upregulated over 200 fold in the differentiated clones (table 4.7A). This occurred with a concomitant decrease in stem cell and cell cycle genes, Sca-1 and Cdk4, which were downregulated 9 and 3 fold, respectively (table 4.7B). There is also a down-regulation in the cytokines HGF (5-fold) and FGF-7 (4-fold), which have a role in proliferation (Forte et al., 2006) (Cho et al., 2008).

Table 4.5 Clones display regulated differentiation. The relative gene expression levels for Sca-1 and PPARγ for 4 BALB/b and 4 BALB/c clones, and the equivalent clone differentiated along the adipogenic pathway was examined via microarray analysis. Strain matched MEFs, BMACs, and BMMNCs were also examined. In comparison to undifferentiated clones (UCs), differentiated clones (DCs) expressed low levels of Sca-1 and alternatively high levels of the adipocyte marker PPARγ. For each strain n = 2 for mouse embryonic fibroblast (MEF), bone marrow mononuclear cells (BMMNCs), and bone marrow adherent cells (BMACs), and n= 4 for UC and DC.

Cell Type	BALB/b		BALB/c	
	Sca-1	PPARγ	Sca-1	PPARγ
UC	1	1	1	1
DC	0.01	4.97	0.22	2.75
MEF	0.64	0.20	0.41	0.21
BMAC	0.06	0.48	0.55	1.64
BMMNC	0.03	0.02	0.02	0.06

Table 4.6 Sca-1 expression decreases upon adipogenic differentiation of MSC clones. The surface antigen profile of BALB/b1 clone 37 was examined by flow cytometry at day 0, and in adipogenic differentiation media at day 1, day 4, and then after 2 weeks when all the cells had differentiated into adipocytes. Samples were scored as '-' if the expression level was in the first decade (same as the isotype control), '±' if it was between the first and second decade, '+' if it was in the second decade, '++' if it was in the third decade and '+++' if it was in the fourth decade, relative to the isotype control.

		Adipogenic Media		
	Day 0	**Day 1**	**Day 4**	**2 Week**
Sca-1	+++	++	++	±
CXCR4	-	-	-	-
SSEA-1	-	-	-	-
MHC-I	±	+	+	+
MHC-II	-	-	-	-
CD3	±	-	±	-
CD11b	-	-	-	-
CD29	-	+	-	±
CD31	-	-	-	±
CD34	±	-	±	-
CD44	+	+	+	±
CD45	-	-	-	±
CD49e	++	++	+	++
CD73	-	±	±	++
CD80	+	+	±	±
CD90	-	-	-	-
CD105	-	-	-	-
CD106	-	-	-	-
CD117	-	-	-	±
CD166	-	-	-	-

Table 4.7 Adipocyte clones are distinct to the undifferentiated clones. The gene expression profile of 4 BALB/b and 4 BALB/c clones, and the equivalent clone differentiated along the adipogenic pathway was examined via microarray analysis. In comparison to undifferentiated clones (UCs), differentiated clones (DCs) expressed high levels of adipocyte genes **(A)**. In comparison, DCs expressed low levels of Cdk4, stem cell marker Sca-1 and cytokines involved in cell proliferation **(B)**. For each strain n = 4 for mouse embryonic fibroblast (MEF), bone marrow mononuclear cells (BMMNCs), and bone marrow adherent cells (BMACs), and n= 8 for UC and DC.

A

Sequence Name	Gene	Accession Number	UC	DC	Fold Change (UC vs DC)
fat specific gene 27	Cidec	BB221402	19	4008	212
hydroxysteroid 11-beta dehydrogenase 1	Hsd11b1	NM_008288	23	3469	151
adipocyte complement related protein	Adipoq	NM_009605	63	9428	149
fatty acid binding protein 4	Fabp4	BC018558	8	836	108
lipase, hormone sensitive	Lipe	NM_010719	17	1349	77
fatty acid Coenzyme A ligase, long chain 2	Acsl1	BI413218	56	4079	73

B

Sequence Name	Gene	Accession Number	UC	DC	Fold Change (DC vs UC)
chemokine (C-C motif) ligand 7	Ccl7	AF128193	1352	113	12
Stem cell antigen- 1/Ly6a	Sca-1	BC002070	9177	1072	9
ELK3, member of ETS oncogene family	Elk3	BC005686	200	32	6
hepatocyte growth factor	Hgf	AF042856	356	76	5
fibroblast growth factor 7	Fgf7	NM_008008	2092	571	4
cyclin dependent kinase 4	Cdk4	NM_009870	3837	1330	3
histone deacetylase 7A	Hdac7a	BB277517	605	223	3

4.10 Discussion

The characteristics of mesenchymal stem cells have not been well clarified. Varying reports about their proliferative ability and surface antigen profiles are perhaps due to variation in cell isolation procedures and cell culture methods used. In addition, a thorough characterisation of MSCs is desirable prior to therapeutic application. In the present study the surface antigen profile of the clones was examined to further characterise these clonally-derived (hence pure), multipotent cell populations. The method used to denote the level of expression for FACS analysis is described in table 4.2. This differs from that used in most previous studies. For example, if the population is to the far right in the fourth decade, the population is denoted +++ meaning very highly expressed. The Peister group denoted a population +++ if it was 100% outside the isotype control and in the second decade (Peister et al., 2004). In our results such a population would be denoted as +.

All clones analysed displayed a remarkably similar surface antigen phenotype, and clones from both mouse strains (BALB/b and BALB/c) were also very similar. All clones consistently expressed high levels of Sca-1 in comparison to the MEFs and BMMNCs. Sca-1 is a glycosyl phosphatidylinositol-anchored cell surface protein (GPI-AP). Sca-1, as its name states (stem cell antigen), is known to be expressed by haematopoietic stem cells and mesenchymal stem cells. In addition, it is also reported to be expressed by stem and progenitor cells in the mammary gland (Welm et al., 2002), prostate (Xin et al., 2005), heart (Oh et al., 2003), liver (Petersen et al., 2003), and skeletal muscle (Lee et al., 2000). The function of Sca-1 has not been fully elucidated, however there are reports that it is involved in cell-cell adhesion and signalling (Holmes and Stanford, 2007). A study of Sca-1 knockout mice found that Sca-1 is required for self-renewal of MSC progenitors and has an indirect role in osteoclast differentiation (Bonyadi et al., 2003).

The results in this study are consistent with previous results in MSCs (Li et al., 2008), (Lamoury et al., 2006), (Peister et al., 2004). Although most studies did not find their MSC- containing populations express Sca-1 to the high intensity that the clones did. One

reason for this may be that the clones are a pure population of MSCs (as discussed in chapter 3), whereas the other studies typically involved use of mixed cell populations. Another group reported their BALB/c MSCs were negative for Sca-1 (Peister et al., 2004). This could be for the reasons just stated. In contrast to Sca-1, clones expressed low levels of the fibroblast marker CD90 which the MEFs expressed at significant levels. The clones and MEFs were positive for the haematopoietic antigen CD34 (Peister et al., 2004), and to a lesser extent, CD45. Although human MSCs are reported to be negative for CD34 and CD45, CD34 expression by murine MSCs has been reported (Peister et al., 2004). Expression of CD45 is more controversial as most groups report murine MSCs as CD45 negative (Jiang et al., 2002). However, the Phinney group reported positive expression of CD45 (Phinney et al., 1999). This group report much higher CD45 expression levels, which they postulate is due to lympho-haematopoietic contamination, whereas the clones in our study expressed relatively low levels. As the cells are clonally-derived and homogeneous, as evidenced by their morphology and the FACS data, we believe contamination is unlikely to be the reason for the low level of CD45 expression. It could simply be due to the fact that pure populations of MSCs cultured at more physiological oxygen tension (5%) do, in fact, express this marker, albeit at low levels.

Many groups have reported gain or loss of markers with culture (Eslaminejad et al., 2007). We found that our clones retained their surface antigen profile when examined up to passage 53. Again this could be due to the reasons mentioned above - our MSCs are clonally-derived and hence pure populations, and furthermore were derived and cultured in more physiologic oxygen conditions.

The ability of multipotent MSCs to expand significantly in culture is one of the reasons that they are such an attractive candidate for tissue engineering (Abdallah and Kassem, 2008). However, there is a paucity of literature on the growth kinetics of clonal adult stem cells as most studies are performed on heterogeneous populations. The growth rate of our clones was followed from passage 0 until passage 58 for the BALB/b and BALB/c clones in 5% oxygen tension (figure 4.3). This was a period of over 6 months by which point the clones reached over 180 population doublings. The results demonstrated that there is a linear increase in the numbers of population doublings of the clones with time in culture. Therefore the growth rate was constant, i.e., there was no sign of a senescence-

associated decrease in growth with passage. Previous studies on the growth rate of MSCs have reported that the initial growth rate was fast, but slowed down with passage and cells began to senescence after 50 population doublings (Muraglia et al., 2000). In contrast, the cell doubling time of our clones at both high and low passage is the same with no sign of senescence.

In a recent study a murine 'MSC' culture called multipotent adult progenitor cell (MAPC) was expanded over 120 population doublings (Jiang et al., 2002). This population was grown in the presence of high concentrations of exogenous growth factors (EGF, LIF, and, PDGF-BB), whereas it is important to point out that no such factors were added to our cultures. A further difference was that we culture the cells in 5% oxygen, whereas the above studies were performed in 20% oxygen. Indeed, cultivation of clones at low oxygen tension had an impact on the growth rate. Clones that were cultured at 5% oxygen tension consistently were more proliferative in comparison to 20% and 1% cultures (figures 4.4- 4.5). Taken together the results demonstrate that low oxygen tension (5%) enhances the growth rate of these clonally-derived, multipotent murine MSCs.

There are two types of cellular senescence, one is 'replicative senescence', i.e., reaching the 'Hayflick limit'. The Hayflick limit is the number of times a cell will divide before senescing due to critical shortening of the telomeres. (52 population doublings) (Hayflick and Moorhead, 1961). The other is 'stress-induced' senescence caused by extrinsic factors. This is 'premature' senescence as it may occur before the Hayflick limit is reached. Culturing cells in 20% oxygen is likely to be an oxidative stress to cells, as MSCs are exposed to much lower oxygen tension *in vivo* (Kofoed et al., 1985). This hypothesis was tested by Moussavi-Harami and colleagues for MSCs and chondrocytes (which are exposed to an even lower oxygen tension *in vivo*, <2% oxygen) (Moussavi-Harami et al., 2004). The group found that cultivation of three different human chondrocyte populations at 5% oxygen was associated with a two-fold increase in growth rate compared to 20% oxygen cultures. A similar increase was found with human MSCs. The investigators also found cells cultured at high oxygen levels were associated with significantly greater oxidant production than low oxygen cultures. Their results support the hypothesis that high oxygen levels (20%) used in standard cell culture conditions are

harmful to cells that are normally exposed to much lower oxygen levels *in vivo*. These conditions induce premature senescence as chondrocytes cultured at 5% oxygen reached an average of 77 population doublings, while cells cultured at 20% oxygen senesced at 38 population doublings. Initial studies by other groups also report enhanced growth of MSCs cultured at low oxygen compared to the standard 20% oxygen tension (Fehrer et al., 2007; Grayson et al., 2006; Lennon et al., 2001). However, we are the first to study the effect of three different oxygen tensions on MSCs so extensively.

As mentioned in the introduction, standard cell culture conditions of 20% oxygen tension were developed for the cultivation of fibroblasts and then cells lines. While high oxygen conditions may be suitable for these cell types, a more specialised environment is required for cells such as MSCs and chondrocytes in order to prevent oxidant-induced premature senescence. In addition, cultivation at low oxygen tension has been reported to cause re-differentiation of chondrocytes (Murphy and Polak, 2004) and maintain the 'stemness' of MSCs (D'Ippolito et al., 2006). As discussed in chapter 3 low oxygen enhanced and maintained the differentiation capacity of the clonal cells in the present study. Thus, oxygen level is an elementary signal to cells, low oxygen tension does not just enhance the growth rate of cells, it also maintains their intrinsic biology. This could be why the growth of clonal cells is enhanced at low oxygen tension (5%). We are minimising culture induced stress, and thus 'premature senescence'. However, it should be noted that growth of clonal cells at 1% oxygen is lower than 5% oxygen. As MSCs are not thought to be exposed to such a low oxygen tension *in vivo*, perhaps the optimal results at the more physiologically relevant 5% oxygen are not surprising.

Stem cells appear to have the ability to prevent or delay replicative senescence, allowing them to continue dividing throughout the lifespan of the organism. One mechanism by which this is achieved is the telomere length is maintained by the enzyme telomerase. Telomeres are guanine-rich DNA repeat sequences on the ends of every eukaryotic chromosome that provide chromosomal stability (Hiyama and Hiyama, 2007). Each round of cellular replication causes their loss due to incomplete replication of linear chromosomes, called the 'end replication problem'. Telomerase is a remarkable enzyme

as it is composed of a reverse transcriptase protein and a template RNA TERC (template RNA component). Telomerase adds telomeric repeats to the ends of the telomere to help maintain the length. The telomere length of the clonal cells was maintained from 18 to 200 population doublings. Furthermore, the clonal cells expressed high levels of telomerase enzyme (compared to MEFs) throughout culture. This could, therefore be one mechanism by which clonal cells avoided the Hayflick limit.

Recent studies have reported that murine MSCs acquire chromosomal abnormalities whilst in culture and subsequently form tumours when injected into immuno-compromised mice (Miura et al., 2006) (Tolar et al., 2007) (Zhou et al., 2006). Hence the karyotype of the clonal cells in this study was examined. Cytogenetic analysis revealed all clonal cells did, in fact have an abnormal karyotype of approximately 69 chromosomes usually with the addition of 1 or 2 mini-chromosomes. The presence of these mini-chromosomes was also reported by Miura and colleagues who called them double-minute chromosomes and speculated that they were associated with elevated levels of c-myc (Miura et al., 2006). Little is known about mini-chromosomes, one hypothesis being that they are amplifications of oncogenes.

Notably, the chromosome number of 69 per cell present in the clones was constant from 18 to180 population doublings. Therefore assuming the initial single bone marrow-derived stem cell had a normal karyotype, the cytogenetic alterations occurred during the first month of culture. Since the MEFs had a normal karyotype and were seeded at a high initial density it is possible that the high selection pressure of seeding cells at a low density to form clones may have caused the chromosomal alteration. However, an abnormal karyotype was also observed in high density, non-clonally derived first passage FVB/N bone marrow adherent cultures. Thus, clonal selection pressure cannot be the only reason for the cytogenetic alterations, and, it seems the transformation was not a strain-specific event. It is possible a combination of low oxygen tension and low density seeding was responsible for the observed chromosomal instability. However, BALB/c BMMNCs that were cultured at a high density in 20% oxygen were tetraploid, whereas the equivalent cells cultured at 5% oxygen were normal, although beginning to show signs of transformation with the presence of a cell with 59 chromosomes and 1 mini-chromosome.

It is difficult to understand the mechanism by which clonal cells gained the abnormal karyotype of 69 chromosomes. Zhou and colleagues also found their MSCs gained a similar number of chromosomes, although theirs ranged from 60-84 (Zhou et al., 2006). The investigators postulated that transformation might have occurred via cell fusion or chromosome replication without cytokinesis. However, this would result in tetraploid cells, therefore there must have been an additional event where approximately 11 chromosomes were lost. The wide range of chromosome number present in the cells of Zhou's group suggests continued chromosomal instability. Whereas, in the present study, once the clonal cells had gained 69 chromosomes this number remained stable through extensive subculture.

Parinello and colleagues suggest that reduced oxygen tension may halt or delay proliferative senescence due to less oxidative stress-induced DNA damage (Parrinello et al., 2003). They also claim that the greater ability of human cells to prevent or repair oxidative DNA damage contributes to the major differences in the incidence of cancer and the rate of ageing between mice and humans. Previous studies have also reported that mouse cells spontaneously immortalize in culture (Todaro and Green, 1963), (Prowse and Greider, 1995). This has been the case as far back as the early studies of Earle and colleagues in 1943 (Earle, 1962). Prowse and colleagues suggest mouse cells are more prone to immortalization and transformation in culture than human cells, due to the presence of high telomerase activity in most adult mouse tissue in comparison to human (Prowse and Greider, 1995). Telomeres in human cells consistently shorten with age *in vivo* and *in vitro*, whereas in the adult mouse the telomeres in different tissues vary in length. The investigators found mouse skin fibroblasts transformed spontaneously in culture and these cells had longer telomeres and higher telomerase activity compared to untransformed cells.

Another reason murine cells acquire transformations quite rapidly is because they have telocentric chromosomes where the centromere is at the end of the chromosome. The biological advantage for this is unknown and it makes identification of mouse chromosomes much more difficult than with human chromosomes. Telocentric chromosomes are more likely to undergo chromosomal translocations, such as

Robertsonian translocation (Kalitsis et al., 2006). In humans only the Y chromosome is telocentric.

In agreement with the above studies we did not detect any chromosomal instability in human MSC cultures (discussed in chapter 6), and indeed few studies report transformation of human cells. In mouse cultures it is a common event, but it was reported in only 1 out of 40 samples in human cultures (Wang et al., 2005b). The investigators reported spontaneous immortalization of a clonally distinct, highly proliferative population at passage 3. Wang and co-workers found higher telomerase activity compared to normal human MSC cultures. However, Rubio and colleagues reported it took place after long-term *in vitro* culture (4-5 months), where cells went through cell crisis phase and then expressed increased levels of c-myc (Rubio et al., 2005). Also there was no telomerase activity detected in normal human MSCs, while it was found in transformed MSCs. In addition, studies reporting chromosomal abnormality in human MSCs usually note the addition and/or translocation of 1 or 2 chromosomes, never a tetraploidy or a 75% increase in chromosome number.

In most studies MSCs are not karyotyped. Interestingly, so-called MIAMI (marrow-isolated adult multilineage inducible) cells (D'Ippolito et al., 2004), which are of human origin show many similarities to the clonal MSC of the present study. Both are established and maintained in low oxygen tension, and display a high proliferative capacity over a prolonged period without evidence of senescence. In addition, neither cell type produced tumours when injected in immuno-compromised mice. The karyotype of MIAMI cells has not been investigated, but it would be interesting to discover if they display any cytogenetic alterations. Multipotent adult progenitor cells (MAPCs) only grow under very specific conditions in the presence of LIF, EGF, PDGF and have a high proliferation capacity. Murine MAPCs (mMAPCs) have been cultured to over 120 population doublings as mentioned above. In addition, the telomere length was maintained at 27 kilo base pairs (kbp) in cells at 40 population doublings to 102 population doublings (Jiang et al., 2002). Murine MSCs have been cultured to 100 population doublings (Meirelles Lda and Nardi, 2003), while our clonal cells were cultured to 180 population doublings, but were found to be karyotypically abnormal. One mMAPC population was hyperdiploid after 45 population doublings. No tumour

formation was found when mMAPCs were transplanted into immuno-deficient mice. Human MSCs tend to proliferate to 40-50 population doublings (Baksh et al., 2004), (Barry, 2003). Human MAPCs can be expanded for more than 80 population doublings (Reyes et al., 2001). Cytogenetic analysis of two MAPC populations revealed a normal karyotype, although this was at 40 population doublings. It will be interesting to see if this was still the case at 80 population doublings since the majority of human MSCs tend to only proliferate to 40-50 population doublings. The telomere length of one donor was also examined from 15 to 35 to 45 population doublings and reported to remain constant. Most studies investigating telomere length in human MSCs report a gradual loss with culture, typically 1kbp with 10 population doublings (Baxter et al., 2004; Parsch et al., 2004). The lack of proliferative senescence and maintenance of telomere length are suggestive of an immortalized cell, and it is possible this may be the mechanism for the high proliferative capacity of the clones.

To retain stem cell properties it would, perhaps, be ideal to avoid tissue culture altogether. However, due to the low frequency of MSCs *in vivo* it is necessary to expand them for research and for tissue repair purposes. One other explanation for the recent evidence of chromosomal abnormality observed in murine and human MSCs could be that *in vivo* the bone marrow MSCs exist in a dynamic environment surrounded by stem and differentiated cells. There is a constant interplay between the cells and the environment. In *in vitro* tissue culture although we are attempting to mimic aspects of the *in vivo* environment by culturing cells at a low oxygen tension we cannot truly mimic the *in vivo* niche. Cultivation at low oxygen seems appropriate for colony formation, proliferation, differentiation, and self-renewal of MSCs as observed in chapter 3. However, we are not adding any growth factors in order not to bias the cell type and presumably MSCs are exposed to a variety in their *in vivo* niche. In addition, we are selecting for highly proliferative MSCs, whilst *in vivo* MSCs are mainly in a quiescent state.

In vitro, the stem cell is out of its original environment, and in consequence is quite possibly under a significant amount of stress and will not behave the same way it would in its natural environment. *In vitro* conditions may also introduce experimental artifacts. Thus, it is not entirely surprising that the cells began to acquire genetic instabilities when

cultivated in such a foreign environment. Results highlight the need for quality control measures for MSCs before clinical use.

As mentioned above, studies which found chromosomal abnormalities in their MSCs, then transplanted them into immuno-compromised mice to investigate whether the cells were malignant (Miura et al., 2006; Tolar et al., 2007; Wang et al., 2005b). The investigators found that sarcomas developed. In the present study, four clonally-derived populations from two mouse strains were injected locally or intra-venously into severe combined immunodeficiency (SCID) mice. No tumour formation was observed at 4½ months (BALB/c) and 7 months (BALB/b) post-transplantation. Although, it cannot be ruled out that tumours may occur at later time-points, others have reported tumour formation within 3-4 weeks following local injection (Miura et al., 2006; Tolar et al., 2007; Wang et al., 2005b). Sarcomas have been reported to have complex karyotypes and are chromosomally unstable *in vivo* and in culture (Tolar et al., 2007). In contrast, the clonal cells in this study had a stable karyotype after the initial transformation. A major difference between our clonal cells and the transformed and malignant cells in the above studies is that our clonal cells were derived and maintained at low oxygen tension. Also the clonal cells did not express significant levels of c-myc. It is possible that culturing cells at low oxygen prevented DNA damage and mutations caused by reactive oxygen species (ROS). Loss of DNA repair mechanisms has been reported to make cells more susceptible to malignant transformation directly or by the occurrence of cancer-prone stem cells (Kenyon and Gerson, 2007).

C-myc is a proto-oncogene, dysregulated expression of c-myc is found in many human cancers (Pelengaris et al., 2002). It is notable that in the Miura study, that there was an increase in c-myc levels and telomerase in the murine MSCs compared to the normal MSCs where negligible levels were present (Miura et al., 2006). Transformed and malignant human MSCs also expressed increased levels of c-myc, as well as VEGF which is found in many tumours, in comparison to normal MSCs (Rubio et al., 2005). Furthermore, c-myc is reported to activate telomerase activity (Wang et al., 1998). However, microarray analysis of clonal cells in this study did not reveal increased c-myc expression compared to controls, i.e., strain-matched BMACs, MEFs, and freshly isolated

BMMNCs. The reason for this could be that our cells, although immortalised, are not malignant. It is possible with extended passaging that c-myc levels may increase and the cells become malignant, however, it should be noted that cells cultured to passage 43 still retained the spindle shaped morphology, ability to self-renew, and capacity to differentiate. The surface antigen profile was also maintained. In addition, the cell doubling time was constant as was the chromosome number per cell.

The malignant murine MSCs reported in other studies typically display a decrease in growth rate when the cell is going through 'crisis phase' and then a morphology change to a flattened cuboidal, cellular morphology at passage 3-5 (Miura et al., 2006). Crisis phase is when the telomere length of cells becomes critically short causing chromosomal instability which leads to apoptosis (Chin et al., 1999). The majority of the cells apoptose, but there are few that do not and they gradually display an increased growth rate as they overcome the crisis period and retain the large cell morphology. Zhou and colleagues report there was an increase in the growth rate of their murine MSCs at passage 3 followed by contact inhibition and growth in 3-dimensional clusters (Zhou et al., 2006). Human MSCs went through crisis phase 4-5 months after isolation (Rubio et al., 2005) which is later than the murine MSCs but in agreement with previous studies (Lloyd, 2002). The cells overcame crisis phase with the increased growth rate described above.

The two significant differences between the clonal cells in this study and the abnormal MSCs in the ones above are; first ours were clonal and derived and cultured at 5% oxygen tension. The second is they did not express elevated levels of oncogene c-myc and of VEGF that some of the cells in the above studies did. Although our clonal cells did express telomerase and the transformed MSCs did also, this is a characteristic of stem cells as well as transformed cells. Based on the above results we can conclude our clonal cells are immortalised but not malignant, as they did not form tumours *in vivo*. The term immortalisation was first used to describe cancer cells that express telomerase and are therefore able to avoid apoptosis and divide continuously. It is more commonly applied to cell lines Hela and Jurkat which are immortalized cancer cell lines, as well as stem and germ cells. It is possible our clonal cells are a type of cell line.

Despite their abnormal karyotype clonal cells did not show characteristics of transformed cells. This is evident by their differentiation which is a highly regulated process. Many studies have reported the dysfunctional patterns of differentiation of transformed cells (Bjerkvig et al., 2005). Our clonal cells expressed significant levels of Sca-1 compared to control cells. Gene expression analysis revealed this virtually disappears in the adipocyte differentiated clones. In contrast, adipocyte differentiated clones expressed significant levels of PPAR-γ compared to undifferentiated clones. This is an adipocyte marker, and is a member of the nuclear hormone receptor superfamily functioning as a transcription factor regulating the expression of adipocyte genes (Tontonoz et al., 1994). In combination with CEBP, PPAR-γ induces expression of adipocyte genes - adipsin, fatty acid synthase, aP2, and GLUT4, which establishes terminal adipogenesis of cells (Wu et al., 1999). These gene expression analysis results were significant as they were the average of four clones and two control cell populations from each mouse strain; both strains exhibiting very similar results.

As mentioned earlier Sca-1 is not only expressed by MSCs but also by HSCs and other tissue-specific stem cells (Holmes and Stanford, 2007). Sca-1 also has a functional role in stem cells. Upon differentiation of the clonal cells there was a dramatic decrease in Sca-1 expression. This was further confirmed at the protein level by flow cytometry, where there was quite a dramatic loss of Sca-1 after just one day in adipogenic differentiation media. This demonstrates how quickly the cells responded to extrinsic signals. After 2 weeks in the differentiation media there were negligible levels of Sca-1 present. In contrast, to Sca-1 there was an increase in CD73 expression with adipogenesis. This is a traditional MSC candidate marker, which was expressed at low levels by the clonal cells. This increased slightly after one day in differentiation media and was expressed at significant levels after complete differentiation. CD73 is, in fact, also expressed on a variety of other cell types. It is also a marker which the International Society for Cellular Therapy (ISCT) (who met to propose a guideline for the identification of MSCs) set as a marker for MSCs (Dominici et al., 2006). Results stress the need for better markers as this one is expressed on adipocytes but not MSCs in this study. Further microarray analysis revealed differentiated cells expressed significantly greater levels (over 70 fold)

of adipocyte genes: fat specific gene 27, adipocyte complement related protein, fatty acid binding protein 4, lipase, and fatty acid coenzyme A ligase. Concomitantly the stem cell and cell cycle (associated) genes: Sca-1, Ccl7, Hgf, Fgf7 and Cdk4 were down regulated in the differentiated clones compared to the undifferentiated clones.

Also, as shown in chapter 3, clonally-derived MSCs exhibited highly regulated patterns of differentiation, and the extent of differentiation was very comprehensive. This is shown histologically not just for adipogenesis, but for chondrogenesis and osteogenesis. All clonal cells examined had this tri-lineage ability and this was maintained with extensive passaging up to passage 43. Also clonal cells demonstrated contact inhibition, as they formed a monolayer. There was no evidence of multiple layers, i.e. lack of contact inhibition which is a characteristic of cancer (stem) cells. In contrast, when Zhou and colleagues injected abnormal MSCs via intra-venous injection in immuno-compromised mice lung tumours formed which contained areas of bone formation and melanocytes (Zhou et al., 2006). The transformed cells differentiated into two different lineages, which in turn were different from the tissue they were in, thus displaying unregulated differentiation. Furthermore, Miura and colleagues found that their transformed MSCs lost their osteogenic potential after they passed through the crisis phase (Miura et al., 2006).

In conclusion, we believe the clonally-derived cells are a very useful model to investigate MSC-specific markers since we observed murine MSC differentiation to be a highly co-ordinated and regulated process, despite chromosomal alterations in the cells. Any candidate markers that are identified would be subsequently assessed in normal multipotent, human MSCs.

Chapter 5

Molecular Signature of MSC Clones

5.1 Introduction

As there is no known MSC marker that can be used to distinguish the rare MSC population from the many other cells in the bone marrow most groups work with a heterogeneous population of bone marrow adherent cells which they call 'MSCs'. Certain groups have attempted to advance the understanding of MSC biology by characterising MSCs using a microarray approach. However, the significance of the results has been limited due to the paucity of the cell model and lack of suitable controls.

The major aim of this study was to identify MSC specific markers and regulators. To this end we have derived multiple clonal populations from mouse bone marrow and subsequently proven their stem cell status through functional analysis, i.e., by demonstrating their ability to self-renew and to differentiate into multiple lineages. FACS analysis demonstrated the cells have a distinct surface phenotype (in comparison to fibroblast controls (chapter 4)).

In an effort to define the molecular signature of these cells an extensive microarray approach was adopted. Potentially MSC specific genes were identified by comparing the gene expression profile of the multipotent, self-renewing, clonal cells to various relevant controls. The implications of our findings are discussed.

5.2 Microarray design

A transcriptomic approach was adopted in an attempt to identify markers unique to MSCs (figure 5.1 shows the design implemented). In total, 28 whole genome-wide arrays were used. The gene expression profile of eight (four per mouse strain) undifferentiated, multi-potent, self-renewing clones were examined. We also utilised four distinct controls to ensure the final gene list would be relevant, i.e., would enable us to better distinguish novel, and potentially MSC-specific genes. The first control group consisted of the same eight clones, but after differentiation into the adipocytes. Therefore these populations had lost their stem cell properties. The second and third controls were strain-matched (i.e., BALB/b and BALB/c) mouse embryonic fibroblasts (MEFs) and bone marrow adherent cells (BMACs). The former do not have the differentiation potential of the clones, while the latter are a heterogeneous population of cells, containing, but not exclusively the

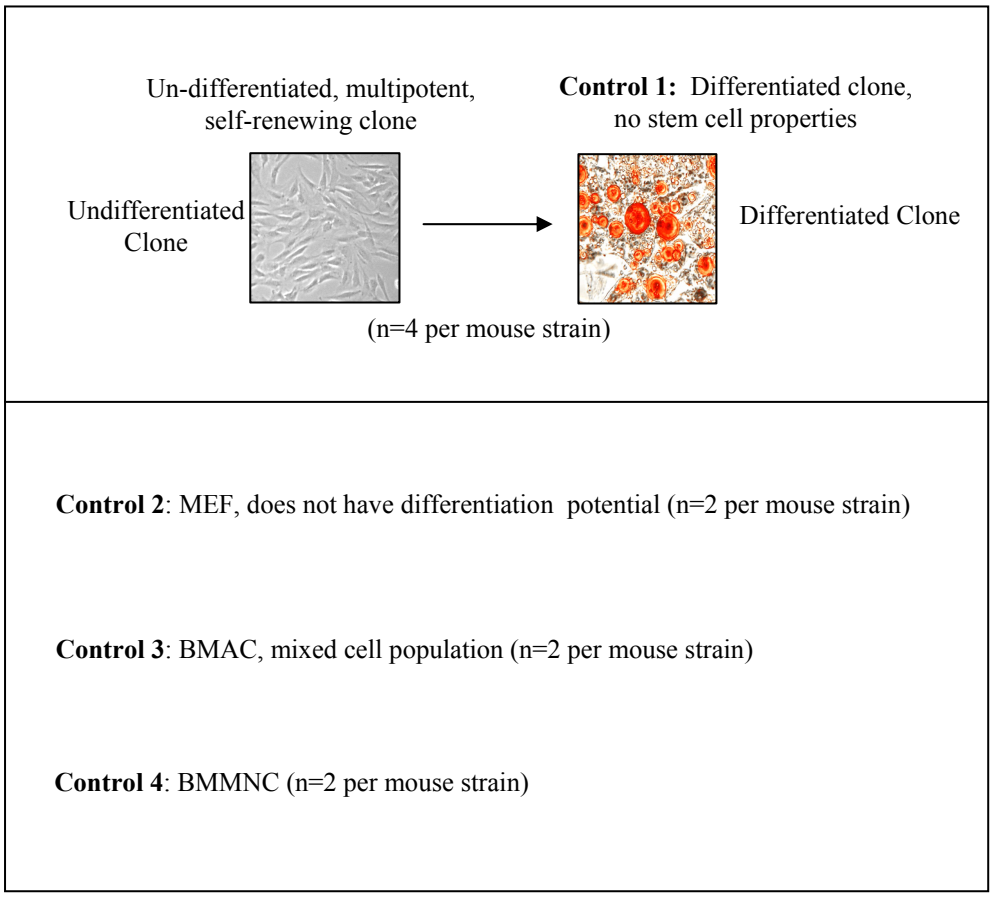


Figure 5.1 Microarray experimental design used to characterise the molecular signature of MSC clones derived for BALB/b and BALB/c mice. The gene expression profile of undifferentiated, multipotent clones (for BALB/b and BALB/c) was compared to 4 distinct controls: the equivalent adipocyte differentiated clones; primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF). Two samples of BMAC, MEF, and BMMNC were used for each mouse strain and four undifferentiated clones and differentiated clones per strain. In total, 28 samples were analysed.

multipotent cells called ‘MSCs’. There were two biological replicates used for these controls for each mouse strain.

5.3 Cell culture and microarray experiments

To avoid oxygen-induced gene expression changes, the cell culture for these experiments was performed in the more physiologically relevant 5% oxygen tension. MEFs were initially cultured at 20% oxygen but prior to microarray analysis cells were cultured at 5% oxygen tension for 4 days. BMMNCs were not cultured - they were lysed immediately after removal of red blood cells. Clonal cells were always cultured in 5% oxygen, as were the adipocyte-differentiated cultures. BMACs were cultured in 5% oxygen for two weeks. Total RNA isolated from these cells was labelled and hybridized to the GeneChip Mouse Genome 430 2.0 Array. The chip contains over 45,000 probe sets representing over 34,000 well substantiated mouse genes as described in Materials and Methods.

5.4 Principal component analysis

Principal component analysis (PCA) is a multivariate analysis technique that is used to reduce multidimensional data sets to lower dimension for analysis. This enables detection of major patterns in the dataset. The PCA of the 28 datasets (figure 5.2) very clearly shows that the 8 undifferentiated clones are very similar and thus cluster very tightly, distinct from each of the control populations including the differentiated clones. Each control sample also clustered tightly together despite differences in mouse strain.

5.5 Unsupervised two-dimensional cluster analysis of microarray data

A two-dimensional (2D) hierarchical unsupervised cluster analysis was also performed on the complete data set of 28 arrays. Results are shown in dendrogram form, and are similar to that of the PCA mentioned above, i.e., each population of cells clustered tightly together (figures 5.3-5.6). The red colour is indicative of relatively high gene expression

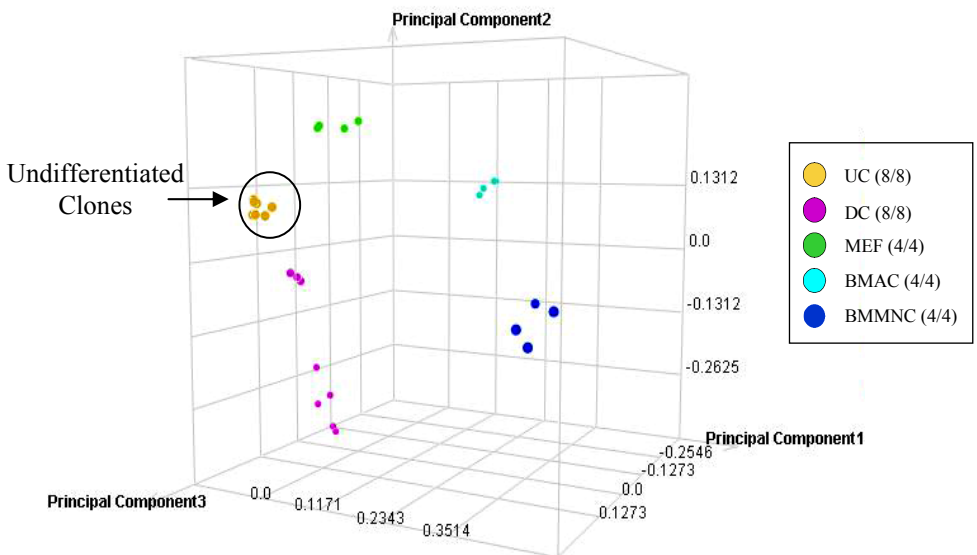


Figure 5.2 Principal component analysis of microarray data. Principal component analysis of the whole-genome expression data of all 28 samples shows that the undifferentiated clone (UC) (ringed) represent a unique cell type, distinct from each of the four controls. Differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

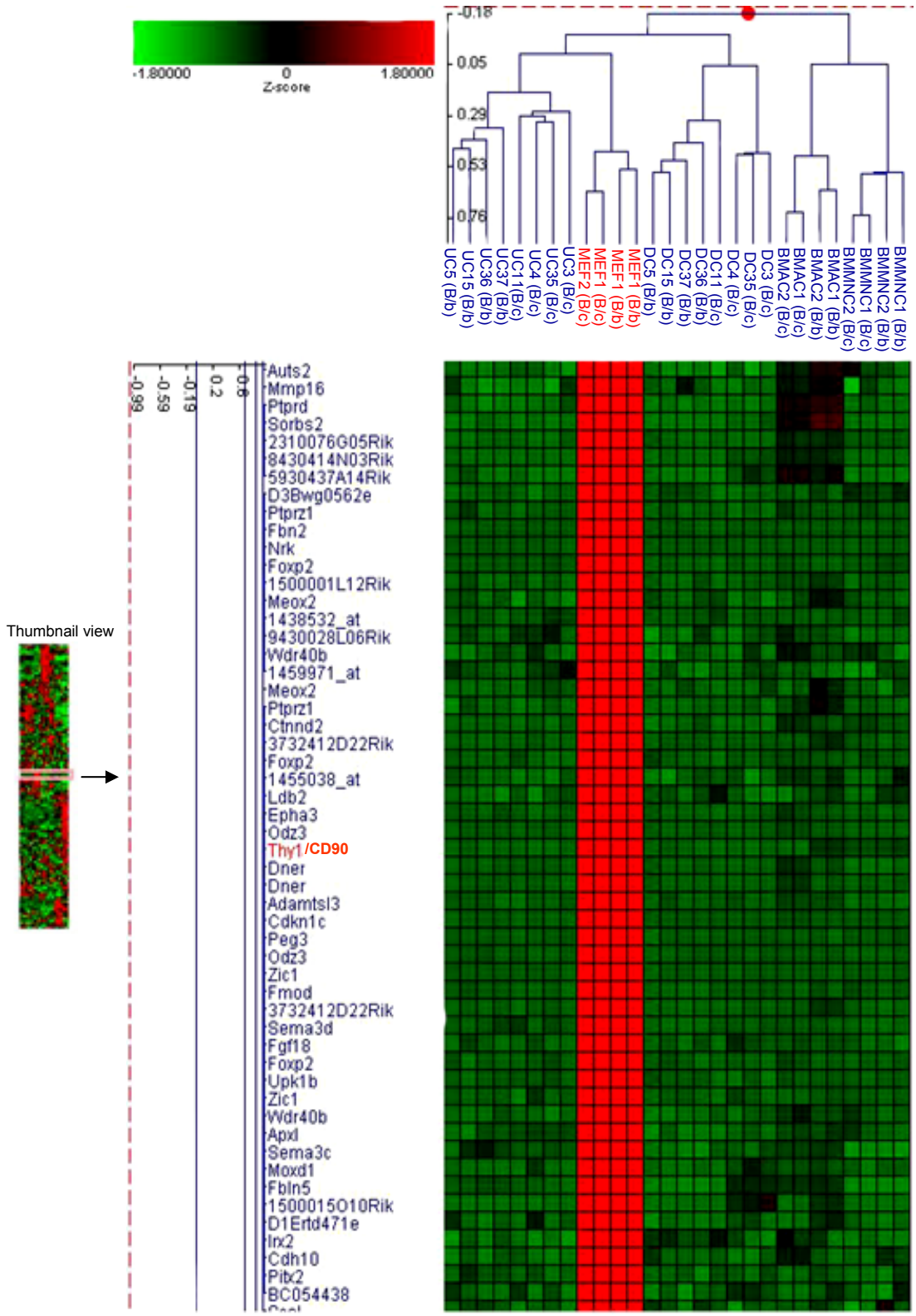


Figure 5.3 Dendrogram showing Thy1/CD90 clustering. An unbiased, genome wide, two-dimensional hierarchical cluster analysis was performed on the complete data set of 28 arrays as described in figure 5.1. Shown are genes clustering closest to fibroblast marker Thy1/CD90. The color scale ranges from saturated red log ratios + 1.8 decreasing to saturated green for log ratios -1.8. Each gene is represented by a single row of colored boxes; each individual control is represented by a single column. Undifferentiated clone (UC); differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF); BALB/b (B/b); BALB/c (B/c).

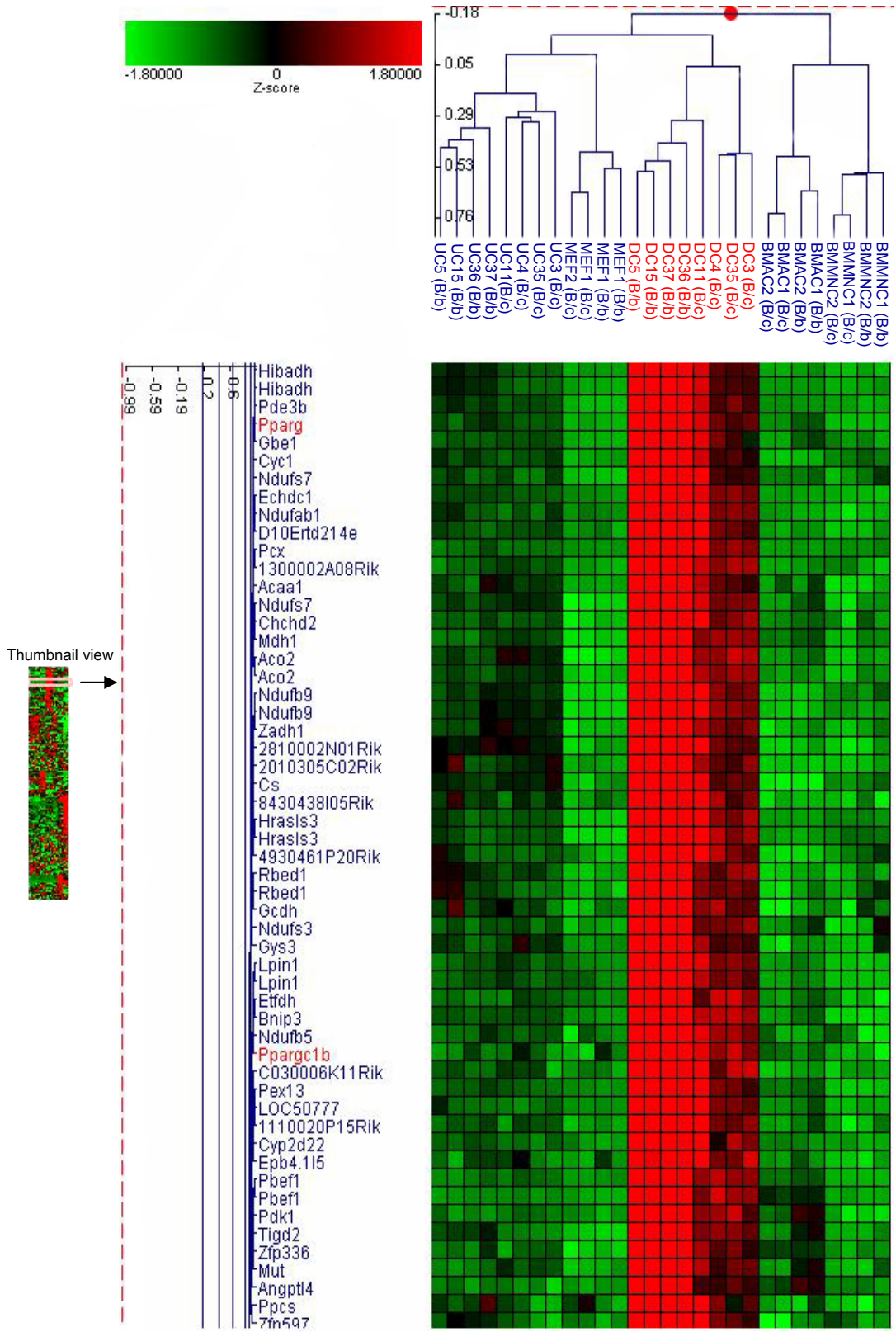


Figure 5.4 Dendrogram showing PPARγ clustering. An unbiased, genome wide, two-dimensional hierarchical cluster analysis was performed on the complete data set of 28 arrays as described in figure 5.1. Shown are genes clustering closest to adipocyte markers PPARγ and PPARγc1b. PPARγ: peroxisome proliferator-activated receptor-gamma. The color scale ranges from saturated red log ratios + 1.8 decreasing to saturated green for log ratios -1.8. Each gene is represented by a single row of colored boxes; each individual control is represented by a single column. Undifferentiated clone (UC); differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF); BALB/b (B/b); BALB/c (B/c).

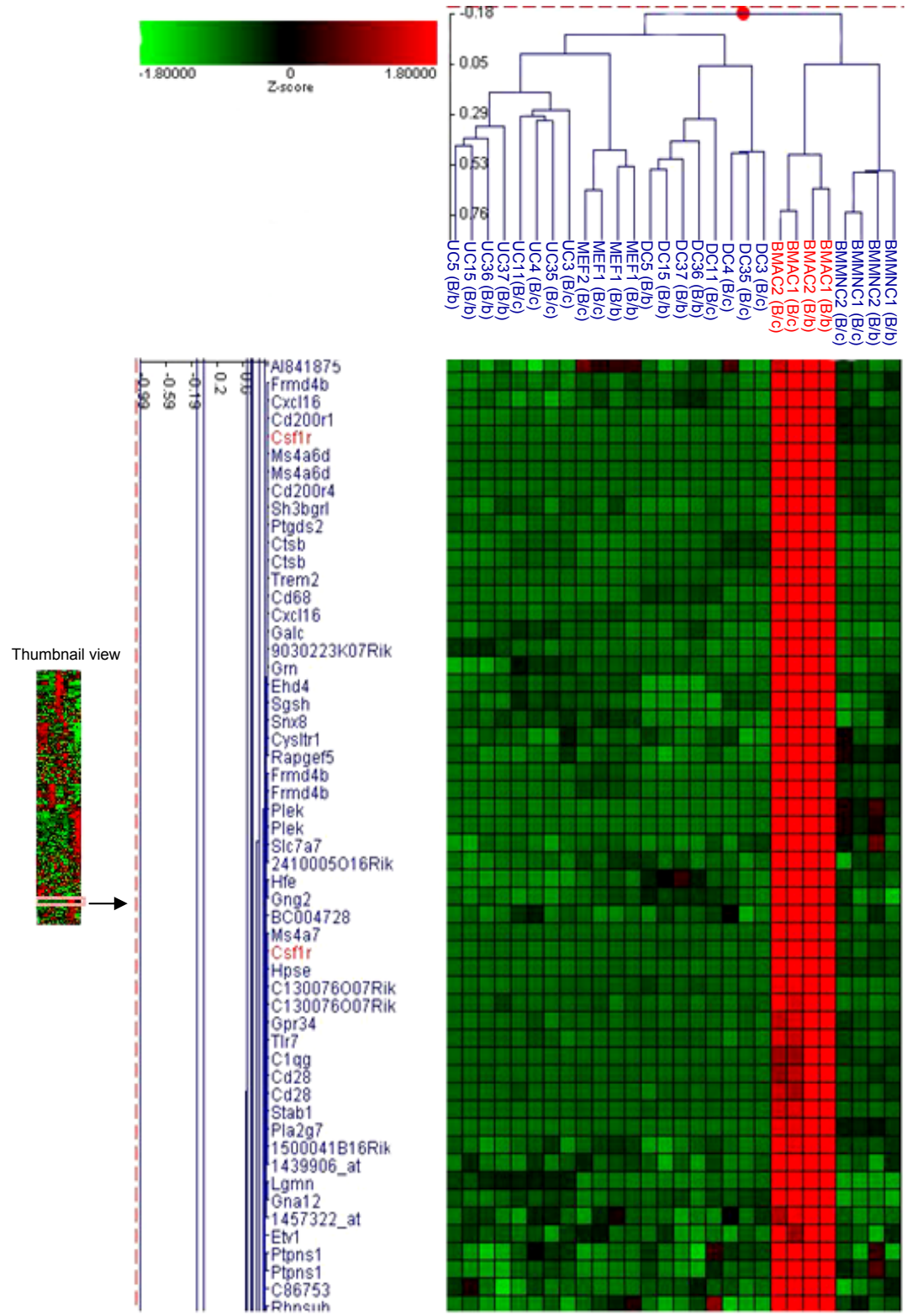


Figure 5.5 Dendrogram showing Csf1r clustering. An unbiased, genome wide, two-dimensional hierarchical cluster analysis was performed on the complete data set of 28 arrays as described in figure 5.1. Shown are genes clustering closest to stromal cell marker Csf1r: colony stimulating factor 1- receptor. The color scale ranges from saturated red log ratios + 1.8 decreasing to saturated green for log ratios -1.8. Each gene is represented by a single row of colored boxes; each individual control is represented by a single column. Undifferentiated clone (UC); differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF); BALB/b (B/b); BALB/c (B/c).

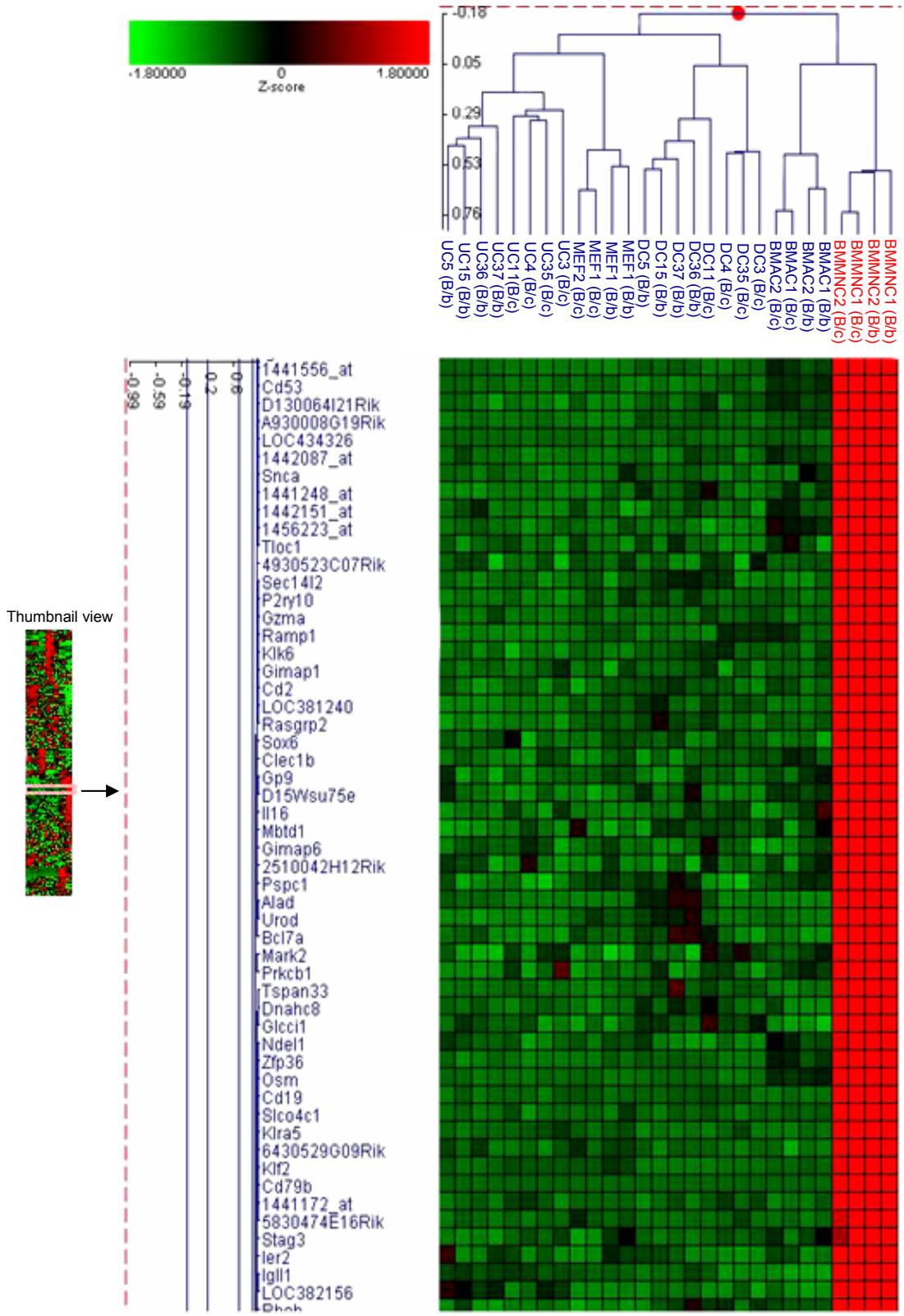


Figure 5.6 Dendrogram showing BMMNC highly expressing genes. An unbiased, genome wide, two-dimensional hierarchical cluster analysis was performed on the complete data set of 28 arrays. The color scale ranges from saturated red log ratios + 1.8 decreasing to saturated green for log ratios -1.8. Each gene is represented by a single row of colored boxes; each individual control is represented by a single column. Undifferentiated clones (UC); differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF); BALB/b (B/b); BALB/c (B/c).

and green of low level expression.

For example, as shown in figure 5.3 where the fibroblast marker CD90 is highlighted, all MEF samples showed high (red) expression, compared to all other cell types. Thus, highlighting, visually the similarity in gene expression profile of the MEF samples, irrespective of mouse strain differences. This striking similarity within control groups is repeated for BMACs taking Csfr1 as an example; and the adipocyte-differentiated clones (PPARγ), and for BMMNCs (figures 5.4-6).

With regard to adipocyte differentiation, BALB/c differentiated clones are less positive compared to the BALB/b for three of the clones (i.e., DC3, DC35 and DC4 – see figure 5.4). This can also be clearly seen in the PCA analysis where three of the DC samples are closer to the UCs than the remaining five DCs (see figure 5.2). Furthermore these findings did indeed match our observation of a more slow differentiation in these specific three cultures (as assessed by Oil Red O staining). Nevertheless, the impressive extent of adipocytic differentiation is highlighted by the fact that MEFs cluster closer to the undifferentiated clones than same clones after undergoing adipogenesis (e.g., see figure 5.4).

5.6 Supervised analysis of microarray data

We performed a stringent analysis selecting for genes expressed on all clones and absent or at least 3 fold lower on the same differentiated clones (figure 5.7A). For the other controls we selected for genes present on all the clones and absent or at least 3 fold lower on each control, e.g. MEF1 and MEF2. This criterion was repeated for the BMAC and the BMMNC samples. For the latter a 10 fold cut-off was chosen since MSCs are thought to be present at a very low level in this population (Castro-Malaspina et al., 1980). This gave a total of 28 comparisons of the clones to the various controls where these conditions had to be met in each case, for cultures from each strain. A final list of 78 and 63 transcripts was obtained for the BALB/b and BALB/c data sets, respectively. An intersection was taken of the resulting genes from each data set. This rigorous analysis

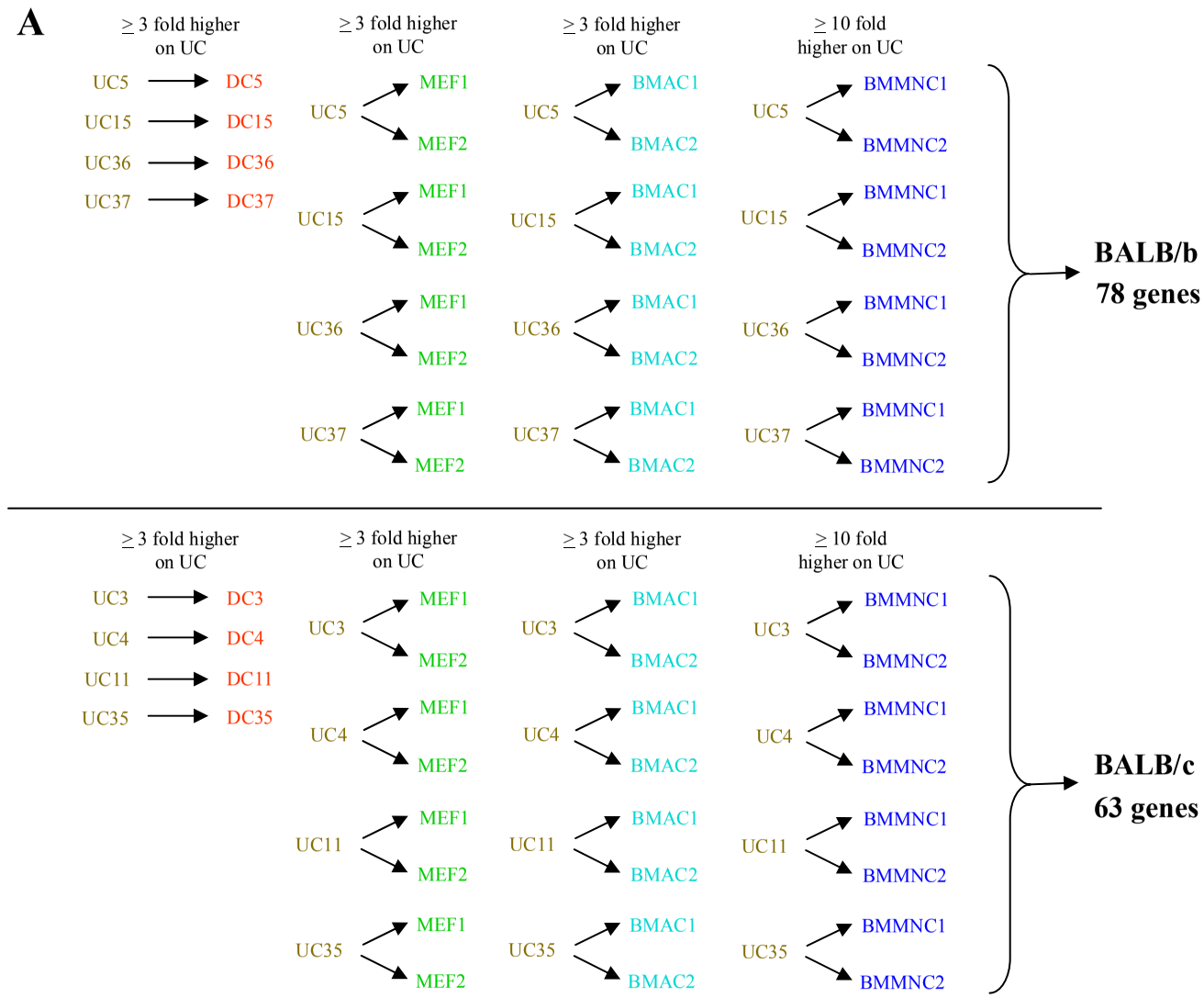


Figure 5.7 Schematic of microarray analysis to find genes specific to the MSC undifferentiated clone. (Figure legend next page)

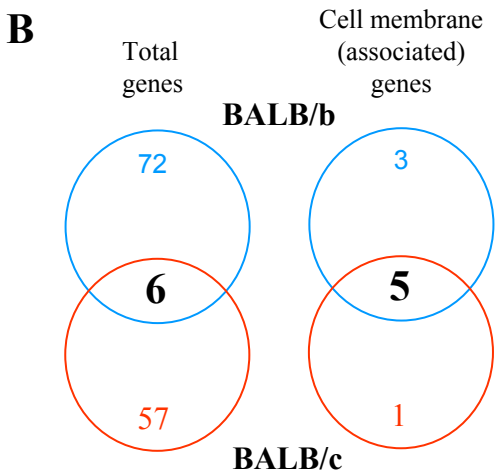


Figure 5.7 (continued) Schematic of Microarray Analysis to Find Genes Specific to the MSC Undifferentiated Clone . Genes were selected which were present on all 4 BALB/b UC and expressed at least 3- fold higher than DC, MEF, and BMAC and 10-fold higher than BMMNC (previous page) (**A**). This gave a final list of 78 genes. This was then repeated for the BALB/c strain and yielded a gene list of 63 genes. (**B**) Venn diagram showing the total number of genes that are shared between the BALB/b and BALB/c strain, yielding a final unique list of 6 total genes specific to the MSC undifferentiated clone. Another venn diagram was derived showing the number of cell membrane (associated) genes with an intensity greater than 100 (arbitary units) shared between the BALB/b and BALB/c strain, yielding a final unique list of 5 genes specific to the MSC undifferentiated clone. Differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF). , and the cell membrane (associated) genes, with intensity greater than 100 (arbitary units)

gave a final list of 6 genes (figure 5.7B). Another venn diagram was derived showing the number of cell membrane (associated) genes with an intensity greater than 100 (arbitrary units) shared between the BALB/b and BALB/c strain. Interestingly, this showed that 5 of the 6 genes are cell membrane (associated) genes. These results are discussed in section 5.7 below.

Classification of the subcellular localisations of the genes based on annotations using the DAVID (**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery) gene ontology software was performed. Several classes of enriched proteins were revealed: surface markers, secreted proteins, cell cycle-associated genes, nuclear proteins, and intracellular proteins. DAVID provides a comprehensive set of functional annotation tools which allows identification of genes not identified by Rosetta Resolver®, as well as information about gene function, and cellular location. Genes with signal intensities greater than 100 (arbitrary units) in the undifferentiated clones are shown (tables 5.1-5). The average signal intensity for each sample type is shown, thus the 3 fold or 10 fold cut-off is not obvious in all cases.

5.6.1 Supervised analysis of microarray data for BALB/b clones

Twenty-four percent of the BALB/b genes were cell membrane or membrane-associated proteins, and these included known MSC genes such as versican and unknown genes such as six-transmembrane epithelial antigen of the prostate 2 (STEAP2), STEAP1 and IL1Ri, and interestingly Ly6f, a member of the Ly6 family which includes Ly6a/Sca-1 (table 5.1).

Secreted proteins vouched for ten percent of the gene list containing, Ccl7, proliferin, and Cxcl5, which have not been reported in MSCs, although Cxcl5 has been found in germ-line stem cells (Mizukami et al., 2008) (table 5.2). Hepatocyte growth factor (HGF) was also expressed by the UCs. Four percent of the genes were cell cycle-associated genes including FGF7 and CXCL1 which have been identified in human MSCs (Djouad et al., 2007; Imabayashi et al., 2003) (table 5.3). A further 11% were nuclear proteins (table 5.4), one of which is vitamin D receptor (Vdr), an osteogenic transcription factor. Another transcription factor, T-box, 18 about which little is known was also identified. A

Table 5.1 BALB/b cell membrane (associated) genes
Cell surface associated genes were selected which were present on all 4 BALB/b UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified using the DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Chondroitin sulphate proteoglycan 2 (versican)	Cspg2	NM_019389	2685	198	264	135	19
Six transmembrane epithelial antigen of the prostrate 2	STEAP2	AK015015	2085	184	522	242	0
Six transmembrane epithelial antigen of the prostrate 1	STEAP1	AF297098	2082	273	307	261	1
Interleukin 1 receptor, type i	Il1ri	NM_008362	395	46	72	0	7
Lymphocyte antigen 6 complex, locus F	Ly6f	NM_008530	267	2	25	6	13
Synaptotagmin xvii	Syt17	AW048713	188	49	26	25	10
Transmembrane protein 100	Tmem100	NM_026433	182	0	8	0	0
Histocompatability 2, m region locus 9	H2-M9	NM_008205	107	8	0	14	0

Table 5.2 BALB/b secreted proteins
Genes encoding for secreted proteins were selected which were present on all 4 BALB/b UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Chemokine (C-C motif) ligand 7	Ccl7	AF128193	1825	20	180	175	14
Proliferin	Plf	X75557	1443	37	0	82	15
Chemokine (C-X-C motif) ligand 5	Cxcl5	NM_009141	869	0	141	21	0
Fibulin 1	Fbln1	BC007140	628	74	138	41	5
Hepatocyte growth factor	Hgf	AF042856	529	32	88	85	0

Table 5.3 BALB/b cell cycle associated genes
Genes associated with the cell cycle were selected which were present on all 4 BALB/b UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Fibroblast growth factor 7	Fgf7	BB791906	2858	189	823	760	0
Chemokine (C-X-C motif) ligand 1	Cxcl1	NM_008176	953	37	109	101	0

Table 5.4 BALB/b nuclear proteins
Genes encoding for nuclear proteins were selected which were present on all 4 BALB/b UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
vitamin D receptor	Vdr	AK018206	992	1	93	138	0
transducin-like enhancer of split 2, homolog of Drosophila E(spl)	Tle2	AK008793	227	10	58	57	2
t-box 18	Tbx18	BQ175889	225	21	84	41	21

Another transcription factor, T-box 18 about which little is known was also identified. A large proportion of the data set (35%) consisted of genes encoding other intracellular proteins (table 5.5). However, the majority had low signal intensity. Ellis van creveld gene homolog (human) had the highest intensity (218) and has not previously been reported in MSCs. The remaining 17% were unknown genes, or hypothetical proteins.

5.6.2 Supervised analysis of microarray data for BALB/c clones

The BALB/c data set share some of the genes present in the BALB/b dataset and are discussed below in 5.7. A large proportion of the data set (27%) were surface markers, including STEAP 1 and -2, versican, and Ly6f (table 5.6). As well as mas-related gpr, membrane f protein, and histocompatability 2, m region locus 9 (H2-M9) which have not previously been identified in MSCs, and also little is known about them.

Nine percent were secreted proteins (table 5.7), and these included insulin-like growth factor binding protein 6 (IGFBP6), which had a high signal intensity in the undifferentiated clones. Angiopoietin 4 and HGF were also identified. Genes encoding nuclear proteins comprised 16% of the final list (table 5.8) - Vdr, cyclin- dependent kinase inhibitor 2a (Cdkn2a), glis family zinc finger 3 (Gfzf3), t-box 18, and transducin-like enhancer of split 2, split homolog of drosphila E (spl) (Tle2) (table 5.8). The last three have not been previously reported in MSCs. Twenty percent consisted of other intracellular proteins, and these included cellular retinoic acid binding protein II (Crabp2), ankyrin 3 epithelial, cystatin E/M (cysteine protease inhibitors) (not previously identified in MSCs) (table 5.9). Twenty-seven percent of the data set represented unknown or hypothetical genes. Interestingly Sca-1 is not present on the surface marker list for either BALB/b or BALB/c data sets. This was due to the stringent criteria set for potential MSC-specific gene detection. FACS analysis from chapter 4 (table 4.2-4.3) showed that there was detectable Sca-1 expression in MEFs, and the microarray data for Sca-1 expression did not make the 3 fold cut-off imposed, thus excluding it from the final list.

Table 5.5 BALB/b intracellular genes
Genes encoding for intracellular proteins were selected which were present on all 4 BALB/b UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
ellis van creveld gene homolog (human)	**Evc**	BC021483	218	44	98	23	10
echinoderm microtubule associated protein like 1	**Eml1**	BC021917	165	7	37	30	1
centrosomal protein 55	**cp55**	NM_021292	119	0	32	33	37
carboxylesterase 1	**Ces1**	BC007140	109	1	10	7	19
coiled-coil containing 21 (tsf)	**Ccdc21**	BB147678	102	40	103	114	0

Table 5.6 BALB/c cell membrane (associated) genes.
Cell surface associated genes were selected which were present on all 4 BALB/c UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Six transmembrane epithelial antigen of the prostrate 2	STEAP2	AK015015	1343	226	337	252	0
Six transmembrane epithelial antigen of the prostrate 1	STEAP1	AF297098	1253	413	156	270	4
Chondroitin sulphate proteoglycan 2 (versican)	Cspg2	NM_019389	395	251	85	28	1
Mas-related gpr, membrane f	Mrgprf	BC019711	344	103	62	54	3
Histocompatability 2, m region locus 9	H2-M9	NM_008205	120	17	0	8	11
Lymphocyte antigen 6 complex, locus F	Ly6f	NM_008530	103	61	17	33	2

Table 5.7 BALB/c secreted proteins

Genes encoding for secreted proteins were selected which were present on all 4 BALB/c UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Insulin-like growth factor binding protein 6	Igfbp6	NM_008344	3492	453	520	487	1
Angiopoietin 4	Angpt4	NM_009641	348	66	32	31	1
Hepatocyte growth factor	Hgf	D10212	162	106	38	56	1

Table 5.8 BALB/c nuclear proteins

Genes encoding for nuclear proteins were selected which were present on all 4 BALB/c UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
vitamin D receptor	Vdr	AV328983	609	154	73	149	0
cyclin-dependent kinase inhibitor 2a	Cdkn2a	BB709140	530	207	87	86	15
glis family zinc finger 3	gfzf3	BB036542	300	172	99	128	3
t-box 18	Tbx18	AK019867	147	158	41	101	11
transducin-like enhancer of split 2, split homolog of drosphila E (spl)	Tle2	NM_007680	133	24	46	5	7

Table 5.9 BALB/c intracellular proteins

Genes encoding for intracellular proteins were selected which were present on all 4 BALB/c UC and absent or expressed at least 3-fold higher than the equivalent DC, MEFs, and BMACs and 10- fold higher than BMMNC as described in figure 5.7. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
cellular retinoic acid binding protein II	Crabp2	AV280494	1812	410	400	101	0
ankyrin 3, epithelial	Ank3	BB709140	465	206	82	138	0
cystatin E/M (cysteine protease inhibitors)	Cst6	BB093349	416	117	41	43	1

5.7 Supervised analysis yields six MSC-specific candidate genes

Comparing the datasets from the two mouse strains, six genes were expressed at a differentially high level in all the UCs compared to the various controls – six transmembrane epithelial antigen of the prostate (STEAP1 and STEAP2), versican, vitamin D receptor, ly6f, and H2-M9. As the graphs in figure 5.8 show, these genes exhibit a strikingly similar gene expression pattern. They are all preferentially expressed in undifferentiated clones and at relatively low levels in all controls suggesting they are potential MSC-specific genes. Amazingly, of the six genes identified, five are cell membrane (or cell membrane associated protein in the case of versican). Indeed, the sixth gene, being vitamin D receptor is also a membrane protein (nuclear). T-box 18 and HGF which are present in both datasets were not present in the final list as the average intensity of both did not fulfil the set criteria.

Of the six genes identified, STEAP1 and STEAP2 are expressed at the highest intensity. These genes were first identified in prostate cancer cells (Hubert et al., 1999; Porkka et al., 2002), and this is the first mention of STEAP 1 and/or 2 in MSCs. Ly6f is a member related to Ly6a/Sca-1, but was expressed at a low level (185 arbitrary units). H2-M9 is a major histocompatability class Ib gene, and also has relatively low signal intensity (114). Vitamin D receptor (Vdr) has a greater signal intensity of 559 in the UCs. It is expressed on many cell types including MSCs, osteoblasts, mammary cells, and cells of the immune system. Versican is relatively highly expressed (1027), but is also known to be expressed on many other cell types including chondrocytes and osteoblasts.

5.8 STEAP1 and -2 cluster with Sca-1

An unbiased, genome-wide, 2D hierarchical cluster analysis was performed on the complete data set of 28 arrays. Genes clustering with Sca-1 are shown in figure 5.9. STEAP1 and -2 and Vdr cluster with Ly6a (Sca-1) and as stated above these genes were also found to be differentially expressed in the UCs following the supervised analysis. Thus two different methods of analysis, (one supervised, and one unsupervised) have identified STEAP1 and -2 and Vdr as differentially expressed on MSC clones.

Several transcripts of cell cycle protein Cdk4 also clustered in this region. Interestingly the hypoxia inducible factor-1 alpha (HIF-1α) was also present in this section.

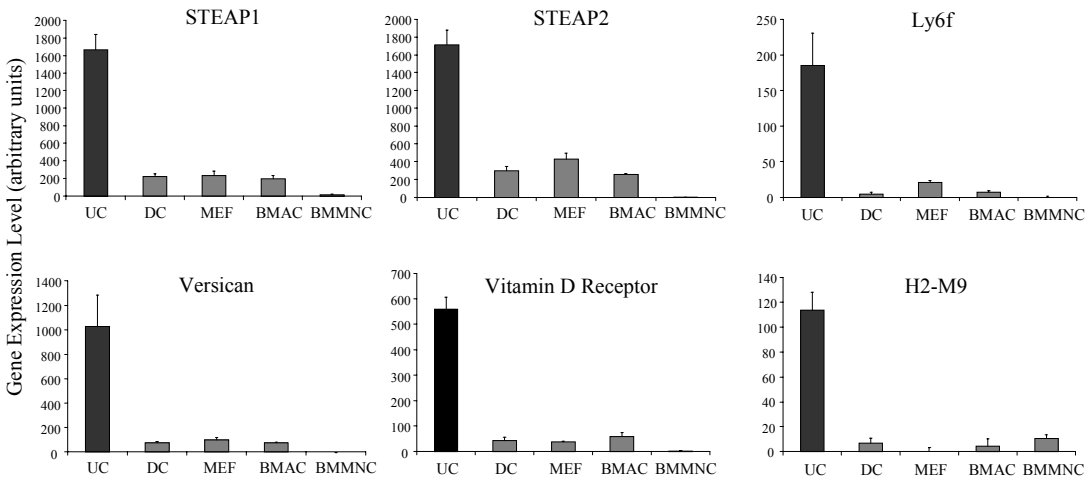


Figure 5.8 Molecular signature of MSC clones. Six genes met the stringent conditions for differentially high expression on all 8 UC compared to all 20 control samples as described in figure 5.7. The average (± SEM) gene expression intensities (arbitary units) from the microarray analysis for these 6 genes are shown (n=8 samples for UC and for DC; n=4 for MEF, BMAC, and BMMNC). Undifferentiated clones (UC); differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

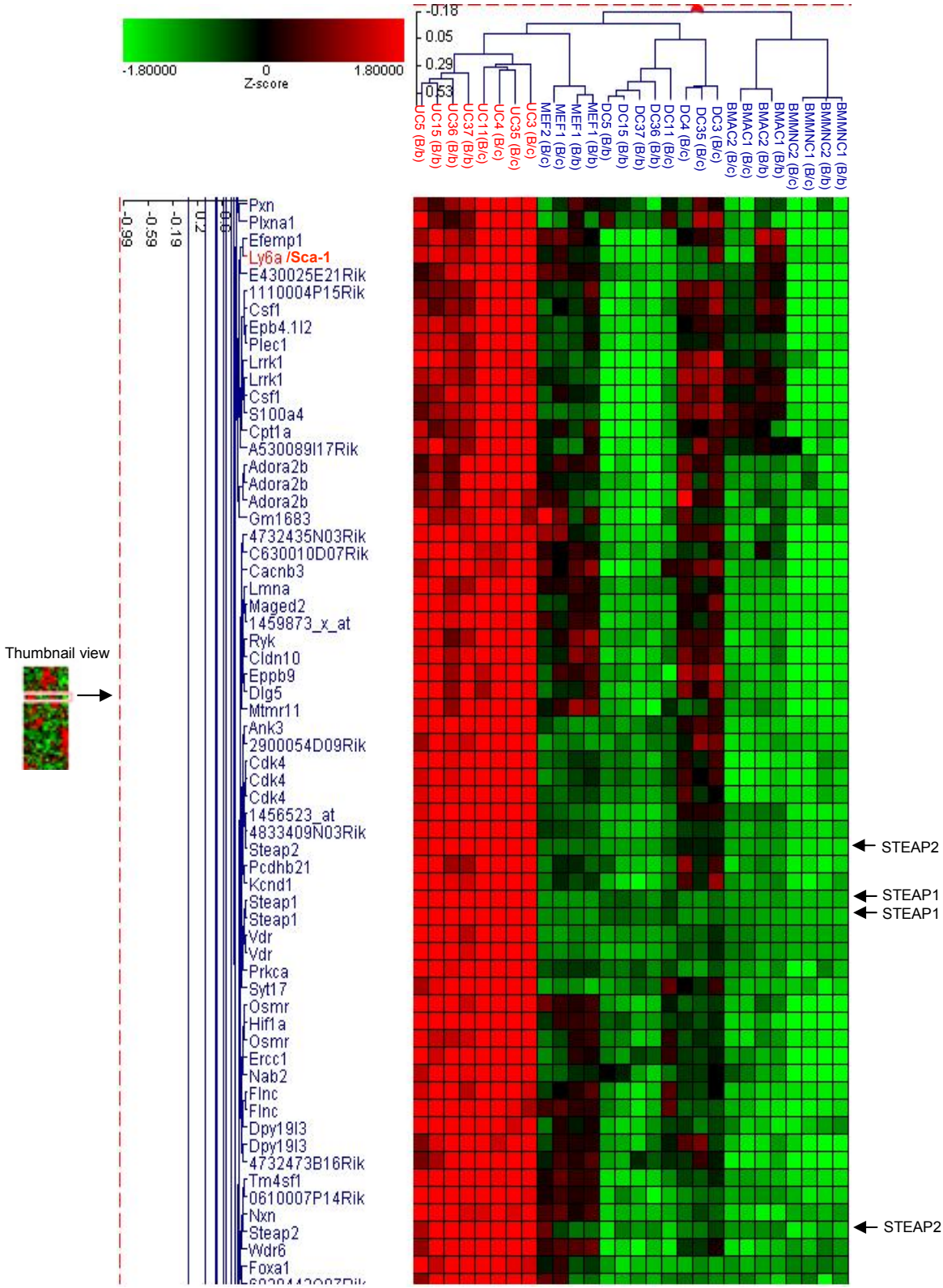


Figure 5.9 Dendrogram showing genes clustering with Sca-1. An unbiased, genome wide, two-dimensional hierarchical cluster analysis was performed on the complete data set of 28 arrays as described in figure 5.1. Shown are genes clustering closest to Sca-1 which include STEAP1 and -2. The color scale ranges from saturated red, log ratios + 1.8 decreasing to saturated green for log ratios -1.8. Each gene is represented by a single row of colored boxes; each individual control is represented by a single column. Undifferentiated clones (UC); differentiated clones (adipocytes) (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF); BALB/b (B/b); BALB/c (B/c).

With regard to the cluster analysis, genes which displayed an expression profile similar to Sca-1 were selected by going up and down one proximal note of Sca-1. This gave a list of 310 genes. Transcripts were categorised into 3 groups based on annotations in the DAVID Gene Ontology software as described above (tables 5.10-12). Table 5.10 shows that Sca-1 is the surface protein with the highest signal intensity 9177 (arbitrary units). This is shown graphically in figure 5.10. Membrane proteins TM4SF1, erythrocyte protein band 4.1-like 2, Maged2, and oncostatin M receptor were also highly expressed in the clones compared to controls (table 5.10). Integrins such as, integrin alpha 5 (fibronectin receptor alpha), ephrin B1, microfibrillar-associated protein 3-like, intergrin alpha 3, were identified in the Ly6a/Sca-1 cluster. Neural cell adhesion molecule 1 (NCAM-1) was also present.

Epidermal growth factor-containing fibulin-like extracellular matrix protein-1 and 2 were also highly expressed in the UC, although also quite high on MEFs. They have not been previously found in MSCs. The undifferentiated clones significantly express the secreted proteins IGFBP6, HGF, FGF7, and Ccl7 in comparison to the controls (table 5.11, figure 5.10). The last three were also identified in the supervised analysis described above.

Table 5.12 shows cell cycle gene Cdk4 was expressed at a high intensity (3837). Growth factor FGF7 was also highly expressed on the undifferentiated MSC clones. Mdm2 was also quite highly expressed, but it was also expressed at a similar level in the MEFs and BMACs (table 5.12). This highlights the danger of sole reliance on unsupervised analysis, and also the advantage of the supervised method which filters out such high, but not MSC-specific gene expression. Thus, STEAP1 and -2 were the only two cell membrane proteins identified by both methods of analysis. From reports in the literature they are not widely expressed in other cell types and they are present at very low levels in all our control cell populations. This makes them very attractive candidates for further analysis.

Table 5.10 Cell membrane (associated) genes clustering with Sca-1.
Genes encoding for cell surface markers were selected which showed an expression profile similar to Sca-1 in an unbiased 2-dimensional hierarchical cluster analysis shown in figure 5.9. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence Name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Lymphocyte antigen 6 complex, locus A/ Sca-1	Ly6a/Sca-1	BC002070	9177	1072	4803	2815	202
Transmembrane 4 superfamily member 1	TM4SF1	BQ177170	3049	287	1794	397	17
Erythrocyte protein band 4.1-like 2	Epb4.1l2	BE951907	2959	878	1717	1948	317
Melanoma antigen, family D, 2	Maged2	AF319976	2587	435	1180	227	6
Oncostatin M receptor	Osmr	AB015978	1927	408	1334	565	5
Six transmembrane epithelial antigen of the prostrate 2	STEAP2	AK015015	1714	298	429	256	1
Six transmembrane epithelial antigen of the prostrate 1	STEAP1	AF297098	1668	223	232	194	13
Angiomotin	Amot	AF297098	1210	299	714	147	12
Transmembrane protein 19	Tmem19	BE951907	1072	425	415	712	199
Histocompatibility 60	H60	BG067039	1069	189	183	421	44
Receptor-like tyrosine kinase	Ryk	BB822862	1030	334	821	320	91
Hyaluronan synthase 2	Has2		455	67	155	262	21
Erythrocyte protein band 4.1-like 3	Epb4.1l3	M98547	454	133	409	171	0
Integrin alpha 5 (fibronectin receptor alpha)	Itga5	BE951907	339	91	173	204	16
Neural cell adhesion molecule 1	Ncam1	BB050303	330	63	175	116	41
Ephrin B1	Efnb1	BB817972	306	119	241	148	28
Solute carrier organic anion transporter family, member 2a1	Slco2a1	NM_008216	298	77	150	309	14
Membrane associated guanylate kinase-related (MAGI-3)	Magi3	AF177146	295	99	243	136	69
Microfibrillar-associated protein 3-like	Mfap3l	NM_008216	258	53	157	157	13
Fyn-related kinase	Frk	BB831986	254	80	138	69	2
Interleukin 1 receptor 1	Ilr1	AB015978	241	68	55	0	0
Intergrin alpha 3	Itga3	BB493533	240	77	202	101	30
Calcium channel, voltage-dependent, beta 3 subunit	Cacnb3	NM_010875	229	64	179	76	1
Angiopoietin 4	Angpt4	BC006797	225	25	0	52	0
Hepatocellular carcinoma-associated antigen 127	Hcca127	NM_033314	180	100	160	119	32
G protein-coupled receptor 85	Gpr85	BF180812	178	66	68	122	5
Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	Edg2	BB529332	160	69	78	65	21
Transmembrane 100	Tmem100	AK017269	135	18	6	4	0

Table 5.11 Genes encoding for secreted proteins clustering with Sca-1.
Genes encoding for secreted proteins were selected which showed an expression profile similar to Sca-1 in an unbiased 2-dimensional hierarchical cluster analysis shown in figure 5.9. The sub-cellular localisation of the genes was classified by DAVID gene ontology software. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Epidermal growth factor-containing fibulin-like extracellular matrix protein 1	Efemp1	BC023060	6128	1180	4635	3486	3
Insulin-like growth factor binding protein 6	Igfbp6	NM_008344	3098	894	633	76	20
Epidermal growth factor-containing fibulin-like extracellular matrix protein 2	Efemp2	NM_021474	2659	887	1573	542	0
Fibroblast growth factor 7	Fgf7	BB791906	2092	571	674	613	2
Chemokine (C-C motif) ligand 7	Ccl7	AF128193	1352	113	149	342	13
Cytokine receptor-like factor 1	Crlf1	NM_018827	878	371	726	363	0
Colony stimulating factor 1	Csf1	M21149	541	177	368	449	65
Hepatocyte growth factor	Hgf	AF042856	356	76	75	77	19
Procollagen, type 1, alpha 2	Col1a2	BB150460	183	36	70	52	3

Table 5.12 Cell cycle associated genes clustering with Sca-1.
Genes associated with the cell cycle were selected which showed an expression profile similar to Sca-1 in an unbiased 2-dimensional hierarchical cluster analysis shown in figure 5.9. The sub-cellular localisation of the genes was classified by DAVID gene ontology website. Genes are ranked by their expression intensity in UC (arbitrary units). Undifferentiated clone (UC); adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF).

Sequence name	Gene	Accession Number	UC	DC	MEF	BMAC	BMMNC
Cyclin dependent kinase 4	Cdk4	NM_009870	3837	1330	1944	963	708
Fibroblast growth factor 7	Fgf7	NM_008008	2092	571	674	613	2
Transformed mouse 3T3 cell double minute 2	Mdm2	AK004719	1578	903	1400	1573	513
Cullin 4B	Cul4b	AW536452	693	383	578	474	362
Histone deacetylase 7A	Hdac7a	BB277517	605	223	362	161	217
Colony stimulating factor 1	Csf1	M21149	541	177	368	449	65
Cyclin-dependent kinase inhibitor 2a	Cdkn2a	NM_009877	530	183	206	108	14
MAD homolog 3	Smad3	B1646741	424	189	313	277	170
Hepatocyte growth factor	Hgf	AF042856	356	76	75	77	19
Eprin B1	Efnb1	BC006797	306	119	241	148	28
Fyn-related kinase	Frk	BB787292	254	80	138	69	2
ELK3, member of ETS oncogene family	Elk3	BC005686	200	32	99	88	22

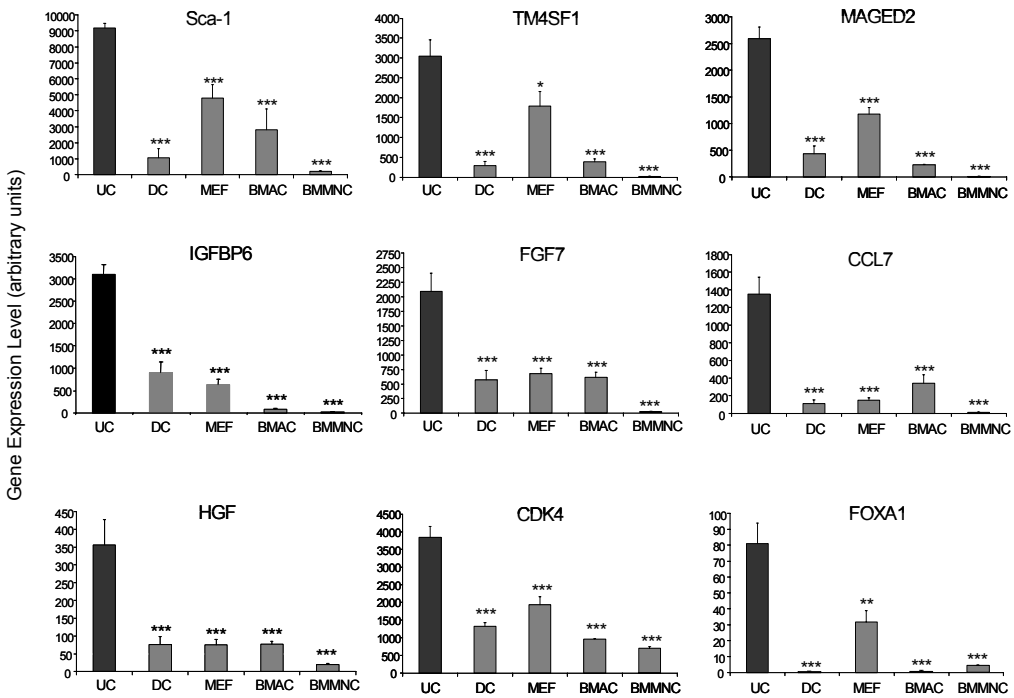


Figure 5.10 Genes highly expressed on MSC clones. Genes which clustered with Sca-1 were selected by going up and down one proximal note of the Ly6a/Sca-1 cluster shown in figure 5.9. This gave a list of 310 genes. Average gene expression intensities in arbitary units (±S.E.M) for 9 of these genes which show differentially high expression on UC are shown. *P<0.05, **P<0.01, ***P<0.001 versus UC expression level. Data were compared using one-way analysis of variance (ANOVA) with Donnet's multiple comparison test using Prism 5 software (Graphpad). Abbreviations: UC - undifferentiated MSC clones; DC – differentiated clones (adipocytes); MEF - mouse embryonic fibroblasts; BMAC - bone marrow adherent cells (primary culture); BMMNC – bone marrow mononuclear cells (freshly isolated), (n=8 samples for UC and for DC; n= 4 for MEF, BMAC, and BMMNC).

5.9 Validation of microarray results

Microarray results were validated by quantitative RT-PCR for STEAP1 and -2, Ly6a/Sca-1, versican, TM4SF1, and adipocyte marker PPARγ. The results confirmed the microarray data (table 5.13).

5.10 Discussion

Many groups have tried to identify MSC specific markers. For example, hybridoma technology has been utilised to obtain monoclonal antibodies raised against a heterogeneous population of adherent bone marrow cells (Haynesworth et al., 1992). Alternatively, investigators have sorted bone marrow cells based on a specific (but arbitrarily chosen) antigen (Jones et al., 2002). Both approaches suffer from major shortcomings, as the antibodies derived in the former may not be MSC specific and the latter method is biased and provides no new information of an MSC marker.

The transcriptome of embryonic stem cells has been well characterised and this has led to greater understanding of the self-renewal and differentiation mechanism of embryonic stem cells (Zhan, 2008). The transcription factors OCT4, Nanog, and SOX4 are known to be essential to the pluripotent and self-renewing phenotype of embryonic stem cells (Chambers et al., 2003; Nichols et al., 1998). Very little however, is known about the mechanisms controlling the 'stemness' of MSCs. Application of microarray technology to further the understanding of MSC biology has been hampered by the lack of physiologically relevant cell models that can be used for characterisation. This is because most groups work with a mixed population of cells they term 'MSCs' (Jia et al., 2002; Song et al., 2006; Wagner et al., 2006; Wieczorek et al., 2003). Furthermore, this heterogeneous cell population is ill-defined. Their ability to both self-renew and differentiate into the mesenchymal lineages of adipocytes, chondrocytes, and osteoblasts has not been well examined. Therefore any subsequent results gained about this heterogeneous population of cells is always going to be limited, and it is difficult to directly compare one such study with another.

Table 5.13 Validation of Microarray Data by Real Time PCR for (A) BALB/b, and (B) BALB/c data sets. Intensities expressed relative to undifferentiated clones (UC) levels. Adipocyte differentiated clones (DC); primary bone marrow adherent cells (BMAC); freshly isolated bone marrow mononuclear cells (BMMNC); and mouse embryonic fibroblasts (MEF); Peroxisome proliferator-activated receptor- γ (PPARγ).

A

	Ly6a/Sca-1		**STEAP1**		**STEAP2**		**TM4SF1**		**Versican**		**PPARγ**	
	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array
UC	1	1	1	1	1	1	1	1	1	1	1	1
DC	0.01	0.01	0.08	0.13	0.06	0.09	0	0	0.06	0.07	6.25	4.97
MEF	0.18	0.64	0.05	0.15	0.07	0.25	0.1	0.56	0.05	0.1	0.06	0.20
BMAC	0.01	0.06	0.04	0.13	0.06	0.12	0.08	0.13	0.03	0.05	0.25	0.48
BMMNC	0	0.03	0	0.01	0	0	0	0.01	0.01	0.01	0.06	0.02

B

	Ly6a/Sca-1		**STEAP1**		**STEAP2**		**TM4SF1**		**Versican**		**PPARγ**	
	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array
UC	1	1	1	1	1	1	1	1	1	1	1	1
DC	0.08	0.22	0.05	0.14	0.3	0.3	0.19	0.26	0.15	0.21	3.85	2.75
MEF	0.17	0.41	0.07	0.12	0.15	0.25	0.45	0.63	0.16	0.22	0.23	0.21
BMAC	0.16	0.55	0.03	0.1	0.06	0.2	0.06	0.14	0.06	0.22	1.50	1.64
BMMNC	0.02	0.02	0	0	0	0	0	0	0.01	0.01	0.38	0.06

One group has examined the gene expression profile of a clonal population of human MSCs and catalogued 2,353 genes (Tremain et al., 2001). However, the significance of the results were limited as only one clone was studied, and no other biological replicates were used. The lack of appropriate control populations is another drawback as it is not possible to distinguish potentially MSC-specific genes from the typically long gene lists that are obtained. Thus these studies have failed to delineate the 'genetic signature' common to MSCs.

The Wagner group used a slightly better approach as they compared the gene expression profile of several cell populations. These included human MSCs isolated from bone marrow, adipose tissue, umbilical cord blood under two different growth conditions; comparing to terminally differentiated human newborn foreskin fibroblasts (HS68) (Wagner et al., 2005). A limitation of the study was that all the MSC populations were heterogeneous, thus making it difficult to distinguish the MSC specific genes from that of the other cell types. Also, only the adipogenic and osteogenic differentiation capacity of the cells was examined. In contrast, in the present study, all the clonal cells studied had tri-lineage differentiation potential as shown in chapter 3. In addition, adipogenesis of umbilical cord blood cells was not convincing in the Wagner study. Another downside of that study is that the different cell populations were cultured in different culture conditions with the addition of various growth factors and chemicals such as dexamethasone which may have selected for distinct cell types. However, the investigators did compare the gene expression profile of the MSC populations to fibroblasts that did not have the differentiation ability of the MSC populations. The group identified 25 genes that were differentially highly expressed on all MSC populations compared to the fibroblasts. However, as already stated, the problem with this is that all the MSC populations were mixed, and each tissue specific MSC was from a different donor of varying ages. The fact that the umbilical cord blood MSCs were not as multipotent as the bone marrow and the adipose MSCs may have affected the results and prevented entry of MSC specific genes into the final gene list.

The Song group examined the gene expression profile of a heterogeneous population of human MSCs, comparing it to that of differentiated MSCs (Song et al., 2006). The major

aim of their study was to identify molecular MSC markers to facilitate the purification of homogeneous MSC populations. The gene expression profile of undifferentiated MSCs was compared to MSCs differentiated into adipo-, chondro-, and osteogenic lineages. Although this was a sound approach there were some limitations; one major one being different donors were used for the different differentiation assays, thus introducing unnecessary biological variability into the dataset, and lessening the power of the results. Also the donors were quite old ranging 59-70 years old. The starting population was again heterogeneous and although they demonstrated the tri-lineage differentiation ability of the MSCs, osteogenic differentiation was only demonstrated through alkaline phosphatase staining which is not, on its own, a robust assay of osteogenesis. Ideally von kossa or alizarin red should be used to directly detect the mineralised matrix secreted by the cells. The investigators compared the gene expression profile of undifferentiated MSCs, differentiated MSCs, and the equivalent de-differentiated MSCs. The analysis resulted in a large gene list of 460 genes which they classed as 'stemness' genes, 91 of which encoded cell surface antigens. It is difficult to examine the function of all these genes. Therefore, the investigators selected 5 based on their previous known functions. Although this is a realistic approach, it is not ideal as potential unknown MSC specific genes may be missed.

Due to these various issues, we therefore very carefully planned our microarray experimental design in order to avoid these different shortcomings, and obtain the most meaningful data possible. As described in chapter one, by combining a low oxygen environment with low density cultures we isolated clonal cells that fulfil the criteria for mesenchymal stem cells. The clones have the capacity to differentiate into the three lineages - adipogenic, osteogenic, and chondrogenic that characterise a MSC. They also have the ability to self-renew. After further characterisation (detailed in chapter 4) we found that the cells were karyotypically abnormal but, we also found they were not malignant, and showed very well-regulated differentiation *in vitro*, and thus could be utilised as a good model to study MSC biology. Our final aim (detailed in the following chapter) was then to use the results from the microarray study to test for the presence of

candidate MSC-specific markers thus identified on (karyotypically) normal human MSCs.

The Song group compared the gene expression profile of undifferentiated MSCs to differentiated MSCs, and the Wagner group compared the gene expression profile of MSCs to fibroblasts (Song et al., 2006; Wagner et al., 2005). We performed an even more extensive analysis. The gene expression profile of the MSC clones in this study was compared to the equivalent adipocyte differentiated clones (which lacked stem cell properties), embryonic fibroblasts (that lacked the differentiation potential of the clones), a mixed population of BMACs, and freshly isolated BMMNCs.

The gene expression profile of the clonal cells was compared to these relevant controls to characterise the molecular signature of the MSCs. It is important to note here that all cells were cultured at 5% oxygen for at least 4 days to ensure no change in transcripts occurred simply due to alterations in the oxygen environment. A previous study has reported a change in gene transcripts in MSCs in response to variation of oxygen levels at different time courses (Ohnishi et al., 2007). Also, all cells were cultured in the same cell culture conditions without the addition of growth factors that may bias the results. This is an important point as studies have reported that differences in cell culture conditions can change the gene expression profile of two parallel populations of MSCs (Wagner et al., 2006). A large percentage of genes were found to be differentially regulated when bone marrow MSCs were cultured in two different media compositions M1 and M2. M1 consisted of DMEM and MCDB201, 2% FCS, supplemented with L-glutamine, 1% insulin transferrin selenium, 1% linoleic acid bovine serum albumin, dexamethasone, L-ascorbic acid 2 phosphate, PDGF-BB, and EGF previously used by Reyes and colleagues (Reyes et al., 2001). M2 was a commercially purchased culture medium from Poietics for human mesenchymal stem cell. MSCs cultured in M1 expressed a greater proportion of genes in the functional categories of metabolism and mitochondria. Whereas genes involved in muscle development, neurogenesis, cell differentiation and morphogenesis, and skeletal developmental were significantly overexpressed in MSCs cultured in M2 media. The results emphasise the huge impact different culture conditions can have on the intrinsic biology of the cells and the need for standardisation if comparisons are going to be made between MSC studies.

In this study, a total of eight undifferentiated clones (from two different mouse strains) were used thus providing the wide range of biological replicates needed for generation of meaningful microarray data. In addition, four different controls were also used, each with their own biological replicates. This thus represented a major undertaking, and in total, the gene expression profile of 28 different samples was examined. Principal component analysis of these 28 genome-wide arrays showed that the undifferentiated clones clustered very tightly together, thus highlighting that they were very similar, irrespective of mouse strain differences. This substantiates the fact that they have a similar morphology, differentiation potential, and surface antigen profile. This was also true for the other cell types examined. Similar findings were also uncovered using an unsupervised 2D-hierarchical cluster analysis of the data. Within the control groups the cell types exhibited very similar expression profiles e.g., the adipocyte differentiated clones preferentially expressed PPARγ which is an adipocyte marker. The MEFs highly expressed the fibroblast marker CD90/Thy-1, which corresponds to the flow cytometry results (table 4.2-4.3) and the BMACs expressed relatively high levels of colony stimulating factor 1- receptor (Csf1r).

We performed two types of analysis, supervised and unsupervised, allowing comparison of the two approaches and strengthening the resultant data. The two analytic methods have different strengths and pitfalls in transcriptional profiling analysis. In the stringent supervised analysis, the minimum cut-off we used when comparing gene expression levels of MSCs to the various controls was 3-fold (and 10-fold for the BMMNCs). The higher value for the latter was applied since MSCs are present at such a low frequency in the bone marrow. Other groups have looked for 2 fold differences (Tuan et al., 2003), but we believe when screening for potentially cell-specific genes, more stringent conditions are necessary. Thus, for cultures from each mouse strain, these conditions had to be met for **all** of the 28 individual comparisons made (see figure 5.7A). This is a very strict regime, but since we are looking for cell-specific genes, we reasoned that these conditions should be met on every occasion, rather than merely using statistical averages. We thus found 63, and 78 genes differentially highly expressed on all the undifferentiated

clones from the BALB/b and BALB/c mice respectively. A final comparison of the genes present in both mouse strains gave only 6 genes. Most strikingly, all six are membrane, or membrane-associated genes, with only one not being associated with the cell membrane itself, i.e., vitamin D receptor (Vdr) being nuclear. The five cell membrane (associated) genes are STEAP1, STEAP2, versican, ly6f, histocompatability 2, m region locus 9 (H2-M9).

Indeed, a very interesting point is that differentially expressed cell membrane (associated) genes were highly conserved between the two mouse strains compared to other genes. For example, 8 genes for cell membrane (associated) markers were differentially expressed from BALB/c MSC clones, and 6 for BALB/c clones, therefore almost all (five) were common to both strains (see figure 5.7B). Indeed, it is noteworthy that only membrane or membrane-associated genes were identified as differentially expressed in the final analysis.

Interestingly, Ly6f is a member of Ly6a/Sca-1 family, which is highly expressed on the undifferentiated clones. Unfortunately little is known about Ly6f protein and no reagents are available. Despite having a low signal intensity, it would be interesting to determine whether it has a functional role in MSC biology, as Sca-1 has been reported to be required for self-renewal of MSC progenitors in a study of Sca-1 knockout mice (Bonyadi et al., 2003). H2-M9 is a major histocompatability class Ib gene, first cloned and sequenced from BALB/c mice (Arepalli et al., 1998) and in our study (like Ly6f) is expressed at a relatively low level. Major histocompatability class 1 genes are expressed on all nucleated cells. These proteins play a major role in the immune system, as they present fragmented antigen parts on the host cell surface. These may be ‘self’ or ‘non-self’. Although there is little known about this protein, based on its function it is likely to be expressed on a variety of cell types. This is the first report identifying the two genes in MSCs. Vitamin D receptor (Vdr) was expressed at a relatively higher level in UCs (than Ly6f and H2-M9). Vdr is also known as the calcitriol receptor, and is a member of the steroid hormone receptor family of nuclear receptor proteins. Vitamin D is required for calcium homeostasis so it is not surprising that Vdr is a transcription factor that plays an important role in regulating osteogenesis in MSCs (Pochampally et al., 2007), and indeed some groups add vitamin D to stimulate osteogenesis of MSCs (Malladi et al., 2006b).

Vdr has also been reported to have a role in stimulating chondrogenesis of chick limb bud mesenchymal cells (Tsonis et al., 1996). Vdr is expressed on many cell types including MSCs, osteoblasts, mammary cells, and cells of the immune system such as T cells and dendritic cells.

Versican also known as chondroitin sulphate proteoglycan core protein-2 (CSPG-2) was found to be highly expressed on the UCs. It is composed of modular protein domains with several glycosaminoglycan side chains. Versican is a component of the extracellular matrix (ECM) of cells and thus plays a role in many cellular processes such as cell adhesion, proliferation, migration, angiogenesis as well as tissue morphogenesis and maintenance (Rahmani et al., 2006). Due to its role in a wide range of cellular processes it is expressed on many cell types and thus may lack the specificity required to be a MSC-specific marker. Studies have found versican expression in MSCs increases upon differentiation into chondrocytes and osteoblasts (Diaz-Romero et al., 2008; Foster et al., 2005). This could be due to an increase in the ECM of both cell types. Therefore, results suggest it does not have a functional role in maintaining the stemness of MSC. As both Vdr and versican are expressed in many cell types in the bone marrow, they are unlikely to be useful markers to distinguish MSCs from other bone marrow-derived cells. H2-M9 and Ly6f are expressed at relatively low signal intensity in the UCs and very little is known about these two membrane proteins, not to mention a lack of reagents available.

Six transmembrane epithelial antigen of the prostate 1 (STEAP1) and STEAP2 were both highly expressed on the undifferentiated clones. STEAP1 and STEAP2 were first discovered relatively recently, in 1999 and 2002 respectively (Hubert et al., 1999; Porkka et al., 2002). They were found in high levels in human prostate cancer. Recent studies have also reported STEAP1 expression in murine prostate cancer (Yang et al., 2001). Based on their secondary structure (that contains six transmembrane domains) they are predicted to function as potential ion channels (Dolly and Parcej, 1996), water channels (Kruse et al., 2006) or transporter proteins (Ohgami et al., 2006). They belong to a family of STEAP proteins that consists of STEAPs 1-4. All STEAPs contain intra-membrane haem-binding sites. STEAPs 2-4 contain a N-terminal domain that is similar to the archael and bacterial $F_{420}H_2$:NADP+ oxidoreductase (FNO) binding proteins (Ohgami et

al., 2006). They are reported to function as ferriductases and cupric reductases that stimulate cellular uptake of both iron and copper *in vitro* and possibly *in vivo*. STEAP1 lacks this FNO-like reductase domain, however STEAP proteins form heteromultimers with STEAP1. Thus, this may be one mechanism by which STEAP1 participates in metal homeostasis. In agreement with these results, STEAP2 was found to co-localise completely with transferrin (Tf) and Tf receptor 1 (TfR1) within a Tf endosome where the reduction of iron takes place. Tf is a major iron chelator and complexes with TfR1 to be endocytosed. In contrast to STEAP2, STEAP1 was only partially co-localised with Tf and TfR1, and was also present in other vesicles that did not contain Tf (Ohgami et al., 2006).

The Ohgami group performed an extensive analysis of STEAP1 and 2 mRNA expression in a variety of human tissue including brain, colon, duodenum, heart, kidney, liver, lung, pancreas, placenta, thymus, ileum, stomach, skeletal muscle, bone marrow, and prostate. They found both STEAP1 and 2 were predominantly expressed in prostate, with low levels in the bone marrow (10 fold less) (Ohgami et al., 2006). In contrast, STEAP4 was predominantly expressed in the bone marrow, with very low levels in the prostate. Initial functional studies by the Challita-Eid group reported STEAP1 function as a transporter of small molecules (<1 kDa), suggesting it may be involved in intercellular communication (Challita-Eid et al., 2007). This hypothesis is further strengthened due to its localisation at cell-cell boundaries (Hubert et al., 1999).
The current study is the first report of STEAP1 and 2 expression in MSCs. We looked for murine STEAP1 and 2 via western blot analysis and FACS, but unfortunately the antibodies were not suitable for detection of murine STEAP1 and STEAP2 proteins.

Both STEAP members showed a strikingly similar expression pattern to stem cell antigen-1 (Sca-1) (figure 5.9). Vdr is also present in the Sca-1 cluster. Hence all four genes showed a very similar expression pattern, as detected by an unsupervised, genome-wide two-dimensional clustering analysis (figure 5.9). Notably, two different analysis methods have identified both STEAP1 and -2 as differentially highly expressed in the UCs, and most importantly these two genes are the only cell membrane genes identified

by both analysis methods. This is particularly remarkable when one is reminded that the analysis is based on the entire genome.

A study by Xin and colleagues reported that Sca-1 enriches for prostate-regenerating cells which possessed stem cells properties (Xin et al., 2005). However, genetic perturbation of PTEN/AKT signalling in these cells led to tumourigenesis with concomitant increase in Sca-1. Results suggest there is an interesting relationship between Sca-1 and the STEAP proteins. In addition, a prostate stem cell antigen (PSCA) which is also found on prostate cancer is located with Sca-1 in a multigene cluster of approximately 18 well conserved genes on chromosome 15 (Gumley et al., 1995). Sca-1 message was very highly expressed as detected in the microarray analysis. Furthermore, the microarray results correspond to the flow cytometry results (in chapter 4), which show Sca-1 is highly expressed in the clones in comparison to MEFs and BMMNCs. Also Sca-1 is highly expressed in UCs compared to BMACs.

The cell cycle gene Cdk4 also clustered with Sca-1/STEAP1/STEAP2. Cdk4 is important for cell cycle G1 phase progression thus indicating the high proliferative capacity of the undifferentiated clones compared to the others cells studied. Interestingly, HIF-1α was also present in this cluster. Since all cell types were cultured in 5% oxygen (except BMMNCs which were not cultured), this indicates that the endogenous steady state HIF-1α mRNA levels are greater in the MSC clones.

Another potential gene of interest present in the Sca-1 cluster is Maged2, which has been previously reported to be expressed by mesodermal cells (Bertrand et al., 2004). Cell surface marker TM4SF1 is also expressed at a high intensity, and is a member of the tetraspanin superfamily of membrane proteins (Virtaneva et al., 1994). Unfortunately there is no reagent available to further study this. In the literature, both Maged2 and TM4SF1 have been implicated in tumour development in the lung (Kidd et al., 2006) and gut (Kao et al., 2003) respectively. Thus, one may speculate that the high levels of Maged2 and TM4SF1 may characterise the transformed nature of the MSCs and potentially distinguish them from their normal counterpart. However, TM4SF1 has also

been reported to be expressed at significant levels in human MSCs (Song et al., 2006; Wagner et al., 2005). Indeed, Song and colleagues report the expression of a number of genes involved in carcinogenesis and metastasis in their normal MSC populations, and hence these genes may not necessarily be representative of abnormal or cancer stem cells.

Cytokine and growth factor signalling is an important determinant of the functional state of cells. Undifferentiated clones significantly express the cytokines, HGF, FGF7, Ccl7, and insulin growth factor binding protein-6 (IGFBP6) in comparison to the controls studied. Both FGF7 and HGF have been previously reported in MSCs (Djouad et al., 2007). FGF7 is also known as keratinocyte growth factor, and high levels of FGF7 expression have been previously reported in human MSCs during their growth phase (Imabayashi et al., 2003). FGF7 is involved in a broad range of activities including cell growth and morphogenesis, tissue repair, and embryonic development. It is typically thought to be an epithelial cell growth factor (Finch and Rubin, 2004). MSCs have been reported to express HGF and its receptor cMET (Rosova et al., 2008). These investigators report HGF is activated at sites of ischemic injury. This is one mechanism proposed by which MSCs migrate to damaged tissue, via a HGF gradient. This is the first mention of Ccl7 (chemokine (C-C motif) ligand 7) in MSCs. Ccl7 expression was also found in the BALB/b clonal cells via the supervised analysis. Ccl7 was previously called monocyte chemotactic protein-3 (MCP-3), and is one of the most pluripotent chemokine, attracting monocytes and regulating macrophage function (Menten et al., 2001). Ccl7 was recently identified as a differentiation factor for neuron development (Edman et al., 2008). Clonal cells also expressed significant levels of IGFBP6. A previous study has reported that secretion of IGFBP6 by stromal cells may be a key modulator of the hematopoietic response to IGFs (Grellier et al., 1995). The transcription factor Foxa1 was also significantly expressed by the clonal cells. Foxa1 is a hepatic transcription factor found in epi-meso progenitor cells (Inada et al., 2008). It is reported to be important in normal prostate development (Mirosevich et al., 2005). Also, Foxa1 and Foxa2 are also involved in regulating midbrain dopaminergic neuron development (Ferri et al., 2007).

Clonal cells also expressed neural cell adhesion molecule -1 (NCAM-1) at over 2 fold greater levels than control samples. It is interesting to note that clonal cells expressed low levels of transcripts that are characteristic of epithelial, neural, and endothelial cell types as discussed above. Thus, indicating the broad differentiation capabilities of MSCs. Although we have only demonstrated the tri-lineage differentiation ability of the clones into the adipogenic, chondrogenic, and osteogenic lineages, it is possible that the cells have a greater differentiation potential. Previous studies that have analysed the gene expression profile of human and murine MSCs have also reported the presence of other lineage specific transcripts (Phinney et al., 2006; Tremain et al., 2001; Wagner et al., 2005) (Silva et al., 2003).

STEAP is an epithelial antigen and may seem an unlikely MSC marker, however it must be noted that MSCs, or more specifically multilineage adult progenitor cells (MAPC), at the single cell level, have been shown to give rise to epithelial lineages of liver, lung and gut (Jiang et al., 2002). We have not investigated the ability of the MSC clones to differentiate into other lineages, but the presence of multilineage transcripts would suggest this is possible given the right environmental cues. As discussed above the murine clones expressed a variety of multilineage transcripts, including hepatic differentiation factors Foxa1, HGF. Although the STEAPs are known as prostate cancer markers, there are other instances where markers that are used to isolate stem cells for one tissue are also used as cancer stem cell markers for another. For example, CD133 is a neural stem cell marker used to isolate neural stem cells (Peh et al., 2008). The watt group also use CD133 to isolate a MSC-enriched population. (Martin-Rendon et al., 2007). CD133 is also used to isolate a small subset of human brain cancer cells which have the potential to form tumours in mice identical to the original tumours (Singh et al., 2004). Also, interestingly a subpopulation of $CD133^{+}$ human prostate epithelial cells have a high growth rate *in vitro* and can reconstitute prostatic-like acini *in vivo* (Richardson et al., 2004). CD34 is the major haematopoietic stem cell (HSC) marker, commonly used in conjunction with other markers to isolate subpopulations of HSCs. However, the first definitive report about cancer stem cells was about a subpopulation of $CD34^{+}/CD38^{-}$ leukemic cells that formed tumours in NOD/SCID mice (Bonnet and Dick,

1997). These results suggest there is a close relationship between stem cell and cancer stem cell markers. This is perhaps because some cancers may be caused by stem cells losing their ability to regulate proliferation. If a stem cell marker has an obvious functional role in the stem cell such as proliferation or self-renewal, and is dysregulated, this may be one way it becomes a marker of both stem and cancer stem cell in the same, or different tissues. Thus, one may speculate that STEAP1 and 2 are markers of cancer cells in the prostate, but also potential markers of normal mesenchymal stem cells in the bone marrow.

The purpose of the next chapter will be to examine their expression in multipotent, karyotypically normal human bone marrow stromal cells.

Chapter 6

Human Bone Marrow Stromal Cells express STEAP1 and -2

6.1 Introduction

Gene expression analysis of murine clonal MSCs revealed that they differentially expressed the cell surface markers STEAP1 and STEAP2, in comparison to relevant controls. The purpose of the work in this chapter was to derive normal, human, multipotent bone marrow stromal cells (BMSCs) and to examine whether they also differentially expressed STEAP1 and 2.

6.2 Isolation of human bone marrow stromal cells

Initially we attempted to isolate clonal human MSCs using the method we had previously developed (described in chapter 3) with murine MSCs. We cultured the cells in the same culture conditions at 5% oxygen tension. Commercially obtained human bone marrow mononuclear cells (hBMMNCs) were seeded at a low density and cells cultured for over a month like the murine cells had previously been. However, there were typically very few adherent cells, and these were representative of senescent cells by their flat, spread-out morphology rather than the highly proliferative murine cells which had a more spindle shaped morphology. Even after several attempts, clonal cells were not apparent, i.e., cells were not present in tight clusters like the murine cells but slightly dispersed. These were isolated using cloning rings and re-seeded into a 3.5 cm tissue culture dish. Most of these cells did not adhere to the tissue culture surface and there was a morphology change in the ones that did from a spindle shape to the flat, spread-out shape. Therefore we decided to culture adherent bone marrow stromal cells (BMSCs) which would contain a population of MSCs and presumably become more enriched for MSCs with subsequent passage as previously reported (Barry, 2003). Human BMMNCs were therefore seeded at a high density and cultured at 5% oxygen. The subsequent cells were spindle shaped although after passage 2 there was an increase in the flatter shaped cells. Cells were cultured until 70% confluence to ensure there was not a morphology change and then re-seeded for expansion. These cells were referred to as hBMSC1 as they were from the first isolation. This method was repeated with a fresh bone marrow aspirate which had been ficoll-separated that we received from a hospital. This bone marrow was different from hBMSC1; it contained whitish-yellow parts floating in the media. This

could be fatty deposits as the sample came from a 60 year old patient and the quantity of adipocytes does increase in the bone marrow with age, thus the bone marrow goes from red to yellow. Human BMSC2 cells from this patient were initially more spindle shaped but soon turned very flat in culture. The cells subsequently had a very slow growth rate compared to hBMSC1, and could only be expanded up to passage three. The third culture (hBMSC3) was derived from a bone marrow aspirate from an 11 year old osteosarcoma patient. Microscopically all these cells were homogeneously spindle shaped and had a faster growth rate. Cells were expanded to passage six and retained their spindle-shaped morphology. The fourth BMSC sample was derived from commercially obtained BMMNCs as described for hBMSC1. The donor was male and 25 years old. These cells were all spindle shaped.

6.3 Growth rate of hBMSC3

To determine the growth rate of hBMSC3, the population doubling time was followed at 5% oxygen. Cells were seeded at a density of 1000-2500 cells/cm^2 and passaged when approximately 70% confluent. Figure 6.1 shows the population doubling time for hBMSC3. Beginning at passage 2 to passage 5 the growth rate is constant. The cell doubling time averaged 3.42 $\pm$ 0.78 days (mean $\pm$ SD), and no cell death was observed during this period.

6.4 Human bone marrow stromal cells are multipotent

The tri-lineage differentiation potential of the hBMSCs was examined to confirm the presence of MSCs in the population. Neonatal human dermal fibroblasts were used as an additional control in differentiation experiments. Cells were cultured in the differentiation media as described in sections 3.4.1-3.4.3 (figure 6.2, table 6.1). All hBMSCs had the ability to differentiate into adipocytes when cultured in the appropriate media, and it took 4 weeks for all the cells to differentiate in the hBMSC3 and hBMSC4 cultures. Only ~ 50% of the cells in hBMSC1 and hBMSC2 cultures differentiated. All hBMSCs

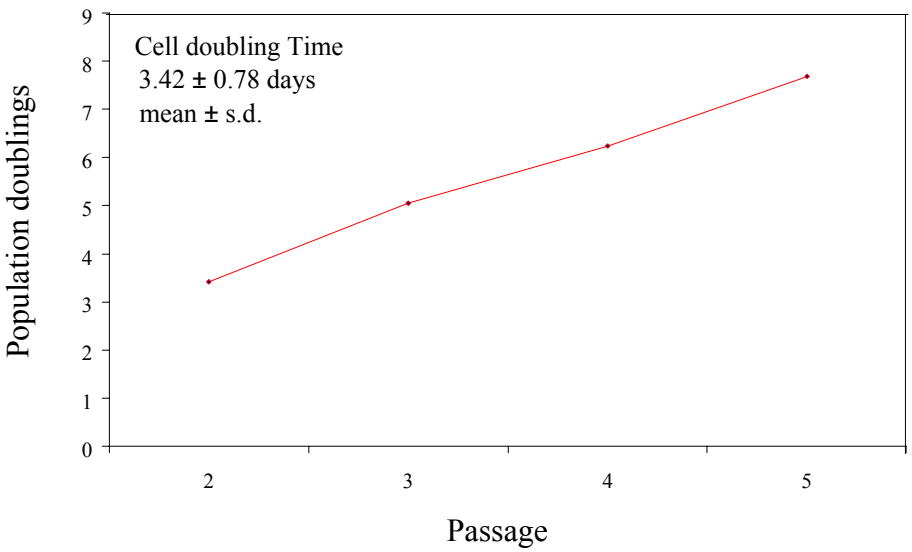


Figure 6.1 Growth rate of human BMSCs. The growth rate of human bone marrow stromal cells from donor 3 (hBMSC3) was followed in 5% oxygen tension. Cells were seeded at a density of 1000-2500 cells/cm^2 and passaged every 5-8 days when they were 70% confluent. Cells were counted at each passage to calculate population doubling time (standard deviation, s.d.).

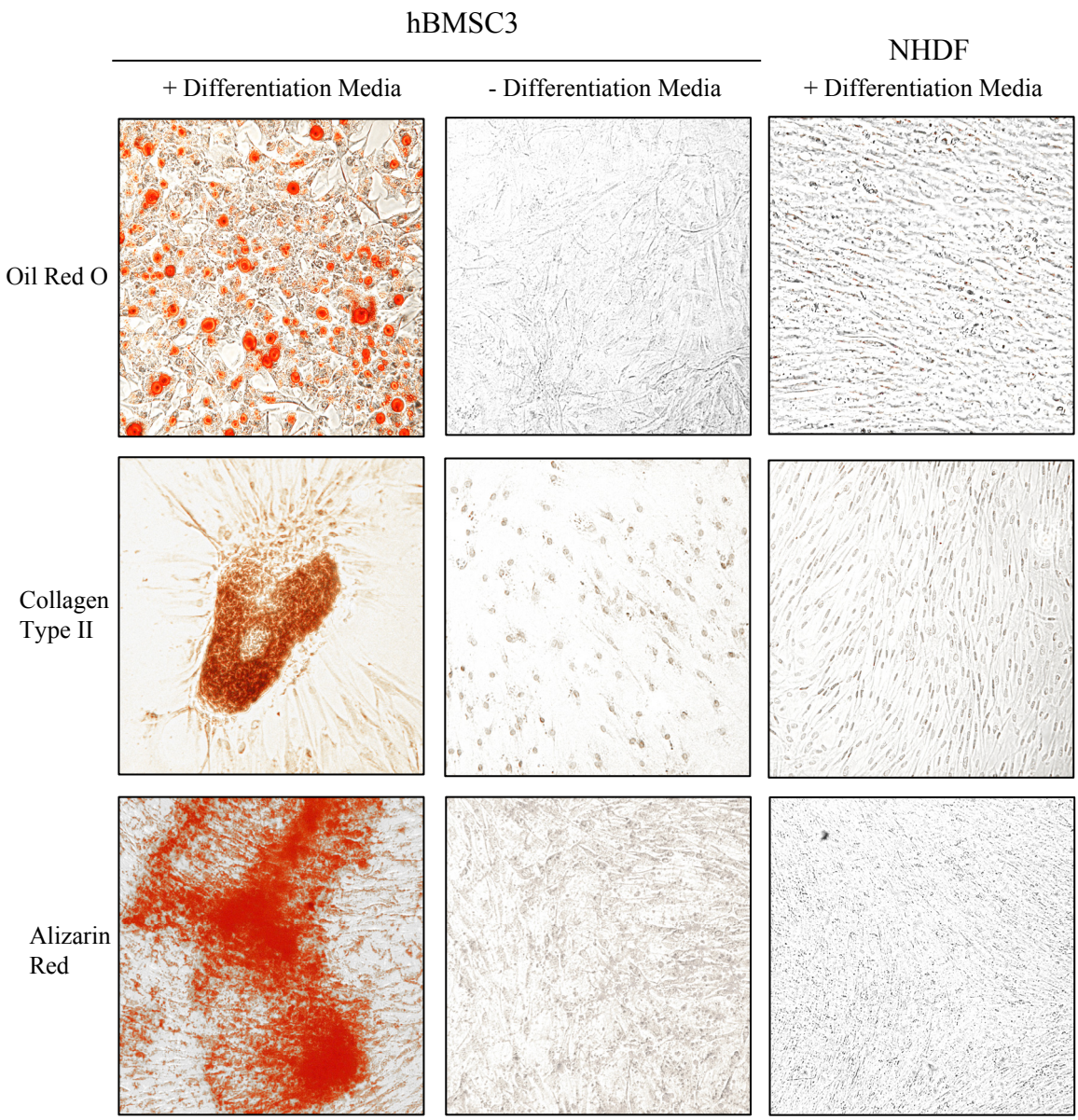


Figure 6.2 Human BMSCs have multilineage differentiation potential. Human bone marrow stromal cells (hBMSCs) and fibroblasts were cultured in the presence or absence of differentiation media as described in figures 3.4- 3.10 to assess their multilineage differentiation potential. Pictures of cultures of hBMSCs from donor 3 (hBMSC3) are shown. NHDF; neonatal human dermal fibroblasts.

Table 6.1 Human BMSCs are multipotent. All human bone marrow stromal cells had the ability to differentiate into adipocytes, chondrocytes, and osteoblasts when cultured under the appropriate conditions, except hBMSC2 where there was not enough cells to perform the osteogenesis experiment. Importantly, under the same conditions neonatal human dermal fibroblasts (NHDFs) failed to differentiate into three lineages

Clone	Adipogenesis	Chondrogenesis	Osteogenesis
hBMSC1	✓	✓	✓
hBMSC2	✓	✓	?
hBMSC3	✓	✓	✓
hBMSC4	✓	✓	✓
NHDF	X	X	X

differentiated into chondrocytes. The osteogenic differentiation of hBMSC2 was not examined as there was not sufficient quantity of cells to perform the differentiation assay. Dexamethasone had to be added to the differentiation media for osteogenic differentiation. The remaining hBMSCs differentiated into osteoblasts when cultured in osteogenic media. Fibroblasts did not differentiate when cultured in the differentiation media, but were fully viable (figure 6.2)

6.5 Surface antigen profile of human BMSCs

The surface antigen profile of the hBMSCs was examined by flow cytometry using the markers listed in table 6.2 and compared to the fibroblasts. Figure 6.3 shows the surface antigen profile of hBMSC3 and the results suggest that it consisted of a homogeneous population of cells, as a single peak was observed in all histograms. The surface antigen profile of hBMSC2 was not examined due to lack of sufficient cell numbers. As shown in table 6.3 all hBMSCs examined were positive for HLA-ABC and negative for HLA-DR in agreement with the literature (Majumdar et al., 2003). Surface antigen profile analysis of hBMSCs revealed hBMSC1 and hBMSC4 expressed STRO-1, the typical 'MSC' candidate marker, while hBMSC3 and fibroblasts did not. All cells expressed the fibroblast marker CD90, and CD13 (a myeloid cell marker), which hBMSC3 and the fibroblasts expressed at greater levels than hBMSC1 and hBMSC 4. hBMSC1 and hBMSC3 were both positive for candidate MSC markers CD29 and CD166 which hBMSC 4 was negative for, while the fibroblasts also expressed CD166. All cells were negative for the haematopoietic markers CD34 and CD45 as well as SSEA1, CD11b, CD133, and CXCR4. CD122, Interleukin 2 receptor beta was expressed by all cells.

6.6 Cytogenetic analysis of human BMSCs

All hBMSCs were karyotypically normal except for two cells in hBMSC2 (figure 6.4 and 6.5). hBMSC2 showed a normal female chromosome complement and banding pattern.

Table 6.2 Surface antigens examined by flow cytometry for human BMSCs.

Antibody	Antigen
STRO-1	STRO-1
SSEA-1	Stage specific embryonic antigen 1
CXCR4	Chemokine (C-X-C motif) receptor 4 (Fusin)
HLA-ABC	Human leukocyte antigen- ABC
HLA-DR	Human leukocyte antigen- DR
CD11b	Leucocyte antigen
CD13	Alanine aminopeptidase
CD29	Integrin β1
CD34	Cluster of differentiation 34
CD45	Leukocyte common antigen, Ly5
CD49e	Integrin α5 (ITGA5) (fibronectin receptor, alpha polypeptide)
CD73	Ecto-5′-nucleotidase
CD90	Thy-1
CD105	Endoglin/ TGF-βR
CD106	Vascular cell adhesion molecule -1 (VCAM-1)
CD122	Interleukin 2 receptor beta
CD133	Prominin 1
CD166	Activated leukocyte cell adhesion molecule (ALCAM)

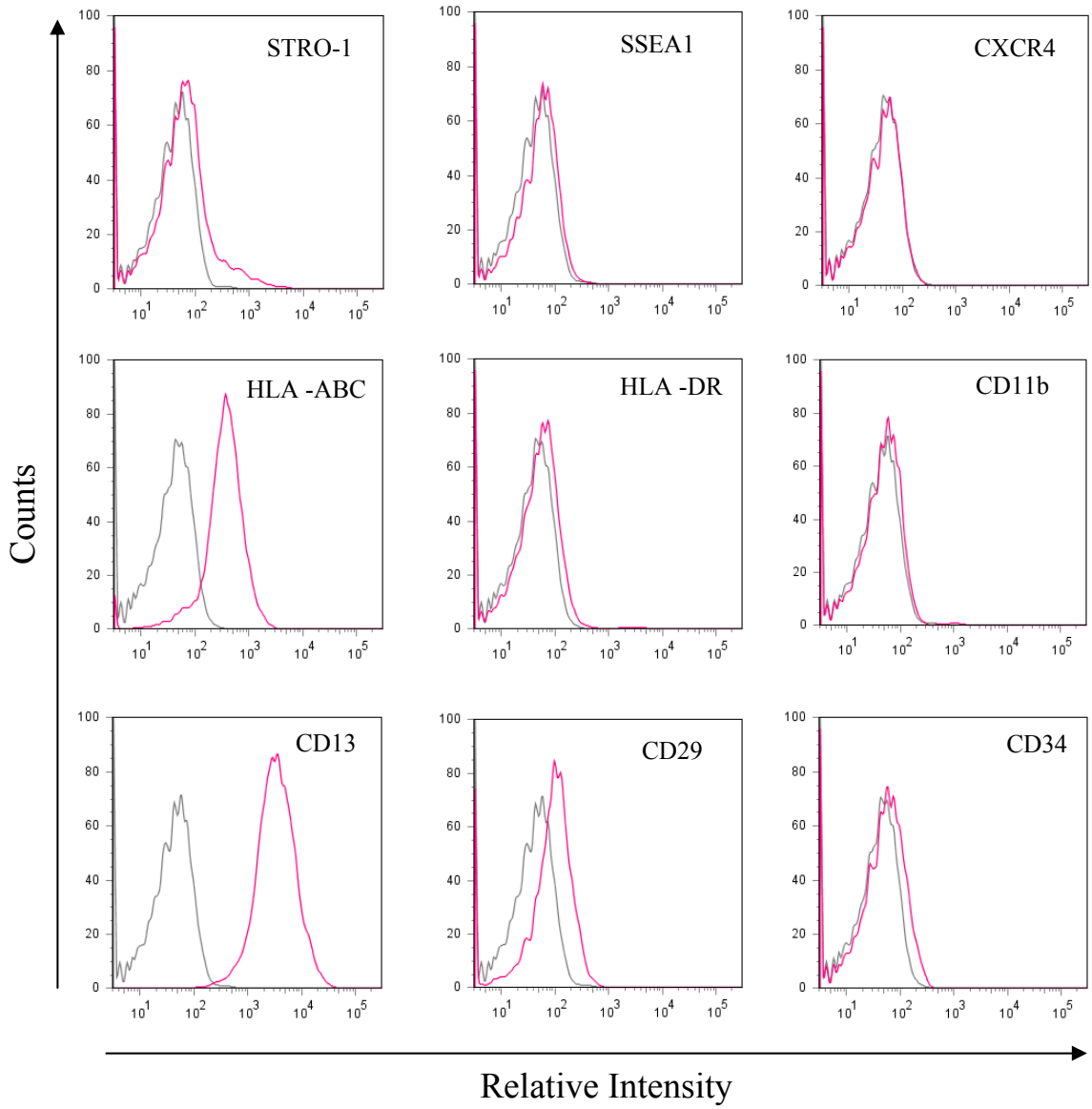


Figure 6.3 Surface antigen profile of human bone marrow stromal cells. Flow cytometric analysis of the surface proteins expressed by human bone marrow stromal cells (hBMSCs) was performed by labelling cells with fluorochrome-conjugated antibody specific for the marker indicated in each histogram. The surface antigen profile of BMSC3 is shown. Labelled cells were analysed by FACS using BD FACSDiva™ software, subsequent results analysed by FLOWJO software. Grey lines and magenta lines in each histogram represent cells labelled with isotype matched control and specific monoclonal antibody, respectively. (Results continued on next page).

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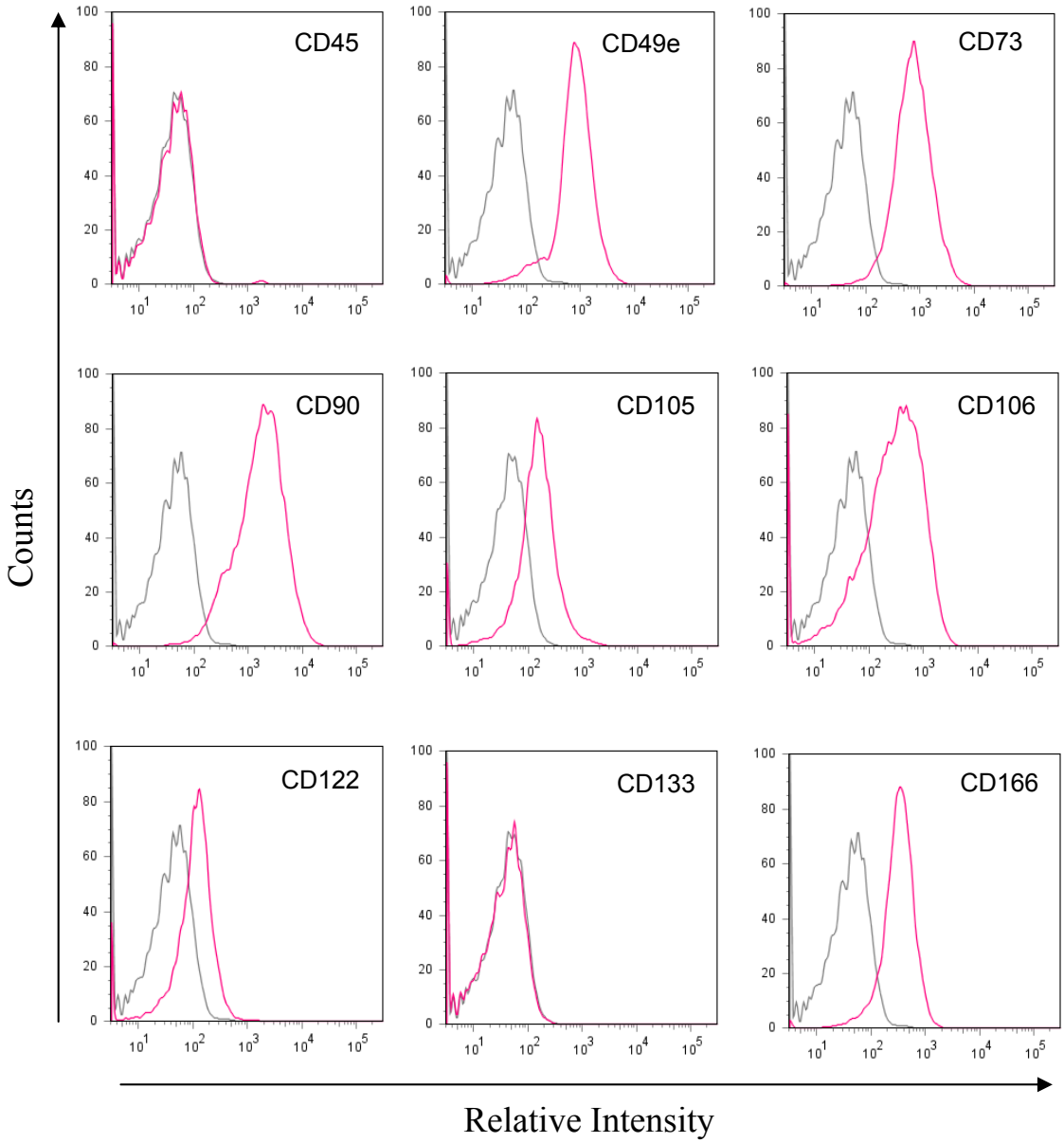


Figure 6.3 (continued) Surface antigen profile of human bone marrow stromal cells.

Table 6.3 Surface phenotype of human BMSCs. Human bone marrow stromal cells (hBMSCs) were harvested at 70% confluence and labelled with PE- or FITC- antibodies or isotype matched controls and analysed by flow cytometry at passage 2-4. The surface antigen profile of neonatal human dermal fibroblasts (NHDFs) was also examined. Symbols refer to intensity of expression. Samples were scored as '-' if the population was in the first decade (same as the isotype control), '±' if it was between the first and second decade, '+' if it was in the second decade, '++' if it was in the third decade, relative to the isotype control.

Antibody	hBMSC1	hBMSC3	hBMSC4	NHDF
STRO-1	+	-	±	-
SSEA-1	-	-	-	-
CXCR4	-	-	-	-
HLA-ABC	±	+	±	±
HLA-DR	-	-	-	-
CD11b	-	-	-	-
CD13	±	++	±	++
CD29	±	±	-	-
CD34	-	-	-	-
CD45	-	-	-	-
CD49e	±	+	±	+
CD73	-	+	-	+
CD90	+	++	±	++
CD105	-	±	-	+
CD106	-	+	-	-
CD122	±	±	±	-
CD133	-	-	-	-
CD166	±	+	-	+

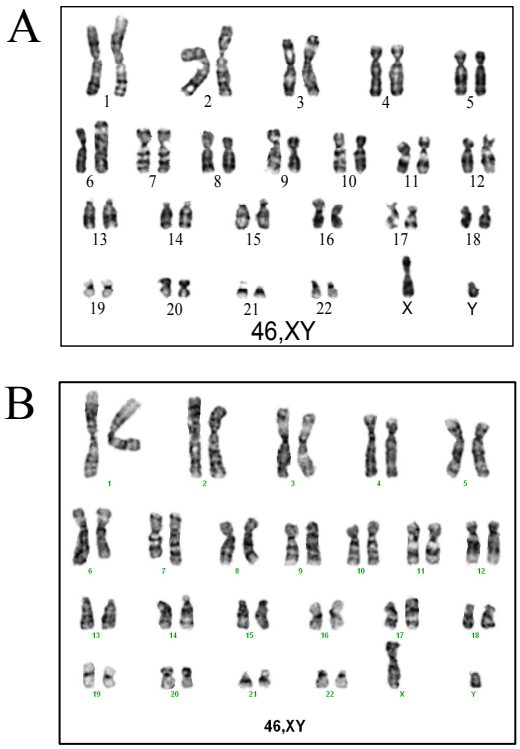


Figure 6.4 Human BMSCs have a normal karyotype. Human bone marrow stromal cells (hBMSCs) from donor 1 displayed a normal diploid karyotype as assessed by G-banding analysis. (A) G-banding pattern is representative of hBMSC3 and 4. (B) Neonatal human dermal fibroblasts. All cultures were examined at passage 3.

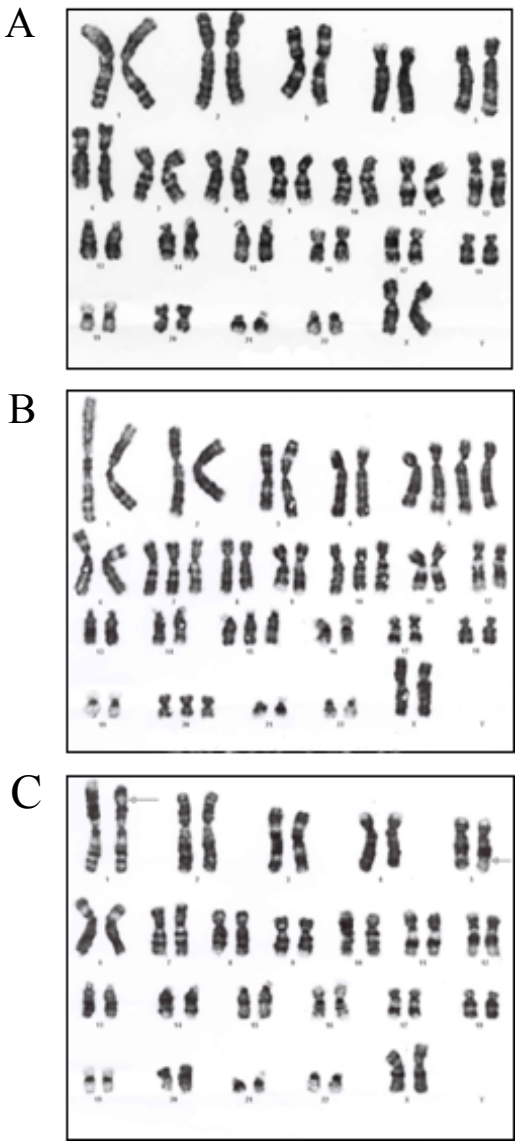


Figure 6.5 Cytogenetic analysis of human BMSC2. The majority (18/20) of human BMSC2 displayed a normal diploid karyotype as assessed by G-banding analysis at passage 3 (A). One cell was hyperdiploid with 52 chromosomes and 4 copies of chromosome 5, 3 copies of chromosome 7, 3 copies of chromosome 10, 3 copies of chromosome 15, and 3 copies of chromosome 20 (B). There was also a single cell with a balanced reciprocal translocation between 1 chromosome 1 at breakpoint p34 and 1 chromosome 5 at breakpoint q31 (C).

However, there was a hyperdiploid cell with a complement of 52 chromosomes and four copies of chromosome 5, three copies of chromosome 7, three copies of chromosome 10, three copies of chromosome 15, and three copies of chromosome 20. Also a single cell with an apparently balanced reciprocal translocation between one chromosome 1 at breakpoint p34 and one chromosome 5 at breakpoint q31 was observed. The neonatal human dermal fibroblasts (NHDFs) were karyotypically normal.

6.7 Human BMSCs express STEAP1

STEAP1 protein expression was examined in the hBMSCs by western blot analysis. Initially we only had hBMSC1 and therefore examined STEAP1 in this population. A band at the correct molecular weight (36kDa) was detected with the Santa-Cruz (sc-10262) antibody and there was negligible level in the fibroblasts (figure 6.6). In addition, to the 36 kDa STEAP1 band there was also a very small band above it at 38 kDa. We next derived hBMSCs from the second donor (hBMSC2), so STEAP1 expression in this cell population was analysed. As shown in figure 6.7 all hBMSCs express STEAP1 at significant levels compared to human fibroblasts and human chondrocytes. The presence of the STEAP bands were not particularly clearly visible in this western blot, and may be due to poor separation as a 12% gel was used. We therefore used a 4-12% gradient gel in the next experiment. At this point we had derived another hBMSC culture, and therefore STEAP1 expression was examined in all three hBMSC cultures. As shown in figure 6.8 all hBMSCs expressed STEAP1 in comparison to human fibroblasts. Interestingly donor 3 expressed greater levels compared to donor 1 and 2. There was also the presence of the 38 kDa band, and this band and the STEAP1 36kD band were differentially expressed by the hBMSCs compared to the fibroblasts.

Examination of STEAP1 protein expression in the hBMSC cultures was also attempted via flow cytometry, however the antibodies were not suitable for this application.

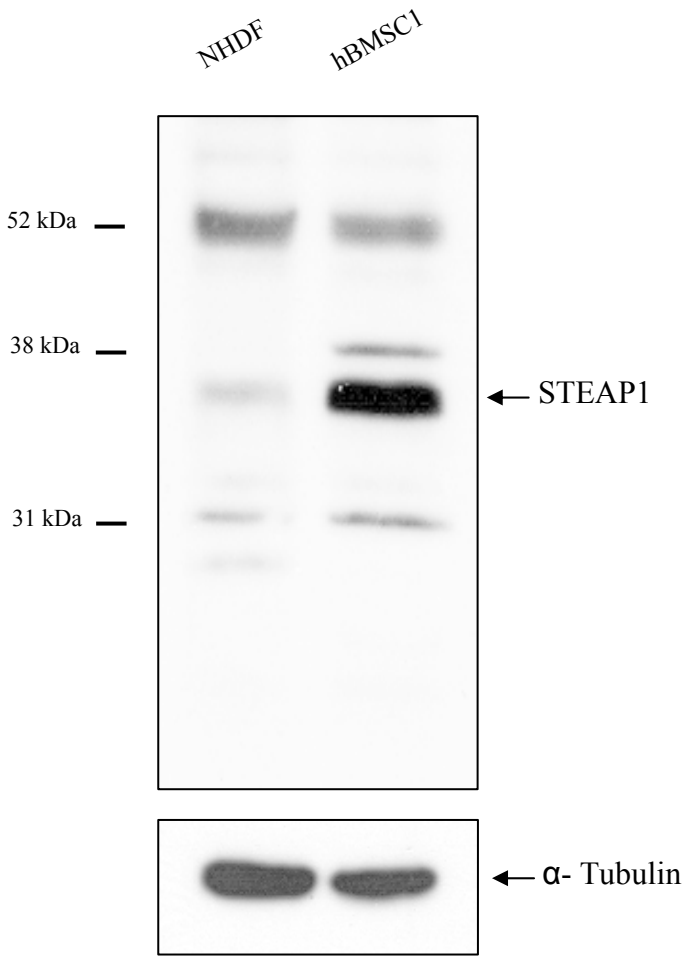


Figure 6.6 Human bone marrow stromal cells (hBMSC1s) express STEAP1. Human bone marrow stromal cells from donor 1 show STEAP1 expression by western blot analysis. In comparison, normal human dermal fibroblasts (NHDFs) express negligible levels. All cells at passage 1-3.

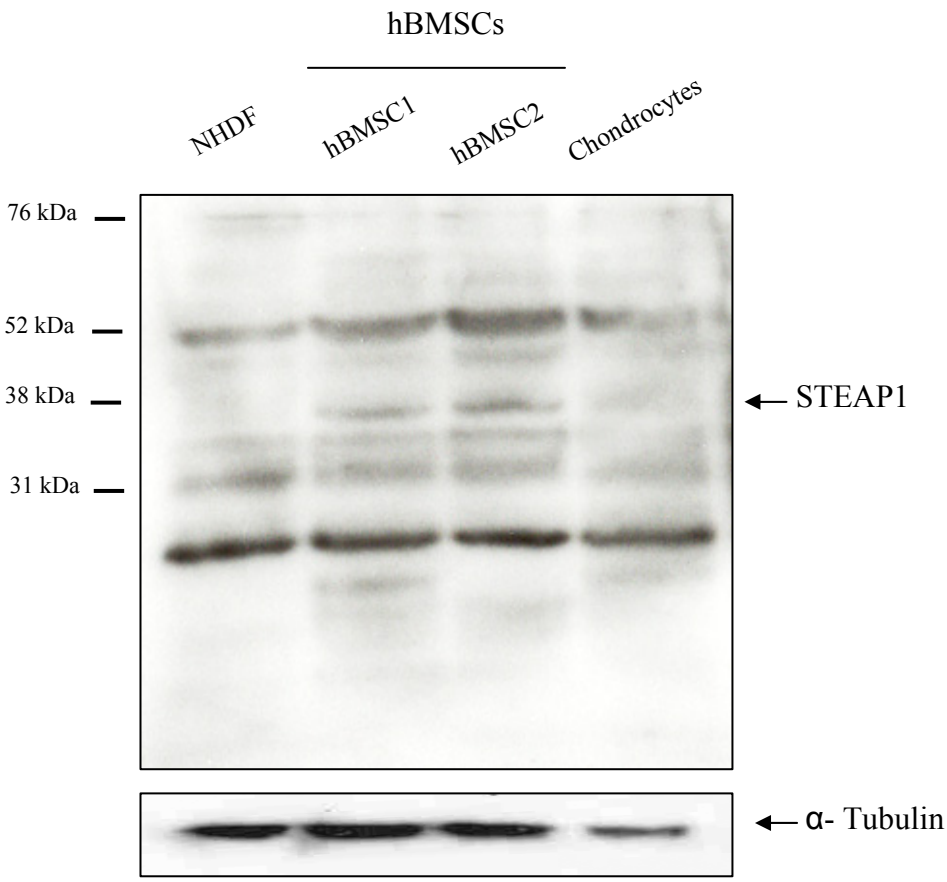


Figure 6.7 Human BMSC1 and 2 express STEAP1. Human bone marrow stromal cells (hBMSCs) from 2 different donors show STEAP1 expression by western blot analysis. In comparison, neonatal human dermal fibroblasts (NHDFs), and human chondrocytes expressed negligible levels. Human chondrocytes were derived from articular cartilage in the femoral condyle. All cells at passage 1-3.

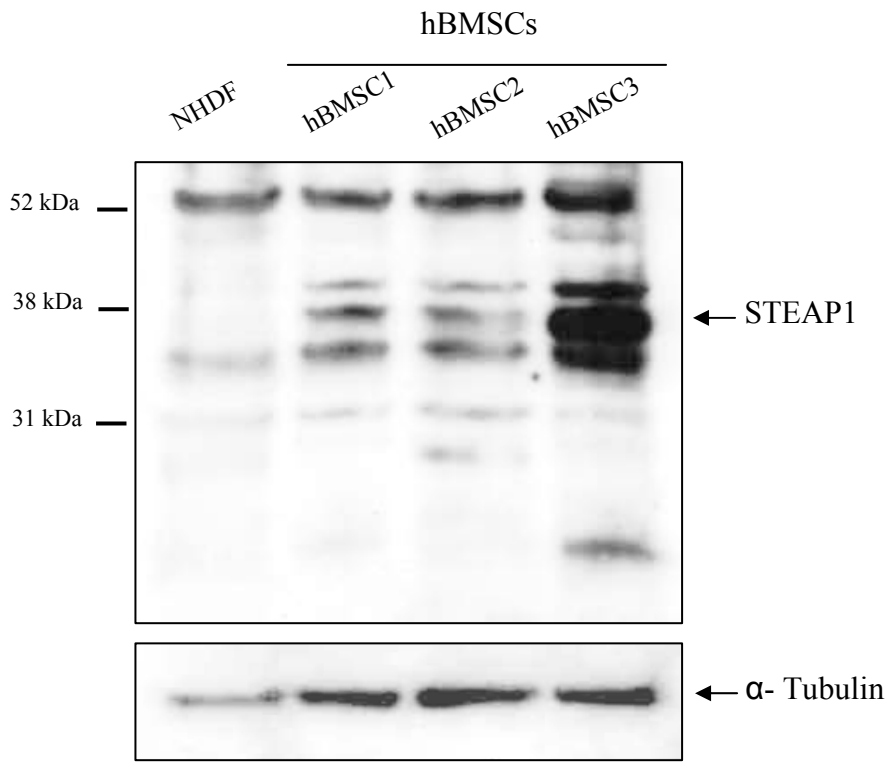


Figure 6.8 Human BMSC1-3 express STEAP1. Human bone marrow stromal cells (hBMSCs) from 3 different donors show STEAP1 expression by western blot analysis. In comparison, neonatal human dermal fibroblasts (NHDFs) express negligible levels. All cells at passage 1-3.

6.8 Human BMSCs express STEAP2

STEAP2 protein expression in hBMSCs was initially examined by western blot analysis. Two antibodies were tested as mentioned in chapter 2: Pro-Sci and Novus –Biological antibodies. Neither antibody was appropriate for western blotting. However, the novus-biological antibody was suitable for FACS analysis. Therefore, flow cytometry was used to examine STEAP2 expression in hBMSCs. Results show both hBMSC3 and hBMSC4 express STEAP2 (figure 6.9). Whilst neonatal human dermal fibroblasts and hBMMNCs were negative, (i.e. did not express it).

The murine clones expressed the stem cell antigen-1 (Sca-1) at a high intensity, which subsequently decreased dramatically when the clones were differentiated into adipocytes. In order to examine whether this expression pattern was the same for STEAP2 in the hBMSCs, hBMSC3 and hBMSC4 were cultured in the presence of adipogenic media for 4 weeks to differentiate them into adipocytes. FACS analysis was performed on the adipocyte differentiated hBMSCs and equivalent control cells cultured in the absence of adipogenic media for the same length of time. As shown in figure 6.10 STEAP2 expression was similar in both adipocyte differentiated hBMSCs and control hBMSCs. STEAP2 expression in hBMSC3s was 50% in normal hBMSCs examined at 70% confluence and remains approximately the same after 4 weeks culture with or without adipogenic media.

STEAP2 expression in hBMSC4 was detected on 35% of the cells when examined when the cultures were 70% confluent (figure 6.9). This increased to 81% for overconfluent cultures, i.e., the controls in the adipogenic differentiation experiments (see figure 6.10). In adipocyte differentiated hBMSC4 cultures, STEAP2 was present in 47% of the cells.

6.9 STEAP1 depletion in human BMSCs

In order to begin to elucidate the function of STEAP1 we attempted to deplete this protein using RNA interference. Bone marrow stromal cells from donor 3 were seeded at approximately 50% confluence and cultured for two days. On day two cells were transfected with siRNA oligos directed against luciferase, a scrambled, and two different

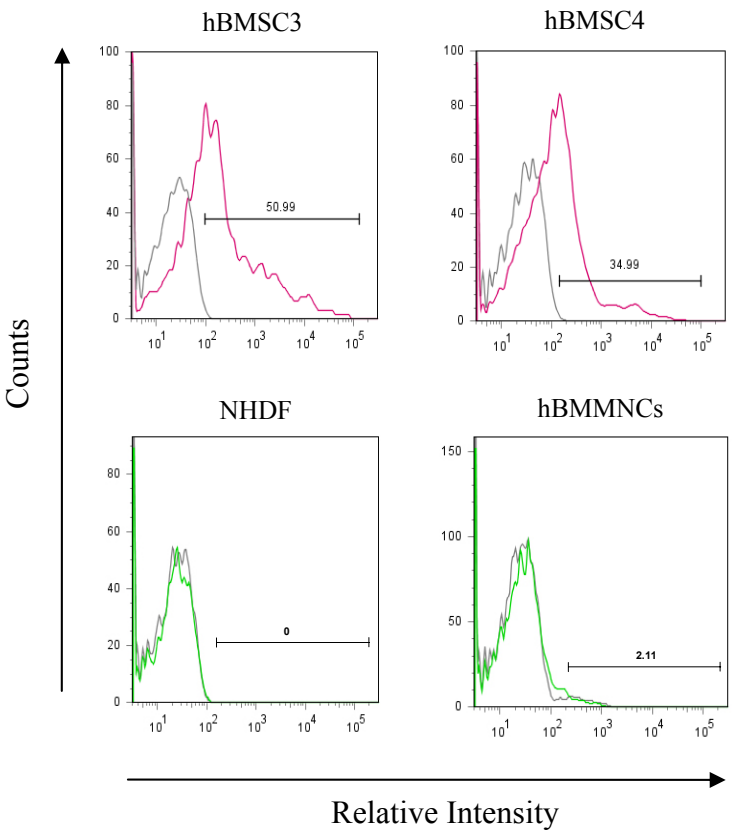


Figure 6.9 Human bone marrow stromal cells express STEAP2. Flow cytometric analysis of STEAP2 expression on human BMSCs was performed by labelling cells with fluorochrome-conjugated antibody specific for STEAP2 protein. Labelled cells were analysed by FACS using BD FACSDiva™ software and subsequent results analysed by FLOWJO software. Grey lines and coloured (magenta and green) lines in each histogram represent cells labelled with isotype matched control and STEAP2 antibody, respectively. Numbers over bars represent STEAP2 percentage expression. STEAP2 was not detected in NHDFs or hBMMNCs. Neonatal human dermal fibroblasts (NHDFs), human bone marrow stromal cells (hBMMNCs).

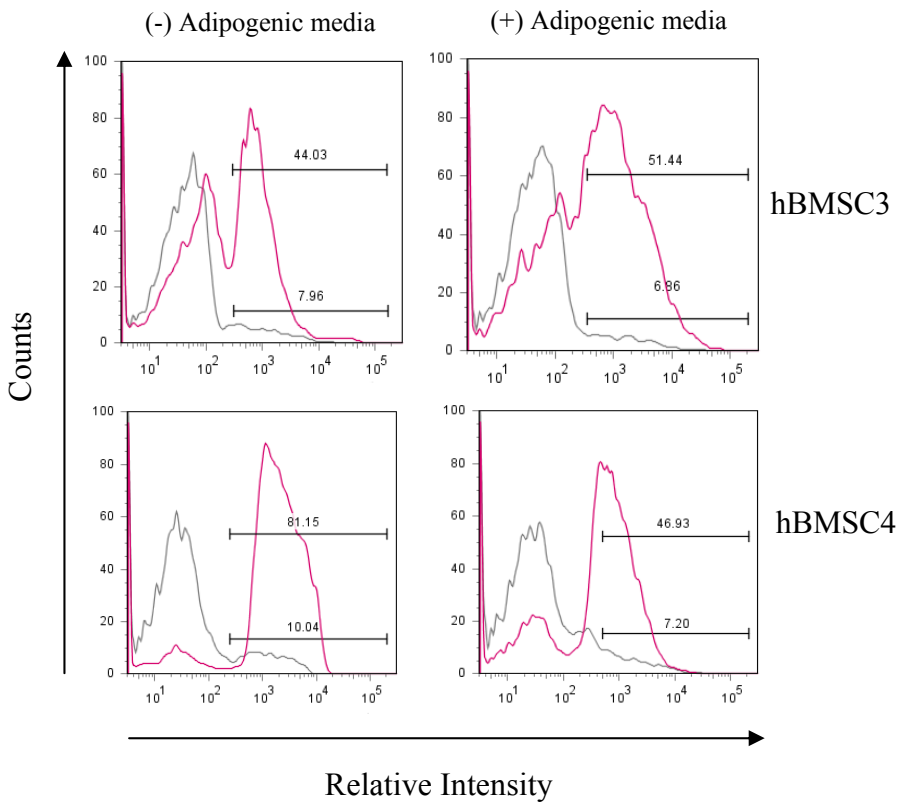


Figure 6.10 STEAP2 expression is retained in adipocyte differentiated human BMSCs. Human bone marrow stromal cells from donors 3 and 4 (hBMSC3 & 4) were cultured for four weeks in adipocyte differentiation media or its absence. Flow cytometric analysis of human BMSCs was performed by labelling cells with fluorochrome-conjugated antibody specific for STEAP2. Labelled cells were analysed by FACS using BD FACSDiva™ software and subsequent results analysed by FLOWJO software. Grey lines and magenta lines in each histogram represent cells labelled with isotype matched control and specific STEAP2 antibody, respectively. Upper bars represent STEAP2 percentage expression and lower bars isotype matched control.

STEAP1 (called STEAP1.1 and STEAP1.2) and STEAP2 (called STEAP2.1 and STEAP2.2) transcripts. Cells were then cultured for two days and then lysed. Knockdown efficiency was then examined by analysing STEAP1 and STEAP2 gene expression via real time RT-PCR. As shown in figure 6.11, STEAP1 expression is depleted by 62% and 80% by STEAP1.1 and STEAP1.2 siRNAs, respectively (relative to control). STEAP2 gene expression is depleted 82% and 83% by STEAP2.1 and STEAP2.2 siRNAs, respectively relative to control. STEAP1 depletion was further examined at the protein level via western blot. Results confirm the real-time PCR data in that there is greater depletion of STEAP1 protein by STEAP1.2 siRNA than by STEAP1.1 siRNA (figure 6.12). In contrast, STEAP1 protein was present in cells transfected with luciferase and scrambled siRNA oligos and using only lipofectamine. The 38kDa band observed in figures 6.6 & 6.8 was faintly visible in the lipofectamine alone and the luciferase lanes. It was absent in the cells transfected with scrambled oligos. However, this could be due to the fact that the lane was underloaded, as revealed by the loading control α-tubulin. This band was also absent in the cells transfected with siRNA oligos against STEAP1.

6.10 Discussion

It is difficult to clonally isolate human MSCs, and there are not many reports of clonally-derived MSCs in the literature. Pittenger and colleagues isolated human clonal cells but these were derived from previously passaged adherent cells. Marrow isolated adult multilineage inducible cells (MIAMI) are clonally isolated in specific culture conditions, with the addition of various growth factors (D'Ippolito et al., 2004). Clonal cells were extensively isolated by the Muraglia group (Muraglia et al., 2000), and one clone was isolated by the Jia group (Jia et al., 2002). Given the plethora of MSC studies this is a very small fraction, therefore highlighting the intrinsic difficulty of the task. Initially we attempted to derive human MSC clones. However, clones formed very rarely and when they did, they became senescent once they were re-seeded for expansion. Unfortunately, time was a limiting factor in this study, and due to the length of time taken to isolate and expand murine clonal MSCs and the subsequent characterisation required we decided to culture adherent human bone marrow stromal cells, which were not clonally isolated.

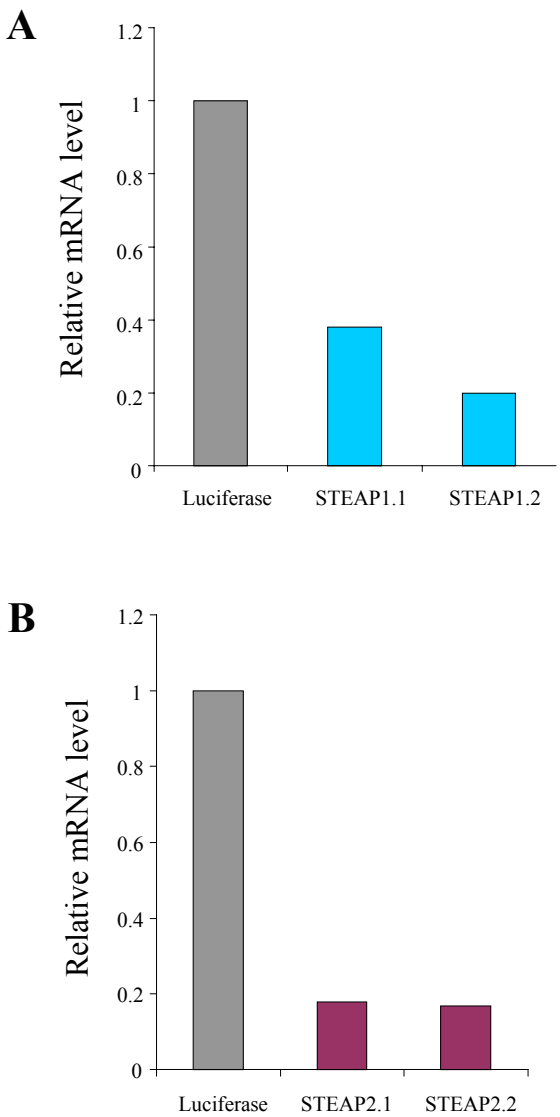


Figure 6.11 Validation of STEAP depletion by real-time PCR. Human bone marrow stromal cells from donor 3 were seeded at 1300cells/cm^2, ~50% confluence. After two days cells were transfected with two specific siRNAs against STEAP1 (S1.1 and S1.2), STEAP2 (STEAP2.1 and STEAP2.2) and controls; luciferase and scrambled oligo, or lipofectamine alone and cultured for two days. Cells were then lysed and STEAP1 (A) and STEAP2 (B) gene expression levels analysed by real-time PCR, normalising expression levels to endogenous control RPLPO. STEAP1 and 2 gene expression levels relative to control luciferase alone sample are shown.

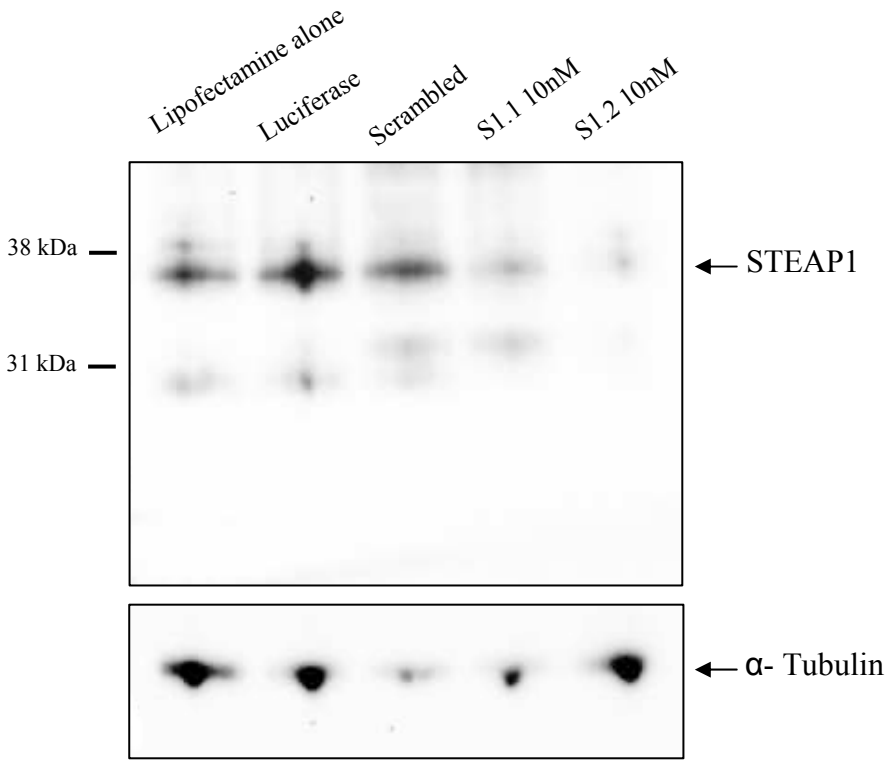


Figure 6.12 STEAP1 depletion in human BMSCs. Human bone marrow stromal cells (hBMSCs) from donor 3 were seeded at a denisty of 1300cells/cm^2, ~50% confluence and transfected with two specific siRNAs against STEAP1 (S1.1 and S1.2) and controls; luciferase, scrambled oligo, or lipofectamine alone and cultured for two days. Cells were then lysed and STEAP1 protein expression examined by western blot analysis.

Studies have reported enrichment of MSCs with passage of such cells (Baksh et al., 2004). We further hypothesised that cultivation in a low oxygen environment may help maintain stem cell properties as this was the case for the murine clones (chapter 3).
Human BMSCs were isolated by seeding human bone marrow mononuclear cells (hBMMNCs) at a high density and removing non-adherent cells the following day. Cells were cultured at 5% oxygen and were subsequently passaged when they were 70% confluent and further expanded. Despite the fact that the hBMSCs are a heterogeneous population to begin with, the morphology of the cells in the hBMSC3 and hBMSC4 cultures was predominantly spindle shaped, while the hBMSC1 and hBMSC2 contained some flattened shaped cells characteristic of senescent cells, which appeared and increased in number (especially in hBMSC2) with time in culture.

The ability of the hBMSCs to differentiate into the adipogenic, chondrogenic, and osteogenic lineages was examined to determine presence of MSCs. The same differentiation media that was used for the murine clones was used in these experiments. All hBMSCs had the potential to differentiate into chondrocytes in a manner similar to the murine clones. However, the osteogenic differentiation media containing A2P, β-GP and BMP2 was not sufficient to stimulate osteogenesis of hBMSCs. Unlike the murine clones, dexamethasone (which is typically added for osteogenesis of both human and murine cells, and for cells from other species) (Peister et al., 2004; Pittenger et al., 1999) had to be added to the media of hBMSC cultures to induce osteogenesis. Dexamethasone is a synthetic glucocorticoid belonging to the steroid hormone family. It is very potent - approximately 20-30 times more so than hydrocortisone. Glucocorticoids have been shown to be potent inducers of osteogenic differentiation *in vitro* (Kirton et al., 2006). Species differences have been previously reported, e.g., it has been shown that large doses of BMP-2 are required to have the same osteogenic effect in human cells than occurs in mouse cells at much lower doses (Rawadi et al., 2003). *In vivo* osteogenic differentiation of MSCs has been widely reported and seems to be the default pathway as it occurs when MSCs are transplanted in ceramic implants and implanted beneath the skin (Lennon et al., 2001). It seems a factor(s) is missing in the differentiation medium if

a potent chemical such as dexamethasone has to be added for *in vitro* osteogenic differentiation of MSCs, when *in vivo* osteogenic differentiation can occur so readily.

The same adipogenic differentiation media used for the murine clones was also used for the human BMSCs. It should be noted that a low level of chemicals was used for this in comparison to previously reported studies. Adipogenesis took longer than the two weeks observed for the murine clones in the present study. It also did not take place to the same extent in hBMSC2 cultures. This could be due to the cells, which were from an older donor (60 years old), and contained the flattened cells characteristic of senescent cells, and were not very proliferative. The default differentiation pathway in murine MSCs is reported to be adipogenesis, while in human MSCs, it is osteogenesis (D'Ippolito et al., 2004). This could be one reason why it took the human BMSCs longer to differentiate. Also, we had optimised the adipogenic media for the murine cells, and thus added a small quantity of the various chemicals. It is likely that if we used a greater quantity as used in most other studies, the cells may have differentiated more quickly.

Previous studies have reported that the age of the donor affects the differentiation potential of BMSCs. There is a decrease in osteoprogenitor cells in the bone marrow from older animals (Quarto et al., 1995). Passage zero clones were isolated by the Muraglia group, in the presence and absence of FGF-2 (Muraglia et al., 2000). An increase in the frequency of bi-potential clones with osteo-chondro potential was found in the bone marrow from younger donors compared to older donors. The donor ages were 5 months to 30 years. This is half the age of donor two in this study, and therefore it is possible this is one reason for the decreased differentiation potential of BMSCs from this donor. The Muraglia group examined the ability of 185 human clones to differentiate, and all clones had the ability to differentiate into the osteogenic lineage.

The growth rate of hBMSC3 was followed at 5% oxygen and was constant between the observed passages (2-5) with an averaged cell doubling time of 3.42 days ± 0.78 days (mean ± standard deviation). The cell doubling time of other human clonal MSCs has been reported to be 1.3 days in the log phase in the initial 20 days in culture and then 4.4 days when they had entered the plateau phase and this remained constant for 100 days

(Muraglia et al., 2000). Typically the growth rate of murine MSCs is quicker than that of human MSCs. This could be due to species differences, and, of course, the lifespan of a mouse is much shorter than a human.

The surface antigen profile of the hBMSCs and fibroblasts was examined using flow cytometry. Human BMSC3 and fibroblasts were positive for CD13 (myeloid cell marker) CD49e (integrin) and CD90 (fibroblast marker). CD105, a putative MSC marker was only expressed by hBMSC3 and by the fibroblasts, and CD166 (another putative MSC marker) was expressed by hBMSC1 and hBMSC3 and the fibroblasts. There did not seem to be much consistency between the cell surface antigen phenotype expression pattern of the hBMSCs. In fact hBMSC3 and the fibroblasts had a more similar surface antigen phenotype. Both populations expressing the markers mentioned above as well as CD73, CD105, and CD166. In particular, both cell populations expressed CD13 and CD90 at greater intensity compared with hBMSC1 and hBMSC4. However, there are significant biological differences between the two cell populations. Human BMSC3 cultures have a tri-lineage differentiation potential which the fibroblasts lack. In addition, hBMSC3 also expressed significant levels of STEAP1 and 2 compared to the fibroblast cultures. Thus, these results highlight the need for more specific MSC markers, as many cell types are positive for the previously suggested markers.

Only the surface marker CD122 was present on all hBMSCs and absent on the fibroblasts. CD122 is interleukin-2 receptor β (IL-2Rβ) which is expressed on immune cells, particularly T-cells. Previously mentioned MIAMI cells which were derived in low oxygen conditions were also positive for this marker (D'Ippolito et al., 2004). CD122 is involved in signalling pathways via the JAK1 protein. JAK1 is reported to activate STAT5a/b transcription factors which stimulate gene expression of D cyclins (Moriggl et al., 1999). Expression of CD122 by the murine clones was not examined, however, microarray analysis did show that the cell cycle gene Cdk4 is differentially expressed by the clones (figure 5.10) and the IL-1 receptor type I by the BALB/b clones (table 5.1). It would be interesting to see if the murine clones express CD122.

Both murine clones and human BMSCs express CD49e which is an integrin α5 also known as ITGA5 or fibronectin receptor. CD49e associates with CD29 (β1 integrin) to

form VLA-5, a well-established fibronectin receptor that is expressed on many cell types including fibroblasts, epithelial cells, endothelial cells, platelets, and peripheral T cells (Huveneers et al., 2007). CD29 was expressed by hBMSC1 and hBMSC3 but not hBMSC4. Murine clones consistently expressed very high levels of Sca-1 and this has been previously reported (Peister et al., 2004). No such stem cell marker has been identified for human MSCs and unfortunately there is no known human homologue of Sca-1. Although, interestingly, over-expression of mouse Sca-1 affects human haematopoietic cells in a similar way to mouse cells, suggesting there may be a protein with a similar role in humans (Bradfute et al., 2005).

All hBMSCs were karyotypically normal except two cells in hBMSC2 cultures (figures 6.5). There was the presence of one cell with a hyperdiploid chromosome number of 52, and another with a normal chromosome number, but with a balanced translocation between two chromosomes. Karyotype analysis was performed on cells at passage three. The transformation is unlikely to be due to tissue culture as the other hBMSCs were normal. It is possible the abnormal cells were present in patient, as the donor was 60 years old and cells may have acquired the genetic abnormalities with time. The ages of the other donors (hBMSC1, 3, and 4) were 25, 11, and 25 years respectively. Or it could be due to fact that older cells are less genetically resilient, when placed out of their *in vivo* environment and exposed to foreign conditions (cell culture), genetic abnormalities can accumulate. However, the majority of hBMSCs were normal, and therefore results are consistent with previous studies reporting the relative resistance to transformation of human MSCs compared to murine cultures (Aguilar et al., 2007).

Now that we had isolated normal, multipotent, human bone marrow stromal cells we examined whether these cells expressed STEAP1 by western blot analysis. As discussed in chapter 5, STEAP1 has been discovered relatively recently in 1999 in human prostate cancer cells (Hubert et al., 1999) and we are the first to report them in MSCs. Western blotting analysis revealed the presence of two differentially expressed bands in the hBMSCs, one large band at 36 kDa (STEAP1), the other smaller one at 38kDa in two out of three western blots. The reason it was not observed in the second western blot could be

due to poor separation. A gradient gel was used in the next experiment and the two bands were clearly observed again. Interestingly, hBMSC3 expressed significantly greater levels of STEAP1 than hBMSC1 and hBMSC2. An observable difference between the cells from this donor and the others is that they were more proliferative, and differentiated into the adipogenic lineage more completely compared to hBMSC1 and hBMSC2. Also all these cells were homogeneously spindle shaped. The specificity of the antibody for STEAP1 was confirmed when cells were depleted of STEAP1, i.e., the 36 kDa STEAP1 band was absent (compared to the controls). The 38 kDa band was also absent, however it was only weakly visible in the controls (lipofectamine alone and luciferase) therefore one cannot conclude whether it was STEAP1 or a non-specific band. The datasheet for the STEAP1 antibody used for the western blot analysis shows a photo of STEAP1 expression in the androgen-sensitive human prostate adenocarcinoma cell line, LNCaP and there does appear to be two very tight bands, with the upper one being larger than the lower one and they both run lower than 36 kDa. However, this may be due to the difference in cell type. The same antibody was also used by the Alves group (Alves et al., 2006) to examine STEAP1 expression in tumour cell lines. There may be two bands in the DU145 (human prostate cancer cell line) and M113 (melanoma cell line) lanes, although greater protein separation is required to say with any certainty. Unfortunately, the LNCaP lane is overexposed so it looks like one very large band. The presence of two bands is not remarked on by the investigators, nor in the datasheet by Santa-Cruz. It will be interesting to elucidate whether the two bands are STEAP1 and what post-translational modifications may be responsible for the shift. Alternatively, it could be a non-specific protein, however this seems unlikely since it is differentially expressed in the hBMSCs and not the fibroblast cultures. Also, the doublet is seen in different cell types, from prostate cancer cells (LNCaP), to hBMSCs. Unfortunately the STEAP1 antibody was not suitable for FACS analysis. Another two were also tested, but they also proved unsuitable.

More importantly, the western blot experiments revealed that STEAP1 is differentially expressed in hBMSCs from three different donors. These cells had also been derived from quite different sources. The first and fourth were from commercially obtained

hBMMNCs. The second hBMSCs from a bone marrow aspirate from an elderly donor (60 years), and the third hBMSCs derived from hBMMNCs from a young (11 years old) donor. The results also revealed the specificity of STEAP1 protein for human BMSCs, in that it was not expressed at detectable levels by both human fibroblasts and human articular chondrocytes. This is particularly interesting, because both cell types are of mesenchymal origin and have been reported to express the most commonly used human MSC marker STRO-1. Although NHDFs in this study did not express it, previous studies have reported STRO-1 expression in human fibroblasts HS68 (Wagner et al., 2005), periodontal ligament fibroblasts (Lallier and Spencer, 2007), and myofibroblasts (Strakova et al., 2008). Flow cytometry analysis of human chondrocytes used in this study also revealed significant levels of STRO-1 expression.

The expression pattern of STEAP1 in the human BMSCs is similar to that of STEAP1 in the murine clonal MSCs, where we first discovered STEAPs. Gene expression analysis showed that STEAP1 was differentially expressed in the murine MSCs compared to the equivalent adipocyte differentiated clones, embryonic fibroblasts, as well as bone marrow adherent cells, and freshly isolated bone marrow mononuclear cells. Although murine MSCs were karyotypically abnormal, they were not malignant as there was no tumour formation when they were injected into immunodeficient mice. Furthermore, we did attempt to derive cytogenetically normal murine MSCs, by culturing BMMNCs in different conditions such as, at a high density, at 20% oxygen, and also a different strain of mice, FVB/N (as described in chapter 4). Unfortunately, all cells developed karyotypic abnormalities.

However, STEAP1 expression in normal human multipotent BMSCs proves it is not a marker of karyotypically abnormal cells. This is the first mention of STEAP1 in MSCs, and its function is unknown. Due to its secondary structure investigators have speculated that it may function as an aquaporin (Kruse et al., 2006), ion channel (Dolly and Parcej, 1996), or more recently in cell-cell communication (Challita-Eid et al., 2007). The Challita-Eid group report STEAP1 function in mediating small molecules between adjacent cells in culture (Challita-Eid et al., 2007). *In vivo* these could be nutrients,

metabolites, and electrolytes. All of which may play an important role in stem cell function. This is further collaborated by immunohistochemical staining of STEAP1 which showed that it is concentrated at cell-cell boundaries in the prostate gland, with much less staining in the luminal side of the epithelia (Hubert et al., 1999).

STEAP2 expression was examined in the hBMSCs by flow cytometry. FACS analysis revealed that STEAP2 is differentially expressed in the two hBMSC cultures studied (hBMSC3 and hBMSC4), compared to both human fibroblasts and hBMMNCs.

Results from chapter 4 showed that Sca-1 is expressed at high intensity in the clones and decreases in adipocyte differentiated clones. We found this expression pattern was not the same for STEAP2 as the protein level was the same in adipocyte differentiated cultures and the control cells cultured for the same length of time. Thus, the results suggest STEAP2 may not have a functional role in maintaining the undifferentiated status of the stem cells. STEAP2 expression by western blot analysis was examined in hBMSCs, but the antibody was not compatible with immuno-blotting. Another antibody was also tested with the same negative results.

It would be interesting to see if STEAP1 expression is altered in the adipocyte differentiated hBMSCs, as it is not present in chondrocytes which is one lineage into which MSCs differentiate. It is possible that the two STEAPs have distinct roles in the MSCs as there are distinct differences in their secondary structure. STEAP2 and other members of the STEAP family (STEAP3 & 4) contain an oxidoreductase domain that STEAP1 does not. These domains are thought to function as ferriductases and cupric reductases that stimulate cellular uptake of both iron and copper *in vitro* and possibly *in vivo* (Ohgami et al., 2006).

In initial studies STEAP1 function was probed using RNA interference. STEAP1 and 2 were successfully depleted in hBMSC3, and although no differences in proliferation were detected, STEAP1 and 2 depleted cells showed reduced adherence to the tissue culture substrate.

In summary, results show that STEAP1 and -2, initially identified via gene expression analysis as candidate MSC markers in murine clonal MSCs are also differentially

expressed by human, normal, multipotent BMSCs. Further work is now needed to more clearly establish their role in MSCs and ascertain their usefulness as cell-specific markers.

Chapter 7

Conclusions and Future Work

7.1 Conclusions and future work

7.1.1 Conclusions

The rationale of this study was to identify potential MSC specific surface markers, as this is needed to enable isolation and purification of MSCs. The intention was to search in murine clonal MSCs and then to verify subsequent findings in human MSCs. Murine cells were chosen due to ready availability of large amounts of freshly isolated murine bone marrow for manipulation and derivation of clonal MSCs. Another advantage was the derivation of different populations of strain-matched control cell populations. This would not have been feasible with human cells.

Murine MSC clones were derived to ensure that results from any experiment would be due directly to the MSCs rather than other cells present, as can be the case if a heterogeneous population is used (as is commonly the case in MSC studies). Another unique and essential aspect of the approach was that the selection and expansion culture protocols were performed under low oxygen conditions (5% oxygen), with the rationale of providing the conditions that more closely resemble the *in vivo* niche of the most primitive marrow-residing mesenchymal stem cells. Subsequent assays proved the clonally-derived cells had the ability to differentiate into adipocytes, chondrocytes, and osteoblasts as well as to self-renew, which demonstrates the (at least *in vitro*) functional criteria for mesenchymal stem cell status. In contrast, mouse embryonic fibroblasts did not have this capacity. Flow cytometry analysis revealed clonal MSCs consistently expressed stem cell antigen-1 (Sca-1). Recent studies have reported that murine cells readily become transformed in cell culture (Todaro and Green, 1963; Tolar et al., 2007). In the present study the MSC clones were indeed found to have an abnormal karyotype. However, they were not malignant, as observed by the fact that there was no tumour formation when these cells were injected into immuno-compromised mice. In addition, the MSC clones did not have other properties described of malignant cells, such as increased c-myc expression (Miura et al., 2006). It appears the murine MSC clonal cells in this study had become immortalised but not malignant. In addition, the differentiation

of the murine MSCs was a highly co-ordinated and regulated process. In particular, in adipocyte differentiated clones there was a significant down-regulation in proliferation related genes such as Cdk4 and stem cell-associated gene Sca-1, with concomitant up-regulation of many adipocyte markers including PPARγ. This strongly suggests the murine clones serve as a very useful model to study MSC biology.

Therefore, the next step was to characterise the molecular signature of the clonal MSCs using a rigorous microarray analysis. This would enable identification of potentially MSC-specific markers by comparing the gene expression profile of MSC clones to relevant controls. Two different analysis methods were used - a supervised analysis examining genes clustering with Sca-1; and an unsupervised analysis where very stringent criteria were set for differential gene expression on the clones (compared to all controls). Thus a genome-wide analysis resulted in the identification (from both methods) of just two cell membrane proteins (STEAP1 and STEAP2) as differentially expressed on the murine MSCs. It was then most important to assess if human MSCs differentially expressed these cell surface proteins.

Human bone marrow stromal cells (hBMSCs) were derived and cultured in 5% oxygen. The human cells were multipotent and had the capacity to differentiate into adipocytes, chondrocytes, and osteoblasts. Furthermore, the cells were karyotypically normal. Both STEAP1 and -2 were differentially expressed in the hBMSCs, therefore strongly suggesting that these antigens are <u>not</u> simply a characteristic of karyotypically abnormal cells. FACS analysis demonstrated that hBMSC cultures expressed significant levels of STEAP2 compared to human fibroblasts and human BMMNCs (where there was no detectable expression). STEAP2 was retained in hBMSCs which had been differentiated into adipocytes, suggesting that STEAP2 did not have a role in maintaining the undifferentiated stem cell phenotype. Human BMSCs also differentially expressed STEAP1 compared to both human fibroblasts and chondrocytes, as shown by immunoblotting. Both of the latter cell types are of mesenchymal origin and one (chondrocyte) is a lineage that MSCs differentiate into. Results tentatively suggest that STEAP1 may have a potential functional role in MSCs as its depletion in hBMSCs by RNA interference resulted in a decrease in cell adhesion.

STEAPs were discovered relatively recently in prostate cancer cells (Hubert et al., 1999; Porkka et al., 2002). Due to their expression on epithelial cancer cells they may seem unlikely markers of mesenchymal stem cells. However, MSCs are reported to differentiate into a wide spectrum of lineages including the epithelial cells (D'Ippolito et al., 2004; Jiang et al., 2002). Future studies are now required to establish the usefulness of STEAP1 and -2 as MSC-specific markers.

7.1.2 Future work

The immediate future work would be to FACS sort adherent human bone marrow stromal cells based on STEAP2 expression. The STEAP2 antibody used in this study was found to be suitable for FACS analysis, and we report that approximately 50% of human BMSC cultures expressed STEAP2. Once these cells are sorted, the next step would be to examine the CFU-f ability and multilineage differentiation potential of both STEAP2 positive and STEAP2 negative populations. Evidence for the usefulness of STEAP2 as a MSC marker would be strengthened if the STEAP2 positive population greatly exceeded the negative population in CFU-f and differentiation assays.

Another immediate future work would be to examine STEAP1 expression in differentiated hBMSCs compared to levels in undifferentiated hBMSCs. In addition, it would be worth examining STEAP2 expression in chondro- and osteo-differentiated hBMSCs. This would allow one to more clearly determine whether STEAPs are involved in maintenance of the undifferentiated stem cell phenotype.

A more long term goal would be to sort human cells freshly isolated from human bone marrow for STEAP2 and then perform the experiments mentioned above to assess the CFU-f ability and tri-lineage differentiation capacity of the sorted cell populations. This would avoid any possible changes in cell surface marker expression due to tissue culture effects and provide clearer evidence about the eligibility of STEAP2 as a MSC surface marker. However, this approach is technically very difficult. STEAP2 expression in the human BMMNCs was negligible (see chapter 6), i.e., there was no detectable STEAP2

positive population. Furthermore, reports of the frequency of MSCs in the BMMNCs vary from 0.001-0.01% (Pittenger et al., 1999) to 0.0001% cells (Castro-Malaspina et al., 1980). All estimates point to the fact that MSCs are an extremely rare population in the bone marrow. Thus if STEAP2 is a potential MSC marker, or even a marker that can be used to enrich for MSCs, these observations suggest that it will still be a rare population that will be sorted, and large amounts of starting material will be required of both human BMMNCs and the STEAP2 antibody in order to isolate a sufficient number of STEAP2 positive cells for subsequent analysis.

A FACS-compatible STEAP1 antibody is not commercially available, although one has been reported in the literature (Challita-Eid et al., 2007). If one was available in the future, it would be important to perform the above mentioned experiments using STEAP1 antibody. In addition, it would be interesting to sort using both antibodies and determine whether this further enriches for MSCs compared to individual STEAP sorted cell populations.

If studies did show that STEAPs were MSC specific cell surface markers there would be many potential applications. An MSC specific marker would enable identification and characterisation of the MSC niche. This would be possible both in animals and humans. Studies on STEAP knock-out mice could be attempted in order to investigate the importance of the marker, i.e. whether the mouse survived if the marker was knocked out. If the knock-out mouse did survive one may then investigate the subsequent physiological effect, for example if there was normal cartilage and bone formation. These studies may lead to a better understanding of the *in vivo* function of the marker and/or MSC. Furthermore, identification of the marker would also enable the cells to be labelled and subsequent cell tracking studies in *in vivo* injury models would also be insightful.
Isolation of a pure population of MSCs would also enable rapid, routine and reproducible *in vitro* expansion of MSCs and the generation of the large cell numbers required for clinical studies.
Identification of a MSC cell surface marker will allow isolation of a pure MSC population. In the clinical setting this would enable comparison to heterogeneous MSC

cell populations in the treatment of specific diseases. It is possible that a pure population of MSCs may enhance the regenerative effect, as all the cells would have a multilineage differentiation potential, and therefore the ability to differentiate into the required cell type. In comparison, a mixed population containing various progenitor cells and committed cells would not. MSCs have the ability to home to sites of tissue damage and have trophic properties which facilitate tissue repair. It is logical to think these are stem cell properties lost upon differentiation. Therefore it is possible a pure population may home to and engraft at sites of tissue damage better than a mixed population and therefore result in greater clinical improvement. Thus, there are many potential applications once a MSC-specific marker is confirmed.

Chapter 8

Bibliography

8.1 Bibliography

Abbondanzo, S.J., Gadi, I., and Stewart, C.L. (1993). Derivation of embryonic stem cell lines. Methods Enzymol *225*, 803-823.

Abdallah, B.M., and Kassem, M. (2008). Human mesenchymal stem cells: from basic biology to clinical applications. Gene Ther *15*, 109-116.

Aggarwal, S., and Pittenger, M.F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood *105*, 1815-1822.

Aguilar, S., Nye, E., Chan, J., Loebinger, M., Spencer-Dene, B., Fisk, N., Stamp, G., Bonnet, D., and Janes, S.M. (2007). Murine but not human mesenchymal stem cells generate osteosarcoma-like lesions in the lung. Stem Cells *25*, 1586-1594.

Alves, P.M., Faure, O., Graff-Dubois, S., Cornet, S., Bolonakis, I., Gross, D.A., Miconnet, I., Chouaib, S., Fizazi, K., Soria, J.C.*, et al.* (2006). STEAP, a prostate tumor antigen, is a target of human CD8+ T cells. Cancer Immunol Immunother *55*, 1515-1523.

Anjos-Afonso, F., and Bonnet, D. (2007). Nonhematopoietic/endothelial SSEA-1+ cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. Blood *109*, 1298-1306.

Arepalli, S.R., Jones, E.P., Howcroft, T.K., Carlo, I., Wang, C., Lindahl, K.F., Singer, D.S., and Rudikoff, S. (1998). Characterization of two class I genes from the H2-M region: evidence for a new subfamily. Immunogenetics *47*, 264-271.

Ashton, B.A., Allen, T.D., Howlett, C.R., Eaglesom, C.C., Hattori, A., and Owen, M. (1980). Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Orthop Relat Res, 294-307.

Azizi, S.A., Stokes, D., Augelli, B.J., DiGirolamo, C., and Prockop, D.J. (1998). Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts. Proc Natl Acad Sci U S A *95*, 3908-3913.

Bab, I., Ashton, B.A., Gazit, D., Marx, G., Williamson, M.C., and Owen, M.E. (1986). Kinetics and differentiation of marrow stromal cells in diffusion chambers in vivo. J Cell Sci *84*, 139-151.

Baddoo, M., Hill, K., Wilkinson, R., Gaupp, D., Hughes, C., Kopen, G.C., and Phinney, D.G. (2003). Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem *89*, 1235-1249.

Bagley, R.G., Rouleau, C., Morgenbesser, S.D., Weber, W., Cook, B.P., Shankara, S., Madden, S.L., and Teicher, B.A. (2006). Pericytes from human non-small cell lung carcinomas: an attractive target for anti-angiogenic therapy. Microvasc Res *71*, 163-174.

Baksh, D., Song, L., and Tuan, R.S. (2004). Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med *8*, 301-316.

Barbero, A., Ploegert, S., Heberer, M., and Martin, I. (2003). Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum *48*, 1315-1325.

Barry, F.P. (2003). Biology and clinical applications of mesenchymal stem cells. Birth Defects Res C Embryo Today *69*, 250-256.

Baxter, M.A., Wynn, R.F., Jowitt, S.N., Wraith, J.E., Fairbairn, L.J., and Bellantuono, I. (2004). Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells *22*, 675-682.

Bembi, B., Parma, A., Bottega, M., Ceschel, S., Zanatta, M., Martini, C., and Ciana, G. (1997). Intravenous pamidronate treatment in osteogenesis imperfecta. J Pediatr *131*, 622-625.

Bertrand, M., Huijbers, I., Chomez, P., and De Backer, O. (2004). Comparative expression analysis of the MAGED genes during embryogenesis and brain development. Dev Dyn *230*, 325-334.

Bianco, P., Riminucci, M., Gronthos, S., and Robey, P.G. (2001). Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells *19*, 180-192.

Bjerkvig, R., Tysnes, B.B., Aboody, K.S., Najbauer, J., and Terzis, A.J. (2005). Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer *5*, 899-904.

Bonnet, D., and Dick, J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med *3*, 730-737.

Bonyadi, M., Waldman, S.D., Liu, D., Aubin, J.E., Grynpas, M.D., and Stanford, W.L. (2003). Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. Proc Natl Acad Sci U S A *100*, 5840-5845.

Bradfute, S.B., Graubert, T.A., and Goodell, M.A. (2005). Roles of Sca-1 in hematopoietic stem/progenitor cell function. Exp Hematol *33*, 836-843.

Brandao-Burch, A., Utting, J.C., Orriss, I.R., and Arnett, T.R. (2005). Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcif Tissue Int *77*, 167-174.

Brighton, C.T., and Heppenstall, R.B. (1971). Oxygen tension in zones of the epiphyseal plate, the metaphysis and diaphysis. An in vitro and in vivo study in rats and rabbits. J Bone Joint Surg Am *53*, 719-728.

Bruder, S.P., Kraus, K.H., Goldberg, V.M., and Kadiyala, S. (1998). The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am *80*, 985-996.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Caplan, A.I. (1991). Mesenchymal stem cells. J Orthop Res *9*, 641-650.

Caplan, A.I. (2007). Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol *213*, 341-347.

Caplan, A.I., and Bruder, S.P. (2001). Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med *7*, 259-264.

Caplan, A.I., and Dennis, J.E. (2006). Mesenchymal stem cells as trophic mediators. J Cell Biochem *98*, 1076-1084.

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

Castro-Malaspina, H., and Jhanwar, S.C. (1984). Properties of myelofibrosis-derived fibroblasts. Prog Clin Biol Res *154*, 307-322.

Challita-Eid, P.M., Morrison, K., Etessami, S., An, Z., Morrison, K.J., Perez-Villar, J.J., Raitano, A.B., Jia, X.C., Gudas, J.M., Kanner, S.B.*, et al.* (2007). Monoclonal antibodies to six-transmembrane epithelial antigen of the prostate-1 inhibit intercellular communication in vitro and growth of human tumor xenografts in vivo. Cancer Res *67*, 5798-5805.

Chamberlain, G., Fox, J., Ashton, B., and Middleton, J. (2007). Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells *25*, 2739-2749.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.

Chin, L., Artandi, S.E., Shen, Q., Tam, A., Lee, S.L., Gottlieb, G.J., Greider, C.W., and DePinho, R.A. (1999). p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell *97*, 527-538.

Cho, J.H., Itoh, T., Sendai, Y., and Hoshi, H. (2008). Fibroblast growth factor 7 stimulates in vitro growth of oocytes originating from bovine early antral follicles. Mol Reprod Dev.

Colter, D.C., Sekiya, I., and Prockop, D.J. (2001). Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci U S A *98*, 7841-7845.

Corcione, A., Benvenuto, F., Ferretti, E., Giunti, D., Cappiello, V., Cazzanti, F., Risso, M., Gualandi, F., Mancardi, G.L., Pistoia, V.*, et al.* (2006). Human mesenchymal stem cells modulate B-cell functions. Blood *107*, 367-372.

Csete, M. (2005). Oxygen in the cultivation of stem cells. Ann N Y Acad Sci *1049*, 1-8.

D'Ippolito, G., Diabira, S., Howard, G.A., Menei, P., Roos, B.A., and Schiller, P.C. (2004). Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci *117*, 2971-2981.

D'Ippolito, G., Diabira, S., Howard, G.A., Roos, B.A., and Schiller, P.C. (2006). Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone *39*, 513-522.

da Silva Meirelles, L., Caplan, A.I., and Nardi, N.B. (2008). In search of the in vivo identity of mesenchymal stem cells. Stem Cells *26*, 2287-2299.

Davani, S., Marandin, A., Mersin, N., Royer, B., Kantelip, B., Herve, P., Etievent, J.P., and Kantelip, J.P. (2003). Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation *108 Suppl 1*, II253-258.

Dazzi, F., Ramasamy, R., Glennie, S., Jones, S.P., and Roberts, I. (2006). The role of mesenchymal stem cells in haemopoiesis. Blood Rev *20*, 161-171.

De Bari, C., Dell'Accio, F., Tylzanowski, P., and Luyten, F.P. (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum *44*, 1928-1942.

De Coppi, P., Bartsch, G., Jr., Siddiqui, M.M., Xu, T., Santos, C.C., Perin, L., Mostoslavsky, G., Serre, A.C., Snyder, E.Y., Yoo, J.J.*, et al.* (2007). Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol *25*, 100-106.

De Ugarte, D.A., Morizono, K., Elbarbary, A., Alfonso, Z., Zuk, P.A., Zhu, M., Dragoo, J.L., Ashjian, P., Thomas, B., Benhaim, P.*, et al.* (2003). Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs *174*, 101-109.

Dennis, J.E., Carbillet, J.P., Caplan, A.I., and Charbord, P. (2002). The STRO-1+ marrow cell population is multipotential. Cells Tissues Organs *170*, 73-82.

Dennis, J.E., Merriam, A., Awadallah, A., Yoo, J.U., Johnstone, B., and Caplan, A.I. (1999). A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. J Bone Miner Res *14*, 700-709.

Dexter, T.M., Wright, E.G., Krizsa, F., and Lajtha, L.G. (1977). Regulation of haemopoietic stem cell proliferation in long term bone marrow cultures. Biomedicine *27*, 344-349.

Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P.D., Matteucci, P., Grisanti, S., and Gianni, A.M. (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood *99*, 3838-3843.

Diaz-Romero, J., Nesic, D., Grogan, S.P., Heini, P., and Mainil-Varlet, P. (2008). Immunophenotypic changes of human articular chondrocytes during monolayer culture reflect bona fide dedifferentiation rather than amplification of progenitor cells. J Cell Physiol *214*, 75-83.

Diefenderfer, D.L., Osyczka, A.M., Garino, J.P., and Leboy, P.S. (2003). Regulation of BMP-induced transcription in cultured human bone marrow stromal cells. J Bone Joint Surg Am *85-A Suppl 3*, 19-28.

Digirolamo, C.M., Stokes, D., Colter, D., Phinney, D.G., Class, R., and Prockop, D.J. (1999). Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol *107*, 275-281.

Djouad, F., Delorme, B., Maurice, M., Bony, C., Apparailly, F., Louis-Plence, P., Canovas, F., Charbord, P., Noel, D., and Jorgensen, C. (2007). Microenvironmental changes during differentiation of mesenchymal stem cells towards chondrocytes. Arthritis Res Ther *9*, R33.

Dolly, J.O., and Parcej, D.N. (1996). Molecular properties of voltage-gated K+ channels. J Bioenerg Biomembr *28*, 231-253.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315-317.

Dowthwaite, G.P., Bishop, J.C., Redman, S.N., Khan, I.M., Rooney, P., Evans, D.J., Haughton, L., Bayram, Z., Boyer, S., Thomson, B.*, et al.* (2004). The surface of articular cartilage contains a progenitor cell population. J Cell Sci *117*, 889-897.

Duncan, R., and McConkey, E.H. (1982). How many proteins are there in a typical mammalian cell? Clin Chem *28*, 749-755.

Earle, W.R. (1962). Some morphologic variations of certain cells under controlled experimental conditions. Natl Cancer Inst Monogr *7*, 213-236.

Edgar, C.M., Chakravarthy, V., Barnes, G., Kakar, S., Gerstenfeld, L.C., and Einhorn, T.A. (2007). Autogenous regulation of a network of bone morphogenetic proteins (BMPs) mediates the osteogenic differentiation in murine marrow stromal cells. Bone *40*, 1389-1398.

Edman, L.C., Mira, H., and Arenas, E. (2008). The beta-chemokines CCL2 and CCL7 are two novel differentiation factors for midbrain dopaminergic precursors and neurons. Exp Cell Res *314*, 2123-2130.

Eslaminejad, M.B., Nadri, S., and Hosseini, R.H. (2007). Expression of Thy 1.2 surface antigen increases significantly during the murine mesenchymal stem cells cultivation period. Dev Growth Differ *49*, 351-364.

Fehrer, C., Brunauer, R., Laschober, G., Unterluggauer, H., Reitinger, S., Kloss, F., Gully, C., Gassner, R., and Lepperdinger, G. (2007). Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. Aging Cell *6*, 745-757.

Ferri, A.L., Lin, W., Mavromatakis, Y.E., Wang, J.C., Sasaki, H., Whitsett, J.A., and Ang, S.L. (2007). Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. Development *134*, 2761-2769.

Field, R.E., Buchanan, J.A., Copplemans, M.G., and Aichroth, P.M. (1994). Bone-marrow transplantation in Hurler's syndrome. Effect on skeletal development. J Bone Joint Surg Br *76*, 975-981.

Finch, P.W., and Rubin, J.S. (2004). Keratinocyte growth factor/fibroblast growth factor 7, a homeostatic factor with therapeutic potential for epithelial protection and repair. Adv Cancer Res *91*, 69-136.

Forbes, S.J., Vig, P., Poulsom, R., Wright, N.A., and Alison, M.R. (2002). Adult stem cell plasticity: new pathways of tissue regeneration become visible. Clin Sci (Lond) *103*, 355-369.

Forte, G., Minieri, M., Cossa, P., Antenucci, D., Sala, M., Gnocchi, V., Fiaccavento, R., Carotenuto, F., De Vito, P., Baldini, P.M.*, et al.* (2006). Hepatocyte growth factor effects

on mesenchymal stem cells: proliferation, migration, and differentiation. Stem Cells *24*, 23-33.

Foster, L.J., Zeemann, P.A., Li, C., Mann, M., Jensen, O.N., and Kassem, M. (2005). Differential expression profiling of membrane proteins by quantitative proteomics in a human mesenchymal stem cell line undergoing osteoblast differentiation. Stem Cells *23*, 1367-1377.

Frank, L., and Massaro, D. (1980). Oxygen toxicity. Am J Med *69*, 117-126.

Frank, L., Summerville, J., and Massaro, D. (1980). Potection from oxygen toxicity with endotoxin. Role of the endogenous antioxidant enzymes of the lung. J Clin Invest *65*, 1104-1110.

Friedenstein, A.J. (1961). Osteogenetic activity of transplanted transitional epithelium. Acta Anat (Basel) *45*, 31-59.

Friedenstein, A.J., Petrakova, K.V., Kurolesova, A.I., and Frolova, G.P. (1968). Heterotopic of bone marrow.Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation *6*, 230-247.

Friedenstein, A.J., Piatetzky, S., II, and Petrakova, K.V. (1966). Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol *16*, 381-390.

Gang, E.J., Bosnakovski, D., Figueiredo, C.A., Visser, J.W., and Perlingeiro, R.C. (2007). SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood *109*, 1743-1751.

Ganz, T. (2000). Paneth cells--guardians of the gut cell hatchery. Nat Immunol *1*, 99-100.

Garcia-Olmo, D., Garcia-Arranz, M., Garcia, L.G., Cuellar, E.S., Blanco, I.F., Prianes, L.A., Montes, J.A., Pinto, F.L., Marcos, D.H., and Garcia-Sancho, L. (2003). Autologous stem cell transplantation for treatment of rectovaginal fistula in perianal Crohn's disease: a new cell-based therapy. Int J Colorectal Dis *18*, 451-454.

Garcia-Olmo, D., Garcia-Arranz, M., Herreros, D., Pascual, I., Peiro, C., and Rodriguez-Montes, J.A. (2005). A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum *48*, 1416-1423.

Gojo, S., Gojo, N., Takeda, Y., Mori, T., Abe, H., Kyo, S., Hata, J., and Umezawa, A. (2003). In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Exp Cell Res *288*, 51-59.

Grant, J.L., and Smith, B. (1963). Bone marrow gas tensions, bone marrow blood flow, and erythropoiesis in man. Ann Intern Med *58*, 801-809.

Grayson, W.L., Zhao, F., Bunnell, B., and Ma, T. (2007). Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. Biochem Biophys Res Commun *358*, 948-953.

Grayson, W.L., Zhao, F., Izadpanah, R., Bunnell, B., and Ma, T. (2006). Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. J Cell Physiol *207*, 331-339.

Grellier, P., Yee, D., Gonzalez, M., and Abboud, S.L. (1995). Characterization of insulin-like growth factor binding proteins (IGFBP) and regulation of IGFBP-4 in bone marrow stromal cells. Br J Haematol *90*, 249-257.

Gronthos, S., Zannettino, A.C., Hay, S.J., Shi, S., Graves, S.E., Kortesidis, A., and Simmons, P.J. (2003). Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci *116*, 1827-1835.

Gumley, T.P., McKenzie, I.F., and Sandrin, M.S. (1995). Tissue expression, structure and function of the murine Ly-6 family of molecules. Immunol Cell Biol *73*, 277-296.

Hanada, K., Dennis, J.E., and Caplan, A.I. (1997). Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. J Bone Miner Res *12*, 1606-1614.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp Cell Res *25*, 585-621.

Haynesworth, S.E., Baber, M.A., and Caplan, A.I. (1992). Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone *13*, 69-80.

Hiyama, E., and Hiyama, K. (2007). Telomere and telomerase in stem cells. Br J Cancer *96*, 1020-1024.

Holmes, C., and Stanford, W.L. (2007). Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells *25*, 1339-1347.

Horwitz, E.M., Gordon, P.L., Koo, W.K., Marx, J.C., Neel, M.D., McNall, R.Y., Muul, L., and Hofmann, T. (2002). Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A *99*, 8932-8937.

Horwitz, E.M., Prockop, D.J., Fitzpatrick, L.A., Koo, W.W., Gordon, P.L., Neel, M., Sussman, M., Orchard, P., Marx, J.C., Pyeritz, R.E.*, et al.* (1999). Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med *5*, 309-313.

Horwitz, E.M., Prockop, D.J., Gordon, P.L., Koo, W.W., Fitzpatrick, L.A., Neel, M.D., McCarville, M.E., Orchard, P.J., Pyeritz, R.E., and Brenner, M.K. (2001). Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. Blood *97*, 1227-1231.

Hubert, R.S., Vivanco, I., Chen, E., Rastegar, S., Leong, K., Mitchell, S.C., Madraswala, R., Zhou, Y., Kuo, J., Raitano, A.B.*, et al.* (1999). STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors. Proc Natl Acad Sci U S A *96*, 14523-14528.

Huveneers, S., Truong, H., and Danen, H.J. (2007). Integrins: signaling, disease, and therapy. Int J Radiat Biol *83*, 743-751.

Imabayashi, H., Mori, T., Gojo, S., Kiyono, T., Sugiyama, T., Irie, R., Isogai, T., Hata, J., Toyama, Y., and Umezawa, A. (2003). Redifferentiation of dedifferentiated chondrocytes and chondrogenesis of human bone marrow stromal cells via chondrosphere formation with expression profiling by large-scale cDNA analysis. Exp Cell Res *288*, 35-50.

Inada, M., Follenzi, A., Cheng, K., Surana, M., Joseph, B., Benten, D., Bandi, S., Qian, H., and Gupta, S. (2008). Phenotype reversion in fetal human liver epithelial cells identifies the role of an intermediate meso-endodermal stage before hepatic maturation. J Cell Sci *121*, 1002-1013.

Jaiswal, N., Haynesworth, S.E., Caplan, A.I., and Bruder, S.P. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem *64*, 295-312.

Jia, L., Young, M.F., Powell, J., Yang, L., Ho, N.C., Hotchkiss, R., Robey, P.G., and Francomano, C.A. (2002). Gene expression profile of human bone marrow stromal cells: high-throughput expressed sequence tag sequencing analysis. Genomics *79*, 7-17.

Jiang, X.X., Zhang, Y., Liu, B., Zhang, S.X., Wu, Y., Yu, X.D., and Mao, N. (2005). Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood *105*, 4120-4126.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M.*, et al.* (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature *418*, 41-49.

Jones, E., and McGonagle, D. (2008). Human bone marrow mesenchymal stem cells in vivo. Rheumatology (Oxford) *47*, 126-131.

Jones, E.A., Kinsey, S.E., English, A., Jones, R.A., Straszynski, L., Meredith, D.M., Markham, A.F., Jack, A., Emery, P., and McGonagle, D. (2002). Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum *46*, 3349-3360.

Jones, P.H., and Watt, F.M. (1993). Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell *73*, 713-724.

Kafienah, W., Mistry, S., Dickinson, S.C., Sims, T.J., Learmonth, I., and Hollander, A.P. (2007). Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. Arthritis Rheum *56*, 177-187.

Kalitsis, P., Griffiths, B., and Choo, K.H. (2006). Mouse telocentric sequences reveal a high rate of homogenization and possible role in Robertsonian translocation. Proc Natl Acad Sci U S A *103*, 8786-8791.

Kanichai, M., Ferguson, D., Prendergast, P.J., and Campbell, V.A. (2008). Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha. J Cell Physiol *216*, 708-715.

Kao, Y.R., Shih, J.Y., Wen, W.C., Ko, Y.P., Chen, B.M., Chan, Y.L., Chu, Y.W., Yang, P.C., Wu, C.W., and Roffler, S.R. (2003). Tumor-associated antigen L6 and the invasion of human lung cancer cells. Clin Cancer Res *9*, 2807-2816.

Kayahara, T., Sawada, M., Takaishi, S., Fukui, H., Seno, H., Fukuzawa, H., Suzuki, K., Hiai, H., Kageyama, R., Okano, H.*, et al.* (2003). Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine. FEBS Lett *535*, 131-135.

Kenyon, J., and Gerson, S.L. (2007). The role of DNA damage repair in aging of adult stem cells. Nucleic Acids Res *35*, 7557-7565.

Kidd, M., Modlin, I.M., Mane, S.M., Camp, R.L., Eick, G., and Latich, I. (2006). The role of genetic markers--NAP1L1, MAGE-D2, and MTA1--in defining small-intestinal carcinoid neoplasia. Ann Surg Oncol *13*, 253-262.

Kirton, J.P., Wilkinson, F.L., Canfield, A.E., and Alexander, M.Y. (2006). Dexamethasone downregulates calcification-inhibitor molecules and accelerates osteogenic differentiation of vascular pericytes: implications for vascular calcification. Circ Res *98*, 1264-1272.

Koc, O.N., Day, J., Nieder, M., Gerson, S.L., Lazarus, H.M., and Krivit, W. (2002a). Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant *30*, 215-222.

Koc, O.N., Gerson, S.L., Cooper, B.W., Dyhouse, S.M., Haynesworth, S.E., Caplan, A.I., and Lazarus, H.M. (2000). Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol *18*, 307-316.

Koc, S., Leisenring, W., Flowers, M.E., Anasetti, C., Deeg, H.J., Nash, R.A., Sanders, J.E., Witherspoon, R.P., Storb, R., Appelbaum, F.R.*, et al.* (2002b). Therapy for chronic graft-versus-host disease: a randomized trial comparing cyclosporine plus prednisone versus prednisone alone. Blood *100*, 48-51.

Kofoed, H., Sjontoft, E., Siemssen, S.O., and Olesen, H.P. (1985). Bone marrow circulation after osteotomy. Blood flow, pO2, pCO2, and pressure studied in dogs. Acta Orthop Scand *56*, 400-403.

Kogler, G., Sensken, S., Airey, J.A., Trapp, T., Muschen, M., Feldhahn, N., Liedtke, S., Sorg, R.V., Fischer, J., Rosenbaum, C.*, et al.* (2004). A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med *200*, 123-135.

Krampera, M., Glennie, S., Dyson, J., Scott, D., Laylor, R., Simpson, E., and Dazzi, F. (2003). Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood *101*, 3722-3729.

Krivit, W., Aubourg, P., Shapiro, E., and Peters, C. (1999). Bone marrow transplantation for globoid cell leukodystrophy, adrenoleukodystrophy, metachromatic leukodystrophy, and Hurler syndrome. Curr Opin Hematol *6*, 377-382.

Kruse, E., Uehlein, N., and Kaldenhoff, R. (2006). The aquaporins. Genome Biol *7*, 206.

Kuznetsov, S.A., Krebsbach, P.H., Satomura, K., Kerr, J., Riminucci, M., Benayahu, D., and Robey, P.G. (1997). Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res *12*, 1335-1347.

Kuznetsov, S.A., Mankani, M.H., Gronthos, S., Satomura, K., Bianco, P., and Robey, P.G. (2001). Circulating skeletal stem cells. J Cell Biol *153*, 1133-1140.

Lafont, J.E., Talma, S., and Murphy, C.L. (2007). Hypoxia-inducible factor 2alpha is essential for hypoxic induction of the human articular chondrocyte phenotype. Arthritis Rheum *56*, 3297-3306.

Lallier, T.E., and Spencer, A. (2007). Use of microarrays to find novel regulators of periodontal ligament fibroblast differentiation. Cell Tissue Res *327*, 93-109.

Lamoury, F.M., Croitoru-Lamoury, J., and Brew, B.J. (2006). Undifferentiated mouse mesenchymal stem cells spontaneously express neural and stem cell markers Oct-4 and Rex-1. Cytotherapy *8*, 228-242.

Lazarus, H.M., Koc, O.N., Devine, S.M., Curtin, P., Maziarz, R.T., Holland, H.K., Shpall, E.J., McCarthy, P., Atkinson, K., Cooper, B.W.*, et al.* (2005). Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic

stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant *11*, 389-398.

Le Blanc, K., Tammik, L., Sundberg, B., Haynesworth, S.E., and Ringden, O. (2003). Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol *57*, 11-20.

Lee, J.Y., Qu-Petersen, Z., Cao, B., Kimura, S., Jankowski, R., Cummins, J., Usas, A., Gates, C., Robbins, P., Wernig, A.*, et al.* (2000). Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol *150*, 1085-1100.

Lennon, D.P., Edmison, J.M., and Caplan, A.I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. J Cell Physiol *187*, 345-355.

Li, Y., Chen, J., Chen, X.G., Wang, L., Gautam, S.C., Xu, Y.X., Katakowski, M., Zhang, L.J., Lu, M., Janakiraman, N.*, et al.* (2002). Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. Neurology *59*, 514-523.

Li, Y., Zhang, C., Xiong, F., Yu, M.J., Peng, F.L., Shang, Y.C., Zhao, C.P., Xu, Y.F., Liu, Z.S., Zhou, C.*, et al.* (2008). Comparative study of mesenchymal stem cells from C57BL/10 and mdx mice. BMC Cell Biol *9*, 24.

Liechty, K.W., MacKenzie, T.C., Shaaban, A.F., Radu, A., Moseley, A.M., Deans, R., Marshak, D.R., and Flake, A.W. (2000). Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med *6*, 1282-1286.

Lloyd, A.C. (2002). Limits to lifespan. Nat Cell Biol *4*, E25-27.

Lodie, T.A., Blickarz, C.E., Devarakonda, T.J., He, C., Dash, A.B., Clarke, J., Gleneck, K., Shihabuddin, L., and Tubo, R. (2002). Systematic analysis of reportedly distinct populations of multipotent bone marrow-derived stem cells reveals a lack of distinction. Tissue Eng *8*, 739-751.

Mackay, A.M., Beck, S.C., Murphy, J.M., Barry, F.P., Chichester, C.O., and Pittenger, M.F. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng *4*, 415-428.

Majumdar, M.K., Keane-Moore, M., Buyaner, D., Hardy, W.B., Moorman, M.A., McIntosh, K.R., and Mosca, J.D. (2003). Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci *10*, 228-241.

Malladi, P., Xu, Y., Chiou, M., Giaccia, A.J., and Longaker, M.T. (2006a). Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. Am J Physiol Cell Physiol *290*, C1139-1146.

Malladi, P., Xu, Y., Yang, G.P., and Longaker, M.T. (2006b). Functions of vitamin D, retinoic acid, and dexamethasone in mouse adipose-derived mesenchymal cells. Tissue Eng *12*, 2031-2040.

Martin-Rendon, E., Hale, S.J., Ryan, D., Baban, D., Forde, S.P., Roubelakis, M., Sweeney, D., Moukayed, M., Harris, A.L., Davies, K.*, et al.* (2007). Transcriptional profiling of human cord blood CD133+ and cultured bone marrow mesenchymal stem cells in response to hypoxia. Stem Cells *25*, 1003-1012.

Martin, I., Muraglia, A., Campanile, G., Cancedda, R., and Quarto, R. (1997). Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology *138*, 4456-4462.

Martinez-Agosto, J.A., Mikkola, H.K., Hartenstein, V., and Banerjee, U. (2007). The hematopoietic stem cell and its niche: a comparative view. Genes Dev *21*, 3044-3060.

Mason, T.M., Lord, B.I., and Hendry, J.H. (1989). The development of spatial distributions of CFU-S and in-vitro CFC in femora of mice of different ages. Br J Haematol *73*, 455-461.

Meirelles Lda, S., and Nardi, N.B. (2003). Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol *123*, 702-711.

Menten, P., Wuyts, A., and Van Damme, J. (2001). Monocyte chemotactic protein-3. Eur Cytokine Netw *12*, 554-560.

Mets, T., and Verdonk, G. (1981). In vitro aging of human bone marrow derived stromal cells. Mech Ageing Dev *16*, 81-89.

Meuleman, N., Vanhaelen, G., Tondreau, T., Lewalle, P., Kwan, J., Bennani, J., Martiat, P., Lagneaux, L., and Bron, D. (2008). Reduced intensity conditioning haematopoietic stem cell transplantation with mesenchymal stromal cells infusion for the treatment of metachromatic leukodystrophy: a case report. Haematologica *93*, e11-13.

Mirosevich, J., Gao, N., and Matusik, R.J. (2005). Expression of Foxa transcription factors in the developing and adult murine prostate. Prostate *62*, 339-352.

Mitsiadis, T.A., Barrandon, O., Rochat, A., Barrandon, Y., and De Bari, C. (2007). Stem cell niches in mammals. Exp Cell Res *313*, 3377-3385.

Miura, M., Miura, Y., Padilla-Nash, H.M., Molinolo, A.A., Fu, B., Patel, V., Seo, B.M., Sonoyama, W., Zheng, J.J., Baker, C.C.*, et al.* (2006). Accumulated chromosomal

instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells *24*, 1095-1103.

Mizukami, T., Kuramitsu, M., Takizawa, K., Momose, H., Masumi, A., Naito, S., Iwama, A., Ogawa, T., Noce, T., Hamaguchi, I.*, et al.* (2008). Identification of transcripts commonly expressed in both hematopoietic and germ-line stem cells. Stem Cells Dev *17*, 67-80.

Modderman, W.E., Vrijheid-Lammers, T., Lowik, C.W., and Nijweide, P.J. (1994). Removal of hematopoietic cells and macrophages from mouse bone marrow cultures: isolation of fibroblastlike stromal cells. Exp Hematol *22*, 194-201.

Montgomery, R.K., and Breault, D.T. (2008). Small intestinal stem cell markers. J Anat *213*, 52-58.

Moriggl, R., Topham, D.J., Teglund, S., Sexl, V., McKay, C., Wang, D., Hoffmeyer, A., van Deursen, J., Sangster, M.Y., Bunting, K.D.*, et al.* (1999). Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity *10*, 249-259.

Mostafa, S.S., Miller, W.M., and Papoutsakis, E.T. (2000). Oxygen tension influences the differentiation, maturation and apoptosis of human megakaryocytes. Br J Haematol *111*, 879-889.

Moussavi-Harami, F., Duwayri, Y., Martin, J.A., and Buckwalter, J.A. (2004). Oxygen effects on senescence in chondrocytes and mesenchymal stem cells: consequences for tissue engineering. Iowa Orthop J *24*, 15-20.

Muraglia, A., Cancedda, R., and Quarto, R. (2000). Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci *113 (Pt 7)*, 1161-1166.

Murphy, C.L., and Polak, J.M. (2004). Control of human articular chondrocyte differentiation by reduced oxygen tension. J Cell Physiol *199*, 451-459.

Nakahara, H., Bruder, S.P., Haynesworth, S.E., Holecek, J.J., Baber, M.A., Goldberg, V.M., and Caplan, A.I. (1990). Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. Bone *11*, 181-188.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., Yamanaka, S. (2008). Gneration of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol *1* 101-6.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.

Noth, U., Osyczka, A.M., Tuli, R., Hickok, N.J., Danielson, K.G., and Tuan, R.S. (2002). Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. J Orthop Res *20*, 1060-1069.

Oh, H., Bradfute, S.B., Gallardo, T.D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L.H., Behringer, R.R., Garry, D.J.*, et al.* (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A *100*, 12313-12318.

Ohgami, R.S., Campagna, D.R., McDonald, A., and Fleming, M.D. (2006). The Steap proteins are metalloreductases. Blood *108*, 1388-1394.

Ohnishi, S., Yasuda, T., Kitamura, S., and Nagaya, N. (2007). Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. Stem Cells *25*, 1166-1177.

Okita, K., Ichisaka, T., Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313-18.

Orkin, S.H. (2000). Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet *1*, 57-64.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S.M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D.M.*, et al.* (2001). Bone marrow cells regenerate infarcted myocardium. Nature *410*, 701-705.

Ortiz, L.A., Gambelli, F., McBride, C., Gaupp, D., Baddoo, M., Kaminski, N., and Phinney, D.G. (2003). Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci U S A *100*, 8407-8411.

Panteleyev, A.A., Jahoda, C.A., and Christiano, A.M. (2001). Hair follicle predetermination. J Cell Sci *114*, 3419-3431.

Park, H.W., Shin, J.S., and Kim, C.W. (2007). Proteome of mesenchymal stem cells. Proteomics *7*, 2881-2894.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. (2003). Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. Nat Cell Biol *5*, 741-747.

Parsch, D., Fellenberg, J., Brummendorf, T.H., Eschlbeck, A.M., and Richter, W. (2004). Telomere length and telomerase activity during expansion and differentiation of human mesenchymal stem cells and chondrocytes. J Mol Med *82*, 49-55.

Peh, G.S., Lang, R., Pera, M., and Hawes, S. (2008). CD133 expression by neural progenitors derived from human embryonic stem cells and its use for their prospective isolation. Stem Cells Dev.

Peister, A., Mellad, J.A., Larson, B.L., Hall, B.M., Gibson, L.F., and Prockop, D.J. (2004). Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood *103*, 1662-1668.

Pelengaris, S., Khan, M., and Evan, G. (2002). c-MYC: more than just a matter of life and death. Nat Rev Cancer *2*, 764-776.

Peters, C., Shapiro, E.G., and Krivit, W. (1998). Hurler syndrome: past, present, and future. J Pediatr *133*, 7-9.

Petersen, B.E., Grossbard, B., Hatch, H., Pi, L., Deng, J., and Scott, E.W. (2003). Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology *37*, 632-640.

Phinney, D.G., Hill, K., Michelson, C., DuTreil, M., Hughes, C., Humphries, S., Wilkinson, R., Baddoo, M., and Bayly, E. (2006). Biological activities encoded by the murine mesenchymal stem cell transcriptome provide a basis for their developmental potential and broad therapeutic efficacy. Stem Cells *24*, 186-198.

Phinney, D.G., Kopen, G., Isaacson, R.L., and Prockop, D.J. (1999). Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem *72*, 570-585.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Pochampally, R.R., Ylostalo, J., Penfornis, P., Matz, R.R., Smith, J.R., and Prockop, D.J. (2007). Histamine receptor H1 and dermatopontin: new downstream targets of the vitamin D receptor. J Bone Miner Res *22*, 1338-1349.

Porkka, K.P., Helenius, M.A., and Visakorpi, T. (2002). Cloning and characterization of a novel six-transmembrane protein STEAP2, expressed in normal and malignant prostate. Lab Invest *82*, 1573-1582.

Post, S., Abdallah, B.M., Bentzon, J.F., and Kassem, M. (2008). Demonstration of the presence of independent pre-osteoblastic and pre-adipocytic cell populations in bone marrow-derived mesenchymal stem cells. Bone *43*, 32-39.

Prowse, K.R., and Greider, C.W. (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proc Natl Acad Sci U S A *92*, 4818-4822.

Quarto, R., Thomas, D., and Liang, C.T. (1995). Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calcif Tissue Int *56*, 123-129.

Rahmani, M., Wong, B.W., Ang, L., Cheung, C.C., Carthy, J.M., Walinski, H., and McManus, B.M. (2006). Versican: signaling to transcriptional control pathways. Can J Physiol Pharmacol *84*, 77-92.

Rawadi, G., Vayssiere, B., Dunn, F., Baron, R., and Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res *18*, 1842-1853.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L., and Verfaillie, C.M. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood *98*, 2615-2625.

Richardson, G.D., Robson, C.N., Lang, S.H., Neal, D.E., Maitland, N.J., and Collins, A.T. (2004). CD133, a novel marker for human prostatic epithelial stem cells. J Cell Sci *117*, 3539-3545.

Rosova, I., Dao, M., Capoccia, B., Link, D., and Nolta, J.A. (2008). Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells *26*, 2173-2182.

Roubelakis, M.G., Pappa, K.I., Bitsika, V., Zagoura, D., Vlahou, A., Papadaki, H.A., Antsaklis, A., and Anagnou, N.P. (2007). Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. Stem Cells Dev *16*, 931-952.

Rubio, D., Garcia-Castro, J., Martin, M.C., de la Fuente, R., Cigudosa, J.C., Lloyd, A.C., and Bernad, A. (2005). Spontaneous human adult stem cell transformation. Cancer Res *65*, 3035-3039.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T.B., Saporta, S., Janssen, W., Patel, N.*, et al.* (2000). Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol *164*, 247-256.

Sarugaser, R., Lickorish, D., Baksh, D., Hosseini, M.M., and Davies, J.E. (2005). Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells *23*, 220-229.

Sekiya, I., Larson, B.L., Smith, J.R., Pochampally, R., Cui, J.G., and Prockop, D.J. (2002). Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells *20*, 530-541.

Shi, S., and Gronthos, S. (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res *18*, 696-704.

Short, B., Brouard, N., Occhiodoro-Scott, T., Ramakrishnan, A., and Simmons, P.J. (2003). Mesenchymal stem cells. Arch Med Res *34*, 565-571.

Silva, W.A., Jr., Covas, D.T., Panepucci, R.A., Proto-Siqueira, R., Siufi, J.L., Zanette, D.L., Santos, A.R., and Zago, M.A. (2003). The profile of gene expression of human marrow mesenchymal stem cells. Stem Cells *21*, 661-669.

Simmons, P.J., and Torok-Storb, B. (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood *78*, 55-62.

Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., and Dirks, P.B. (2004). Identification of human brain tumour initiating cells. Nature *432*, 396-401.

Song, L., Webb, N.E., Song, Y., and Tuan, R.S. (2006). Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. Stem Cells *24*, 1707-1718.

Sotiropoulou, P.A., and Papamichail, M. (2007). Immune properties of mesenchymal stem cells. Methods Mol Biol *407*, 225-243.

Strakova, Z., Livak, M., Krezalek, M., and Ihnatovych, I. (2008). Multipotent properties of myofibroblast cells derived from human placenta. Cell Tissue Res *332*, 479-488.

Studer, L., Csete, M., Lee, S.H., Kabbani, N., Walikonis, J., Wold, B., and McKay, R. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J Neurosci *20*, 7377-7383.

Tae, S.K., Lee, S.H., Park, J.S., and Im, G.I. (2006). Mesenchymal stem cells for tissue engineering and regenerative medicine. Biomed Mater *1*, 63-71.

Taupin, P. (2006). OTI-010 Osiris Therapeutics/JCR Pharmaceuticals. Curr Opin Investig Drugs *7*, 473-481.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E., and Scott, E.W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542-545.

Todaro, G.J., and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol *17*, 299-313.

Tolar, J., Nauta, A.J., Osborn, M.J., Panoskaltsis Mortari, A., McElmurry, R.T., Bell, S., Xia, L., Zhou, N., Riddle, M., Schroeder, T.M.*, et al.* (2007). Sarcoma derived from cultured mesenchymal stem cells. Stem Cells *25*, 371-379.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. (1994). mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev *8*, 1224-1234.

Tremain, N., Korkko, J., Ibberson, D., Kopen, G.C., DiGirolamo, C., and Phinney, D.G. (2001). MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. Stem Cells *19*, 408-418.

Tropel, P., Noel, D., Platet, N., Legrand, P., Benabid, A.L., and Berger, F. (2004). Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. Exp Cell Res *295*, 395-406.

Tsonis, P.A. (2004). Stem cells from differentiated cells. Mol Interv *4*, 81-83.

Tsonis, P.A., Sargent, M.T., Del Rio-Tsonis, K., and Jung, J.C. (1996). 9-cis retinoic acid antagonizes the stimulatory effect of 1,25 dihydroxyvitamin D3 on chondrogenesis of chick limb bud mesenchymal cells: interactions of their receptors. Int J Dev Biol *40*, 1053-1059.

Tuan, R.S., Boland, G., and Tuli, R. (2003). Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res Ther *5*, 32-45.

Virtaneva, K.I., Emi, N., Marken, J.S., Aruffo, A., Jones, C., Spurr, N.K., and Schroder, J.P. (1994). Chromosomal localization of three human genes coding for A15, L6, and S5.7 (TAPA1): all members of the transmembrane 4 superfamily of proteins. Immunogenetics *39*, 329-334.

Wagers, A.J., Christensen, J.L., and Weissman, I.L. (2002). Cell fate determination from stem cells. Gene Ther *9*, 606-612.

Wagner, W., Feldmann, R.E., Jr., Seckinger, A., Maurer, M.H., Wein, F., Blake, J., Krause, U., Kalenka, A., Burgers, H.F., Saffrich, R.*, et al.* (2006). The heterogeneity of human mesenchymal stem cell preparations--evidence from simultaneous analysis of proteomes and transcriptomes. Exp Hematol *34*, 536-548.

Wagner, W., and Ho, A.D. (2007). Mesenchymal stem cell preparations--comparing apples and oranges. Stem Cell Rev *3*, 239-248.

Wagner, W., Roderburg, C., Wein, F., Diehlmann, A., Frankhauser, M., Schubert, R., Eckstein, V., and Ho, A.D. (2007). Molecular and secretory profiles of human

mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. Stem Cells *25*, 2638-2647.

Wagner, W., Wein, F., Seckinger, A., Frankhauser, M., Wirkner, U., Krause, U., Blake, J., Schwager, C., Eckstein, V., Ansorge, W.*, et al.* (2005). Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol *33*, 1402-1416.

Wakitani, S., Mitsuoka, T., Nakamura, N., Toritsuka, Y., Nakamura, Y., and Horibe, S. (2004). Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. Cell Transplant *13*, 595-600.

Wakitani, S., Saito, T., and Caplan, A.I. (1995). Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve *18*, 1417-1426.

Wang, G., Bunnell, B.A., Painter, R.G., Quiniones, B.C., Tom, S., Lanson, N.A., Jr., Spees, J.L., Bertucci, D., Peister, A., Weiss, D.J.*, et al.* (2005a). Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. Proc Natl Acad Sci U S A *102*, 186-191.

Wang, J., Xie, L.Y., Allan, S., Beach, D., and Hannon, G.J. (1998). Myc activates telomerase. Genes Dev *12*, 1769-1774.

Wang, Q.R., and Wolf, N.S. (1990). Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp Hematol *18*, 355-359.

Wang, Y., Huso, D.L., Harrington, J., Kellner, J., Jeong, D.K., Turney, J., and McNiece, I.K. (2005b). Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture. Cytotherapy *7*, 509-519.

Watt, F.M., and Hogan, B.L. (2000). Out of Eden: stem cells and their niches. Science *287*, 1427-1430.

Watt, F.M., Lo Celso, C., and Silva-Vargas, V. (2006). Epidermal stem cells: an update. Curr Opin Genet Dev *16*, 518-524.

Weissman, I.L., Anderson, D.J., and Gage, F. (2001). Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol *17*, 387-403.

Welm, B.E., Tepera, S.B., Venezia, T., Graubert, T.A., Rosen, J.M., and Goodell, M.A. (2002). Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol *245*, 42-56.

Wieczorek, G., Steinhoff, C., Schulz, R., Scheller, M., Vingron, M., Ropers, H.H., and Nuber, U.A. (2003). Gene expression profile of mouse bone marrow stromal cells determined by cDNA microarray analysis. Cell Tissue Res *311*, 227-237.

Williams, J.T., Southerland, S.S., Souza, J., Calcutt, A.F., and Cartledge, R.G. (1999). Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. Am Surg *65*, 22-26.

Wilson, A., Oser, G.M., Jaworski, M., Blanco-Bose, W.E., Laurenti, E., Adolphe, C., Essers, M.A., Macdonald, H.R., and Trumpp, A. (2007). Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci *1106*, 64-75.

Wognum, A.W., Eaves, A.C., and Thomas, T.E. (2003). Identification and isolation of hematopoietic stem cells. Arch Med Res *34*, 461-475.

Wright, G.J., Jones, M., Puklavec, M.J., Brown, M.H., and Barclay, A.N. (2001). The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. Immunology *102*, 173-179.

Wright, N.A. (2000). Epithelial stem cell repertoire in the gut: clues to the origin of cell lineages, proliferative units and cancer. Int J Exp Pathol *81*, 117-143.

Wu, Z., Rosen, E.D., Brun, R., Hauser, S., Adelmant, G., Troy, A.E., McKeon, C., Darlington, G.J., and Spiegelman, B.M. (1999). Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell *3*, 151-158.

Wynn, R.F., Hart, C.A., Corradi-Perini, C., O'Neill, L., Evans, C.A., Wraith, J.E., Fairbairn, L.J., and Bellantuono, I. (2004). A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood *104*, 2643-2645.

Xin, L., Lawson, D.A., and Witte, O.N. (2005). The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proc Natl Acad Sci U S A *102*, 6942-6947.

Xu, Y., Malladi, P., Chiou, M., Bekerman, E., Giaccia, A.J., and Longaker, M.T. (2007). In vitro expansion of adipose-derived adult stromal cells in hypoxia enhances early chondrogenesis. Tissue Eng *13*, 2981-2993.

Yang, D., Holt, G.E., Velders, M.P., Kwon, E.D., and Kast, W.M. (2001). Murine six-transmembrane epithelial antigen of the prostate, prostate stem cell antigen, and prostate-specific membrane antigen: prostate-specific cell-surface antigens highly expressed in prostate cancer of transgenic adenocarcinoma mouse prostate mice. Cancer Res *61*, 5857-5860.

Ying, Q.L., Nichols, J., Evans, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.

Young, R.G., Butler, D.L., Weber, W., Caplan, A.I., Gordon, S.L., and Fink, D.J. (1998). Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res *16*, 406-413.

Yu, J., Vodyanik, MA., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, JL., Tian, S., Nie, J., Jonsdottir, GA., Ruotti, V., Stewart, R., Slukvin, II., Thomson, JA. (2007). Induced Pluripotent stem cell lines derived from human somatic stem cells. Science *318*, 1917-20.

Zhan, M. (2008). Genomic studies to explore self-renewal and differentiation properties of embryonic stem cells. Front Biosci *13*, 276-283.

Zhou, Y.F., Bosch-Marce, M., Okuyama, H., Krishnamachary, B., Kimura, H., Zhang, L., Huso, D.L., and Semenza, G.L. (2006). Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. Cancer Res *66*, 10849-10854.

Zwartouw, H.T., and Westwood, J.C. (1958). Factors affecting growth and glycolysis in tissue culture. Br J Exp Pathol *39*, 529-539.