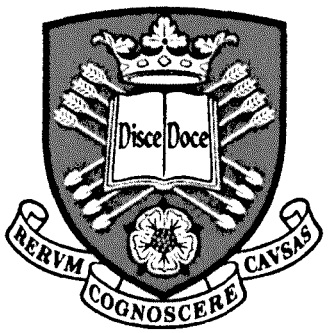
Large scale *in vitro* expansion of Mesenchymal Stem Cells



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Declaration

This is a declaration to state that this thesis is an account of the author's work which was conducted at the University of Sheffield, U.K. This work has not been submitted for any other degree or qualification.

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List of Publications

Conferences

- "Suspension cultures of Mesenchymal Stem Cells" 1st UK Mesenchymal Stem Cell Meeting, York, United Kingdom 2006. (Poster presentation)

- "Large scale suspension cultures of Mesenchymal Stem Cells" 3rd World Congress for Regenerative Medicine, Leipzig, Germany 2007. (Poster presentation)

- "Different approaches for *in vitro* expansion of Mesenchymal Stem Cells" BESG Young Researchers Meeting, Sheffield, United Kingdom 2009. (Poster presentation)

Contents

Abbreviations

2DE	two-dimensional gel electrophoresis
ALP	alkaline phosphatase
Å	ångström
AMP	adenosine monophosphate
ARF1	ADP-ribosylation factor1
ASNS	asparagine synthetase
ATP	adenosine triphosphate
BM	bone marrow
BMC	bone marrow cell
Bp	base pairs
C	carbon
CaF_2	calcium fluoride
CAP	adenylate cyclase-associated protein1
CD	cluster of differentiation
CFU-f	colony-forming-units fibroblasts
cm	centimetre
cm^2	square centimetre
CO_2	carbon dioxide
CRP2	cysteine and glycine rich protein2
Dex	dexamethasone
DLC1	dynein light chain1
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eEF1B	eukaryotic translation elongation factor 1 beta 2
EGF	epidermal growth factor
EPM	electrophoretic mobility
ER	endoplasmic reticulum
ESC	embryonic stem cell
ESI	electrospray ionisation
FBS	foetal bovine serum
FCS	foetal calf serum
FTIR	fourier transform infrared spectroscopy
g	gram
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H	hydrogen
hnRPA1	heterogeneous nuclear ribonucleoprotein A1
HPLC	high performance liquid chromatography
hs	heparin sulphate
HSC	haematopoietic stem cell
Hz	hertz
IL	interleukin
IMS	industrial methylated spirit
IR	infrared
iTRAQ	isobaric tag for relative and absolute quantitation
KCl	potassium chloride
kDa	kiloDalton
KH_2PO_4	monopotassium phosphate

l	litre
LC	liquid chromatography
M	molar
MALDI	matrix-assisted laser desorption / ionisation
MEM	minimum essential medium
mg	milligram
ml	millilitre
mm	millimetre
mm^3	cubic millimetre
mM	millimolar
MS	mass spectroscopy
MS/MS	tandem mass spectroscopy
MSC	mesenchymal stem cells
m/z	mass-to-charge ratio
N	Nitrogen
NCBI	National Center for Biotechnology Information
NSC	neural stem cell
ng	nanogram
O_2	oxygen
OD	optical density
°C	degree Celsius
P	phosphate
p	probability
PALS	Phase Analysis Light Scattering
Panther	Protein ANalysis THrough Evolutionary Relationships
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PS	penicillin / streptomycin
PTMA	prothymosin alpha
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SCF	stem cell factor
SCX	strong cation exchange
S.D.	standard deviation
SPP	Single pass perfusion
TGF-β	transforming growth factor beta1
TOF	time of flight
TPT1	tumour protein, translationally-controlled 1
TRC	tissue repair cells
UV	ultraviolet
V	volt
VitD	1α,25-Dihydroxyvitamin D_3
YB1	Y box protein1
£	pound sterling
µg	microgram
µl	microlitre
µm	micrometre

Summary

Mesenchymal stem cells (MSCs) can undergo self-renewal and differentiation into a variety of mature cell types. Thus, using MSCs for tissue engineering and other medical applications holds many promising advantages over normal somatic cells. However, exactly these characteristics make MSCs more difficult to grow and control *in vitro*. The aim of this research project was to investigate different culture systems for their utility to expand bone marrow derived MSCs in large quantities. A large scale expansion of MSCs is especially of interest since only a small number of bone marrow derived MSCs are present in donor derived samples, which do not meet the demand for medical applications.

In this thesis three different culture systems, static monolayer cultures, stirred suspension cultures, and pour-off cultures, were compared with each other for their ability to support MSCs proliferation while allowing them to keep their full differentiation capacity. Cell samples derived from these cultures were used for cell count, to start a CFU-f assay and to start osteogenic and adipogenic differentiation assays. The highest MSC numbers obtained from the static monolayer cultures were about 460% of the initial cells. The differentiation capacity of these cells was restricted, so they only formed osteoblasts. Furthermore, MSC samples obtained from this culture system were used for proteomic analysis on an electrospray ionisation quadrupol (ESI-qQ-STAR) mass spectrometer with the isobaric tag for relative and absolute quantitation (iTRAQ) method. This analysis revealed a difference in the proteome of MSCs from different passage levels which is involved in a changing proliferation and differentiation behaviour.

In stirred suspension cultures, the increase in MSC number varied for the different culture media. The best result was achieved in MyeloCult® medium with Pluronic F-68, IL-3 and SCF, however, reaching only 140% of the initial cell density, this result was significantly worse than in the control monolayer cultures. The pour-off cultures supported an increase in MSC number, which resulted in 860% of the initial cell number. In addition, MSCs expanded in this culture system were able to differentiate into ostoblasts and adipocytes. Thus, pour-off cultures are the most promising culture system for large scale expansion of MSCs with high differentiation potential.

Chapter 1

Introduction and Hypothesis Statement

1.1 Introduction

This introductory chapter gives a general background to the research described in this thesis and the context in which such research will contribute to a better understanding of the field of stem cell science and engineering. Furthermore, the overall goal of the studies conducted will be detailed and finally, the subject of each chapter of this thesis will be summarised.

1.2 Tissue engineering and cell culture

Organ transplantation and implantation of synthetic prosthesis is the state-of-the-art medical treatment available for patients with injured or lost organs and body parts. However, the current quantity of organs available for transplantation cannot meet the needs of patients', resulting in organ waiting lists (Fentiman, 1994). Furthermore, not all organs can be replaced by a synthetic prosthesis, and tissue engineered constructs are believed to be more efficient and reliable (Fentiman, 1994). Hence, there is a growing need for biologically active tissues, which is not being met by transplantation. This has led to the development of tissue engineering.

The engineering of living tissue aims to develop biological constructs for implantation into the body, and also to support the regeneration of tissue after damage or injury. The purpose is to repair, replace or maintain the function of a particular tissue or organ. Tissue engineered constructs include e.g. artificial skin, engineered cardiovascular substitutes, metabolic organs such as the artificial pancreas and liver, and even artificial neurons used for nerve regeneration and pain killers. The functional basic element of a tissue engineered construct is that the living cells have a desired, system specific function. This can be secretion of extracellular matrix, biosynthesis and secretion of proteins in response to specific stimuli, or interaction with other cells or extracellular polymers to construct specific three dimensional structures. Cell sources include human and animal tissues, from where the cells are isolated before being amplified *in vitro*.

The challenges arising from cell culture for tissue engineering are to harvest cells in sufficient quantity to fabricate bio-artificial tissues for clinical applications, maintain

the cell function throughout the entire *in vitro* culture time, and a sufficient period of time after being implanted, and not to cause immune reactions in the patient when implanting the engineered construct. Cells originating from human tissues have the advantage of good immunocompatibility, especially if the patient's own cells are used as autografts after isolation from another part of their body (Fentiman, 1994). The cell availability depends not only on the numbers of cells present in the original tissue, but also on the extent to which the isolated cells can be grown in culture. Cell growth can be stimulated by identifying and supplying the necessary growth factors and controlling the state of cellular differentiation (Zandstra and Nagy, 2001). This, however, can be challenging, especially if the control mechanism for the differentiation pathways is poorly understood. A typical example in this area is the attempt to grow *ex vivo* a bone marrow analog for tissue engineering a haematopoietic system *in vivo* (Koller *et al.*, 1993).

Cells within the human body reside in a complex environment which is controlled biochemically and biomechanically. The biochemical impact of culture condition has long been recognised. However, many tissues or organs function in the presence of a mechanical stress environment, and therefore the biomechanical influence should not be underestimated either. It is the totality of the environment that influences the cell function.

In this thesis, existing cell culture techniques will be used to investigate the possibility for amplifying bone marrow derived mesenchymal stem cells (MSCs) *in vitro*. Mesenchymal stem cells are multipotent and can develop into a variety of fully differentiated connective tissues, thus they are ideal candidates for tissue engineering. Developing and optimising a protocol to produce large numbers of MSCs in an economical way, will allow their multisided usage in clinical applications.

The following chapters will highlight the main challenges in the development of different mesenchymal stem cell (MSC) cultures, and focus on the improvement of the culture conditions in order to obtain high quantities of mesenchymal stem cells with full differentiation potential. Given the extent of the parameters important in the control of mesenchymal stem cell self-renewal and differentiation, an exhaustive

analysis of all potential culture parameters was not possible. However, the results obtained by the following studies can help to improve and develop clinically relevant mesenchymal stem cell–based technologies.

1.3 Research aims

The overall goal of this thesis is to establish an improved culture system for the *in vitro* expansion of MSCs. With this culture system, a greater fold increase in MSC numbers should be obtained than compared with existing culture methods. This goal will be achieved through the following key objectives:

- Determine the proliferation and differentiation potential of MSCs in static monolayer cultures under different culture conditions
- Establish a protocol for expanding MSCs in stirred suspension bioreactors
- Optimise the growth of MSCs in stirred suspension cultures by changing the culture conditions
- Determine the proliferation and differentiation capacity of MSCs in pour-off cultures under different culture conditions and compare these findings with the results from the static and stirred suspension cultures
- Implement an iTRAQ method for analysing the proteome of MSCs
- Identify possible proteomic changes of sub-cultured MSCs

Chemical as well as physical stimuli can trigger differentiation of mesenchymal stem cells into early progenitor cells. These progenitor cells may have a similar phenotype and can still differentiate into one fully matured cell type only. However, this would limit the multisided usage of MSCs and since the stem cells themselves are the desired product, the expansion process must not affect or change the cell phenotype and function. This requires the development of a culture system that has low stress levels for MSCs.. Therefore, the improved culture technique has to fulfil three criteria: 1) It must support the amplification of large cell numbers, 2) the cells must maintain a high stem cell quality and 3) the process must be able to be automated and upscale. The aim, however, was not only to prove the possibility of a large scale

MSCs expansion but rather to implement a protocol that exhibit an improvement to the already existing culture systems and would allow the production of MSCs for the application in regenerative medicine. Therefore, the main focus was also on the cost efficiency of the process.

1.4 Thesis outline

An outline of the thesis structure with experiments carried out in the chapters is given in Figure 1.1. An overview of each chapter is summarised below.

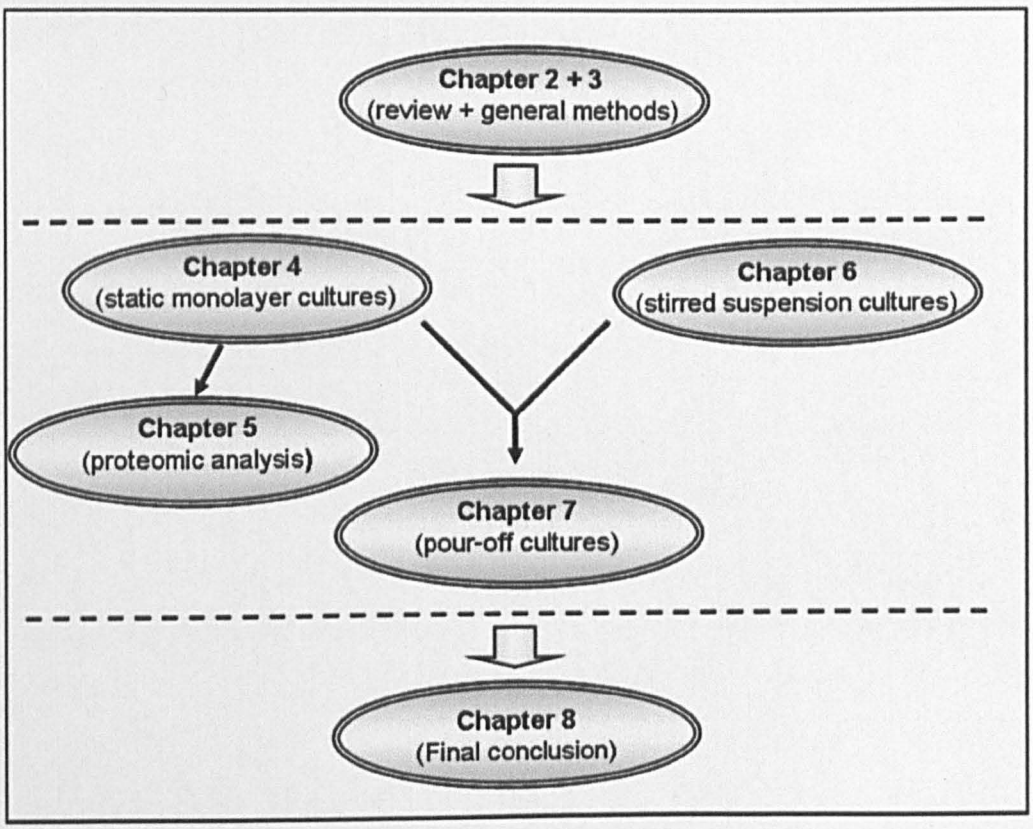

Figure 1.1: An overview of the format of studies in this thesis.

Chapter 2: The aim of this chapter is to describe the nature of mesenchymal stem cells, and summarise the research conducted on them so far. This is achieved by

giving a brief introduction on the historical development of (stem) cell culture and on the definition and classification of stem cells in general. This is followed by a review on MSCs, including their *in vivo* niche, their characteristics, culture systems, and therapeutic applications. The last section presents a discussion on the proteome analysis of MSCs, and the latest findings achieved with this analysis.

Chapter 3: The materials and the general methods for culturing and analysing MSCs used throughout this thesis are described in this chapter. This includes the protocols for isolation MSCs from rat femurs and tibia, conducting cell and colony counts, and running differentiation tests.

Chapter 4: The traditional method of expanding MSCs is the static monolayer culture, which forms the basis of this chapter. MSCs were expanded under different culture conditions, and their changes in proliferation and differentiation capacity were recorded. The results of this chapter are used as a control to compare the findings from the suspension and pour-off cultures against this standard technique.

Chapter 5: In this chapter, the proteome of MSCs expanded under the same conditions as in chapter 4 was analysed using high throughput differential proteomic techniques. This was of interest because researchers theorise that MSCs lose their stem cell character and slow down in proliferation when expanded too much on plastic. To verify this theory, the proteome of MSCs after passage 2 and 4 were compared.

Chapter 6: The development and the implementation of a protocol for stirred suspension culture of MSCs is described in this chapter. This protocol was based on the culture conditions for static monolayer cultures, developed in chapter 4. However, the aim of this chapter was not only to develop but also to optimise the protocol to achieve the possibility of scale-up, automation and high stem cell quality. This required the usage of different medium additives which increased the cost, a problem every published study of MSCs in suspension cultures has reported. The results were compared with the findings from the static monolayer cultures in chapter 4, and the conclusion for further improvement let to the usage of pour-off cultures, as mentioned in chapter 7.

Chapter 7: To address the problems arising from MSCs expansion in static and suspension cultures, and also to prove the assumption of suspension based proliferation, pour-off culture experiments were conducted. This chapter explains the methods, and presents the results achieved with this culture method. The aim was to be able to use the cheap culture medium and conditions of the static monolayer cultures, while being able to scale-up, automate and monitor the cultures in a similar way as it is possible with stirred suspension based bioreactors. The results were compared with the findings from both static and suspension derived MSCs.

Chapter 8: The results and conclusions in this thesis are summarised here, allowing a direct comparison of the performance of the different culture systems. Finally, future research is suggested.

Chapter 2

General Background and Review

2.1 Introduction

The history of stem cell research started in 1963, when the first descriptions of the self-renewing activities of transplanted mouse bone marrow cells were documented by Ernest McCulloch and James Till (Becker *et al.*, 1963). Further success was achieved when scientists at the beginning of the 1980's derived mouse embryonic stem cells from the inner cell mass (Martin, 1981). With this finding, research into animal and human stem cells began, and has been ongoing since this time. In the 1980's and 1990's, improved methods for growing mammalian cells *in vitro* allowed for the intensification and deepening of the analysis of stem cells, which in 1998, led to the establishment of the world's first human embryonic stem cell line by James Thomson (Thomson *et al.*, 1998). Thomson successfully removed cells from spare embryos at fertility clinics and cultured them *in vitro*. Since this discovery, stem cells, with their regeneration potential and differentiation capacity, have become more and more attractive for many applications in tissue engineering, cellular therapies and drug screening, especially since research has shown that stem cells exist in most, if not all, tissues (Ortiz-Gonzalez *et al.*, 2004), (Woodward *et al.*, 2005), (Mimeault *et al.*, 2006) (Trounson, 2006).

Implementation of stem cells for tissue engineering requires a technique that is able to cultivate large quantities of readily available stem cells or their differentiated cell types. Unlike most traditional biotechnological processes in which cells are cultivated to produce a certain product (e.g. protein or virus) this new technology aims to generate the cells themselves as the product. For haematopoietic stem cell transplantation, for example, the undifferentiated stem cells have to be expanded in cell cultures (Kwon *et al.*, 2003), whereas for other applications (e.g. transplantation of skin) the production of a specific differentiated cell type with defined characteristics is required (Sun *et al.*, 2005). While expanding undifferentiated stem cells, it is crucial to maintain their self-renewal and full differentiation capacity. For the production of specific differentiated cell types, on the other hand, it is important to carefully control the differentiation into a very specific lineage and eliminate all undesirable cell types that could arise during the differentiation process. *In vitro* differentiation of stem cells usually produces a mixed population of different cell lineages (Humphries *et al.*, 1981). Both pose a challenge in the cultivation technique

of stem cells for clinical applications, making it necessary to clearly understand and determine the exact role of all parameters important for the control of the stem cell fate during the cultivation process. A further challenge for using stem cells in tissue engineering is the large number of cells required (about 7.5 x 10^9 MSCs to help healing a 10 cm defect human femur). Stem cells are derived in small numbers form a donor, (e.g. only 0.01 - 0.001% of the nucleated bone marrow cells are mesenchymal stem cells) and have to be amplified before being further used for medical applications. Hence, a culture system for expanding stem cells *in vitro* needs to allow the control of the stem cell fate while producing large numbers of stem cells.

The purpose of this study is to determine the potential of different culture techniques for the large scale expansion of one specific adult stem cell type, mesenchymal stem cells (MSCs). In this chapter, a review on stem cells and more specifically about MSCs is presented. The review will highlight the main achievements in mesenchymal stem cell research and focuses on the culture systems for MSCs and suggests where research is needed in order to better understand the control mechanisms of these cells.

2.2 Definition and classification of stem cells

A cell has to fulfil three criteria in order to be categorised as a stem cell. It must be an undifferentiated cell which, first, is capable of self-renewal by either symmetric or asymmetric division (Figure 2.1), second, is capable of multilineage differentiation and third, reconstitutes a given tissue *in vivo* (Potten and Lajtha, 1982) (Hall and Watt, 1989) (Weissman *et al.*, 2000). These properties make stem cells highly attractive for many applications in tissue engineering, cellular therapies and drug screening (Mimeault and Batra, 2006). However, the degree to which a stem cell differentiates through all the progenitor cell phases up to the fully differentiated cell is largely determined by the microenvironment in which the individual stem cell develops. Therefore, stem cell proliferation is very sensitive to various environmental stresses, and poses a challenge in their cultivation (Weissman *et al.*, 2000).

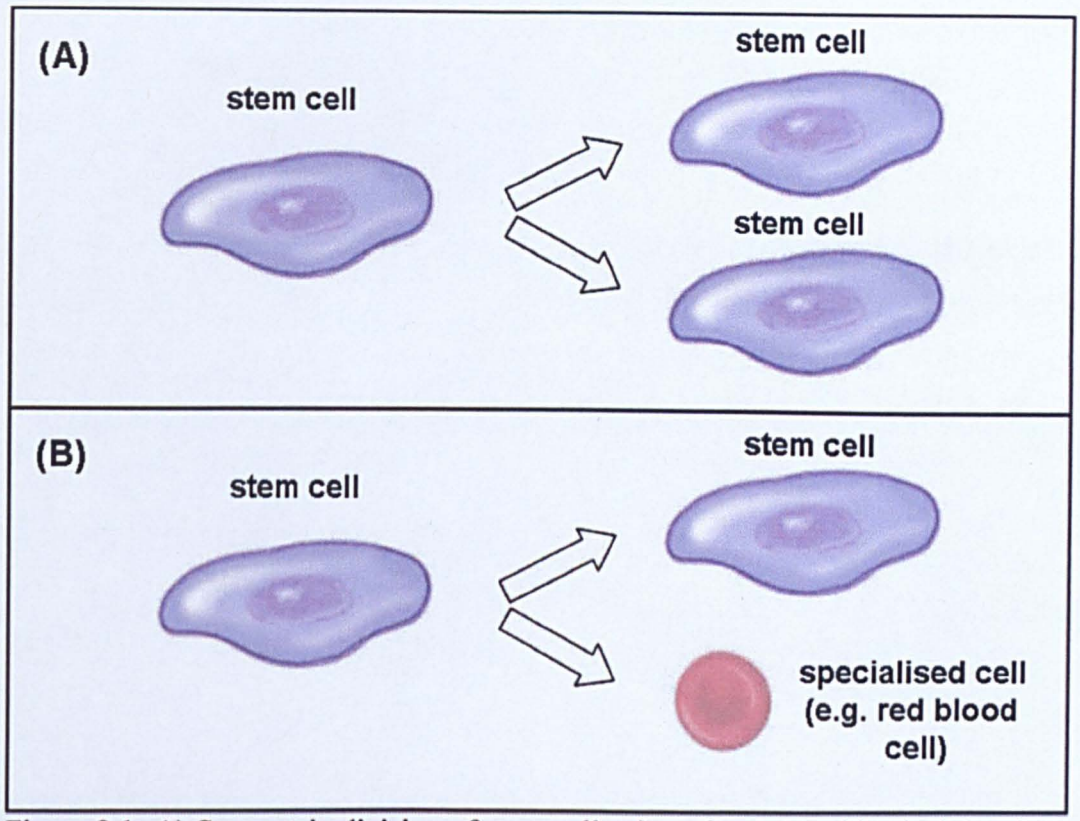

Figure 2.1: **A)** Symmetric division of stem cells gives rise to two identical daughter cells, both fully functional stem cells. **B)** Asymmetric cell division of stem cells gives rise to one stem cell and one mature or specialised cell which has limited or no self renewal. (Modified from: 4. The Adult Stem Cell . In *Stem Cell Information*. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2009 Available at http://stemcells.nih.gov/info/2001report/chapter4)

Distinct stem cell types have been established from embryos and identified in the foetal tissues and umbilical cord blood, as well as in many adult mammalian tissues and organs (Ortiz-Gonzalez *et al.*, 2004) (Rollini *et al.*, 2004) (Trounson, 2006). Stem cells are classified according to their differentiation capability into totipotent, pluripotent and multipotent and, according to their differentiation stage, into embryonic and adult stem cells (Ulloa-Montoya *et al.*, 2005). Totipotent stem cells can be found in early embryonic development up to the 8 cell stage. They give rise to all embryonic somatic tissues and germ cells including the extraembryonic tissue and thus are the only cells with the potential to develop into a complete individual (Yeom *et al.*, 1996). Pluripotent stem cells can still differentiate into the three somatic germ layers that comprise an organism: mesoderm (muscle, bone, etc.), ectoderm (neurons, skin, etc.) and endoderm (hepatocytes, pancreatic beta cells, etc.), but are not able to form trophoblasts and therefore no longer have the capacity to develop into a

complete individual. Multipotent stem cells are the most developed stem cells with the capability to differentiate only into cells of the one tissue or germ layer from which they originated from (Ulloa-Montoya *et al.*, 2005).

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocysts. Their pluripotency was first identified by the formation of tumours consisting of different types of tissue, such as skin, hair and muscle, when embryonic stem cells were transplanted into post-natal animals (Wobus *et al.*, 1984) (Reubinoff *et al.*, 2000). The presence of cells from all three germ layers in one tumour demonstrated their pluripotency. Furthermore, human ESCs have been differentiated into ectoderm, endoderm, and mesoderm cells *in vitro* (Yao *et al.*, 2005). More specifically, they express many cell markers that are common to pluripotent cells such as CD9, CD24, octamer-binding protein (Oct-4), Nanog, LIN28, Rex-1 and Thy-1 (Trounson, 2006) (Richards *et al.*, 2004) (Chambers, 2004). All ESCs have high levels of telomerase expression and activity for long-lasting periods in culture, giving them the ability to undergo unlimited self-renewal. Although they are able to generate large numbers of any cell type for transplantation into patients, and can proliferate indefinitely in culture, there are ethical considerations restricting the use of ESCs (Orkin and Morrison, 2002) (Pflegerl *et al.*, 2008) (Ohara, 2003) (Hug, 2005).

Adult stem cells are undifferentiated cells located in a differentiated adult tissue such as bone marrow, skin, liver, heart, brain, kidneys, eyes, gastrointestinal tract, pancreas, lungs, breast, ovaries, prostate and testis (Brittan *et al.*, 2002) (Tumbar *et al.*, 2004) (Watts *et al.*, 2005) (Woodward *et al.*, 2005) (Guettier, 2005) (Heissig et al., 2005) (Leri *et al.*, 2005) (Mimeault *et al.*, 2006) (Seaberg *et al.*, 2004). Even though they differentiate into progenitor, precursor and mature cells of multiple lineages, they are not pluripotent. Adult stem cells contribute to ongoing tissue maintenance or repair (Bach *et al.*, 2000). In this situation, they are very few in number, but have a high proliferation potential, and the capacity to generate fully mature, tissue specific cell types. The exact numbers of stem cells are unknown and vary from tissue type to tissue type. Adult stem cells do not replicate indefinitely in culture, and their degree of self-renewal is more restricted compared to embryonic stem cells. Some concepts have been developed to explain the balance between

proliferation and differentiation (Fuchs *et al.*, 2004) (Griffiths et al., 2005) (Wilson *et al.*, 2006). During stem cell self-renewal, the cells undergo symmetric division into two identical daughter cells both with full stem cell characteristics (Figure 2.1A). In contrast, during differentiation the stem cells undergo asymmetric division into one daughter stem cell and one daughter cell which continues on a differentiation pathway (Figure 2.1B). The differentiating daughter cell may give rise to other progenitor cells and, ultimately, to more differentiated cells. These differentiated cells can constitute tissues or organs either where they originate or other distant tissues by performing cell migration.

The three most used adult stem cells for tissue engineering and cellular therapies are neural stem cells, haematopoietic stem cells and mesenchymal stem cells. The most extensively of these cells are the haematopoietic stem cells (HSC) (Wilson *et al.*, 2006) (Fried and Johnson, 1968) (Weimann *et al.*, 1974) (Spangrude *et al.*, 1988). They give rise to all the blood cell types of both the myeloid and lymphoid. This includes monocytes and macrophages, neutrophils, basophils, eosinophils, T-cells, B-cells, NK-cells, microglia, erythrocytes (red blood cells), megakaryocytes (e.g. platelets), and dentritic cells. Furthermore, when being transplanted, HSCs repopulate the haematopoietic system of a recipient with a removed or damaged system (Bhatia *et al.*, 1997). Haematopoietic stem cells originate from the bone marrow. Another type of adult stem cells is neural stem cells (NSC). They have the ability to generate all the cells found in the adult central nervous system (e.g. neurons, astrocytes, oligodendrocytes) (Gage, 2000). They are present from birth, and now are believed to persist into adult life in the brain and spinal cord (Gage, 2000). Mesenchymal stem cells (MSCs), which will be described in detail in section 2.4, are also adult stem cells. Amongst other sources of origin, MSCs can also be isolated from the bone marrow, making the bone marrow home to two different adult stem cell types.

2.3 Bone marrow, primary location of Mesenchymal Stem Cells

Bone marrow is a complex, well organised tissue filling the space between vascular sinuses and bone surfaces of the pores in cancellous bones. It is composed of two distinct but interdependent compartments: the haematopoietic system and the bone marrow stroma (Weiss, 1970). The haematopoietic system consists of developing haematopoietic cells, also called haematopoietic stem cells (HSCs). They can give rise to all blood cell types, and are retained in the bone marrow until they have matured and are released into the vascular system (Fliedner, 1998). This assures the continuous replenishment of all types of circulating haematopoietic cells. HSCs can be found within discrete cellular spaces termed 'niches' (Moore and Lemischka, 2006). In these niches, the HSCs are in close contact with their microenvironment (Arai *et al.*, 2005). At least three niches with specialised functions can be defined: the bony niche, the stromal niche and the adipocyte niche. Figure 2.2 shows the cellular composition of these three niches and their positions within the bone marrow.

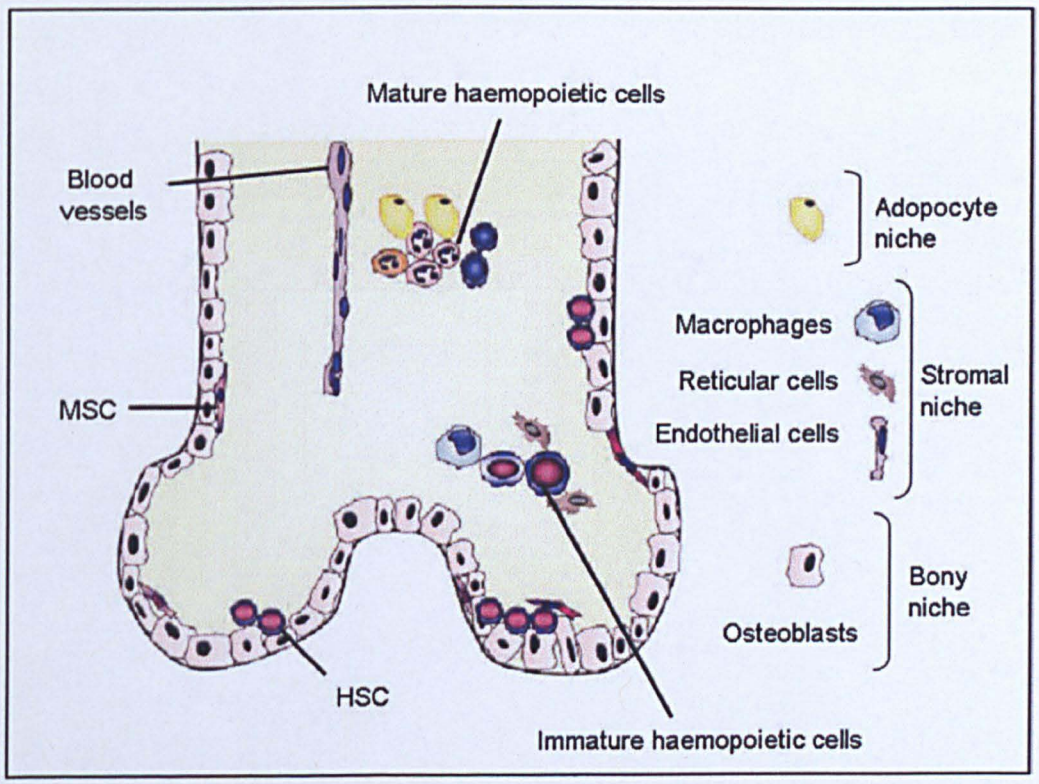

Figure 2.2: The three haematopoietic niches and their cellular components according to Dazzi *et al.*, 2006.

Cells from the second compartment, the bone marrow stroma, generate and maintain the haematopoietic microenvironment of these three niches (Neiva *et al.*, 2005). The bony niche is lined with osteoblasts and preosteoblasts, both of which are found close to the endosteal surface (Arai *et al.*, 2004) (Calvi *et al.*, 2003) (Zhang *et al.*, 2003). The stromal niche contains reticular cells, fibroblasts, endothelial cells and macrophages, and is found stretching into the medulla of the bone. Reticular cells are the dominant cells of the marrow stroma. They encase sinuses and branch into the surrounding haematopoietic space, where they form a spongework on which haematopoietic cells are arranged (Bianco *et al.*, 1993). Reticular cells are considered "fibroblastic" in nature since they are defined by the expression of high levels of membrane-associated alkaline phosphatase activity, collagen types I and III and osteonectin. They originate from the primitive osteogenic periosteum of developing bones and co-migrate, along with ingrowing blood vessels, to the interior of developing bones, where they actively proliferate and provide the reservoir of immediate osteogenic precursors (proliferating pre-osteoblasts) for pre-natal osteogenesis (Krebsbach *et al.*, 1999). It has been shown, that mature endothelial cells also play an important role in supporting haematopoiesis after radiation exposure as progenitors of the marrow stroma (Li *et al.*, 2004) (Gaugler *et al.*, 2001). The adipocyte niche is located in the non-haematopoietic space within the long bone, also known as "yellow marrow" (Dazzi *et al.*, 2006). In post-natal human marrow, stromal cells demonstrate characteristics of pre-adipocytes (Bianco *et al.*, 1988). It has been suggested that alkaline phosphatase (ALP) positive reticular cells and adipocytes are alternative phenotypes (Weiss, 1976). An inverse relationship between ALP-positive stromal cells and adipocytes can be demonstrated at any age or marrow site. The number of adipocytes increases in the bone marrow when there is a pathological or age related reduction in haematopoietic tissue or during skeletal development when there is space in excess of what is needed for haematopoiesis (Weiss, 1976).

Investigations into the interactions between the haematopoietic and the stroma compartments have shown that a close relationship exists between osteogenesis and haematopoiesis (Taichman, 2005). Thus, haematopoietic cells influence the activity of the stromal compartment, and in return some cell types of the marrow stromal tissue (reticular cells, adipocytes, osteoblasts and macrophages) not only serve as a

mechanical support for differentiating haematopoietic cells, but also support haematopoiesis by regulating the survival, proliferation, migration, and differentiation of HSCs through several mechanisms (Taichman, 2005). This includes cell contact interactions or the production of growth factors, chemokines and extracellular matrix molecules. Several examples have been mentioned for these interactions. Reticular cells, for example, sort the differentiating haematopoietic cells into characteristic locations in their spongework (Weiss, 1976). Adipocytes can regulate haematopoiesis via secretion of leptin, which has been shown to correlate with white blood cell numbers (Wilson *et al.*, 1997). Furthermore, the stromal osteoblasts express diverse soluble factors, such as granulocyte colony-stimulating factor (G-CSF), IL-6 and the Notch ligand jagged 1 that can influence the proliferation and differentiation of HSCs (Murphy *et al.*, 2005). There is also evidence that cytokine production by mesenchymal stem cells (MSCs), osteoblasts and stromal cells can be influenced by 'cross-talk' between these cells and HSCs (Taichman *et al.*, 1997) (Ahmed *et al.*, 2001). In this matter, the work by Beachy *et al.* (2004) indicated that specific signals induced by the hedgehog (Hh) pathway and the Wnts pathway may be involved in the control of the bone marrow stem cell homing, migration and differentiation.

As well as haematopoietic stem cells, the bone marrow is home to stem-like cells that are precursors of the stromal tissue. The majority, if not all, of the stromal cells derive from a common mesenchymal progenitor, referred to as the mesenchymal stem cell (MSC). Among the MSCs, there are primitive mesodermal progenitor cells (MPCs) or multipotent adult progenitor cells (MAPCs). Together, they lead to the generation of different bone marrow and bloodstream cell lineages (Arai *et al.*, 2004). Thus far, the location and lineage commitment of MSCs *in vivo* are much less characterised than those of HSCs. The origin of mature osteoblasts and haematopoietic cells has been directly proven (Krebsbach *et al.*, 1997) (Kuznetsov *et al.*, 1997). However, the origin of the MSCs has only been surmised, in a perivascular region in the bone marrow tissue (Shi *et al.*, 2003). As no specific antigens for MSCs have been described yet, it is not possible to determine the exact anatomical location within the bone marrow.

2.4 Mesenchymal Stem Cells

Mesenchymal stem cells were first reported by Friedenstein *et al.* (1968), who referred to them as colony-forming-units fibroblasts (CFU-f), according to their ability to adhere to tissue culture plastic, and grow into colonies when cultured *in vitro* (Friedenstein *et al.*, 1968 and 1976) (Hung *et al.*, 2002). CFU-fs can develop, under appropriate experimental conditions, into a variety of fully differentiated connective tissues, such as cartilage, bone, adipose tissue, fibrous tissue, and myelosupportive stroma, both *in vitro* and *in vivo* (Friedenstein *et al.*, 1974) (Owen, 1988) (Pittenger *et al.*, 1999), while retaining a high proliferation capacity (Friedenstein *et al.*, 1987). Based on such observations, researchers developed the concept that osteoblasts, chondrocytes, adipocytes and other connective tissue cells derive from a common ancestor, which was found to be a stromal stem cell persisting within the bone marrow of postnatal and adult organisms. It was then, that the name marrow stromal cell, or mesenchymal stem cell (MSC) was formed (Rickard *et al.*, 1996) (Prockop, 1997) (Colter *et al.*, 2001). MSCs are harvested most commonly from bone marrow, where they represent about 0.01 - 0.001% of the nucleated cells, but they can also be found in other different tissues such as umbilical cord blood, synovial membrane of knee joints, adipose tissue and muscle. All these tissues contain MSC like cells with the ability to proliferate extensively in culture and to differentiate into multiple lineages under specific conditions *in vitro* (De Bari *et al.*, 2001) (Chang et al., 2006) (Zuk *et al.*, 2001) (Nesti *et al.*, 2008) (see Figure 2.3).

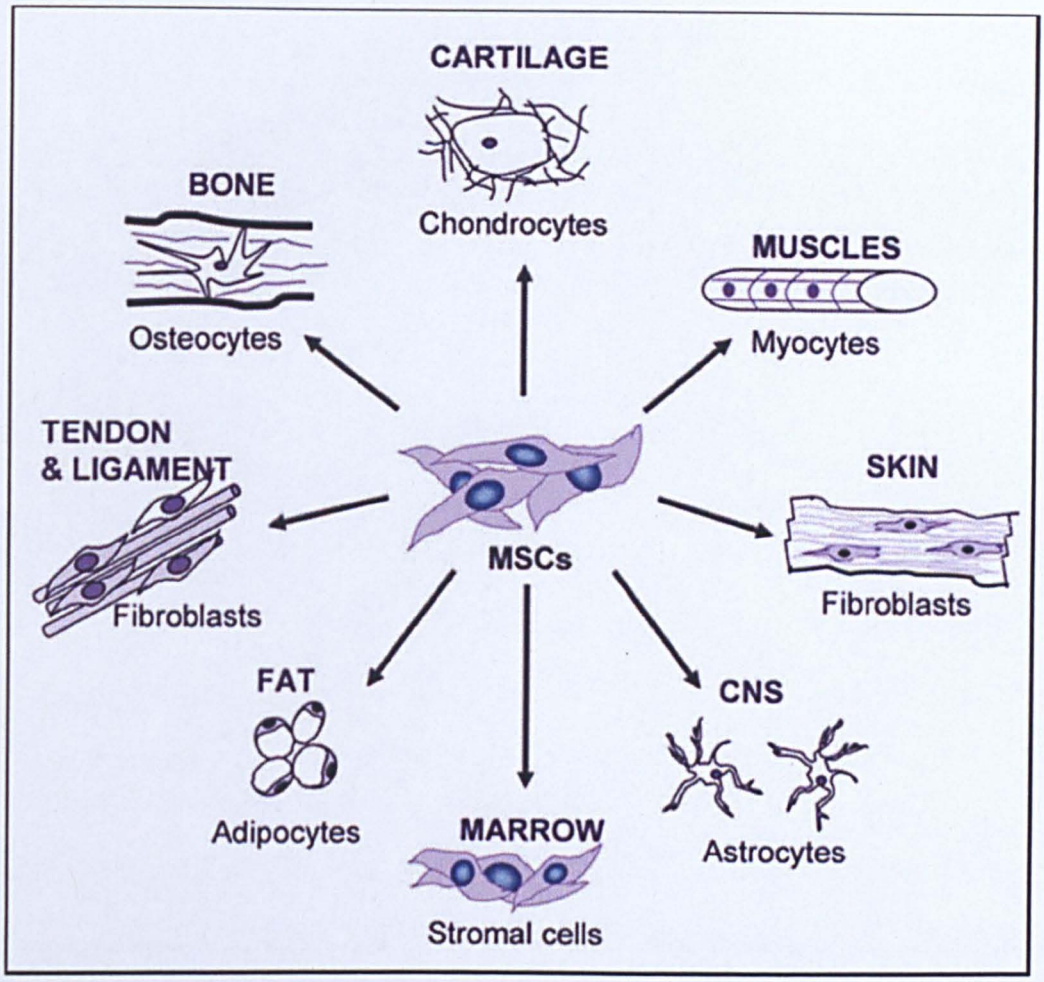

Figure 2.3: Differentiation potential of adult mesenchymal stem cell. They can differentiate into multiple lineages such as osteocytes, chondrocytes, fibroblasts, adipocytes, astrocytes and myocytes (Grassel and Ahmed, 2007).

The term mesenchymal stem cell can describe a vast variety of multipotent adult stem cells, since the only way to define MSCs is by their differentiation capacity. Although, the CFU-f assay has traditionally been used to obtain the numbers of bone marrow derived MSCs, this assay on its own does not directly detect differentiation or self-renewal potential. In order to determine these cell properties, time consuming differentiation assays have to be conducted, in which MSCs are cultured in the presence of specific growth factors or differentiation agents (Bosnakovski *et al.*, 2005). Kuznetsov *et al.* (1997) found that, upon transplantation, bone marrow derived CFU-fs form bone and even support haematopoiesis. However, the CFU-fs were heterogeneous with respect to their osteogenic differentiation potential and their capacity to support haematopoiesis (Kuznetsov *et al.*, 1997). Using *in vitro* differentiation assays, a clonal study of human MSCs also revealed varying

differentiation potential of the MSC clones (Muraglia *et al.*, 2000). All of these clones had osteogenic potential, whereas 60–80% had osteo- and chondrogenic potential, and only 33% displayed osteo-, chondro- and adipogenic potential altogether. Furthermore, Muraglia *et al.* (2000) showed that, upon static monolayer culture, MSCs gradually lose their adipo- and chondrogenic potential. These findings clarify that a single type of differentiation potential of MSCs is not a reliable method to describe them.

There are remarkable differences not only at the differentiation capacity of MSCs into multiple lineages, but also the cell morphology and rates of proliferation. Bruder *et al.* (1997) evaluated the growth kinetics of MSCs by serial subculture over 15 passages, which corresponded to 38 population doublings. During subculturing, the cells showed changes in morphology from thin spindle-shaped cells to broad and flattened cells. The proliferation potential and the final cell number after a fixed period in culture also decreased with time (Bruder *et al.*, 1997). In a similar study, the growth kinetics of bulk and clonal MSCs were reported for up to five passages, corresponding to 24 population doublings for the bulk population and 19 – 23 population doublings for the clones (Banfi *et al.*, 2000). During subculturing, the cell doubling time increased in the bulk population and a loss of the osteogenic differentiation potential *in vivo* was observed. These results prove that MSCs, in contrast to ESCs, cannot proliferate indefinitely in culture. Their limited life-span *in vitro* is thought to be due to their lack of telomerase activity, which prevents shortening of the telomeres. Banfi *et al.* (2002) showed that the telomere shortening in MSCs during *in vitro* expansion (50–100 bp/doubling) falls within the range of telomere shortening for telomerase-negative somatic cells, which only have a very limited life span. In addition they also found that the gene expression profile of late passage MSCs revealed a commitment towards the osteogenic lineage.

Thus far, a definitive demonstration of both self-renewal and multilineage differentiation of a MSC at the single-cell level is missing, and the molecular mechanisms that control MSC self-renewal and differentiation are not fully understood. This is in part because of the heterogeneous MSC populations isolated from the bone marrow, which also vary among different laboratories (Baksh *et al.*, 2004). Therefore, investigations at the single cell level are necessary (Kemp *et al.*,

2005). In order to analyse MSCs at this level, they have to be identified and isolated first. However, neither their differentiation capacity nor their morphology are parameters that allow a satisfying identification. The fibroblastic morphology in culture cannot be used to distinguish them from more differentiated cell phenotypes since both osteoblasts and fibroblasts are morphologically nearly indistinguishable (Ducy *et al.*, 2000). Hence, several molecular markers and certain antigens, in particular surface proteins, have been used as an attempt to characterise MSCs and distinguish them from more mature cells such as fibroblasts and osteoblasts (Ishii *et al.*, 2005) (Haynesworth *et al.*, 1992) (Bruder *et al.*, 1998) (Gronthos *et al.*, 2003) (Honczarenko *et al.*, 2006).

Several research groups have employed proteomic studies on MSCs in order to characterised both changes in the intracellular pathways involved in differentiation processes and changes in the expression of protein surface markers in MSCs before and after the induction of differentiation (Baharvand *et al.*, 2007). Foster *et al.* (2005) characterised changes in membrane protein markers of human MSCs undergoing osteoblast differentiation. Samples from the cells membrane were analysed using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) in order to find surface markers that help to distinguish undifferentiated MSCs from early osteogenic progenitor cells. 463 unique proteins were identified, including all known markers of human MSCs. 83 identified proteins, such as alkaline phosphatase, increased in expression levels by at least twofold and 21 proteins, including fatty acid synthase, decreased upon osteoblast differentiation. Foster *et al.* (2005) concluded that mass spectrometry-based proteomics can reveal novel markers of human MSCs. Another group (Zhang *et al.*, 2007) identified differently expressed proteins responsible for osteoblast differentiation in human MSCs by using two-dimensional gel electrophoresis (2DE), MALDI-TOF-MS and peptide mass fingerprinting. Proteome maps were generated from undifferentiated human MSCs and from osteogenic induced MSCs. In total, 102 proteins with at least two-fold changes in expression were revealed with 2DE, whereof 52 could be successfully identified by MALDI-TOF-MS. An experiment using adipocytes, differentiated from human MSCs, for proteomic analysis with 2DE and MALDI-TOF-MS were conducted by Lee *et al.* (2006). The MSCs were cultured as undifferentiated cells in static monolayer cultures for two passages before adipogenic differentiation was

induced. Cell samples from undifferentiated cells and adipogenic cells after different time length in adipogenic differentiation culture were used for the proteomic analysis. 32 protein spots were shown to have different expression levels. Among the up-regulated proteins, four were associated with adipogenesis.

Although at present several markers are known to be expressed by human MSCs (e.g. CD13, CD49e, CD73 (SH3 and SH4), CD105 (SH2), Mac1, PDGF-R and Stro-1), their antigenic phenotype is not unique, and hence no single marker has been found to be specific for MSCs (Conget and Minguell, 1999) (Barry and Murphy, 2004). A combination of surface markers coexpressed on one single cell represents a promising strategy for distinct characterisation of MSCs (Kemp *et al.*, 2005) (Schieker *et al.*, 2007). However, the expression of various markers, similar to the differentiation and proliferation potential, is variable between different cell populations, and also within a cell population, as a function of time in culture.

2.5 Therapeutic applications of Mesenchymal Stem Cells

It is surprising that the lack of definition has not prevented the widespread use of the term mesenchymal stem cell, as well as the enthusiasm about their value for therapeutic applications (Colter *et al.*, 2000). The wide differentiation potential of mesenchymal stem cells (MSCs) and their more differentiated progenitors allows them to be used for applications such as tissue engineering and gene therapy. The maintenance of their multipotential capacity after transplantation, which has been proven by Liechty *et al.* (2000), is the essential requirement for the usage in a huge variety of cell therapies. Liechty *et al.* (2000) transplanted human bone marrow derived mesenchymal stem cells in foetal sheep. The cells were first expanded in a static monolayer culture for one passage, and then stored in liquid nitrogen until they were injected into the peritoneal cavity of either 65 or 85 day old foetal sheep, which were in different development stages for haematopoiesis and immune response. The foetal lamb model was chosen because it is immunologically tolerant, and because human and sheep DNA and proteins are widely disparate, which allows the use of human-specific markers for the detection and characterisations of the transplanted human MSCs. The foetal sheep were killed at several time points after injection

(longest was 13 months), tissue and blood samples were collected and analysed for the presence of human cells by DNA isolation and immunohistochemistry. All analyses showed the presence of human MSCs in pre- and post-natal haematopoietic and lymphopoietic tissues like foetal liver, bone marrow, spleen and thymus as well as in nonlymphohaematopoietic sites like adipose tissue, lung, articular cartilage, pervascular areas of the central nervous system and cardiac and skeletal muscle. These results have caused great interest in developing and expanding populations of MSCs which are able to repopulate and repair damaged tissues.

Disorders that might benefit from MSC based therapies include diabetes, heart failure, muscular disorders, arthritis, and vision disorders (Mimeault and Batra, 2006). Several *in vivo* studies have been conducted to assess the therapeutic potential of MSCs for these disorders. For instance, Oh *et al.* (2004) examined the use of MSCs to treat pancreatic diseases. The cells were trans-differentiated in insulin producing cells *in vitro* before they were transplanted subcapsular into hyperglycemic mice. This resulted in a significant reduction of blood glucose levels which was maintained for up to 90 days post-transplantation. Thus, it also could be possible to develop cell therapies for human diabetic patients by using the human MSC derived β-cells. Nevertheless, additional *in vivo* work is needed to fully understand the migration and differentiation process of MSCs into β-cell-like progenitors.

Another investigation indicated that the transplantation of mice bone marrow stem cells into the infracted heart of mice resulted in the differentiation of myocytes and the formation of coronary vessels, which improved cardiac function (Kajstura *et al.*, 2005). Hence, it appeared that MSCs could represent a more accessible source of stem cells for the treatment of cardiac disorders than cardiac stem cells.

In further studies, MSCs were seeded onto ceramic scaffolds before being transplanted subcutaneously into mice in order to demonstrate their osteogenic differentiation capacity *in vivo* (Diao *et al.*, 2009). Matsushima *et al.* (2009) also transplanted ceramic scaffolds loaded with human bone marrow derived MSCs into rats to determine the bone forming potential of the stem cells. In this study, the freshly harvested MSCs were expanded in static monolayer cultures for 2 passages

before being seeded either on hydroxyapatite ceramics or on β-tricalcium phosphate ceramics. Both ceramics were then transplanted subcutaneously into nude rats and recovered after 8 weeks. Bone formation was measure by analysing the scaffolds using microcomputer tomography (micro CT) and automated picture analysing software. The results suggested that human MSCs cultured on ceramics could retain their osteogenic differentiation capacity even after ectopic implantation.

In another transplantation experiment, MSCs were harvested from the bone marrow of rhesus monkeys, expanded and differentiated into neural lineage cells *in vitro* before being transplanted into the injured spinal cord of the rhesus monkeys (Deng *et al.*, 2006). 3 month after transplantation, the primates achieved nearly normal sensory responses and 10% of the transplanted MSCs showed the presence of neural cell markers. Deng *et al.* (2006) concluded, that the implantation of differentiated MSCs activated neurogenesis and functional recovery in the primate spinal cord injury model.

Moreover, human MSCs from healthy donors were also used to reconstruct chemically burned rat corneal surfaces (Ma *et al.*, 2006). The cells were first expanded in culture on human amniotic membrane in Dulbecco's modified Eagle's medium (DMEM) before being transplanted into rat corneas, 7 days after chemical burns. Ma *et al.* (2006) observed that transplantation of MSCs successfully reconstructed the damaged corneal surface, however, they concluded that the therapeutic effect may be due, in part, to an inhibition of inflammatory and angiogenic processes rather than the epithelial differentiation of MSCs. This opinion was also shared by Popp *et al.* (2009) who believed that mesenchymal stem cells are promising candidates for immunomodulation therapy. Popp *et al.* (2009) stated that MSCs suppress the immune response in a variety of *in vitro* and disease models and thus may be of benefit for patients suffering from autoimmune disorders or transplant rejection. The mechanism by which MSCs modulate the immune response has still to be investigated, but it most likely involves expression of local factors such as indoleamine 2,3-dioxygenase, inducible nitric oxide synthase, and others, as well as interactions with dendritic or antigen-presenting cells (Popp *et al.*, 2009).

The clinical transplantation procedures of MSCs, which depend on patient state and diagnosis, generally involve the intravenous injection or subcutaneous administration of a specific number of stem cells directly into therapeutically targeted areas. However, transplantation is not the only way to use MSCs in cell therapy. The high plasticity and migratory potential of bone marrow derived MSCs, offer the possibility of injecting these stem cells into the circulation or mobilising them *in vivo* using signalling factors to regenerate the particular damaged tissue (Blau *et al.*, 2001; Orlic, 2005). These procedures are often applied when studying the MSC's repair potential of infracted myocardium (Vandervelde *et al.*, 2005).

Besides their use for tissue engineering, MSCs can also be used for gene therapy. Gene-based strategies involve modifications or replacement of a particular gene product in the stem cells before their transplantation into the patient. These therapies might be considered for the treatment of diverse genetic and incurable diseases such as bleeding disorders. For instance, it has been reported that bone marrow stem cells transfected with a gene for human coagulation factor IX (hF1X) secreted the biologically active hF1X form of this factor in *in vitro* experiments. When transduced stem cells were subcutaneously transplanted into immunocompromised mice, secretion of hF1X into the circulation could be proven (Krebsbach *et al.*, 2003).

Before MSCs can fulfil their expected role in cell therapies, the underlying mechanisms of the MSC biology, which are involved in self renewal and differentiation, have to be better understood. In addition, efforts have to be made to optimise the production of bone marrow derived MSCs for a variety of technological applications (Colter *et al.*, 2000).

2.6 Culture systems for Mesenchymal Stem Cells

A variety of culture techniques for MSC cultivation has been described in the literature, and is described below. Although all of these techniques are suitable for *in vitro* expansion of MSCs, each has its own advantages and disadvantages. The problems of providing ideal culture conditions for MSCs are based in the lack of appropriate surface markers associated with identifying them, and the limited knowledge of their requirements to maintain the full differentiation and proliferation capacity. A proteomic study on MSCs by Wagner *et al.* (2006) pointed to the lack of common standard for cultivation protocols of MSCs *in vitro*. Wagner *et al.* (2006) compared the proteome of human MSCs, which were expanded in two different culture media. This work demonstrated the influence of different culture conditions on the proteome and cellular organisation of MSCs. Human bone marrow derived MSCs were either expanded in 58% Dulbecco's Modified Eagles Medium–Low Glucose and 40% MCDB 201 from Sigma, supplemented with 2% fetal calf serum, or in a commercially available Poietics Human Mesenchymal Stem Cell Medium (PT-3001) from Cambrex. After 6 passages, the MSCs were harvested, and the proteome was analysed by matrix assisted laser desorption / ionisation time of flight mass spectrometry (MALDI-TOF-MS). In total, 136 proteins were identified. Proteins involved in metabolism were more up regulated in the mixed culture medium, whereas proteins involved in development and differentiation were more up regulated in the commercially available medium. This study proves that culture conditions exert a prominent impact on the proteome of MSCs, and thus it is necessary to have a common standard for expanding MSCs *in vitro* when comparing results from different laboratories. This underpins why the application of MSCs is not yet routinely used for medical applications.

2.6.1 Static monolayer cultures

The general protocol for expanding bone marrow derived mesenchymal stem cells involves isolation of the mononuclear cells and seeding these cells on tissue culture plates, such as T-flasks in a medium containing foetal bovine serum (FBS). After the adherent cell fraction attaches to the plastic surface and forms a monolayer, the

complete medium is changed for the first time to remove all non-adherent cells. The adherent cells are allowed to proliferate until they reach about 90% confluency. At this point, they have to be lifted off the plastic surface by incubating them with trypsin/EDTA and re-plated into new culture plates to ensure further proliferation as a monolayer. The adherent cells, which consists mainly of MSCs are normally expanded for a limited number of passages, and then used for further applications or assays such as various differentiation assays (Ulloa-Montoya *et al.*, 2005).

Different strategies have been implemented to improve the isolation and *in vitro* expansion of MSCs, including optimisation of culture temperature, initial plating density and initial selection of a more purified and homogeneous MSC population based on cell surface markers or cell size (Stolzing and Scutt, 2006) (Colter *et al.*, 2000) (Gronthos *et al.*, 2003). Extensive expansion of MSCs leads to *in vitro* aging, the accumulation of oxidative damage and, as a result of both, to reduced proliferation and differentiation capacity (Sugiura *et al.*, 2004). Stolzing and Scutt (2006) have shown that a lower culture temperature of 32°C improved the differentiation potential of MSCs and reduced apoptosis by decreasing the effects of oxidative damage to the cells. Furthermore, Shui and Scutt (2001) demonstrated that mild heat shock (repeated exposure to 39 / 41°C) stimulated alkaline phosphatase activity in bone marrow derived MSCs and their differentiation into osteoprogenitor cells. Both studies hypothesise that the up-regulation of several heat shock proteins might be involved in the differentiation process of MSCs. Hence, a carefully controlled and regulated culture temperature is crucial for MSCs to keep their full differentiation and proliferation potential whilst being expanded in culture.

The seeding cell density has been proven to influence the proliferation potential of MSCs in static cultures (Colter *et al.*, 2000). Cells, initially plated at low densities (1.5 – 3 cells/cm^2), grew more rapidly and expanded an average of 600 fold (about 50 population doublings), whereas cells at higher densities (12 cells/cm^2) grew more slowly and expanded only 3 – 5 times (about 15 population doublings) (Colter *et al.*, 2000). These findings were confirmed by another study examining initial seeding cell densities in a larger interval of 10 – 1000 cells/cm^2 (Sekiya *et al.*, 2002). They also showed that the lowest cell density produced the highest fold increase in cell number. In addition, changes in cell morphology were found with different cell

densities, and were correlated with varied differentiation potential. Low cell density cultures favoured the appearance of thin, spindle shaped MSCs (called RS-1A) that differentiated into adipocytes. Plating MSCs at higher cell densities promoted appearance of wider spindle-shaped cells that could be differentiated into cartilage (Sekiya *et al.*, 2002).

A purification of the initial MSCs population can also improve their proliferation *in vitro*. An initial selection of purified and homogeneous MSCs was achieved by cell sorting of human bone marrow mononuclear cells using the cell surface markers STRO-1 and vascular cell adhesion molecule 1 (VCAM-1) (Gronthos *et al.*, 2003). The purified MSC population expressed telomerase and could be expanded for over 40 population doublings in a serum free media. They showed a 50% cloning efficiency for fibroblast colonies, and tri-lineage mesenchymal differentiation potential *in vitro* but heterogenous differentiation capacity *in vivo*. Simmons *et al.* (1991) first showed that the cell surface marker STRO-1 identified a population of bone marrow cells that had the capacity to form fibroblast colonies, gave rise to cells with smooth muscle phenotype and had adipogenic and osteogenic differentiation potential.

As mentioned at the beginning of the section 2.6.1, bone marrow derived MSCs are isolated by plating the mononuclear cells from bone marrow on tissue culture plates in medium containing FBS. However, slight variations in the composition of the serum are unavoidable, since FBS is obtained from different sources and at different times. These variations in FBS appear to have a major effect on MSC cultures, affecting the plating efficiency and subsequent expansion. In order to minimise the effects, different lots of FBS are mixed which each other, thus the differences in the serum composition are minimised. Nonetheless, the only way to avoid these effects completely is by using serum free media. Gronthos *et al.* (1994) was able to expand STRO-1^+ and STRO-1^+ VCAM-1^+ MSC populations in serum free medium, and found that in these conditions dexamethasone and L-ascorbate were essential for colony formation, whereas epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) supported the colony growth.

Another downside of the static monolayer culture technique, besides the use of serum in the culture medium, was observation that serial subculturing of the MSCs affected their proliferation capacity (Sun *et al.*, 2006) (Celebi and Elcin 2008). Both research groups conducted proteome analysis on subcultured MSCs to investigate these effects. Sun *et al.* (2006) analysed the proteome of hMSCs using 2DE and MALDI-TOF-MS. They were interested in finding different regulated proteins during serial subculturing, which might be involved in proliferation and differentiation related processes. Indeed, several proteins associated with cell proliferation, cell cycle and apoptosis were shown to be differentially regulated, indicating that passage-specific protein regulation can play a role in the decrease of proliferation and osteogenic differentiation potential. Celebi and Elcin (2008) were also interested in the effect of serial subculturing on rat bone marrow derived MSCs. They expanded the MSCs in static monolayer cultures for up to 10 passages before extracting the proteins and separating them by 2DE. About 1000 protein spots were detected, and of these 31 proteins were successfully identified by MALDI-TOF-MS. Amongst them were 18 differentially expressed proteins that were involved in cell proliferation and differentiation through different signaling pathways.

The advantage of the static monolayer culture system and the reason why this method is commonly used for MSCs expansion, is the surface based growth of these cells. In static monolayer cultures, MSCs can easily adhere to the culture plastic surface and proliferate in relatively simple culture conditions. However, this simplicity also has its disadvantages. Although bone marrow derived MSCs were successfully expanded for several passages in static monolayer cultures, these culture systems have a poor reproducibility, lack of mixing (gradients for pH, dissolved oxygen, cytokines and metabolites) and do not allow online monitoring or control of the culture conditions (Ohgushi et al., 1999). However, a better control of the culture condition can improve the expansion of MSCs, as mentioned above. Being able to amplify MSCs in large numbers is very important, since the donor derived cell samples only consist of a small number of MSCs (about 0.1% or ~40,000/ml), which cannot meet the expected demands for therapeutic applications (about 7.5 x 109 MSC to help healing a 10 cm defect in a human femur). Therefore, a technique is required which is able to cultivate large quantities of stem cells in a controlled and well defined environment.

2.6.2 Stirred suspension cultures

Bioreactors provide a controlled *in vitro* environment, allowing the study of stem cell proliferation and differentiation. The main advantage of the bioreactor is not only the production of larger quantity of cells and easier handling, its use also allows for the permanent monitoring and control of the physical and chemical environment, thus providing the cells with optimised culture conditions which are crucial for their cultivation. In a bioreactor, different culture parameters can be investigated (e.g. O_2 tension, cytokine concentration, medium exchange rates, glucose concentration, etc) that may influence the viability, proliferation and differentiation of MSCs (Ulloa-Montoya *et al.*, 2005) (Baksh *et al.*, 2003). For example, reduction in glucose concentrations led to decreased apoptosis, an increased rate of MSC proliferation and increased the number and size of fibroblastic colonies in the CFU-f assay (Stolzing *et al.*, 2006). Low oxygen concentrations *in vitro* can also increase proliferation rates and enhance differentiation along the different mesenchymal lineages (Das *et al.*, 2009). The observation that more mature cells may be less sensitive to changes in their oxygen environment than their immediate precursors (Cipolleschi *et al.*, 1993) suggests that the definition of an "optimal" oxygen tension may depend not only on the kind of stem cells used but also on the developmental potential of the cells being stimulated.

A variety of bioreactors for cell cultivation already exists and can be easily adapted for large scale MSC cultivation. However, first, it has to be proven that MSCs can proliferate in suspension, in order to be amplified in suspension based bioreactors. Cells with similar properties to mesenchymal stem cells can be isolated from the circulation (Zvaifler *et al.*, 2000) and thus may be able to survive and proliferate in suspension. Furthermore, studies have shown that MSCs do not necessarily need adherence to a surface to grow and proliferate (Baksh *et al.*, 2003). Therefore, an alternative and more attractive method for their cultivation can be the stirred suspension culture. Promising results on the growth of haematopoietic (Kwon *et al.*, 2003), neural (Sen *et al.*, 2002), and embryonic (Fok *et al.*, 2005) stem and progenitor cells in stirred suspension cultures have been obtained. These protocols may be used to develop and improve the cultivation of MSCs in stirred suspension cultures. Baksh *et al.* (2003) and Chen *et al.* (2006) have proven that marrow derived

stromal cells (detected as CFU-f) can also be expanded in stirred suspension cultures. In both studies MSCs were grown in culture medium supplemented with stem cell factor (SCF) and interleukin 3 (IL-3) and showed osteogenic differentiation potential.

As mentioned in section 2.3, there is a close interaction of all bone marrow cells *in vivo* either by cell - cell contact or by secretion of cytokines and other signalling molecules. These complex influences on MSC development make it very difficult to find the optimum culture condition and require time intensive investigations of these interactions. One possibility to create a culture condition, which offers a similar microenvironment to MSCs in stirred suspension cultures than the niche of MSCs in the bone marrow, is to culture whole bone marrow cells. So far, however, the suspension culture systems do not mimic the complex three-dimensional microenvironment of adult bone marrow. According to Zandstra and Nagy (2001), it is likely that these "adult" microenvironments are highly damped in their ability to initiate extensive stem cell self-renewal. They argue that, although many studies have shown that lineage commitment and mature cell function are stabilised by cell – cell and cell - extracellular matrix interactions, stem cell self-renewal may be more efficient in the absence of these signals. In fact, direct evidence of feedback-induced differentiation has been shown in embryonic stem cells (Klug *et al.*, 1996). Furthermore, the effect of mature cell populations or their products is well documented on haematopoietic stem cell differentiation (Cashman *et al.*, 1999).

In addition to the physicochemical parameters, it is clear that the protein microenvironment also has significant effects on stem cell development. Stem cell properties are the result of expression of a specific subset of genes. Changes in their expression will determine development from the stem cell state into functional cell lineages. Although there is still much to learn about the genes involved in such changes and how they are regulated, it is clear that stem cells interact with many molecules in their extracellular environment via transmembrane receptor complexes to maintain their viability, and to change their differentiated state. A key feature of any stem cell culture system is the combination of cytokines it delivers to the microenvironment of the cells, and how the concentrations of these cytokines and their associated receptors are maintained over time. All publicised experiments with MSCs in suspension cultures used quite expensive additives of cytokines for their

culture medium (Baksh *et al.*, 2003) (Chen *et al.*, 2006), which will raise a financial problem for the scale-up to large scale production of these cells. The dynamic nature of cytokine supplementation also needs to be taken into account, as the number of ligand-receptor complexes depends on 1) the number of unoccupied receptors available and 2) the ligand concentration, which both change with time (Lauffenburger *et al.*, 1987). In stem cell cultures, not only does the dynamic requirement of cytokines effect the MSCs development, but the production of cytokine and growth factors by the cells themselves are likely to effect the outcome of these cultures as well, as observed in other stem cell cultures e.g. on haematopoietic stem cells (Zandstra *et al.*, 1997 and 1999). Ideally, when critical numbers of particular cells are generated, the culture system may no longer require exogenous growth factors to maintain optimised MSCs expansion.

Summarising, the advantages of stirred suspension cultures over static monolayer cultures are promising. Not only can higher MSC numbers be obtained from stirred suspension cultures, but, more importantly, a suspension based proliferation is necessary for the application of bioreactors as culture vessels. Bioreactors allow control of the culture environment, and thereby enable the investigation of how different parameters effect the MSC proliferation and differentiation. The downside of stirred suspension cultures is the fact that expensive growth factors have to be added to the culture medium in order to achieve increase in MSC number. These growth factors will increase the production costs for MSCs at a large scale, and thereby may restrict the usage of these cells for medical applications. Furthermore, by stirring the culture medium, shear forces are exerted on the MSCs, and these shear forces can damage or kill the cells.

A compromise between stirred suspension culture and surface adherent culture system are solid or macroporous microcarriers. Solid microcarriers are made of cross-linked dextran, cellulose or polystyrene and support cell growth on their external surface, whereas macroporous microcarriers, typically made of gelatin, collagen or other soft material with high oxygen diffusion characteristics, have open pores to provide larger growth surface area (Bancel and Hu, 1996). MSCs are allowed to adhere to the microcarriers, which are then put into a stirred suspension culture to amplify the cells. The suspension culture with microcarriers is maintained

in a similar way than the suspension cultures of MSCs. Regular medium change provides the MSCs with enough nutrients, and stirring will keep the microcarriers in suspension (Schop *et al.*, 2008). The advantages of this culture system are the combination of adherent growth of MSCs on the surface of the microcarriers with a large scale expansion, considering the large number of microcarriers which can be suspended in the culture medium (Weber *et al.*, 2007). However, this system also combines the disadvantages of static monolayer cultures and stirred suspension cultures. Although the MSC expansion is supported by adherence to the surface of the microcarriers, the extensive mixing in the stirred suspension culture might still disturb the formation of a normal microenvironment which would be formed by those cells when grown on a stationary surface. In addition, any surface bound system has the disadvantage that trypsin treatment is necessary to detach the cells (Weber *et al.*, 2007). Hence, by using microcarriers in stirred suspension cultures to expand MSCs, both shear stress and chemical stress will be exerted to the cells.

2.6.3 Further culture systems

Single pass perfusion (SPP) is an alternative way to expand mesenchymal stem cells as monolayer cultures without the shear stress caused by any stirring system. In SPP cultures, cells are seeded into a chamber in which the culture medium is continuously replaced by fresh medium at a slow, controlled rate without disturbance or removal of cells. This allows a controlled exchange of nutrients and metabolic by-products and an optimal maintenance of the microenvironment. The beneficial effect of medium perfusion has been demonstrated on the improved productivity and viability of bone marrow cultures (Schwartz *et al.*, 1991), and also on the metabolic activity and growth factor production rates of marrow stromal cells (Caldwell *et al.*, 1991). The usage of the SPP technology led to the development of an automated perfused bioreactor system for the clinical-scale expansion of human primary cells (Lundell *et al.*, 1999). Bone marrow mononuclear cells cultured in a perfused bioreactor go through a major change in the composition of cell populations, resulting in a mixed lineage cell product, termed Tissue Repair Cells (TRCs), that has been used for patient-specific haematopoietic reconstitution (Pecora *et al.*, 200) (Stiff *et al.*, 2000). TRCs are a mixed cell population containing cells of haematopoietic, mesenchymal, and endothelial lineages.

Another culture method, named "pour-off culture", can be used to show that MSCs proliferate in suspension. It was applied by three different research groups to obtain bone marrow derived MSCs with high proliferation potential and full differentiation ability (Oreffo *et al.*, 1996) (Zhang *et al.*, 2009) (Baksh *et al.*, 2007). Pour-off cultures were first described by Oreffo *et al.* (1996) who used this culture system to examine the non-adherent bone marrow stromal fraction from human bone marrow samples. Pour-off cultures can be seen as a combination between static monolayer cultures and suspension cultures. Oreffo *et al.* (1996) cultured whole bone marrow cells in a cell culture dish for 24 hours before transferring the non-adherent cells into a new dish. Thus, only the non-adherent cells were kept in the cultures, whereas the adherent cells were used to analyse the colony forming potential of the non-adherent cells at the time point of re-plating. Zhang *et al.* (2009) applied the pour-off culture technique to establish the presence of non-adherent MSCs in adult bone marrow. They expanded bone marrow derived MSCs in pour-off cultures by seeding 1×10^7 whole bone marrow cells in a culture plastic dish and re-plating the non-adherent cells every 24 hours. An increase in MSCs number of 2.3 fold was achieved with this technique after only 4 days compared to the initial cell seeding density. The expanded MSCs were then used for transplantation to establish their migration and differentiation potential *in vivo*. Zhang *et al.* (2009) concluded that the adult bone marrow harbours pluripotent non-adherent MSCs which can migrate *in vivo* to any organ of the body through the circulation and, in the appropriate microenvironment, can adhere, proliferate and differentiate into specialised cells of the target tissue, and thus function in tissue regeneration. Baksh *et al.* (2007) also used this culture system to characterise suspension-derived MSCs for therapeutic strategies requiring systemic infusion. They found that the bone marrow contains a $CD49e^{low}$ subpopulation of MSCs, which are present in the non-adherent cell fraction and which are able to proliferate in suspension while maintaining a single and round morphology. However, Baksh *et al.* (2007) reported that the remaining adherent cells of the pour-off cultures did not form fibroblastic colonies when the non-adherent cell fraction was removed after 24 hours. Therefore, they concluded that the adherent cells were either a mature cell type which is not able to proliferate and form colonies, or they require some stimulation from the non-adherent cell types to initiate their cell cycle.

Pour-off cultures combine the advantages of the static monolayer cultures and the stirred suspension cultures but avoid the disadvantages of both. In other words, using pour-off cultures to expand MSCs from bone marrow cells, allows the cells from the adherent fraction of the bone marrow to adhere to a plastic surface whilst the non-adherent cells can stay and proliferate in suspension. Furthermore, this culture technique does not require the use of trypsin or of an agitation method, and therefore minimises the stress exerted on the cells. Thus, a more MSC friendly culture environment is created which supports the amplification of MSCs *in vitro*.
However, the disadvantages of the pour-off technique are the extremely work intensive handling of the cultures and the fact that no medium is changed during the whole culture period. Both will limit the potential for a large scale MSC expansion in pour-off cultures. In order to use the pour-off culture method to amplify large numbers of MSCs the automation of this technique including a regular medium change is necessary.

2.7 Conclusions

In this chapter, the wide field of stem cell research was briefly introduced by presenting a general background to the definition and classification of all stem cells with focus on one particular cell type, the mesenchymal stem cells (MSCs). Different *in vitro* expansion methods for MSCs are investigated in this thesis, and therefore the *in vivo* niches and several *in vitro* culture methods for these cells are described. The potential usage of MSCs in therapeutic applications such as tissue engineering and gene therapy is mentioned to explain the widespread interest in MSC research. By describing the complexity of the *in vivo* microenvironment of MSCs and the lack of definition for these cells, difficulties with the culture methods resulting from it are provided.

Despite the reported achievements in successfully expanding MSCs in suspension cultures, there are still many obstacles to overcome before MSCs can be used for regenerative medicine on a wide spread base. For instance, the addition of cytokines to the culture medium in MSC suspension cultures, which was reported by all research groups, will pose an enormous cost factor and thus, the use of these cells

will be less appealing. Therefore, the optimisation of the culture conditions is still of great importance. Avoiding the addition of growth factors is just one way to optimise the cultivation method. The aim of this study is to find a cheap and practical way to produce large numbers of MSCs by expanding them in large scale cultures. Based on the literature available, the following chapters will introduce three methods for MSC culture techniques. The results obtained from these techniques will be investigated and compared to each other and to the literature in detail. Table 2.1 summarises the advantages and disadvantage of these culture methods for MSC expansion as described in the literature.

Table 2.1: Summary of the advantages and disadvantages for *in vitro* MSC expansion in static monolayer cultures, suspension cultures or pour-off cultures as described in this chapter.

Culture method	**advantage**	**disadvantage**
static monolayer cultures	• well established • similar conditions to natural niche • easy handling	• lack of mixing • no online monitoring / control • poor reproducibility
suspension cultures	• large expansion rates • possibility for online control • easy scale-up	• need of growth factors • shear forces
pour-off cultures	• suspension based proliferation • supporting adherent cells • large expansion rates	• extremely work intensive • no medium change • no online control

Chapter 3

General Materials and Methods

3.1. Introduction

In this chapter, the general materials and commonly used methods for the experiments throughout all chapters are described and explained. The source of the cells and their extraction methods are detailed. Assays and techniques to quantify cell number, cell viability and colony forming behaviour are described, and different ways to measure cell differentiation are explained. However, materials and specific cell culture methods relating to individual culture techniques are described in the relevant chapter dealing with the particular technique.

3.2. Reagents and consumables

Unless stated otherwise, all general chemicals were purchased from Sigma-Aldrich (Poole, Dorset U.K.), tissue culture media, foetal calf serum and phosphate buffered saline were purchased from Lonza (Wokingham, U.K.), cytokines from PeproTech EC Ltd (London, U.K.), and plasticware from Nunc (Nottingham, U.K.) or Greiner Bio One (Gloucester, U.K.).

3.3 Culture medium

Two different standard media, Dulbecco's Modified Eagles Medium (DMEM) and MyeloCult® medium, were used to cultivate bone marrow derived rat MSCs *in vitro*. High glucose (4500 mg/l) DMEM with non-essential amino acids consists of 2 mM Glutamax, 2 mM penicillin / streptomycin (PS) and 10% foetal calf serum (FCS). MyeloCult® medium consists of Alpha Minimum Essential Medium (α-MEM) without L-glutamine, supplemented with 12.5% horse serum, 12.5% FCS, 2 mM Glutamax, 2 mM penicillin / streptomycin, 0.16 mM i-Inositol, 0.016 mM folic acid and 0.1 mM 2-mercaptoethanol. Both media were further modified in several experiments as determined in chapter 4, 6 and 7 (section 4.2, 6.2 and 7.2).

3.4 Isolation of rat bone marrow cells (Scutt et al., 2003)

The rat bone marrow cells (BMCs) were obtained from male Wister rats (200 – 250g) from the Field Laboratories at the University of Sheffield. The rats were killed by cervical dislocation and sprayed with 70% Industrial Methylated Spirit (IMS) to kill fungal spores etc, before the dissection. The back legs were removed cleanly at the hip joint, transported to the cell lab and all soft tissue were removed from the bones under sterile conditions. The growth plates were removed from both the femur and the tibia. Using a bone cutter, the tibia at the tibial/fibula junction and the femur a little below the hip joint were cut. The cut bones were placed cut side down, on inserts in 1.5 ml Eppendorf tubes and were spun at 2000 rpm (900 g) for 1 minute. The inserts help to ensure that the bone marrow is deposited in the bottom of the Eppendorf tube. The bones and inserts were removed and the marrow was resuspended in 1 ml culture medium which was then made up to 10 ml per bone in culture medium, forcing the cells through a 1000 µl pipette tip to break down the big cell clusters. Figure 3.1 illustrates the workflow beginning with the Wistar rats, then showing the removed femurs and tibias and finally the flushed out bone marrow cells.

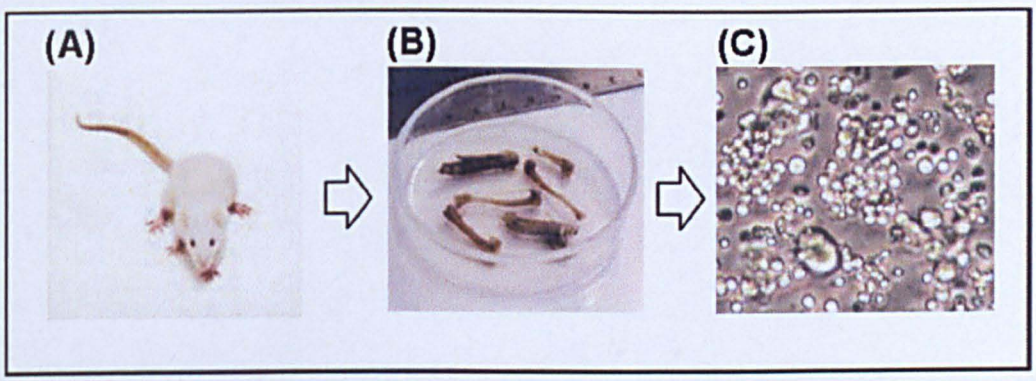

Figure 3.1: Key stages in the isolation of rat bone marrow cells. (**A**) 200 – 250 g Wistar rats were used to obtain (**B**) the femurs and tibias, from which in turn (**C**) the bone marrow cells were collected.

3.5 Cell count and cell viability

The determination of the overall health of a cell culture requires measurements of both the total cell concentration, and the ratio of viable and dead cells, i.e. the viability. This data is essential to monitor the cells and find the optimum culture conditions. Three methods were used to determine the cell viability in the samples taken from all the cultures and they are discussed in turn below.

3.5.1 Haemocytometer

MSCs in solution have a diameter of about 20 µm, therefore they are big enough to be analysed with a light microscope and a haemocytometer using the trypan blue exclusion method (Guo *et al.*, 2002). When cells die, their membranes become permeable allowing for the uptake of the trypan blue dye. As a result, the dead or non-viable cells appear to be blue under a light microscope. Counting both stained and unstained cells allows the investigator to determine the viability of a cell culture.

In this studie, 100 µl of a sample, taken from a cell culture, was mixed with 100 µl of trypan blue (Sigma 93590) to stain the dead cells. The solution was incubated for 5 minutes at room temperature before 10 µl of the stained cell solution were transferred onto a haemocytometer. The stained and unstained cells in the large middle square (consisting of 25 small squares) of the counting grid were counted with an inverted light microscope from Novex Kolland (K – Range) using a 100 x magnification. The volume between coverslip and middle square equals 0.9 mm^3. Therefore multiplying the counted cell number with 2 x 10^4 will result in the total cell concentration [cells/ml] of the sample taken from the culture before it was diluted with trypan blue. For example, if the analysis of a 100 µl sample taken from a 12 ml cell solution resulted in 30 counted cells, the total cell concentration in the 12 ml cell solution was 6 x 10^5 cells/ml. The total cell number in the 12 ml solution was then 7.2 x 10^6 cells.

30 (cells counted) x 2 (dilution factor with trypan blue) x 10^4 (volume correction) = 6 x 10^5 cells/ml in culture.

6×10^5 (cells/ml in solution) x 12 (ml solution)
$= 7.2 \times 10^6$ (total number of cells)

3.5.2 ViCell™ XR Cell Viability Analyser

A similar way to count cells and measure their viability is to use the video imaging system ViCell™ XR Cell Viability Analyser (Beckman Coulter®). It automates the trypan blue dye staining protocol by taking pictures from stained cell samples which are automatically recognised and counted by the ViCell™ XR software. The software also determines which cells have or have not absorbed the trypan blue according to the gray scale of the cells in the pictures. Cells absorbing the trypan blue dye appear darker, hence have lower gray scale values.

Before the first use, general information about the cell type (e.g. minimum and maximum cell size) had to be added into the database of the ViCell™ XR software. Then, 0.5 ml of the cell sample was pipetted into a sample cup provided with the ViCell™ system. The cup was placed into the ViCell™ analyser and the sample ID was entered into the computer. For each sample, 3 replicates were prepared. Mixing the cells with the trypan blue staining solution and sample loading into the counting chamber were done automatically by the machine. Results included information about the total cell count, the viability and the average cell size.

3.5.3 Guava Personal Cell Analysis (PCA) System

Viability tests based on the principle of flow cytometry were carried out on the Guava Personal Flow Cytometer (Guava Technologies, Hayward, CA) using the Viacount software package. All cells to be analysed were stained with the ViaCount™ reagent, which consist of a combination of two fluorescent nucleic acid dyes. These dyes stain viable and non-viable cells differently according to the permeability of the cell membranes. The nuclear dye stains only nucleated cells,

while the viability dye stains dying cells. Both dyes are detected at different wavelength thus enabling measurement of both live and dead. Debris is excluded from results based on negative staining with the nuclear dye.

Prior to the analysis, the cell samples were prepared by washing in 200 µl phosphate buffered saline (PBS) and disrupting any cell clumps using a 200 µl pipette tip. A 50 µl aliquot of the cell sample was diluted 1:6 with the Guava ViaCount™ reagent and incubated for 5 minutes at room temperature. Then the samples were loaded into the Guava Personal Flow Cytometer, the dilution factor was entered into the computer and total cell concentration as well as the percent viability were measured with the Guava ViaCount™ software package.

3.6 Fibroblastic colony forming unit (CFU-f) assay

This assay is based on a technique reported by Friedenstein *et al.* (1974). It measures the colony forming ability of single cells by allowing them to adhere to a plastic surface and proliferate to form fibroblastic colonies. From all bone marrow cells, only MSCs are known to have this fibroblastic colony forming ability. Therefore the CFU-f assay is the traditional method used to determine MSC numbers in BMC suspensions. Furthermore, by checking the size of a colony as well as the shape and size of the cells, which are forming the colony, it's possible to qualitatively describe the stem cell character of the MSCs.

All CFU-f cultures were conducted in triplicate with either 0.3 ml freshly harvested BMCs or about 4 x 10^3 MSCs in 56 cm^2 cell culture dishes. They were incubated in 10 ml DMEM or MyeloCult® medium at 37°C in an atmosphere with 7.5% CO_2. The first medium change was after 5 days and thereafter it was changed twice weekly for up to 14 days. The cultures were then stopped by washing with Phosphate Buffered Saline (PBS) and fixing with 70% Industrial Methylated Spirit (IMS). After fixing, the plates were washed 3 times in tap water.

3.6.1 Colony staining and imaging

Fixed colonies were stained for 30 minutes with borate buffer at a pH 8.8 containing 1% 1,9-Dimethyl-Methylene Blue (Sigma 341088). The plates were then washed in tap water until no more dye was eluted and allowed to air-dry overnight. To count the colonies, the dishes were photographed using a Pixera Professional digital camera at a resolution of 800/600 pixels. Due to the uneven surface of the individual colonies, the image analysis software 'Gene Tools' (Syngene, Cambs.) could recognize larger colonies as several distinct colonies. To prevent this, differences between neighbouring pixels were eliminated and isolated pixels, whose values differ from those of their surroundings, were removed. This may lead to data loss in not well or unstained areas of the CFU-f plates. The pictures were then converted into an 8-bit greyscale TIFF image using Adobe Photoshop. Gene Tools image analysis software was applied to obtain the number of colonies per dish and the mean colony size. Only colonies of 20 to 10000 pixels and having an intensity of 20 grey levels above the background were recognised (Dobson *et al.*, 1999). Figure 3.2 shows the workflow of the colony analysing process. First a picture was taken from a stained CFU-f culture, which was then converted into 8-bit greyscale TIFF image. This grey image was used with the Gene Tools image analysis software to obtain the colony number on the plate.

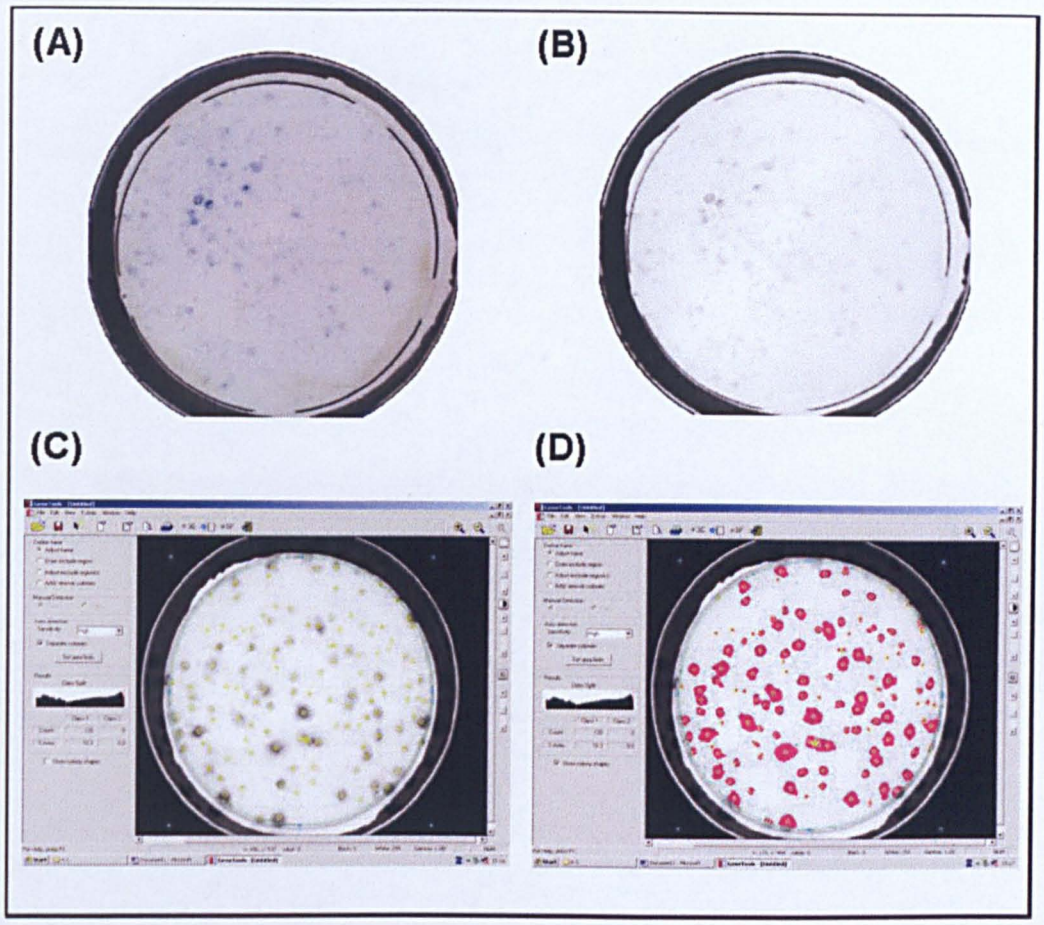

Figure 3.2: (A) A methylene blue stained CFU-f culture dish. (B) The same culture dish as in A but converted into an 8-bit greyscale TIFF image. (C) and (D) Screen shots of the Gene Tools image analysis software, showing how the colony numbers on the plate were obtained.

However, the counted colony number does not equal the number of CFU-fs in a sample taken from the suspension or monolayer culture, because the samples were diluted in order to start every CFU-f culture always with 4×10^3 cells. To acquire the total number of the CFU-fs in a suspension or monolayer culture, at the time point the sample was taken, the counted colony number had to be multiplied by the dilution factor and a factor correcting the relation between sample and culture size. For example, if 1 ml sample was taken from a 40 ml suspension culture and diluted 3 times, in order to start the CFU-f assay with 4×10^3 cells, the counted colony number (let's say 120 colonies per dish) has to be multiplied with 3 x 40.

120 (colonies) x 3 (dilution factor) x 40 (culture size)
= 14,400 CFU-f (in the suspension culture)

3.7 Differentiation assays

To demonstrate the differentiation potential of the cultured MSC populations, the cells of interest were differentiated into osteoblasts and adipocytes by culturing them in standard osteogenic and adipogenic medium as detailed below (Maniatopoulos *et al.*, 1988) (Janderova *et al.*, 2003).

3.7.1 Osteogenic differentiation

Osteogenic differentiation was demonstrated by staining three different markers present in osteoblasts but not MSCs. Alkaline phosphatase was stained with naphtholphosphate and fast red, calcium was stained with alizarin red and collagen was stained with Sirius red (Drury *et al.*, 1967).

Osteogenic cultures were conducted in triplicate in 56 cm^2 cell culture dishes. They were started with either 0.3 ml freshly harvested BMCs or about 4 x 10^3 MSCs taken from a suspension culture (chapter 6.2) or a monolayer culture (chapter 4.2) and incubated in 10 ml DMEM or MyeloCult® medium for approximately 4 to 5 days. At the first medium change the culture medium was replaced by 10 ml osteogenic medium consisting of standard DMEM with the addition of 10^{-8} M dexamethasone and 50 µg/ml 2-ascorbate-phosphate (Maniatopoulos *et al.*, 1988). For MSCs cultured using the pour – off method (chapter 7), cells were differentiated by adding osteogenic medium directly into the culture dish after the pour off. After incubating for 14 days with media change twice a week, the cultures were washed with PBS and fixed with 70% IMS. After fixing, the plates were washed 3 times in tap water and were ready for staining.

3.7.1.1 Alkaline phosphatase assay

The enzyme alkaline phosphatase can hydrolyse organic phosphate under alkaline conditions and this phosphate can then combine with free calcium ions to form insoluble calcium phosphate. The presence of the enzyme alone can already be considered as a marker for osteoblasts. However, monocytes, embryonal stem cells

and gut epithelial cells are also known to express this enzyme (Heinemann *et al.*, 2000) (O'Connor *et al.*, 2008) (Vaishnava and Hooper, 2007). To confirm the differentiation into an osteoblast, the presence of calcium and collagen in the osteogenic cell has to be shown as well. The alkaline phosphatase assay measures the breakdown of the substrate naphthol phosphate AS-BI under alkaline conditions to insoluble naphthol. This can be stained by the dye fast red to produce a red colour.

Fixed and washed colonies were used for alkaline phosphatase staining. A solution of 20 mM Tris, pH 8.5, containing 50 µg/ml naphthol phosphate AS-BI (Sigma N2125) and 1 mg/ml Fast Red TR Salt hemi(zinc chloride) (Sigma F8764) was freshly prepared and added to the cultures. Even though the staining takes place within hours, the plates were incubated overnight at room temperature before the solution was removed, the plates were washed with tap water and photographed as soon as they were dry. All colonies producing alkaline phosphatase appeared red. Gene Tools image analysis software was applied to count them as described in section 3.6.1. After the pictures were taken, the colonies were destained with 0.2 M sodium hydroxide in 70% IMS to be ready for the calcium staining assay.

3.7.1.2 Calcium staining assay

Alizarin Red is widely used to determine the presence of calcium deposition in osteogenic cells by forming an Alizarin Red S-calcium complex. However, it also can interfere with magnesium, manganese, barium, strontium, and iron but these elements usually do not occur in sufficient concentration (Drury *et al.*, 1967).

To stain the osteogenic cells for calcium, 1 mg/ml Alizarin Red S (Sigma A5533) was dissolved in distilled water and the pH was adjusted to pH 5.5 with ammonium hydroxide. Cells were incubated in this solution overnight at room temperature. The next day, the stain was removed; the plates were washed and photographed before they were destained in 5% perchloric acid to be ready for the collagen staining assay. The pictures were analysed using the Gene Tools image analysis software (section 3.6.1) to count any dark red stained colonies which have produced calcium.

3.7.1.3 Collagen staining assay

Sirius Red stain was used to show any collagen formation in the differentiated cells. 1 mg/ml Sirius Red, also known as Direct Red 80 (Sigma 43665), was added to saturated picric acid. The osteogenic cells were incubated overnight in the Sirius Red stain, before they were washed. Photographs were taken from the plates. The colonies with cells containing collagen appeared red, and were counted using the Gene Tools image analysis software (section 3.6.1). Subsequently, cells were destained with 0.2 M sodium hydroxide/methanol (50:50).

3.7.2 Adipogenic differentiation

Adipogenic differentiation was demonstrated by staining the lipid vesicles with Oil Red O. The lipids were stained by Oil Red O because the solubility of the dye in the lipoid substances is greater than the solubility of it in the hydroalcoholic dye solvents. To avoid the staining of non-adipogenic cells, Oil Red O has to be dissolved in isopropanol.

An Oil Red O stock was prepared by dissolving 0.7g Oil Red O (Sigma O-0625) in 200 ml 100% Isopropanol, stirring it slowly overnight and filtering it with a 0.2 µm filter. The stock solution was kept at 4°C for up to half a year. To obtain an Oil Red O working solution, 6 parts of the Oil Red O stock were mixed with 4 parts distilled water. This mixture had to be kept for 20 minutes at room temperature and filtered again with a 0.2 µm filter before it was ready to use.

Adipogenic cultures were conducted in triplicate in 56 cm^2 cell culture dishes. They were started with either 0.3 ml freshly harvested BMCs or about 10 x 10^3 MSCs taken from a suspension culture (chapter 6.2) or a monolayer culture (chapter 4.2) and incubated in 10 ml DMEM or MyeloCult® medium for 8 days. At the first medium change the culture medium was replaced by 10 ml adipogenic medium consisting of standard DMEM with the addition of 10^{-6} M dexamethasone, 10 µg/ml Insulin, 500 mM isobutyl-methylxanthine and 0.2 mM indomethacin. For MSCs cultured using the pour – off method (chapter 7), cells were differentiated by adding

adipogenic medium directly into the culture dish after the pour off. After incubating for at least 3 weeks with media change twice a week, the cultures were confluent and fixed in 4% Paraformaldehyde. To fix the cells, they were washed in PBS, about 5 ml Paraformaldehyde was added to the dish and incubated at least for 1 hour up to overnight at room temperature. After all the Paraformaldehyde was removed, the cells were washed in 60% isopropanol and 5ml of the Oil Red O stain (working solution) was added when the dishes were dried. The cultures were stained for 1 hour at room temperature, and the stain was washed off under running water before pictures were taken of the differentiated cells with a digital camera. Adipocytes were identified on the basis of lipid droplets, which had formed within the cells, stained red with the Oil Red O stain.

3.8 Data handling

Three biological replicates of all suspension culture and pour – off experiments were performed. In addition, each result of cell or CFU-f analyses was calculated as a mean +/- standard deviation (S.D.) of triplicate counts (technical replicates). The data in the figures are presented as mean +/- standard deviation of the biological replica. However, if biological replica were not available (e.g. due to contamination problems), data are presented as a mean +/- standard deviation of the technical replica. Significance between 2 treament groups was asseed by using an unpaired Student's T test, whereas the significance between more than 2 treatment groups was assessed by using analysis of variance (ANOVA). Differences are considered significant at a probability of <0.05.

Chapter 4

Growth of Mesenchymal Stem Cells in Static Culture Systems

4.1 Introduction

Friedenstein *et al.* (1968) discovered that bone marrow contains a cell type capable of self renewal and differentiation into osteoblasts. These cells could be expanded *in vitro* by allowing them to adhere to a plastic surface, where they would grow as a monolayer covered with culture medium. Nowadays these cells are referred to as mesenchymal stem cells (MSCs). Static monolayer cultures in plastic flasks (T-flasks), specially treated to increase the adherence of cells, are still the standard method used to expand MSCs *in vitro* and are the subject of this chapter.

The most widely used culture medium for MSCs in static cultures is DMEM with 10% serum (Bruder *et al.*, 1997) (Guo *et al.*, 2001) (Mok *et al.*, 2003). However, to compare the results from the experiments in suspension and pour-off cultures (see chapters 6 and 7) with the results from the standard experiments in this chapter, the same culture medium from the suspension and pour-off cultures, MyeloCult® medium with various additives, was also used for the monolayer cultures. The results from this chapter were taken as a control to determine which system worked best, the traditional monolayer culture method, the stirred suspension culture method or the "pour-off" culture method. Furthermore, testing new media constituents in a well studied and well defined culture system also enables the effect of these compounds on the proliferation and differentiation behaviour of MSCs to be investigated.

One of the medium additives was Pluronic F-68, which is known to bind to the cell membrane and thereby strengthen it (Zhang *et al.*, 1992). Since it was mainly used in stirred suspension cultures, more details about PluronicF-68 will be given in section 6.1. In order to determine a potential side effect of Pluronic F-68 on the membrane proteins of MSCs, the infra red spectra from cells in static cultures containing Pluronic F-68 were compared to the infra red spectra from cells in static cultures without Pluronic F-68 using Fourier Transform Infrared Spectroscopy (FTIR). A change in the membrane protein quantity can have an effect on the infra red spectrum of the cells, which can be measured by FTIR (Cricenti *et al.*, 2003). FTIR identifies types of chemical bonds in molecules which absorb infrared light by the production of an infrared absorption spectrum. Absorption of infrared radiation by a typical organic molecule results in the excitation of vibrational, rotational and bending

modes. The absorption will be detected as a symmetric interferogram and translated into a spectrum by fourier transformation. Although FTIR is not a method to detect changes of specific membrane proteins, it is a simple and fast analysis technique which can confirm the presence or absence of organic compounds in a sample. Furthermore it has been shown that it is possible to obtain a FTIR spectrum of MSCs, and use this information to identify the differentiation state of individual human mesenchymal stem cells (Krafft *et al.*, 2007)

Another fast and easy way to detect an effect of Pluronic F-68 on the membrane proteins of MSCs grown in static cultures is the measurement of their electrophoretic mobility (EPM). Cells in solution have a surface charge, which is either negative or positive and a diffusion layer, which electrically screens the surface charge (Dukhin and Semenikhin, 1970). If an electric field is applied both surface charge and diffusion layer exert opposite directed electrostatic forces on the cell (Dukhin and Semenikhin, 1970). Since the force of the surface charge is stronger, the cell moves in the direction of this force, resulting in what is called electrophoretic mobility. So far several general experiments to determine the EPM of some types of mammalian tissue cells as well as tumour cells have been conducted (Simon-Reuss *et al.* 1964) (Vassar, 1963), and it was found that the electrophoretic properties of cells were mainly caused by ionogenic groups present at the cell peripheries. More interestingly, it has been shown that EPM can be used to discriminate between MSCs and cells with similar surface antigens (Lee *et al.*, 2006). Lee *et al.* (2006) stated that although cell electrophoresis cannot determine the specific surface protein, it can reflect the net surface charge density of the cell membrane. A modification of the functional groups of peripheral membrane proteins can change this net surface charge which then can be measured.

4.2 Materials and methods

The general methods for cell culture have already been given previously in chapter 3. In this chapter methods are described for the culturing and expansion of rat bone marrow derived mesenchymal stem cells specifically in static culture systems. Two different basic culture media were applied, Dulbecco's Modified Eagle Medium (DMEM) and MyeloCult® medium, and both were supplemented with various additives such as Pluronic F-68, dexamethasone, Interleukin-3 (IL-3) and Stem Cell Factor (SCF). Furthermore, the use of additional analysing techniques such as FTIR and EMP are also described.

4.2.1 Expansion of Mesenchymal Stem Cells in static culture

Mesenchymal stem cells were derived from rat bone marrow as described in chapter 3 (section 3.4). 10 ml of freshly harvested bone marrow cells (BMCs) were suspended in 10 ml culture medium and seeded into a T75 flask, having a final density of about 1 x 10^6 cells/ml. Cell culture medium was either normal DMEM, DMEM with 0.05% Pluronic, DMEM with 10^{-8} M dexamethasone, normal MyeloCult® medium, MyeloCult® medium with 10^{-8} M dexamethasone or MyeloCult® medium with 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF. The cells were incubated at 37°C in an atmosphere of 7.5% CO_2. The first medium change was after 5 days and thereafter it was changed twice weekly until ~90% confluence was reached. Then the cells were passaged and again expanded until ~90% confluence. To detach the adherent cells, the medium was removed and the flask was washed with phosphate buffered saline (PBS) before 3 ml of 0.25% trypsin / 1 mM EDTA solution was added. The culture was incubated for 5 minutes at 37°C. The detachment of the cells was carefully monitored by phase microscopy using an inverted microscope (Novex Kolland). The sides of the flask were tapped to help loosen the cells. To stop the incubation, 9 ml of culture medium, containing 10% foetal calf serum (FCS), were added and a 100 µl sample was taken to determine the cell number in the suspension using a haemocytometer. The detached cells then were transferred into new sterile T75-flasks, which were seeded each with 1 x 10^6 cells. A smaller sample of the cells was used to start fibroblastic colony forming unit (CFU-f)

and differentiation assays as described in chapter 3 (section 3.6 and 3.7). However, differentiation assays started with cells from passage level 3 and 4 were seeded at a higher cell density than described in section 3.8. Instead of 4 x 10^3 MSCs per dish, about 8 x 10^4 cells were used. To calculate the fold increase in total cell number, the counted cells were compared to the number of adherent cells in the bone marrow sample used to start the first culture. This number was determined by using 0.3 ml from the bone marrow cells to start a CFU-f assay and count the formed colonies (see section 3.5.1 and 3.6.1 for haemocytometer and CFU-f assay). Figure 4.1 shows the schematic diagram of the workflow.

For example, the CFU-f assay, started with 0.3 ml bone marrow cells, had 250 colonies and 75 cells were counted with the haemocytometer in the 100 µl sample taken when passaging the cells.

The initial number of colony forming cells in 10 ml bone marrow was 8 x 10^3 cells.

250 (colonies) / 0.3 (volume to start CFU assay) x 10 (volume to start static culture) = 8 x 10^3 MSCs (initial MSCs number in static culture)

The number of MSCs when the culture was passaged was 13.5 x 10^6 cells.

75 (cells counted) x 2 (dilution factor with trypan blue) x 10^4 (volume correction) = 1.5 x 10^6 cells/ml detached MSCs .
1.5 x 10^6 (cells/ml detached MSCs) x 9 (ml suspension) = 13.5 x 10^6 (total number of MSCs)

The fold increase in this case was 1.6 x 10^3

13.5 x 10^6 (MSC number when cells were harvested) / 8 x 10^3 (initial MSC number) = 1.6 x 10^3

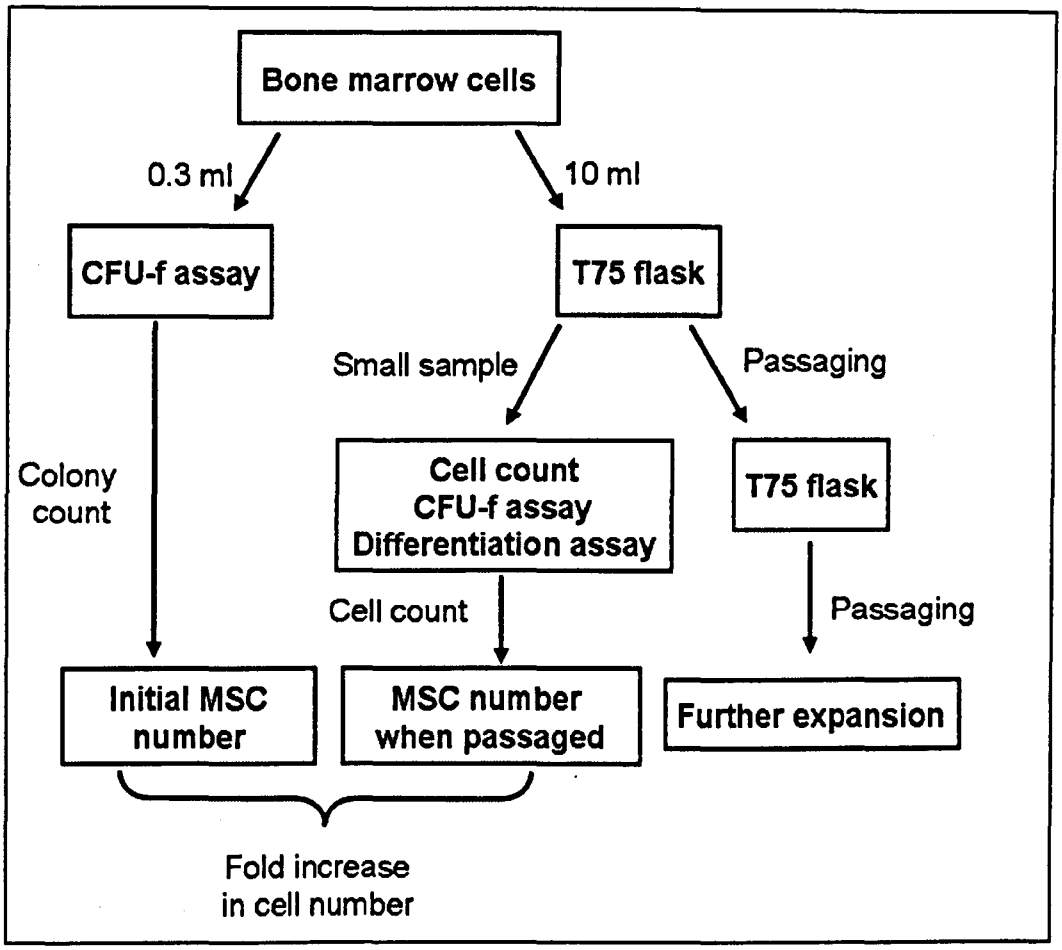

Figure 4.1: Diagram of the workflow for static monolayer cultures of MSCs.

4.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

The MSCs used for FTIR analysis were expanded in cell culture flasks as monolayer cultures for 2 passages using DMEM as control and DMEM with 0.05% Pluronic as a test condition. When cultures were 90% confluent after the second passage, cells were trypsinised, centrifuged at 1200 rpm for 5 minutes to remove the trypsin solution and resuspended in 1 ml normal DMEM. 100 µl of this cell solution was made up to 1 ml with DMEM before placed on a calcium fluoride (CaF_2) glass window. The MSCs on the CaF_2 window were then placed in a cell culture dish, transferred to an incubator and cultured overnight at 37°C and 7.5% CO_2, to allow the cells to attach to the glass surface. Care was taken that the MSCs did not dry out. Just before the FTIR analysis the CaF_2 window was taken out of the incubator, washed with phosphate buffered saline (PBS) and transferred to the FTIR

spectrometer. FTIR measurements were performed on a Perkin Elmer Spectrum One Fourier transformation infrared spectrometer. For liquid samples in transmission mode, the cells were monitored using a liquid infrared flow cell (Sigma-Aldrich) with CaF_2 windows. A total of 100 scans with a resolution of 4 cm^{-1} and wave number range from 4000 to 950 cm^{-1} were collected for the samples in transmission mode using the flow cell. The spectra of PBS were used as a background and the baseline shift of the spectra was corrected using the Spectrum One software (Perkin-Elmer). Micro-FTIR spectra images were also collected on a Perkin Elmer Spotlight FT-IR Imaging System over the 4000-700 cm^{-1} wave number range, taking eight (8) scans per pixel at a resolution of 4 cm^{-1} and a beam diameter of 6.25 μm.

4.2.3 Electrophoretic mobility of Mesenchymal Stem Cells

The electrophoretic mobility is only a very small movement, which can be detected with Phase Amplitude Light Scattering (PALS) (Tscharnuter, 2001) using for example the zetaPALS instrument (Brookhaven Instruments Corporation). The zetaPALS determines zeta potential and electrophoretic mobility measuring small phase shifts in the scattered light that arise due to the movement of particles in an applied electric field. The measured phase change is proportional to the change in the position of the particles. Although this machine is more commonly used to determine the zeta potential of small colloidal samples (Najafi *et al.*, 2007), it also should be able to be applied to larger objects such as MSCs (Zhang *et al.*, 2009).

MSCs used for zetaPALS analysis were cultured in cell culture flasks as a monolayer for either 2 or 4 passages using DMEM as control and DMEM with 0.05% Pluronic as test condition. When the cultures were 90% confluence after the second passage, cells from one T75 – flask were harvested using trypsin/EDTA, centrifuged at 1200 rpm for 5 minutes to remove the trypsin solution, washed 2 times in PBS and split into 4 aliquots before finally being resuspended in 1 ml of running buffer (10mM, 30mM, 60mM and 100mM of either PBS or potassium chloride). The cell concentration in each aliquot should be around 5 x 10^5 cells. A 10 μl aliquot of MSCs resuspended in 0.1 M PBS and in 0.1 M potassium chloride (KCl) was taken out to analyse the cell viability using the trypan blue staining method to make sure that the cells do not break apart in the buffer conditions the zetaPALS analysis. The

experiment was carried out at a frequency of 2.0 Hz and a voltage of 2.5 V. Each sample was analysed 10 times to get a mean value for the electrophoretic mobility.

4.3 Results and discussion

The following results demonstrate the expansion capacity of MSCs in static cell cultures. The total cell number of each monolayer culture was monitored over a period of 28 days (5 passages) by analysing the cell samples with the methods described in section 3.5 when the cultures were passaged. To determine the stem cell character of the expanded MSCs, cell samples were taken at each passage and analysed using CFU-f and differentiation assays. The CFU-f assay was applied to measure the number of colony forming cells at each passage level of the monolayer cultures. In Figures 4.3 and in Figure 4.4 plates from the CFU-f assays with cells expanded in DMEM and MyeloCult®, respectively, are shown as an example for all conducted assays in this thesis. The information obtained from the CFU-f and the differentiation assays for this chapter is summarised in Table 4.3 in the conclusion section of this chapter. The differentiation assay was used only in a qualitatively way to show the presents of MSCs with the ability to differentiate into osteoblasts or adipocytes. The reason, that the quantity of these cells was not counted, was the difficulty in harvesting a sufficient number of MSCs with these abilities. This was less of a problem for the static culture method than it was for the suspension culture (chapter 6) and pour – off culture method (chapter 7). The effect of different basic culture media (DMEM and MyeloCult® medium) as well as different medium additives (pluronic, dexamethasone, interleukin-3 (IL-3) and stem cell factor (SCF)) on the proliferation capacity and the differentiation potential of MSCs in monolayer cultures was assessed. A possible effect of Pluronic F-68 on the cell membrane was analysed with FTIR spectroscopy and measurements of the electrophoretic mobility of MSCs were made.

4.3.1 DMEM versus MyeloCult® medium in static monolayer cultures

A commonly used and cheap (about £30 / litre) culture medium for MSC expansion *in vitro* is DMEM containing 10% foetal calf serum, glutamine and penicillin / streptomycin (Bruder *et al.*, 1997). MyeloCult® medium compared to DMEM is considerably more expensive (about £70 / litre), contains more serum (25%), more amino acids and vitamins (L-ascorbic acid, D(+)biotin and D-pantothenic acid). For expansion of MSCs MyeloCult® is used as culture medium in stirred suspension cultures (Chen *et al.*, 2006) (Baksh *et al.*, 2003). The experiments in this thesis conducted in static cultures with DMEM and MyeloCult® medium allowed for a comparison of the effect of these two media on the growth behaviour of MSCs and enabled an assessment of a possible beneficial influence of MyeloCult® medium in a well established culture system.

MSCs expanded in MyeloCult® medium grew faster than comparable cultures of MSCs kept in normal DMEM. In order to passage both set of cultures at the same time, and thus take cell samples to count the total cell number in both cultures at the same time points, the MyeloCult® cultures were seeded at a slightly lower starting cell density after every passage (about 0.7×10^6 cells/ml) and passaged after 100% confluence. The fold increase in total cell number after 9 days, at the first passage, in both cultures was about the same, 0.9×10^3 fold in DMEM and 1.0×10^3 fold in MyeloCult® medium (Figure 4.2). However at the end of the experiment, after 29 days (5 passages) the fold increase in the MyeloCult® cultures was clearly higher, 3.9×10^5 fold, compared to 0.2×10^5 fold ($p = 0.001$) in DMEM cultures. That means, for the DMEM cultures, starting with a bone marrow sample which contained for example 3400 plastic adherent cells, the final total cell number would be about 0.07×10^9 cells, whereas in the MyeloCult® cultures, the final total cell number would be about 1.3×10^9 cells, if started with the same bone marrow sample.

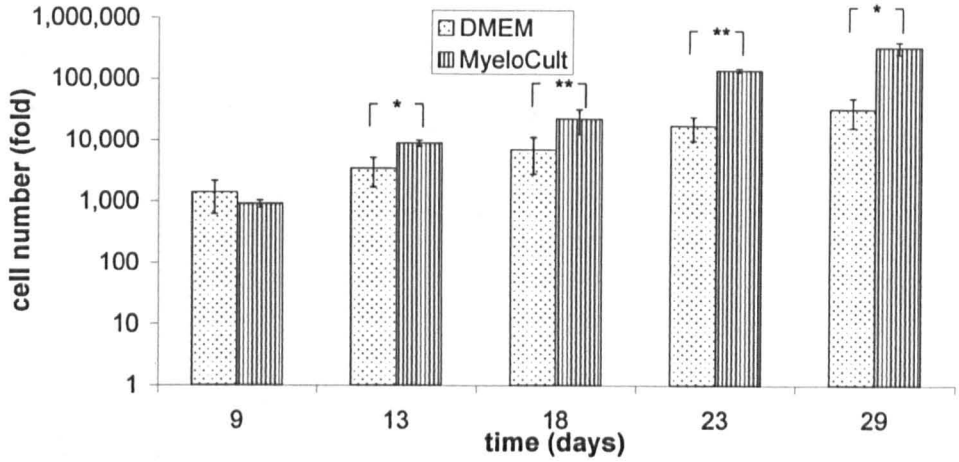

Figure 4.2: Increase in total cell number verses time. Cells were derived from rat bone marrow and expanded in static cultures with either DMEM or MyeloCult® medium. Results for the DMEM culture are mean +/- S.D. from 3 different cultures whereas results for MyeloCult® cultures are mean +/- S.D. from 3 cell counts from just 1 culture due to contamination problems. *$p < 0.01$; **$p < 0.05$, DMEM compared with MyeloCult®

Despite this massive increase in total cell number for both cultures, and the significant ($p = 0.001$) beneficial effect of the MyeloCult® medium on the cell proliferation, the more interesting question was if all these cells still retained their full differentiation potential. Therefore, cells amplified in DMEM and MyeloCult® medium were used for osteogenic, adipogenic and colony forming (CFU-f) assays. The colony forming behaviour is an indicator of the stem cell character of plastic adherent MSCs. As displayed in the Figures 4.3 and 4.4 cells expanded in static cultures with DMEM for as long as 2 passages formed recognisable colonies during the CFU-f assays. The colonies were rather small and did not stain very well. MSCs passaged more than twice formed even smaller colonies, which were barely visible on the plates. In contrast, the stained cells in the CFU-f assays started with MSCs derived from MyeloCult® monolayer cultures stained stronger and were easier to identify (Figure 4.4). Due to cell growth, a monolayer rather than single colonies were formed in the dishes from the passage level 1 and 2, but after 3 passages single colonies were recognisable. However, using the colony detection software with the parameters mentioned in chapter 3.6.1, colonies were detected on all plates.

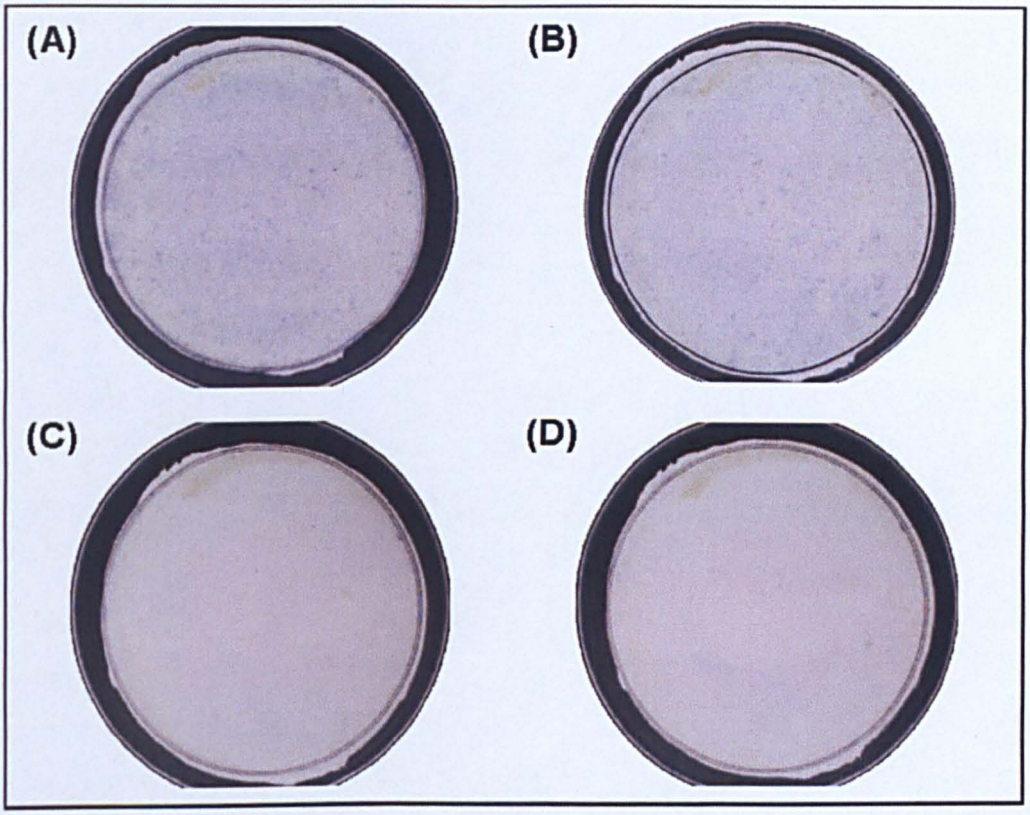

Figure 4.3: Methylene blue stained CFU-f dishes started with MSCs from monolayer cultures with DMEM, after one passage (A), two passages (B), three passages (C) and four passages (D).

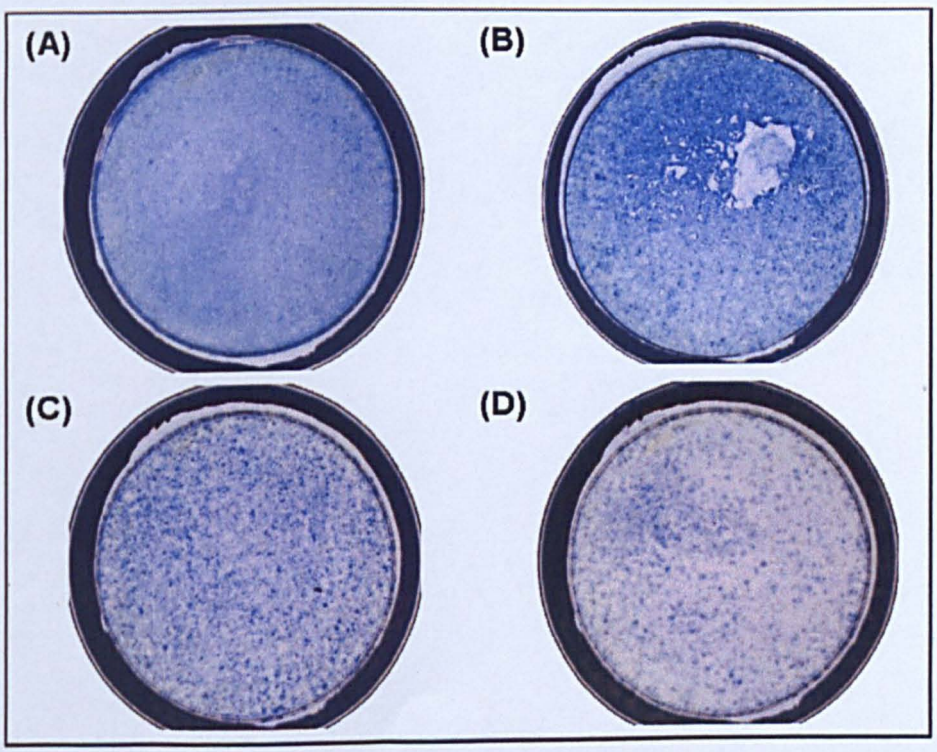

Figure 4.4: Methylene blue stained CFU-f cultures started with MSCs from monolayer cultures with MyeloCult® medium, after one passage (A), two passages (B), three passages (C) and four passages (D).

The number of colony forming cells in the monolayer cultures with DMEM increased 4.6 fold after 9 days (Figure 4.5). That means, the static cell culture which was started with 3,400 CFU-fs grew to about 15,600 CFU-fs after 9 days. The increase in CFU-f number of the static culture with MyeloCult® medium for the same time was similar at 4.5 fold. Over time, the number of CFU-fs in both cultures continuously decreased, resulting in a final 2 fold increase since the initial starting culture for DMEM, and a 2.3 fold increase since the initial starting culture for MyeloCult® medium after 23 days (Figure 4.5). This is only a very small increase in colony forming cells compared to the development of the total cell number, and means that the final CFU-f number would be about 6800 cells in the DMEM cultures and about 7800 cells in the MyeloCult® cultures. More interestingly, the highest increase in CFU-fs was not at the end of the culture period but after 9 days (the first passage). The pictures taken from the CFU-f assays of MSCs expanded in DMEM cultures further suggest, that MSCs harvested from later passages lost their colony forming ability (Figure 4.3). Although the development of the CFU-f number in DMEM and MyeloCult® cultures was the same, an obvious difference was the colony size. While the colonies derived from the DMEM experiment were barely recognisable, the colonies from the MyeloCult® cultures stained very strongly, and even formed a confluent monolayer in the background of the plates which were started with MSCs from the first two passage levels (Figure 4.4). This indicates that the proliferation capacity of the cells forming a colony must be higher in cells derived from MyeloCult® cultures compared to cells derived from DMEM cultures.

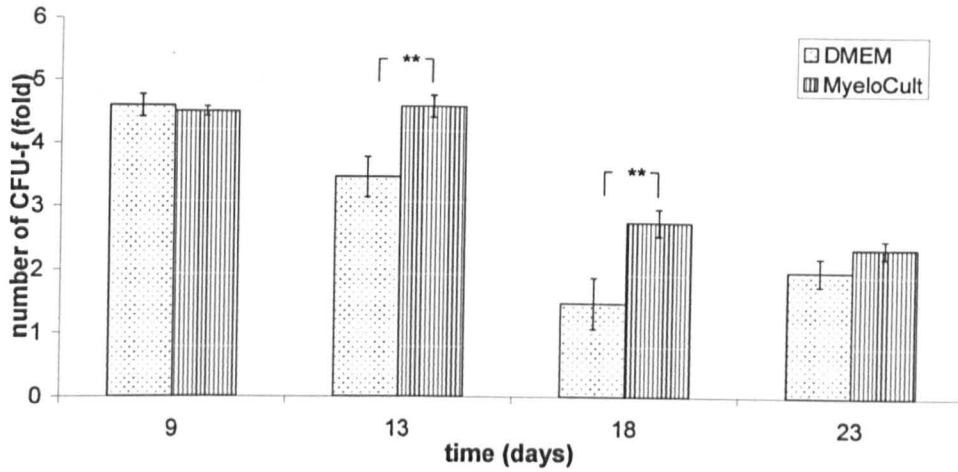

Figure 4.5: Development of CFU-f verses time. Cells were derived from rat bone marrow and expanded in static cultures with either DMEM or MyeloCult® medium. Results for both cultures are mean +/- S.D. from 3 different CFU-f cultures. *$p < 0.01$; **$p < 0.05$, DMEM compared with MyeloCult®

The CFU-f assay however is only an indicator of the stem cell character of MSCs. To confirm the real differentiation capacity, cell aliquots from both static cultures were taken after every passage and grown in either osteogenic or adipogenic differentiation medium for 14 days. The formation of osteoblasts was shown by staining the cells for alkaline phosphatase, calcium and collagen. Although the CFU-f number decreased over time, MSCs with osteogenic differentiation capacity were found throughout all the passages for both types of monolayer cultures. Figures 4.6 and 4.7 show one example plate used for the osteogenic differentiation assay of MSCs harvested from the DMEM and the MyeloCult® monolayer culture, respectively, after 23 days. The colonies on the plate were stained with naphtholphosphate and fast red for alkaline phosphatase (picture A), with alizarin red for calcium (picture B) and with sirius red for collagen (picture C). All three of these osteogenic markers were present. The plates from the osteogenic differentiation assay of cells derived from DMEM and MyeloCult® cultures seemed to be very similar in colony density and staining intensity, which could be evidence for a similar osteogenic differentiation potential for MSCs expanded in both types of medium. The adipogenic differentiation of MSCs harvested from any monolayer culture failed, indicating that this differentiation ability got lost during the *in vitro* expansion.

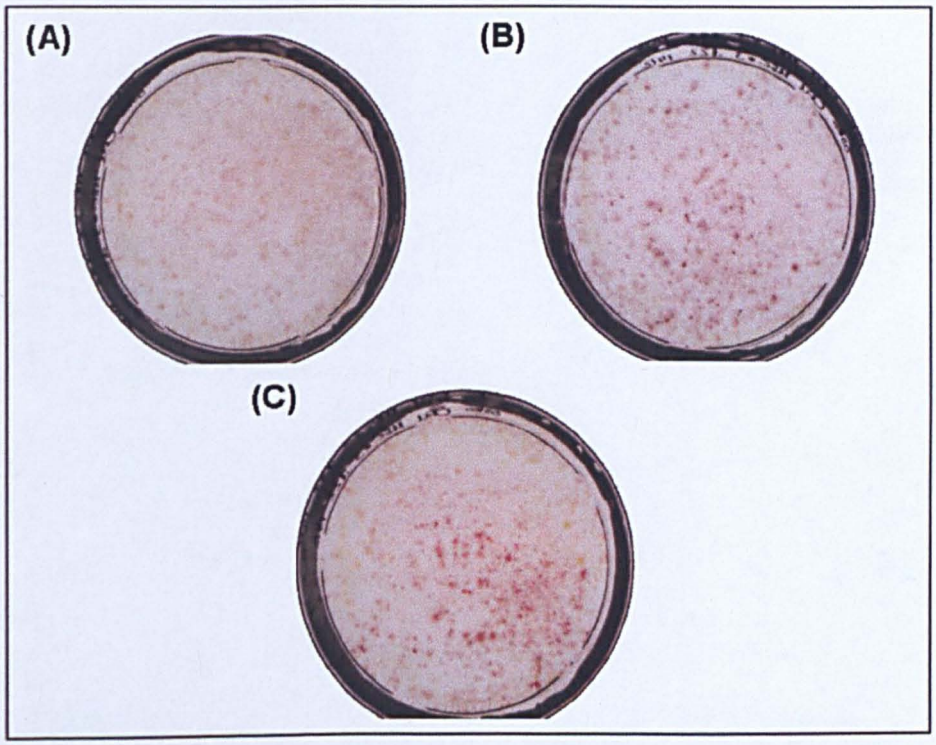

Figure 4.6: Differentiation assay of MSCs harvested from monolayer cultures with DMEM after 4 passages. The plate was stained for alkaline phosphatase (A), calcium (B) and collagen (C).

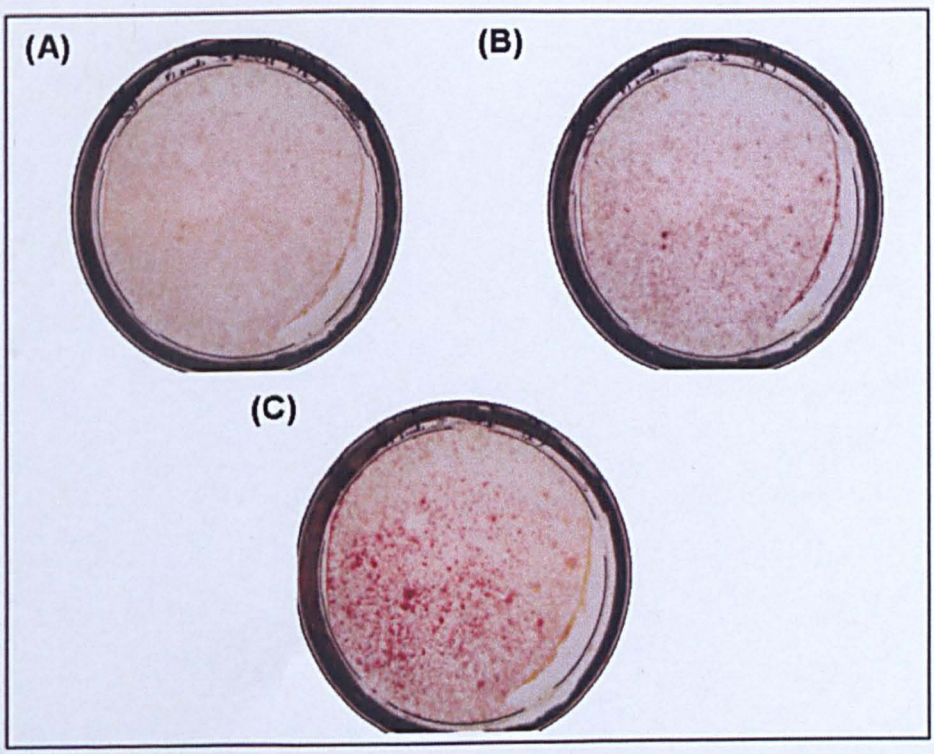

Figure 4.7: Differentiation assay of MSCs harvested from monolayer cultures with standard MyeloCult® medium after 4 passages. The plate was stained for alkaline phosphatase (A), calcium (B) and collagen (C).

Summarising the results observed for monolayer expansion of MSCs in DMEM and MyeloCult® medium, it is obvious that the proliferation capacity of MSCs in MyeloCult® medium after 23 days was higher than in DMEM ($p = 0.001$) but this increased proliferation did not result in a significantly ($p = 0.137$) higher CFU-f number or better osteogenic differentiation potential compared to the CFU-f number and the differentiation potential of DMEM derived MSCs. For both media conditions, the increase in CFU-f number was small compared to the increase in total cell number and adipogenic differentiation could not be seen at any time. A possible explanation was found by Sekiya *et al.* (2002) saying that a MSC monolayer culture comprises of different progenitor cell types, which have different proliferation and differentiation capacities. Therefore, the tested culture conditions (i.e. MyeloCult® medium) could have supported the expansion of early osteogenic precursor cells with limited differentiation and colony forming capacity rather than supported the growth of fully undifferentiated mesenchymal stem cells. These finding support the theory, that MSCs lose their stem cell character when cultured for a long period in static monolayer cultures (Sun *et al.*, 2006).

4.3.2 Effect of interleukin-3 and stem cell factor on Mesenchymal Stem Cell expansion in monolayer cultures

The cytokines Interleukin-3 (IL-3) and stem cell factor (SCF) act as growth factors for MSCs and improve their proliferation capacity (Wang *et al.*, 1990) (Yamada *et al.*, 2000). This benefit was of interest for the stirred suspension culture experiment (chapter 6) and the studies using the pour-off technique (chapter 7) and since in both culture systems MyeloCult® was the main culture medium, the effect of IL-3 and SCF was determined in static monolayer cultures with MyeloCult® only. A more detailed introduction on IL-3 and SCF will be given in chapter 6 section 1. The results from the studies in this chapter were used to investigate how important the support from the growth factors was for the proliferation capacity, using a less stressful culture method.

MSCs grown in MyeloCult® medium with IL-3 and SCF achieved an increase of 2.3 x 10^3 fold after 9 days whereas the control in MyeloCult® medium increased by 1.0 x 10^3 fold. After the final passage at day 29, the increase in cell number of the culture with growth factors was 6.1 x 10^5 fold compared to the control with 3.9 x 10^5 fold (Figure 4.8). This result indicates that the addition of IL-3 and SCF could enhance the growth rate of MSCs in static cultures over a period of 29 days ($p = 0.001$), but at some time points in between the period of the experiment, the control culture had higher increase rates, e.g. after 13 days (10.1 x 10^3 fold for control and 6.9 x 10^3 fold for the culture with growth factors) (Figure 4.8).

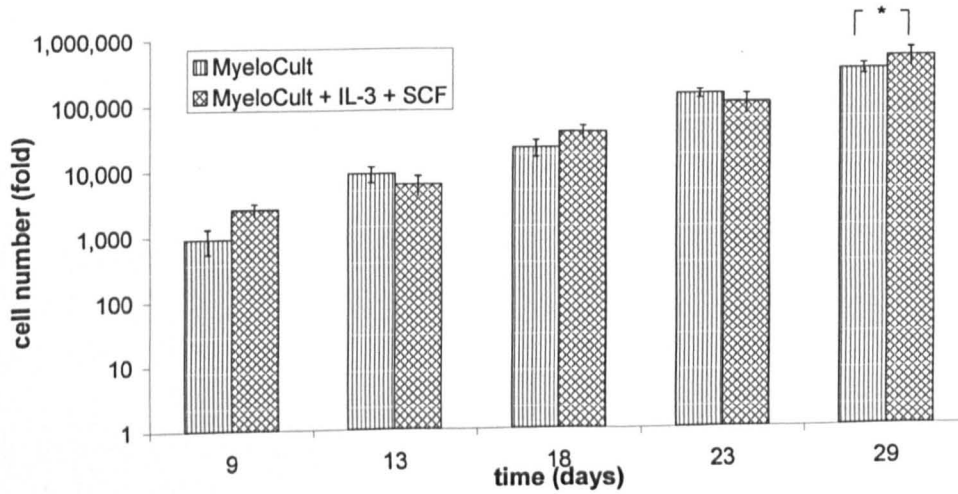

Figure 4.8: Increase in cell number verses time. Cells were derived from rat bone marrow and expanded in static cultures with either MyeloCult® or MyeloCult® with 2 ng/ml IL-3 and 10 ng/ml SCF as culture medium. Results for both cultures are mean +/- S.D. from 3 cell counts from only 1 culture each, due to contamination problems. *$p < 0.01$, MyeloCult® compared with MyeloCult® + IL-3 + SCF

The effect of IL-3 and SCF in MyeloCult® medium on the colony forming ability was determined with the CFU-f assay. Only the plate with MSCs from the first passage displayed single colonies, the other plates seemed to have a monolayer of cells. Nevertheless, colonies were detected in all plates using the colony detection software detailed in section 3.6.1. The development of the colony number over time is presented in Figure 4.9. An increase of 4.1 fold was achieved after 9 days and a 4.6

fold increase after 13 days. After 23 days, the total increase in CFU-f compared to the initial CFU-f number was only 2.2 fold (Figure 4.9).

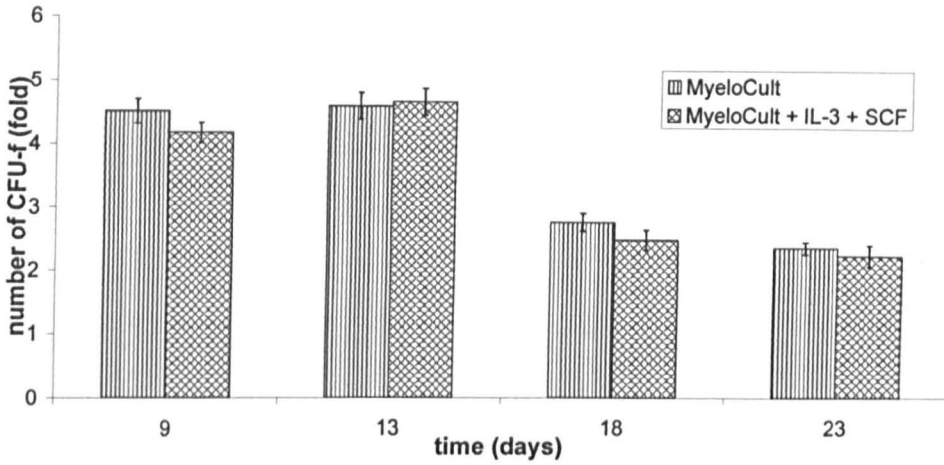

Figure 4.9: Development of CFU-f over time. Cells were derived from rat from bone and expanded in static cultures with either MyeloCult® medium only or with MyeloCult® medium containing dexamethasone, IL-3 and SCF. Results for the three conditions are mean +/- S.D. from 3 different CFU-f cultures. No significant differences between the CFU-f numbers in either culture.

MSCs harvested from monolayer cultures with MyeloCult® medium containing IL-3 and SCF were able to differentiate into osteoblasts and could be stained for alkaline phosphatase, calcium and collagen, respectively. Adipogenic differentiation of these cells could not be achieved.

Although, the addition of the growth factors was supposed to have a beneficial impact on the proliferation rate of MSCs (Baksh *et al.*, 2003), neither the overall total cell number ($p = 0.80$) nor the overall number of colony forming cells ($p = 0.87$) was significantly increased in static cultures with IL-3 and SCF (Figures 4.8 and 4.9). Furthermore, the differentiation potential of these cells was unchanged, as no adipogenic differentiation was detected. Considering the higher costs caused by use of the cytokines (about £200 / litre culture medium), the benefits in static culture systems are negligible.

4.3.3 Effect of dexamethasone on Mesenchymal Stem Cell expansion in monolayer cultures

The effect of dexamethasone on the proliferation and differentiation of MSCs in static cultures was determined in both DMEM and MyeloCult® medium. Dexamethasone is a glucocorticoid, which can effect the proliferation and the differentiation behaviour of MSCs (Song *et al.*, 2009) The results from the experiment in MyeloCult® medium will be used later as a control for the stirred suspension cultures in MyeloCult® medium with dexamethasone (chapter 6). The outcome from the experiments in DMEM were compared with those in MyeloCult® medium to see if a potential positive effect of dexamethasone is medium independent and consequently to test whether dexamethasone should be used as a standard medium additive.

The increase in total cell number in the DMEM cultures with dexamethasone was 1.2 x 10^3 fold after 9 days, compared to 0.9 x 10^3 fold in the controls in DMEM without dexamethasone and a final increase of 9.2 x 10^3 fold in the dexamethasone cultures after 23 days compared to an increase of 11.8 x 10^3 fold in the controls (Figure 4.10). The numbers obtained from the MyeloCult® cultures with dexamethasone were similar, 1.7 x 10^3 fold increase after 9 days compared to 1.0 x 10^3 fold in the controls in MyeloCult® without dexamethasone and 3.7 x 10^5 fold increase at the end of the experiment compared to 3.9 x 10^5 fold of the controls (Figure 4.11).

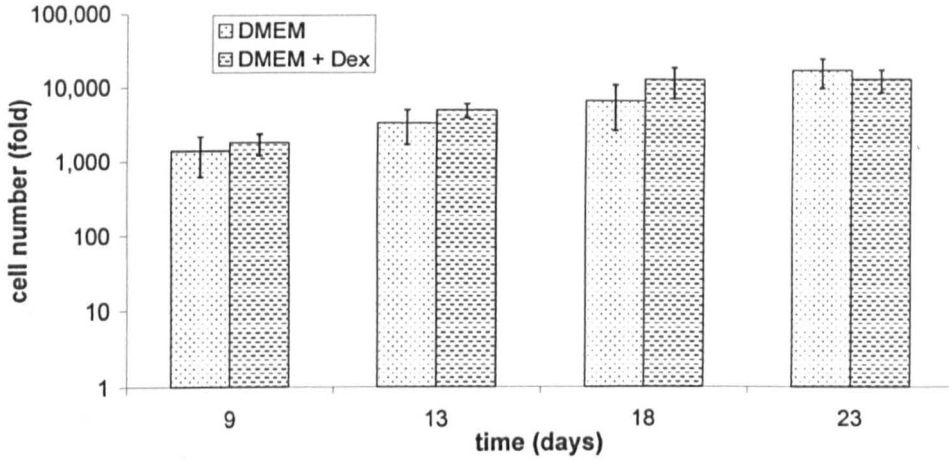

Figure 4.10: Increase in total cell number verses time. Cells were derived from rat bone marrow and expanded in static cultures with either DMEM only or DMEM with 10^{-8} M dexamethasone. Results are mean +/- S.D. from 3 different cultures. No significant differences between the cell numbers in either culture.

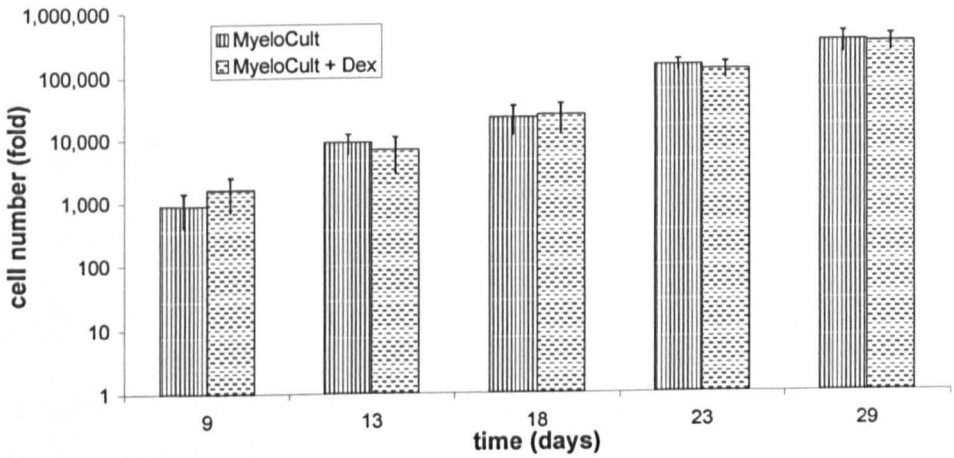

Figure 4.11: Increase in total cell number verses time. Cells were derived from rat bone marrow and expanded in static cultures with either MyeloCult® or MyeloCult® with 0.05% pluronic as culture medium. Results for all cultures are mean +/- S.D. from 3 different cultures. No significant differences between the cell numbers in either culture.

The CFU-f assay from MSCs expanded in monolayer culture with DMEM containing 10^{-8} M dexamethasone showed numerous colonies on the plates from passage 1 and 2, some of them were easy to recognise. The plates of the CFU-f assay started with MSCs from passage 3 and 4 on the other hand seemed to have only very few and very small colonies, which were not visible on the picture. MSCs harvested

from monolayer cultures with MyeloCult® medium containing 10^{-8} M dexamethasone produced recognisable colonies in the CFU-f assay after the first passage. However, the CFU-f culture plates from later passages did not display colonies. Nevertheless, colonies were detected using the colony detection software detailed in chapter 3.6.1. The development of the CFU-f number in monolayer cultures with DMEM and 10^{-8} M dexamethasone resulted in a 4.6 fold increase after 9 days and still a 3.5 fold increase after 13 days. Yet, after 23 days, the colony number dramatic decreased to 0.1 fold of the initial number of colonies (Figure 4.12). The CFU-f numbers in the monolayer culture with MyeloCult® medium containing 10^{-8} M dexamethasone at the different passage levels is presented in Figure 4.13. After 9 days, an increase in CFU-f number of 3.8 fold was achieved, compared to the initial colony number of the monolayer culture at day 1. The maximum of 4.4 fold increase was reached after 13 days. Towards the end of the monolayer culture period, the CFU-f number slightly decreased again to an overall increase of 2.3 fold after 23 days (Figure 4.13).

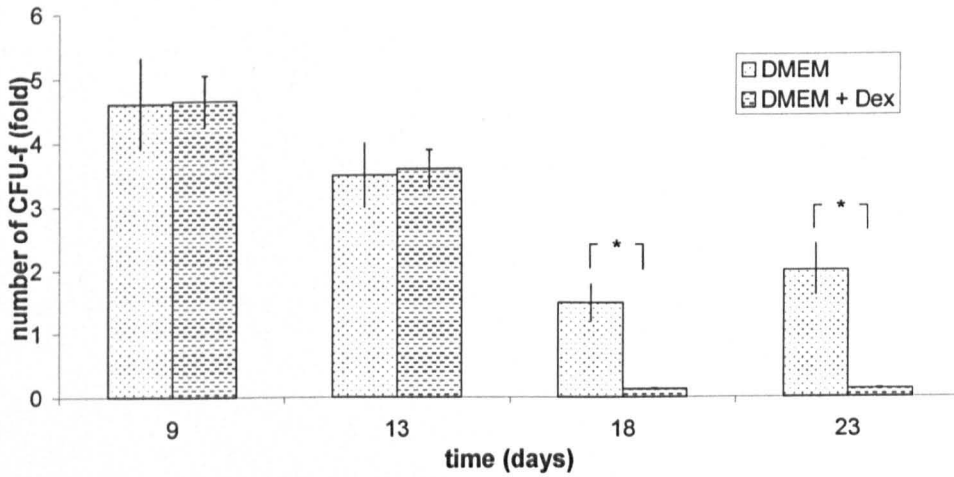

Figure 4.12: Development of CFU-f verses time. Cells were derived from rat bone marrow and expanded in static cultures with either DMEM or DMEM and dexamethasone. Results for both cultures are mean +/- S.D. from 3 different CFU-f cultures.*$p < 0.01$, DMEM compared with DMEM + Dexamethasone

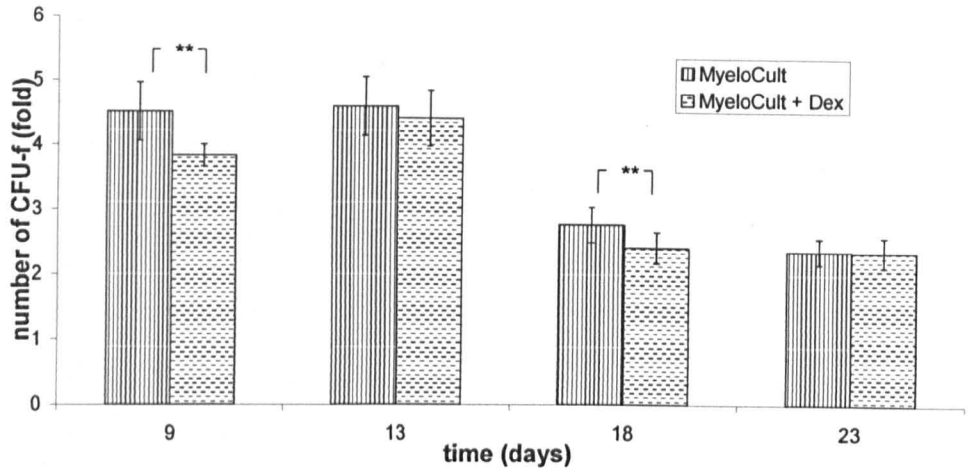

Figure 4.13: Development of CFU-f verses time. Cells were derived from rat bone marrow and expanded in static cultures with either MyeloCult® medium only or MyeloCult® containing 0.05% pluronic. Results for all cultures are mean +/- S.D. from 3 different CFU-f cultures. **p < 0.05, MyeloCult® compared with MyeloCult® + Dexamethasone

In both media types, DMEM and MyeloCult®, the addition of dexamethasone did not effect the proliferation or colony formation potential of the MSCs in static monolayer cultures. The increase in total cell number was about the same for the static cultures with DMEM ($p = 0.83$) and MyeloCult® medium ($p = 0.92$) containing dexamethasone compared to the respective control (Figures 4.10 and 4.11). This is also true for the development of the CFU-f number in these static cultures. The MSCs harvested from static cultures in MyeloCult® medium with dexamethasone seemed to form less obvious colonies, but rather a monolayer of cells compared to the cells of the control in MyeloCult® only. Counting the colonies of both CFU-f assays revealed the same increase in colony forming cells at the end of the culture period, a 2.3 fold increase in both the MyeloCult® dexamethasone culture and the control ($p = 0.641$). The two CFU-f assays started with MSCs expanded in DMEM containing dexamethasone and DMEM only looked similar in regard to colony formation and staining intensity, but the CFU-f count showed that the colony forming cells in the static culture with dexamethasone decreased faster (0.1 fold of the initial cell number) than in the control (2 fold of the initial cell number) at the end of the culture period ($p = 0.012$).

Differentiation into osteoblasts could be shown for cells growing in both culture media DMEM with dexamethasone and MyeloCult® medium containing dexamethasone (Table 4.3). The adipogenic differentiation assay, in contrast, did not generate any adipocytes.

Summing up, the addition of dexamethasone to static cultures of bone marrow derived rat MSCs in either DMEM or MyeloCult® medium did not increase the proliferation behaviour of these cells. Although dexamethasone is known to play an important role in the osteogenic differentiation of MSCs (Song *et al.*, 2009) as well as in the adipogenic differentiation (Jing *et al.*, 2009) and furthermore has an effect on the apoptosis and proliferation behaviour of MSCs *in vitro* (Song *et al.*, 2009) (Akavia *et al.*, 2006), no adipogenic differentiation was observed in this study.

4.3.4 Effect of Pluronic F-68 on Mesenchymal Stem Cell expansion in monolayer cultures

Pluronic F-68 was mainly used in stirred suspension cultures (chapter 6) with MyeloCult® as culture medium. Since the results from the monolayer cultures were a control for the findings in suspension cultures, Pluronic F-68 was only tested in monolayer cultures with MyeloCult® medium. Pluronic F-68 is known to strengthen the cell membrane (Zhang *et al.* 1992) thereby protecting the cells from shear stress, which is a beneficial effect for cells in stirred suspension cultures. In order to determine any other positive or negative effect of pluronic on MSCs, the proliferation and differentiation behaviour of these cells was recorded in a culture system where no shear stress was exerted on the cells.

Comparing the static monolayer cultures of MSCs grown in MyeloCult® medium with or without Pluronic F-68, it is clear that the cell proliferation at the end of the culture time in the pluronic culture is slower ($p = 0.001$). 0.8×10^3 fold increase after 9 days, and a final increase of 0.1×10^5 fold after 29 days in pluronic cultures compared to 0.9×10^3 fold at day 9 and 3.9×10^5 fold at day 29 in the control (Figure 4.14).

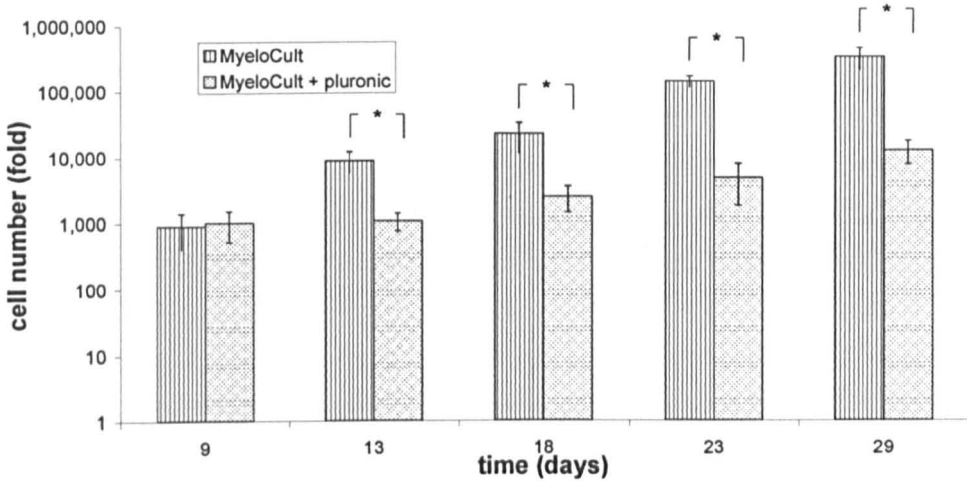

Figure 4.14: Increase in total cell number verses time. Cells were derived from rat bone marrow and expanded in static cultures with either MyeloCult® or MyeloCult® with 0.05% Pluronic as culture medium. Results for all cultures are mean +/- S.D. from 3 different cultures. *p < 0.01, MyeloCult® compared with MyeloCult® + Pluronic F-68

The colonies formed by MSCs harvested from static cultures in MyeloCult® with 0.05% pluronic stained only very faintly however, on the CFU-f plates with MSCs from the first passage colonies were recognisable. On the plates with cells from the second passage no colonies, but a monolayer was found, and on plates with cells from third passage no cells were visible at all. This observation was confirmed by the CFU-f count conducted with the colony detection software (chapter 3.6.1), showing that the number of colonies decreased with increasing passage number. At the first passage, the CFU-f number increased by 4.3 fold compared to the initial CFU-f number in the monolayer culture, but at the end of the monolayer culture after 18 days, the number of CFU-f decreased to 0.1 fold of the initial value (Figure 4.15).

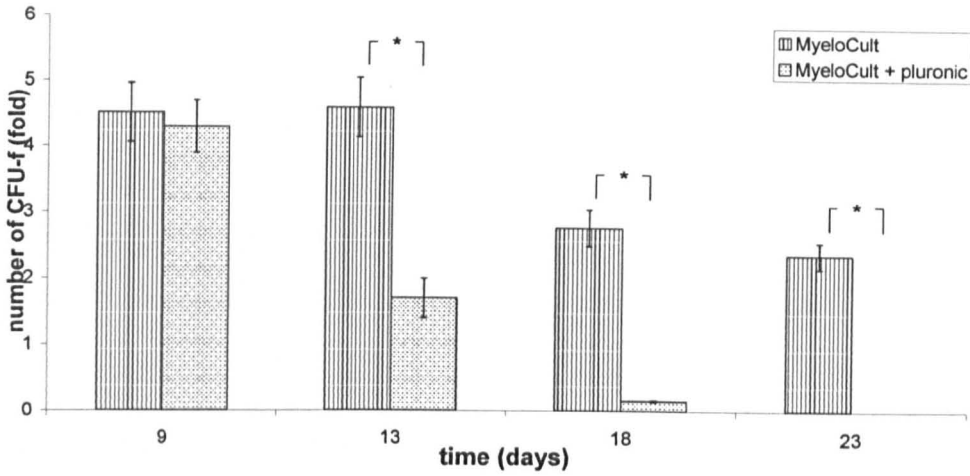

Figure 4.15: Development of CFU-f verses time. Cells were derived from rat bone marrow and expanded in static cultures with either MyeloCult® medium only or MyeloCult® containing 0.05% pluronic. Results for all three cultures are mean +/- S.D. from 3 different CFU-f cultures. *$p < 0.01$, MyeloCult® compared with MyeloCult® + Pluronic F-68

Regardless of the dramatic drop in CFU-f number, MSCs taken from static cultures in MyeloCult® medium with pluronic at passage 2 differentiated into osteoblasts. Adipogenic differentiation was not observed. The proliferation data in this study showed a slow increase of total cell number over time in the static monolayer culture containing pluronic (Figure 4.14), but comparing these numbers with the fold increase achieved in the control cultures it is obvious that the proliferation rate was significantly reduced ($p = 0.001$) (Figure 4.14). The effect of pluronic on the colony forming ability of MSCs in static culture is even more dramatic (Figure 4.15). Although the proliferation and colony forming ability of MSCs *in vitro* was effected negatively by pluronic, the results from the differentiation assay suggest that the differentiation capacity remained unchanged as MSCs harvested after the third passage had already experienced a decrease in CFU-f number, but still formed osteoblasts in the osteogenic differentiation assay. With these experiments, it could be shown that pluronic affected the proliferation and colony forming potential of MSCs, but not their differentiation capacity. Pluronic did not kill or destroy the cells, it merely slowed down their growth rate. A possible reason for the effect on the

proliferation capacity can be the fact that pluronic attaches to the cell membrane, and thereby may replace the same functional groups which are essential for the cell proliferation. To find some evidence for this theory, FTIR analysis on MSCs was conducted and their electrophoretic mobility was measured. Results and discussion are presented in the following section.

4.3.5 FTIR spectroscopy on Mesenchymal Stem Cells

The propose of the FTIR analysis of MSCs was to determine a possible effect of pluronic on the cell membrane proteins, and to test how useful different bench scale FTIR spectrometers were for analysing MSCs in general. Much of the FTIR analysis on biological material was completed using synchrotron radiation (Jamin *et al.*, 1998) (Holman *et al.*, 2002). The results presented here demonstrate that an IR absorption spectrum can be obtained from MSCs (Figure 4.16), and that several specific peaks, typical for any biological sample, can be identified in this spectrum (Table 4.1). Using the FTIR spectrometer (Perkin Elmer Spotlight FTIR Imaging System) which has a built in microscope and digital camera, spectral measurements of single cells, or specific regions of the MSC could be taken. Figure 4.17-A is such a picture, with three *in vitro* expanded MSC attached to the calcium fluoride (CaF_2) window. Scanning the whole area of this picture during the FTIR analysis resulted in the infrared (IR) picture of these cells (Figure 4.17-B). The blue background of the picture means that there was very little absorption of IR light, while the red area indicates the highest absorption of IR light. The spectrum in Figure 4.17-C is the spectrum from the red area of the IR picture. Although it looks similar to the spectrum from the green area of the IR picture (Figure 4.17-D), there were slight differences in the P=O stretching of PO_2- (peak 5 and 6 in Table 4.1) and in the symmetric stretching of carboxylic acid groups C-O of COO- (peak 3 in Table 4.1).

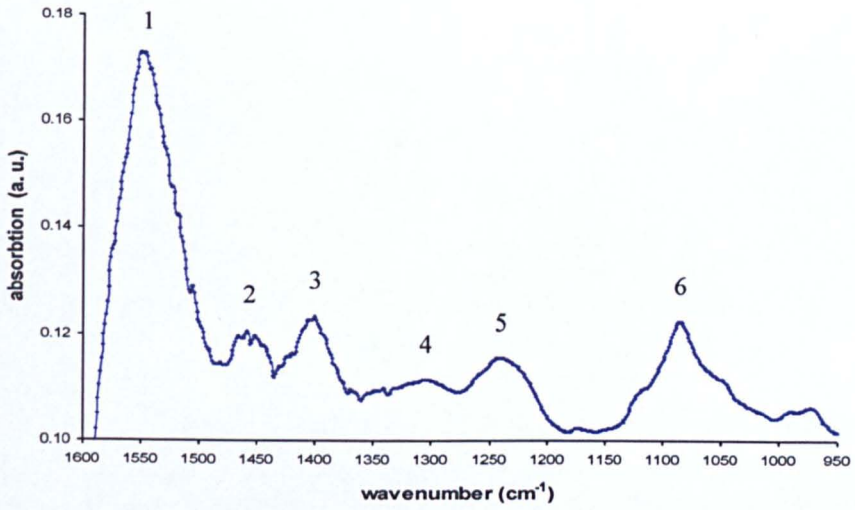

Figure 4.16: FTIR absorption spectrum of bone marrow derived rat MSCs in phosphate buffered saline. The cells were expanded for 2 passages as monolayer cultures with DMEM as culture medium before being analysed.

Table 4.1: Explanation of the peaks in the FTIR spectrum obtained from MSCs in Figure 4.24. Band assignments according to: Naumann *et al.* (1991), Jiang *et al.* (2004), Yee *et al.* (2004) and Dittrich *et al.* (2005)

peak number	**description**	**typical wave number (cm^{-1})**
peak 1	N-H bending of amides associated with proteins (amide II)	1584
peak 2	asymmetric deformation of CH_3 /CH_2 of proteins	1468
peak 3	symmetric stretching of carboxylic acid groups (C-O of COO- groups)	1400
peak 4	Amide III components of proteins	1310-1240
peak 5	P=O asymmetric stretching of PO_2-	1250-1220
peak 6	P=O symmetric stretching of PO_2-	1088-1084
at 1690 cm^{-1}	different amide I band components	1695

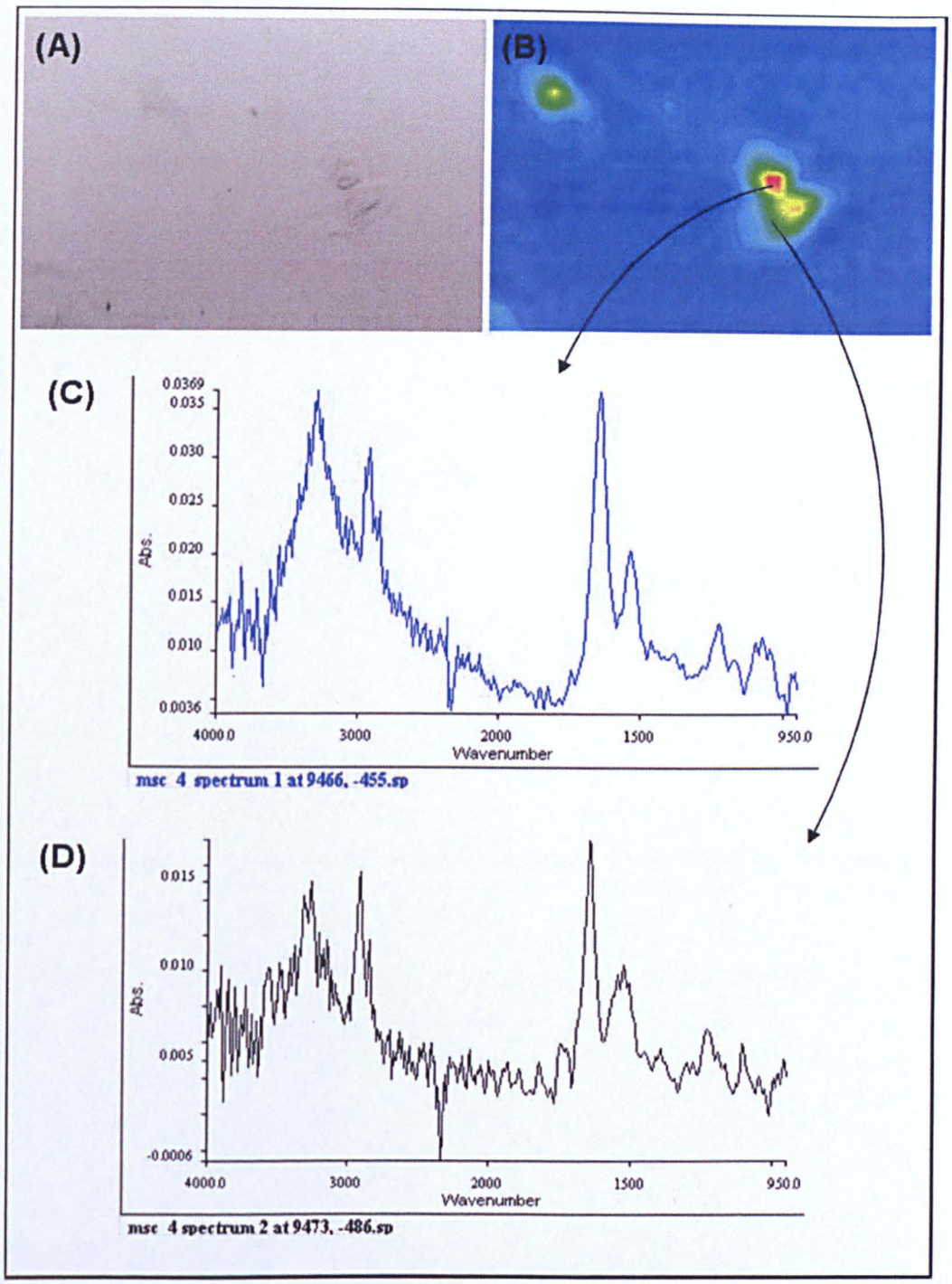

Figure 4.17: Picture of MSCs attached to the CaF_2 window of the FTIR spectrometer. The picture was taken with the digital camera connected to the microscope of the spectrometer. (**A**) IR picture of the same cells as in picture A. The picture was generated after scanning the area of the picture A. (**B**) FTIR absorption spectrum of a MSC. This spectrum originated from the red area of the IR picture. (**C**) FTIR absorption spectrum of the same MSC as in picture C. This spectrum originated from the green area of the IR picture. (**D**)

In addition to the absorption spectra, the IR pictures themselves can be a valuable source of information. As can be seen in Figure 4.18, different IR pictures can be taken from the same cell sample. Taking an IR picture at the wavelength where amides absorb the IR light (1695 cm^{-1}, 1548 cm^{-1} and 1310-1240 cm^{-1}) revealed the regions with the most proteins (red areas in the Figure 4.18-B). Repeating the same process but at the wavelength where either OH-bonds (~3500 cm^{-1}) or polysaccharides (1200-900 cm^{-1}) absorb the IR light highlighted regions in the pictures where either alcohols or polysaccharides were located (red areas in the Figure 4.18 C and D).

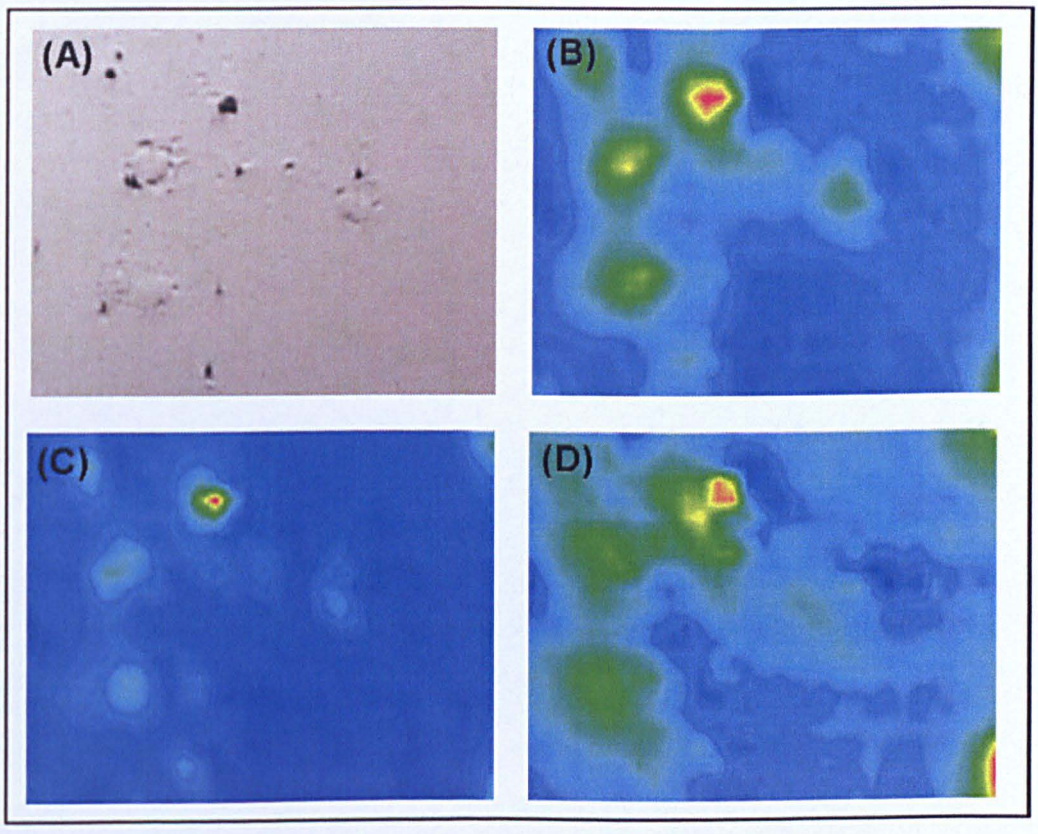

Figure 4.18: Picture of MSCs attached to the CaF_2 window of the FTIR spectrometer. The picture was taken with the digital camera connected to the microscope of the spectrometer. (**A**) IR picture of the same cells as in picture A. This picture was generated after scanning the area of the picture A and displaying only the absorption of IR light caused from the amides (1695 cm^{-1}, 1548 cm^{-1} and 1310-1240 cm^{-1}). (**B**) IR picture of the same cells as in picture A. This picture was generated by displaying only the absorption of IR light caused from OH bonds in alcohol, etc (~3500 cm^{-1}). (**C**) IR picture of the same cells as in picture A. This picture was generated by displaying only the absorption of IR light caused from the polysaccharides (1200-900 cm^{-1}). (**D**)

These results show that IR images of single MSCs and spectra of different cell compartments could be obtained using the Perkin Elmer Spotlight FTIR Imaging System in conjunction with a microscope and a digital camera (Figure 4.17). This method also enabled different regions in the IR picture, showing areas with high levels of proteins, polysaccharides and alcohols / sugars to be highlighted (Figure 4.18). Applying this technique, a bench size FTIR spectrometer can be used to analyse IR pictures of single MSCs, and compare them to each other to obtain information about differences in their composition.

Although the pictures are a nice visual feature to illustrate that FTIR is a useful tool to analyse MSCs, the spectra obtained from the spectrometer Perkin Elmer Spotlight FT-IR Imaging System were not sensitive enough to reveal any differences in the cell membrane (protein region of the IR spectrum) of MSCs expanded in culture medium with or without Pluronic F-68 (results not shown). To find these differences another spectrometer (Perkin-Elmer Spectrum One Fourier transformation spectrophotometer (Perkin-Elmer, UK) was used. It was unable to take pictures of the cells, but the signal / noise ratio was higher and thus the analysis resulted in more sensitive spectra. Two spectra from these analyses are displayed in Figure 4.19. The brown spectrum (A) is from MSCs expanded in DMEM containing pluronic, the black spectrum (B) is from cells expanded in DMEM only. At three different wavelength (1360 cm^{-1}, 1170 cm^{-1} and 980 cm^{-1}), peaks were present in the brown spectrum (A) but not in the black spectrum (B). These peaks were in the Amide III region of the IR spectrum and in the region where vibration of carbohydrates (C-O-C, C-O) absorb the IR light, and are highlighted with red circles (Figure 4.19).

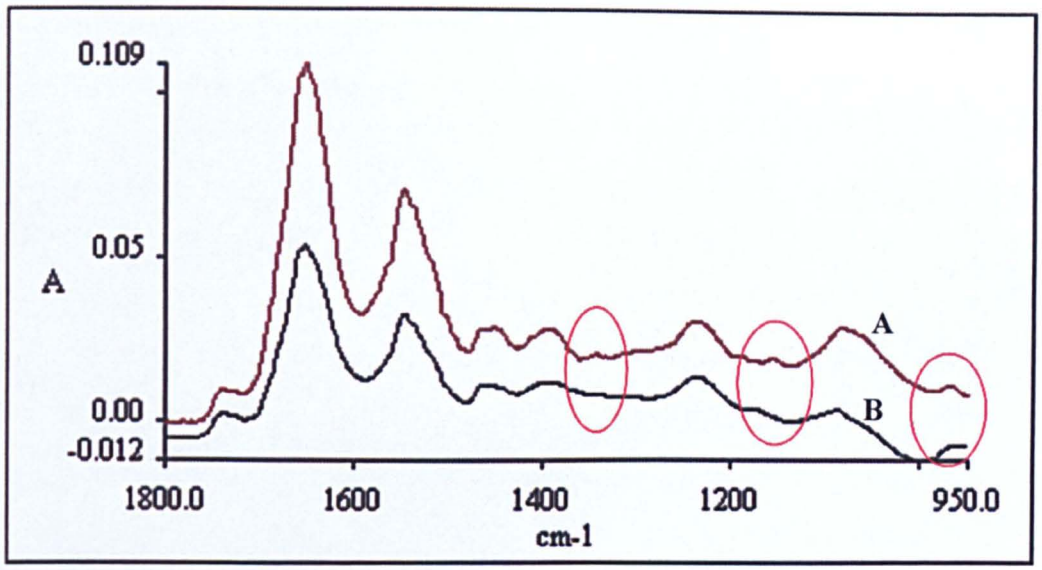

Figure 4.19: Normalised FTIR absorption spectra of rat bone marrow derived MSCs in phosphate buffered saline. The MSCs causing the brown spectrum (**A**) were expanded for 2 passages as monolayer cultures with standard DMEM as culture medium. The cells responsible for the black spectrum (**B**) were cultured the same way but in DMEM + 0.05% pluronic. The differences in the two spectra are highlighted with red circles.

The difference in the Amide III peak (1360 cm^{-1}) was associated with proteins, as was the difference in the vibration of carbohydrates (C-O-C, C-O) (1170 cm^{-1}). These differences associated with proteins would fit with the theory of a change in functional groups in the MSC membrane. Furthermore, the observed change in proteins could have appeared anywhere in the cell as FTIR does not measure only the surface of the cells but the cell as a whole. The spectrum does not give any information about where in the MSC this has occurred. In general, FTIR is a fast and easy method to analyse differences in the DNA and protein ratio of different cell populations, and can also be used to reveal high and low levels in proteins, DNA or alcohol/sugars of different cells. FTIR has been used on mammalian cells to track chemical changes in the cells, to distinguish between cancerous and noncancerous cells (Mourant *et al.*, 2002) to map the distribution of functional groups within a cell (Jamin *et al.*, 1998) or to observe different growth stages (Mourant *et al.*, 2003). With MSCs, FTIR was used to identify the differentiation state of single cells (Krafft *et al.*, 2007). However, to obtain more detailed information about the effect of Pluronic F-68 on the membrane proteins of MSCs, more specific techniques have to be applied, e.g. proteomic methods.

4.3.6 Electrophoretic mobility of Mesenchymal Stem Cells

In this experiment, the measurement of the electrophoretic mobility (EPM) should reveal if pluronic changes the surface charge of MSCs by attaching to the cell membrane. Showing the incorporation of pluronic into the cell membrane would strengthen the theory that pluronic slows down the proliferation rate of MSCs in *in vitro* cultures by replacing functional groups in the cell periphery. Brooks and Seaman (1973a, d) demonstrated that the electrokinetic potential of red blood cells changed when suspended in solutions containing several polymers of various molecular weights, including dextran and Pluronic F-68. Through the use of radioactive dextran and a combination of theoretical and experimental calculations of the zeta potential, they demonstrated that this change is the result of adsorption of these polymers onto the cell membrane.

The zetaPALS experiments on MSCs were conducted in static cultures with DMEM only and DMEM with 0.05% pluronic as culture medium. Two different running buffers were tested to resuspend the harvested MSCs, phosphate buffered saline (PBS) and Potassium chloride (KCl). PBS was used as standard buffer for all MSCs handling in this thesis, whereas KCl was used as running buffer for zetaPALS studies on *E.coli* (Eboigbodin *et al.*, 2006). In order to decide if both buffers were suitable, a simple viability test using trypan blue staining was conducted. MSCs resuspended in 0.1 M PBS had a viability of 90.6% after 20 minutes in the buffer solution, whereas MSCs after the same time in 0.1 M KCl only had a viability of 23.1%. The outcome can be seen in Figure 4.20. Figure 4.20-A shows a sample of MSCs resuspended in 0.1 M PBS. The cells in this picture appear healthy. Figure 4.20-B shows the same cells stained with trypan blue. Most of the cells did not take up the dye, which means they were not damaged or dead. The MSCs resuspended in KCl appeared to be damaged (Figure 4.20-C). This was confirmed by the staining in Figure 4.20-D. Most of the cells were blue, and therefore either dead or damaged.

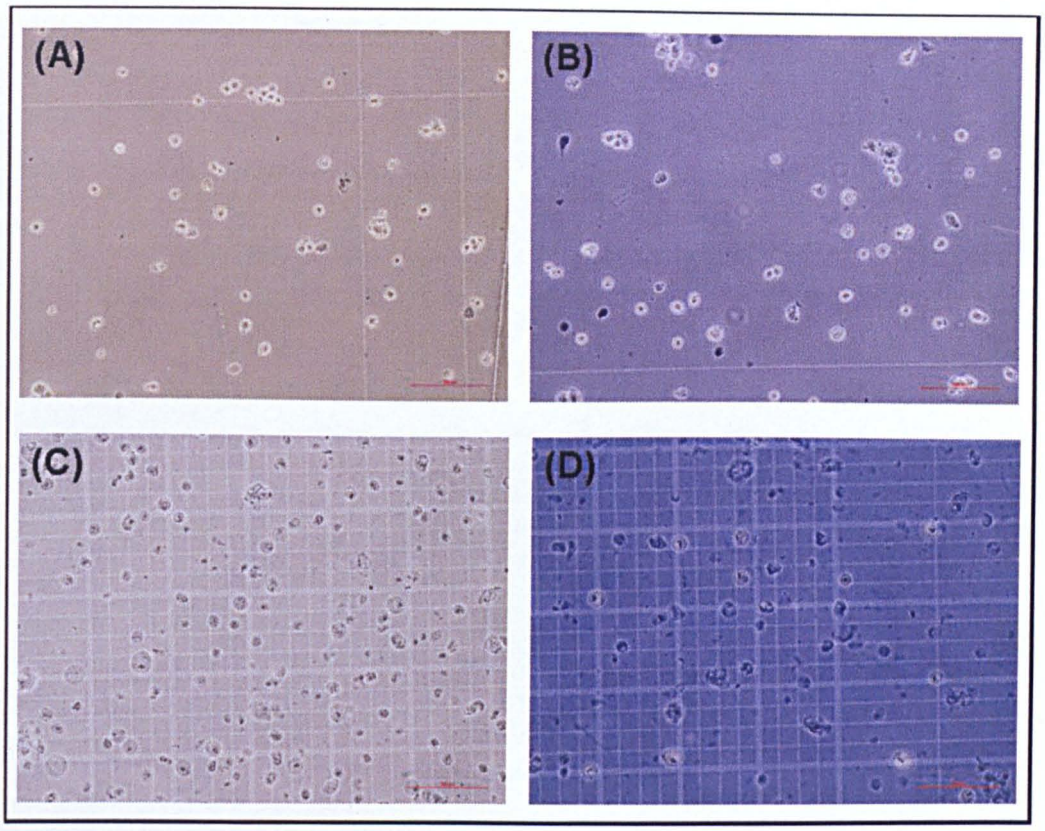

Figure 4.20: 40 x magnifications of MSCs resuspended in 01. M PBS after being harvest from a static culture. (**A**) The same cells as in A but stained with trypan blue. (**B**) 40 x magnifications of MSCs resuspended in 01. M KCl after harvesting from the same static culture as the MSCs in picture A. (**C**) The same cells than in C but stained with trypan blue. (**D**)

Since the electrophoretic mobility of cells is affected by a variety of chemical and physical factors (Simon-Reuss, 1964), all MSCs were resuspended in the same running buffer. The viability experiment conducted showed that PBS, even at the lowest ionic strength used for the measurement, did not damage the MSCs, and therefore is a suitable buffer (Figure 4.20). Potassium chloride was tested as well, but the results revealed that KCl caused massive damage to the cells (Figure 4.20). Although Simon-Reuss (1964) found that viability and nonviability had no significant difference on electrophoretic mobility, the MSCs resuspended in KCl burst into small fragments, and are therefore not likely to be measuring the EPM of whole cells. Hence PBS was chosen as running buffer for all measurements. MSCs from the same sample were split into 4 aliquots and analysed at different molarities (10 mM, 30 mM, 66 mM and 100 mM). All cells exhibited negative mobility values, implying that the surface of these cells had a net negative charge. The results are presented in Table 4.2, indicating an increasing EPM with a decreasing molarity.

These finding are not surprising, since there are less free ions available to form the diffuse layer around the cell in a buffer with low ionic strength. The diffuse layer however, decreases the EPM. To see if the electrophoretic mobility of MSCs changes when they are kept in culture medium containing pluronic, the values for the EPM at all molarity levels were compared with the control, but no significant difference was found ($p = 0.383$). Thus, there was no evidence that pluronic alters the functional groups in the cell membrane when attached to it.

Table 4.2: Mean EPM values at different running buffer concentrations. The MSCs used for this experiment were expanded for 2 passages in two different medium conditions, DMEM only and DMEM + 0.05% pluronic.

condition	PBS concentration (mM)	mean mobility (μm/s) / (V/cm)	Std. error (μm/s) / (V/cm)
DMEM	10	-3.10	0.04
DMEM	30	-2.30	0.05
DMEM	66	-1.93	0.07
DMEM	100	-1.91	0.06
pluronic	10	-3.11	0.09
pluronic	30	-3.01	0.10
pluronic	66	-2.01	0.08
pluronic	100	-1.95	0.10

4.4 Conclusions

In this chapter bone marrow derived rat MSCs were expanded in static monolayer cultures using different culture media. The static culture system is the most widely accepted standard method to expand MSCs *in vitro* (Ulloa-Montoya *et al.*, 2005). Thus, the results from the experiments conducted in this chapter were used to test the effect of various medium compounds on the proliferation and differentiation capacity of the expanded MSCs. In addition, the results will be used later on as a control for the experiments with MSCs in suspension cultures (chapter 6) and the so-called pour-off cultures (chapter 7) in order to compare the efficiency of these methods. Furthermore, MSCs expanded in monolayer cultures were analysed using FTIR spectroscopy and zetaPALS measurement in order to study a possible effect of pluronic on the cell membrane.

Table 4.3: Summarised information from the pictures of the CFU-f and differentiation assays conducted in the experiments mention in this chapter.

Medium	initial cell density	Time [days]	Colony formation	Osteogenic differentiation	Adipogenic differentiation
DMEM	1 x 10^6 cells/ml	9	small	medium	-
DMEM	1 x 10^6 cells/ml	13	small	medium	-
DMEM	1 x 10^6 cells/ml	18	barely visible	medium	-
DMEM	1 x 10^6 cells/ml	23	barely visible	small	-
MyeloCult	0.7 x 10^6 cells/ml	9	confluent	small	-
MyeloCult	0.7 x 10^6 cells/ml	13	confluent	small	-
MyeloCult	0.7 x 10^6 cells/ml	18	small	confluent	-
MyeloCult	0.7 x 10^6 cells/ml	23	medium	confluent	-
MyeloCult + IL-3 + SCF	0.7 x 10^6 cells/ml	9	confluent + medium	small + medium	-
MyeloCult + IL-3 + SCF	0.7 x 10^6 cells/ml	13	confluent	small + medium	-
MyeloCult + IL-3 + SCF	0.7 x 10^6 cells/ml	18	confluent	small + medium	-
MyeloCult + IL-3 + SCF	0.7 x 10^6 cells/ml	23	confluent	small + medium	-
DMEM + Dex	1 x 10^6 cells/ml	9	small	medium + big	-
DMEM + Dex	1 x 10^6 cells/ml	13	small	medium + big	-
DMEM + Dex	1 x 10^6 cells/ml	18	not visible	small	-
DMEM + Dex	1 x 10^6 cells/ml	23	not visible	small	-

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MyeloCult + Dex	0.7×10^6 cells/ml	9	small	confluent	-
MyeloCult + Dex	0.7×10^6 cells/ml	13	confluent	confluent	-
MyeloCult + Dex	0.7×10^6 cells/ml	18	confluent	confluent	-
MyeloCult + Dex	0.7×10^6 cells/ml	23	confluent	confluent	-
MueloCult + pluronic	0.7×10^6 cells/ml	9	small	medium	-
MueloCult + pluronic	0.7×10^6 cells/ml	13	confluent	medium	-
MueloCult + pluronic	0.7×10^6 cells/ml	18	not visible	-	-
MueloCult + pluronic	0.7×10^6 cells/ml	23	not visible	-	-

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The studies in this chapter have confirmed that it is possible to expand MSCs in static monolayer cultures with all tested media conditions and achieve high increases in the total cell number after 29 days (5 passages). However, there was a large discrepancy between the increase in total cell number and the increase in colony forming cells.

The highest increase in CFU-f number was found after one or two passages as summarised in Table 4.4. Thereafter, the increase reduced to a lower level than at the beginning. This was true for all medium conditions. A possible reason could be the treatment with trypsin when passaging the MSCs. This treatment is known to stress the cells (Sun *et al.*, 2006), and could reduce their colony forming capacity as well as their differentiation potential.

Table 4.4: Summary of the results for the fold increase in CFU-f achieved by the different static culture methods. Since there are no significant differences between the medium conditions, cultures with DMEM are at least the cheapest to perform.

Media type	Static cultures max increase in CFU-f (fold)	Costs (£) / fold increase
DMEM	4.6	1.3
DMEM + Dex	4.6	1.3
MyeloCult®	4.6	3.0
MyeloCult® + Dex	4.4	3.2
MyeloCult® + pluronic	4.3	3.3
MyeloCult® + IL-3 + SCF	4.7	11.5

The addition of dexamethasone, IL-3 and SCF did not have any significant positive effect on the proliferation or differentiation behaviour of colony forming MSCs. If any of these additives provided an advantage, it was not evident in static culture systems. Maybe in more stressful environments like a stirred suspension culture the benefits of these additives will result in increased proliferation and survival rates.

0.05% Pluronic F-68 in the culture medium was sufficient to decrease the proliferation rate and the colony forming ability of MSCs in monolayer cultures. However, using FTIR spectrometry and zetaPALS analysis no evidence could be found that this effect was caused by pluronic changing functional groups in the cell membrane. In addition, pluronic did not seem to change the osteogenic differentiation capacity of the cells.

MSCs taken from all static cultures at any time point differentiated into osteoblasts when incubated in osteogenic medium. Furthermore, clearly visible colonies were formed in all osteogenic differentiation assays, as long as the assays were started with a sufficient number of MSCs. This indicates that at least some MSCs kept their full osteogenic differentiation and colony forming capacity.

In contrast to the osteogenic differentiation, no adipocytes were found in any adipogenic differentiation assay started with MSCs from any monolayer culture. This implies that the MSCs expanded in any of the static cultures did not have their full differentiation potential. Together with the results from the CFU-f assay, this is evidence that the static culture system is not the best way for *in vitro* expansion of MSCs.

Chapter 5

Proteomic Analysis of Mesenchymal Stem Cells

5.1 Introduction

In chapter 4, studies on the *in vitro* expansion of Mesenchymal Stem Cells (MSCs) in static monolayer cultures were described, and the results were presented. With this culture system, an increase in MSC number with osteogenic differentiation potential was achieved after a culture period of two weeks. The data indicated that a MSC population with the capacity to form colonies on culture plastic and to differentiate into osteoblasts can be expanded in adherent culture systems. However, a discrepancy between the increase in total cell number and in colony forming cells, led to the hypothesis that this culture method exerted chemical stress on the MSCs, and thus restricted their proliferation potential and their full differentiation capacity as indicated by a reduced capacity of colony formation, and the inability to differentiate into adipocytes. The chemical stress might be caused by using trypsin when subculturing the cells. To analyse the biological changes within stressed MSCs, and understand the reason for the loss of their proliferation and differentiation capacities, analysis of the global proteomic response using an isobaric labelling technique called iTRAQ™ were conducted on rat bone marrow (BM) MSCs expanded in static monolayer cultures for either 2 passages or 4 passages.

As highlighted in chapter 2, several groups have worked on the proteomic analysis of MSCs, and built up a comprehensive understanding of the MSC proteome in addition to the sequenced genome. Such information can be used to discover biomarkers and identify the normal proteomic pattern, in comparison to changes in activated or suppressed pathways, which occur during proliferation, differentiation, or other experimental conditions (Wagner *et al.*, 2006). Thus far, the majority of MSC proteomic research has primarily used 2-dimensional gel electrophoresis (2DE), which visualises parts of the cell proteome of MSCs, and then identifies single proteins from the gels by various forms of mass spectrometry (MS). Of special interest are the changes of the MSCs proteome caused by differentiation, especially osteogenic and adipogenic differentiation, by various cytokines and by *in vitro* aging.

Foster *et al.* (2005), Salasznyk *et al.* (2005) and Zhang *et al.* (2007) identified differently expressed proteins responsible for osteoblast differentiation in human mesenchymal stem cells (hMSCs) using 2DE and Matrix Assisted Laser Desorption /

Ionization - Time Of Flight (MALDI-TOF) mass spectrometry. Lee *et al.* (2006) analysed the proteome of adipocytes differentiated from hMSCs applying the same techniques and found 4 proteins with different expression levels, which were associated with adipogenesis. Wang *et al.* (2004) and Kratchmarova *et al.* (2005) studied the mechanism of divergent growth factor effects in hMSCs using 2DE and liquid chromatography - tandem mass spectrometry (LC-MS/MS). More interesting proteomic research related to the project described in this chapter was conducted by Sun *et al.* (2006) and Celebi and Elcin (2008) both using 2DE and MALDI-TOF-MS. Sun *et al.* (2006) analysed the proteome of hMSCs during serial subculture and osteogenic differentiation and showed that the proliferation and osteogenic capacity of these cells decrease during serial subculturing. Several proteins associated with cell proliferation, cell cycle, morphological changes, and apoptosis were shown to be differentially regulated during serial subculture. Moreover, further proteins were identified, which were differentially regulated during osteogenic differentiation, indicating that differentially regulated passage-specific proteins may play a role in the decrease of osteogenic differentiation potential under serial subculturing. Celebi and Elcin (2008) aimed to generate proteome maps of undifferentiated rat BM-MSCs, and to identify differentially regulated proteins during serial subcultures. They extracted proteins from Wistar rat BM-MSCs, and detected about 1000 proteins of which 31 proteins were successfully identified and 18 were differentially expressed. Some of these proteins were associated with proliferation and differentiation capacity such as N-myc downstream regulated protein, adenylate kinase isoenzyme 1 and T-complex protein 1 alpha subunit.

Although the majority of MSC proteomic research has primarily used 2DE, this technique has a major limitation since not all proteins can be visualised in this way. Alternative non-gel-based quantitative MS approaches in proteome research are available. One technique is referred to as isobaric tag for relative and absolute quantitation (iTRAQ), which permits more accurate quantification and improved sequence coverage of proteins (Wiese *et al.*, 2007). Furthermore, it allows the direct comparison of entire protein networks and the uncovering of critical differences that are capable of changing the fates of MSCs. iTRAQ technology is based on labelling peptides after enzymatic digestion of the proteins. iTRAQ labels (tags) are designed to form a covalent link with primary amines found at the lysine side chain and on the

N-terminus of tryptic peptides generated from protein digests (Ross *et al.*, 2004). The iTRAQ workflow is shown in Figure 5.1. Different labelled samples are combined, fractionated by nano liquid chromatography (nanoLC) and analysed by tandem mass spectrometry (MS/MS). Due to the isobaric mass design of the iTRAQ reagents, differentially labelled peptides appear as single peaks in MS scans, thus reducing the probability of peak overlapping. When iTRAQ-tagged peptides are subjected to MS/MS analysis, the fragmentation of the tags attached to the peptides liberates a low molecular mass, isotope-encoded reporter ion that is unique for each group of tags. Relative quantification requires integrating the area under the MS peak for each reporter ion and calculating the different ratios of reporter ion peak areas. These ratios correspond to the ratios of peptide concentrations in the tested experimental conditions and the proteins from which they originate. Database searching of the fragmentation data from the peptides results in the identification of the labelled peptides and the corresponding proteins. Because there are up to eight different iTRAQ reagents available, comparative analysis of a set of 2 – 8 samples is possible within one single MS run. Several research groups have used the iTRAQ technique to analyse and compare the proteome of different mammalian cells (Unwin *et al.*, 2005) (Xu *et al.*, 2009), including human MSCs (Seshi, 2006).

One of the major hurdles that have to be overcome in large-scale proteomic studies in general is the reduction of sample complexity. As yet, there is no single method that allows identification of all proteins present in a sample. Furthermore, differences in culture methodologies applied in different laboratories are likely to induce variations in protein expression (Wagner *et al.*, 2006). Thus, the different protein separation techniques, proteome analysis methods, and culture conditions, of various individual research groups, result in the generation of a variety of proteome data sets that are difficult to compare directly. Nevertheless, the information obtained through different methods should prove to be complementary, and provide a better understanding of the biological system. In this chapter, the proteomic differences in bone marrow derived rat BM MSCs from different passage levels were analysed in order to determine their influence on the proliferation and differentiation capacity of these cells. The protocol for culturing MSCs in static monolayer pour-off cultures was the same as that described in chapter 4.

5.2 Materials and methods

The methods for analysing the proteome of MSCs, which were expanded in static monolayer cultures, are described. The analysis was conducted with an electrospray ionisation quadrupole time-of-flight tandem mass spectrometer (ESI-qQ-TOF-MS/MS) using an iTRAQ based proteomic workflow.

5.2.1 Mesenchymal Stem Cell static culture

MSCs were derived from rat bone marrow as described in chapter 3.4. 10 ml of freshly harvested BMCs were suspended in 10 ml culture medium and seeded into a T75 flask, having a final density of about 1 x 10^6 cells/ml. Cell culture medium was normal DMEM. The cells were incubated at 37°C in an atmosphere of 7.5% CO_2. The first medium change was after 5 days and thereafter twice weekly until ~90% confluence was reached. Then the cells were passaged and again expanded until ~90% confluence. To detach the adherent cells, the medium was removed and the flasks were washed with phosphate buffered saline (PBS) before 3 ml of 0.25% trypsin/1mM EDTA solution was added. The culture was incubated for 5 minutes at 37°C. To stop the incubation, 9 ml of culture medium, containing 10% FCS, were added and a sample was taken to determine the cell number in the solution using a haemocytometer. The lifted cells then were split 1:2 and transferred into new sterile T75-flasks. At passage 2 and 4 cells from 3 confluent flasks were combined and centrifuged (Scientific Laboratory Supplies Sigma 4K15) at 300g for 5 minutes to pellet the cells. To remove all traces of medium, cells were washed with 3 ml Dulbecco's Phosphate Buffered Saline (PBS) (Cambrex Bio Science Ltd Wokingham).

5.2.2 Protein extraction

Cells were re-suspended in 300 µl PBS by dissociating the pellet with a 200 µl pipette, forcing the cells into and out of the tip in a controlled manner. To break the cells open, they were sonicated for 10 – 15 seconds using the Sonifier 450 (Branson). Proteins were recovered by centrifugation at 21,000g for 30 minutes at 4°C (Heraeus

Model Multifuge 3S-R, Germany). The total protein concentration in the supernatant was quantified using the Bradford test (OD_{595}) (BioRad) according to the manufacturer's instructions. Two times 100 µg of protein (biological replicates) from both conditions (cells harvested at passage 2 and at passage 4) were precipitated in 4°C cold acetone by mixing one volume of protein solution corresponding to 100 µg of protein with six volumes of acetone, incubating at -20°C for 4 hours and decanting the acetone.

5.2.3 Protein reduction, cysteine blocking and protein digestion

The following two steps mentioned in section 5.2.3 and 5.2.4 were carried out using the iTRAQ™ 4 plex kit from Applied Biosystems.

To reduce the proteins, 20 µl dissolution buffer, 1µl denaturant and 2 µl reducing reagent were added to each sample before they were incubated for 1 hour at 60°C. After spinning the samples down (2,000g for 2-3 seconds), 1 µl cysteine blocking reagent was added and they were incubated at room temperature for another 10 minutes. Finally 10 µl trypsin solution (containing 25 µg trypsin and 222 µg calcium chloride) was mixed into each sample which were then incubated at 37°C overnight.

5.2.4 Labelling the protein digests with the iTRAQ™ reagents

Each iTRAQ™ reagent (114 - 117) was dissolved in 70 µl ethanol, transferred to the sample tubes containing the cell digests and incubated at room temperature for 1 hour. The two biological replica samples from passage 2 were labelled with reagents 114 and 115 and the samples from passage 4 were labelled with the reagent 116 and 117. Afterwards, the labelled samples were combined and vacuum concentrated prior to fractionation using strong cation exchange.

5.2.5 Strong cation exchange (SCX) fractionation

Separation was performed on a BioLC HPLC column (Dionex, Surrey, U.K.) using a Polysulfoethyl A column (PolyLC, Columbia, MD) 5 μm of 100 mm length × 2.1 mm i.d. and 200 Å pore size. Buffer A consisted of 10 mM KH_2PO_4 and 25% acetonitrile at pH 3.0, filter sterilised and buffer B consisted of 10mM KH_2PO_4, 25% acetonitrile, and 500 mM KCl at pH 3.0, filter sterilised. The 60 minute gradient consisted of 100% buffer A for 5 minutes, 5 to 30% buffer B for 40 minutes, 31 to 100% buffer B for 5 minutes, 100% buffer B for 5 minutes, and finally 100% buffer A for 5 minutes. A flow rate of 0.2 ml/minute was maintained with an injection volume of 200 μl. A UV detector (UVD170U) and Chromeleon Software v6.50 (Dionex/LC Packings, The Netherlands), was used to monitor the chromatogram as fractions were collected every minute using a Foxy Jr. Fraction Collector (Dionex). Fractions were dried in a vacuum centrifuge and stored at -20°C prior to mass spectrometric analysis.

5.2.6 Mass spectrometry

Mass spectrometry (MS) was performed using a QStar XL Hybrid ESI Quadrupole time-of-flight tandem mass spectrometer, ESI-qQ-TOF-MS/MS (Applied Biosystems, Framingham, MA; MDS-Sciex, Concord, Ontario, Canada) coupled with an online capillary liquid chromatography system (Famos, Switchos and Ultimate from Dionex/LC Packings, Amsterdam, The Netherlands). 60 μl of 3% acetonitrile and 0.1% formic acid (Switchos buffer) was used to resuspend the dried samples ready for the MS. 10 – 15 μl (depending on the peptide concentration as seen in the peak intensity of the SCX chromatogram) were injected to the nano-LC-ESI-MS/MS system for each analysis. Initial separation took place on a PepMap C-18 RP capillary column (LC Packings) with a constant flow rate of 0.3 μl/min. LC buffers A and B were made up as 3% acetonitrile, 0.1% formic acid and 97% acetonitrile, 0.1% formic acid, respectively. The gradient was started as 97% buffer A and 3% buffer B for 3 minutes, followed by 3 to 25% buffer B for 120 minutes, 90% buffer B for 7 minutes and finally 97% buffer A for 7 minutes. Data acquisition in the mass spectrometer was set to the positive ion mode, with a selected mass range

of 350-1800 *m/z*. Tandem mass spectrometry was performed on peptides with +2, +3 charge states across a scan range of 65-2000 *m/z*.

5.2.7 Protein identification

Protein Pilot software v.2.0 (Applied Biosystems, MDS-Sciex) was used for protein quantification and identification. Identifications were made on the 8th July 2008, using NCBI and swiss prot protein database. Search parameters were set at a mass tolerance suitable for QSTAR instruments and Peptides with above 80% confidence were used for identification and quantification (Figure 5.1). The Panther (Protein ANalysis THrough Evolutionary Relationships) classification system was used to assign molecular function and biological process for the set of proteins identified (and relatively quantified). The functional relationship of differentially expressed proteins was further investigated using MetaCore™ (GeneGo Inc.) software.

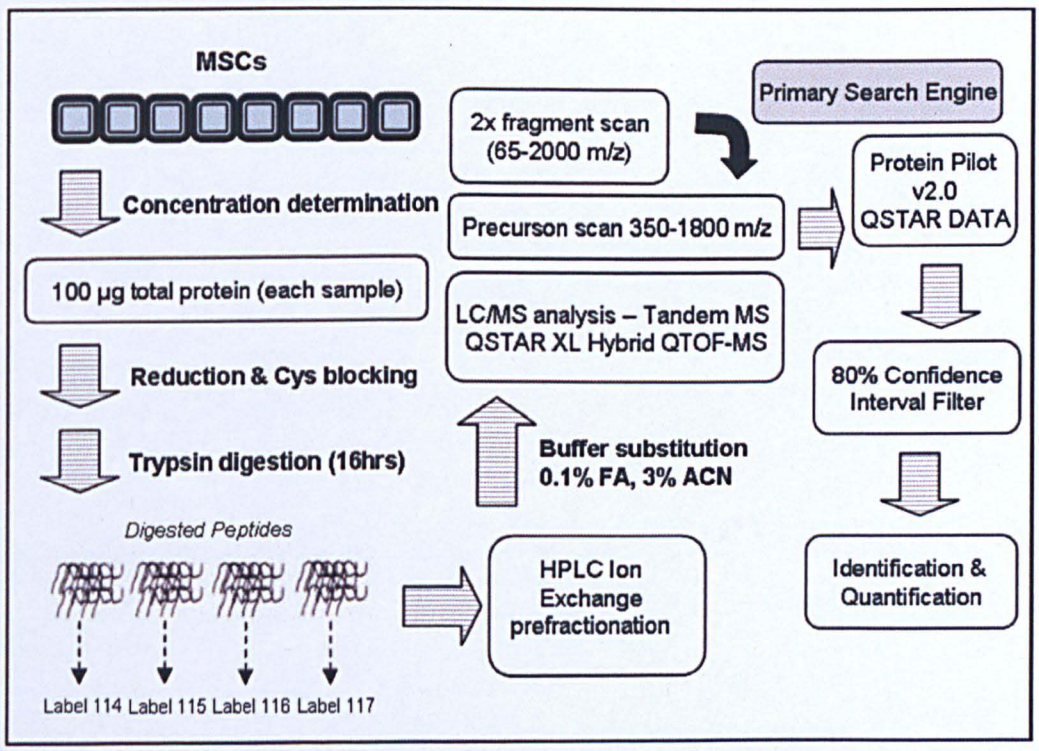

Figure 5.1: Diagram to show the sample preparation and the proteomic workflow for iTRAQ based analysis of mesenchymal stem cells.

5.3 Results and Discussion

The results are presented and discussed in three parts. Firstly, the biological phenotype i.e. growth and colony forming behaviour, as well as the morphology of mesenchymal stem cells at the different passage levels is described. Secondly, the differentially regulated proteins are identified, characterised and compared to other proteomic studies of bone marrow MSCs. Finally, only proteins which have been identified for the first time as proteins of interest, in terms of MSC biology are described in detail.

5.3.1 Morphology and proliferation rate of Mesenchymal Stem Cells

MSCs were expanded in static monolayer cultures as mentioned in paragraph 5.2.1. They were always plated at the same density (~ 1 x 10^6 cells / flask) and subcultured at similar confluence levels. The proliferation rate of MSCs during the 4 passage period decreased only slightly. The time to reach confluence varied for the different passage levels (Figure 5.2). The p-value for significant differences between the times to reach confluence from passage 2 and 4 is $p = 0.024$. More interestingly, after an initial increase, the colony forming potential decreased over the same time period. Figure 5.3 displays the number of colonies formed when 4 x 10^3 MSCs were plated into cell culture dishes and grown as CFU-f culture after each passage. At passage 2, 11,900 colony forming cells were present, whereas at passage 4 only 5,000 CFU-fs could be found. The p-value for significant differences between these numbers is $p = 0.047$.

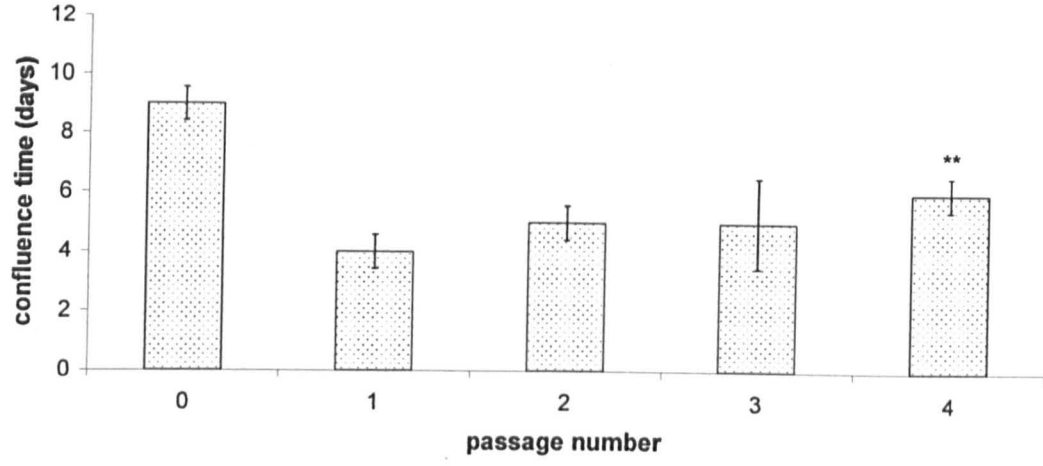

Figure 5.2: Proliferation rate of rat bone marrow derived MSCs, in terms of reaching confluence level during subcultures. Cells were expanded in static cultures with standard DMEM. Plating density was about 1 x 10^6 cells per T-75 flask. Data are presented as mean +/- S.D. from 3 different cultures. **$p < 0.05$, confluence time at each passage level compared with confluence time at passage number 2.

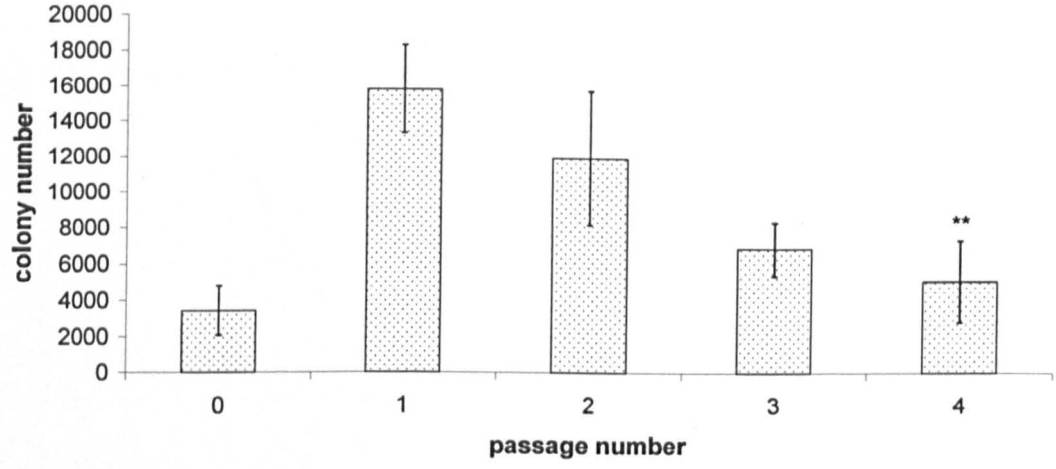

Figure 5.3: Development of CFU-f verses passage number. Cells were derived from rat bone marrow and expanded in static cultures with DMEM. Data are presented as mean +/-S.D. from 3 different CFU-f assays. **$p < 0.05$, colony number at each passage level compared with colony number at passage number 2.

Furthermore, the average size of the colonies also decreased after serial subculturing. MSCs expanded in static cultures for as long as 2 passages formed small but recognisable colonies during the CFU-f assay. The colonies formed by MSCs passaged more than twice were barely visible on the plates (Figure 5.4). In addition,

the morphology of the single MSC in the monolayer cultures changed over time as well, and some phenotypic differences were observed between primary and subcultured cells. As it can be seen in Figure 5.5, the shapes of the adherent rat bone marrow cells in the primary culture (passage 0 cells) were heterogeneous, consisting of both fibroblastic cells and polygonal cells (Figure 5.5A). The majority of the cells demonstrated spindle morphology in ongoing subcultures. At passage level 2, they spread and demonstrated typical elongated fibroblastic phenotype (Figure 5.5B). Figures 5.5C and 5.5D show a representative confluent culture after 3 and 4 passages, respectively. In both cultures, the accumulation of the extracellular matrix can be observed as the cells became wider.

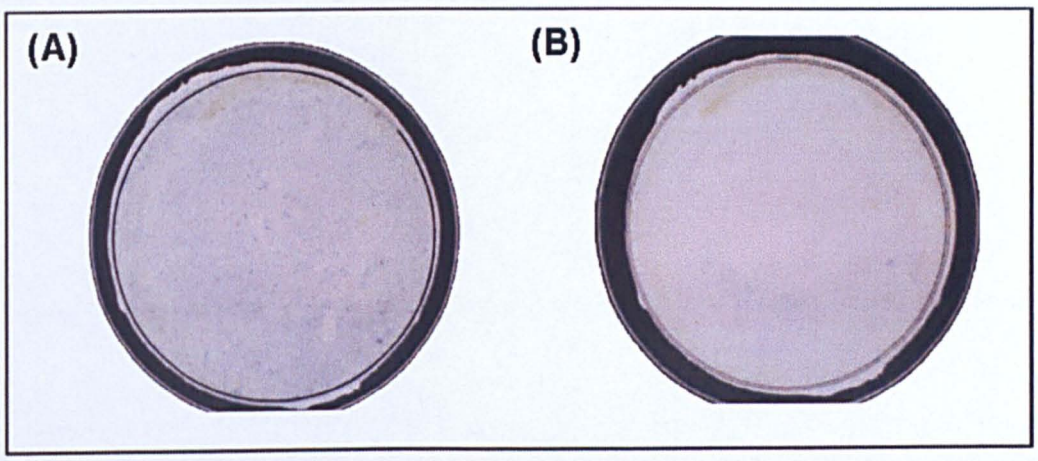

Figure 5.4: Methylene blue stained CFU-f cultures started with MSCs which were expanded in standard monolayer cultures. Visible colony formation with MSCs after passage level 2 (A) and no visible colonies after 4 passages (B).

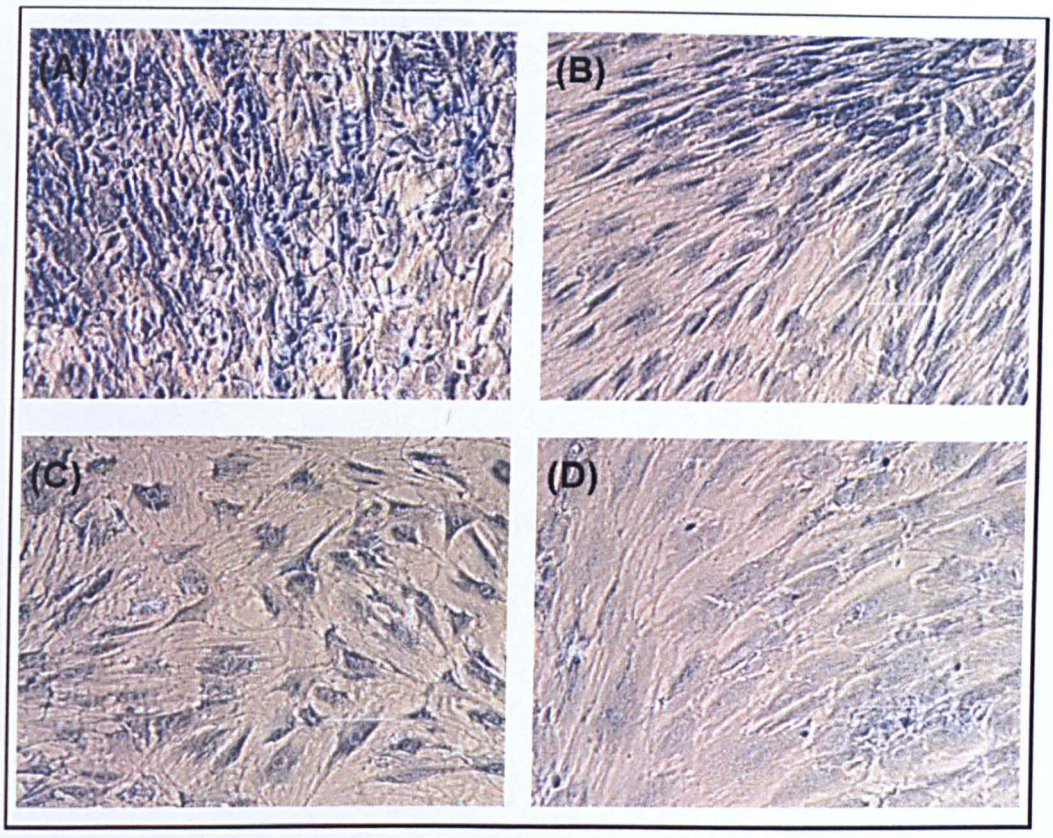

Figure 5.5: Methylene blue stained MSC static cultures. Confluent primary culture of rat bone marrow derived cell, passage 0 (A). Thin spindle shaped like MSCs after passage 2 (B). Bigger and wider MSCs at passage 3 (C) and passage 4 (D).

5.3.2 Mass spectrometry analysis

The proteins extracted from MSCs harvested after passage 2 and passage 4 were identified and relatively quantified using the iTRAQ technique (see material and methods in this chapter). A complete list of all identified proteins can be found in Appendix A. In order to calculate the different expression levels of the proteins at passage 2 and passage 4, the $\log_{10}$ ratio of the peptides labelled from both replicas at passage 2 (115:114) were compared to the $\log_{10}$ ratios of the peptides labelled from passage 4 and passage 2 (116:114) and (117:114), using the equation 5.1.

$$\text{Fold change} = 10^{\left(\frac{\log \frac{(115:114)}{(116:114)} + \log \frac{(115:114)}{(117:114)}}{2} \right)} \qquad 5.1$$

Proteins were considered up-regulated for a fold change rate of a minimum of 1.5 or bigger and down-regulated for a rate of maximum of 0.5 or smaller. In total, 381 proteins were identified and of these 105 were differently expressed with 48 proteins up-regulated and 57 down-regulated. In a study conducted by Seshi (2006), the iTRAQ technique was applied to identify differently expressed proteins in normal and leukemic bone marrow stromal cells. In this study 900 proteins were identified in total, and 73 of these were differentially expressed in the leukemic cells. Comparing both iTRAQ results with other liquid chromatography / tandem mass spectrometry based studies of MSCs, similar numbers of identified and regulated proteins (between 104 and 113 proteins) were identified (Foster *et al.*, 2005) (Kratchmarova *et al.*, 2005). However, most of the studies analysing the MSC proteome used 2 dimensional gel electrophoresis (2DE) and matrix assisted laser desorption / ionisation time of flight mass spectrometry (MALDI-TOF-MS). Applying these techniques between 1000 and 1700 protein spots were separated in 2DE gels and between 12 and 63 proteins were identified with MALDI-TOF-MS (Sun *et al.*, 2006) (Wagner *et al.*, 2006). Celebi and Elcin (2008) also investigated the effect of subculturing on the proliferation potential of bone marrow derived rat MSCs. They detected 1000 proteins spots on 2DE gels of which only 31 proteins were identified with MALDI-TOF-MS. Compared to the findings by Celebi and Elcin (2008), the iTRAQ approach in this study allowed identification of a higher percentage of the proteins in the samples and a greater number of differentially expressed proteins.

5.3.3 Functional categories and identification of regulated proteins

In total, 79 of the regulated proteins in this study could be classified into nine functional categories (cell structure and cytoskeleton, ion transport, protein synthesis and degradation, metabolic enzymes, translation and transcription, signalling pathways, cell adhesion, cell cycle and proliferation, and stress response) (Tables 5.1 and 5.2). The largest group of regulated proteins were the cell structure and cytoskeleton proteins (25%), followed by the metabolic enzymes (20%) and the proteins involved in protein synthesis and degradation (14%). Figure 5.6 shows the percentage of all categorised proteins. These numbers, however, changed when either only the up-regulated or only the down-regulated proteins were considered. Amongst the up-regulated proteins, 26% were involved in protein synthesis and degradation, whereas for the down-regulated proteins the largest group were the cell structure and cytoskeleton proteins (27%). Furthermore, all identified cell cycle proteins were down-regulated, whereas all identified proteins engaged in cell adhesion were up-regulated (Figures 5.7 and 5.8). 30 of these 79 classified proteins were found in earlier proteomic studies of bone marrow derived MSCs (Wagner *et al.*, 2006) (Zhang *et al.*, 2007) (Sun *et al.*, 2006). Hence, 49 proteins being regulated in MSCs during subculturing are mentioned in this study for the first time. A list of all regulated and classified proteins with the corresponding reference of previous studies (if applicable) can be found in Table 5.1.

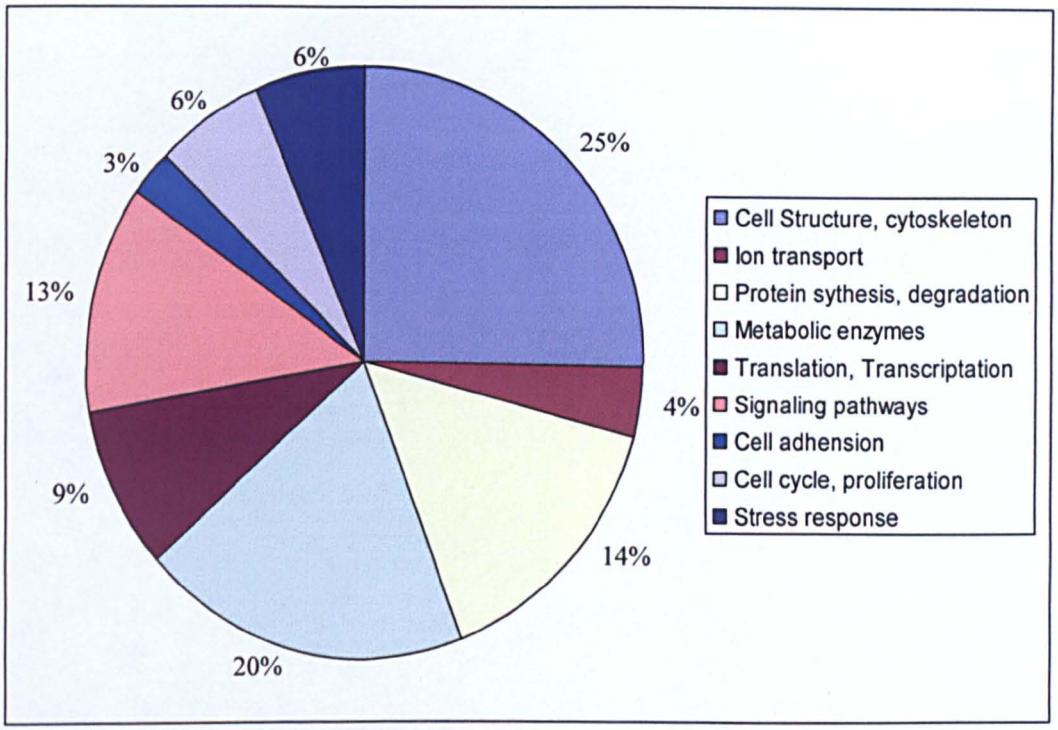

Figure 5.6: Functional categories of all identified and regulated proteins. The majority belong to cell structure and cytoskeleton, protein synthesis and degradation, and metabolic enzymes.

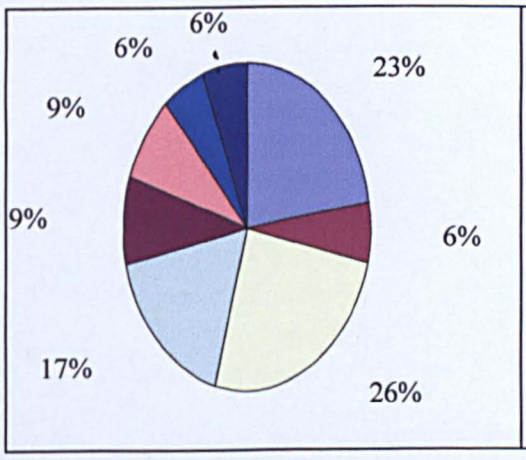

Figure 5.7: Functional categories of up-regulated proteins. The largest group consists of protein synthesis and degradation.

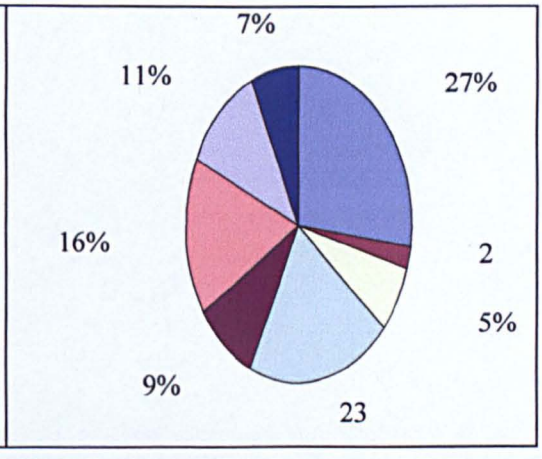

Figure 5.8: Functional categories of down-regulated proteins. The largest group consists of cell structure and cytoskeleton proteins.

Table 5.1: Differentially expressed proteins (up-regulated) in rat bone marrow MSCs identified by Protein Pilot software v.2.0 (Applied Biosystems, MDS-Sciex) and NCBI / swiss prot protein database. Proteins were categorised using PANTHER software. The references mention the study in which the proteins were described before. A blank entry means indicates a novel protein found in MSCs.

Accession no.	Protein identity	fold change	Category	References
gi|71681130	ATP synthase, H+ transporting	5.35	Ion transport	Sun et al. 2006
gi|63101555	Sec61 alpha 1 subunit (S. cerevisiae)	4.72	Cell Structure	-
gi|728810	ADP/ATP translocase 2 (ANT 2)	4.45	Ion transport	-
gi|83300587	ATP synthase subunit alpha	3.57	Metabolic enzymes	Foster et al. 2005
gi|92373398	Y box protein 1	3.36	Translation, Transcriptation	-
gi|9845234	annexin A2	3.09	Cell Structure	Sun et al. 2006; Nengshend et al. 2006; Zhang et al. 2007; Wang et al. 2004
gi|85541036	Myosin-Id (Myosin heavy chain myr 4)	3.05	Cell Structure	Mareddy et al. 2009
gi|62512124	Ribophorin II (RPN-II)	2.84	Protein sythesis	-
gi|57012987	PRA1 family protein 3	2.73	Protein sythesis	-
gi|157823041	integrin, alpha 11	2.73	Cell adhesion	Foster et al. 2005
gi|68565369	Isocitrate dehydrogenase (NAD(+)-specific ICDH)	2.71	Metabolic enzymes	Wagner et al. 2006
gi|145566928	Polymerase I and transcript release factor (cav-p60)	2.65	Translation, Transcriptation	-
gi|6981052	heat shock 10 kDa protein 1	2.55	Protein sythesis	Sun et al. 2006; Wagner et al. 2006; Mareddy et al. 2009; Nengsheng et al. 2006

gi|55824765	Serpinh1 protein	2.55	Protein sythesis	-
gi|149015978	fibronectin 1, isoform CRA_a	2.51	Cell adhesion	-
gi|763181	annexin VI	2.47	Signaling pathways	Kratchmarova et al. 2005
gi|58865778	dolichyl-di-phosphooligosaccharide-protein glycotransferase	2.44	Cell Structure	-
gi|48675371	complement component 1	2.39	Stress response	-
gi|33086478	Ab1-205	2.24	Metabolic enzymes	-
gi|6822247	Nogo-A protein	2.24	Metabolic enzymes	-
gi|50403574	60S ribosomal protein L10	1.94	Protein sythesis	-
gi|8393206	cysteine and glycine-rich protein 1	1.89	Cell Structure	-
gi|157823877	cytoskeleton-associated protein 4	1.89	Cell Structure	-
gi|3914232	Transmembrane emp24 domain-containing protein 2	1.89	Signaling pathways	-
gi|55977470	Tubulin alpha-1A chain (Alpha-tubulin 1)	1.83	Cell Structure	Kratchmarova et al. 2005; Wagner et al. 2006
gi|77993298	signal sequence receptor, alpha	1.81	Protein sythesis	-
gi|154816168	heat shock 70kDa protein 9A	1.79	Stress response	-
gi|38454246	ribosomal protein L3	1.75	Protein sythesis	Wagner et al. 2006
gi|157820787	NADH dehydrogenase (ubiquinone) 1	1.73	Metabolic enzymes	-
gi|57858	ribosomal protein S24	1.72	Protein sythesis	-
gi|42476181	malate dehydrogenase, mitochondrial	1.71	Metabolic enzymes	Wang et al. 2004
gi|44888251	Phosphatidylinositol-binding clathrin assembly protein	1.68	Translation, Transcriptation	-
gi|78214352	cysteine and glycine-rich protein 2	1.68	Cell Structure	-
gi|71795613	ribosomal protein, large P2	1.61	Protein sythesis	Wagner et al. 2006
gi|56971262	Hnrpa1 protein	1.61	Translation, Transcriptation	-

Table 5.2: Differentially expressed proteins (down-regulated) in rat bone marrow MSCs identified by Protein Pilot software v.2.0 (Applied Biosystems, MDS-Sciex) and NCBI / swiss prot protein database. Proteins were categorised using PANTHER software. The references mention the study in which the proteins were described before. A blank entry means indicates a novel protein found in MSCs.

Accession no.	Protein identity	fold change	Category	References
gi|77404363	nucleosome assembly protein 1-like 1	0.12	Cell cycle, proliferation	-
gi|6981326	S100 calcium-binding protein A4	0.21	Stress response	-
gi|8393610	karyopherin (importin) beta 1	0.29	Signaling pathways	-
gi|157786926	actin related protein 2/3 complex, subunit 3	0.32	Cell Structure	-
gi|81884568	Macrophage-capping protein (CAP-G)	0.32	Cell Structure	-
gi|14549433	calcium binding protein NEFA	0.33	Ion transport	-
gi|157819651	chaperonin subunit 7 (eta)	0.33	Cell Structure	Zhang et al. 2007
gi|157786744	dihydropyrimidinase-like 2	0.34	Metabolic enzymes	Zhang et al. 2008
gi|6911221	CArG-binding factor A	0.36	Translation, Transcriptation	-
gi|55562869	Tumor protein, translationally-controlled 1	0.38	Cell cycle, proliferation	Wagner et al. 2006
gi|56585024	Phosphoglycerate kinase 1	0.41	Metabolic enzymes	Ye et al. 2006
gi|70912366	ARP3 actin-related protein 3 homolog	0.41	Cell Structure	Kratchmarova et al. 2005
gi|51316981	ADP-ribosylation factor 1	0.44	Signaling pathways	-
gi|52783577	Dynein light chain 1	0.44	Cell Structure	-
gi|54036318	S100 calcium-binding protein A11 (Calgizzarin)	0.45	Cell cycle, proliferation	-
gi|6093729	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (LH1)	0.45	Metabolic enzymes	-
gi|407164	heat shock protein 70	0.46	Stress response	Wagner et al. 2006

gi|74355722	Capping protein (actin filament) muscle Z-line, alpha 2	0.46	Cell Structure	Sun et al. 2006
gi|38382858	Protein disulfide isomerase associated 3	0.47	Protein sythesis	Sun et al. 2006; Wagner et al. 2006
gi|59709467	CAP, adenylate cyclase-associated protein 1	0.48	Signaling pathways	-
gi|2498032	Myosin regulatory light chain 2	0.48	Cell Structure	Mareddy et al. 2009
gi|149030883	glutathione synthetase, isoform CRA	0.49	Metabolic enzymes	Wagner et al. 2006
gi|149034971	rCG42612, isoform CRA_b	0.49	Cell Structure	-
gi|6978505	annexin A5	0.50	Metabolic enzymes	Wagner et al. 2006
gi|40254781	GDP dissociation inhibitor 2	0.50	Signaling pathways	Wagner et al. 2006
gi|399660	aldehyde reductase	0.51	Signaling pathways	Zhang et al. 2007
gi|81889864	Tubulin alpha-4A chain	0.52	Cell Structure	Kratchmarova et al. 2005; Wagner et al. 2006
gi|149029682	rCG42490, isoform CRA_f	0.53	Cell Structure	-
gi|149036910	actin related protein 2/3 complex, subunit 4	0.54	Cell Structure	-
gi|157818179	eukaryotic translation elongation factor 1 beta 2	0.55	Translation, Transcriptation	Sun et al. 2006
gi|157819753	reticulocalbin 1	0.56	Signaling pathways	-
gi|6981712	tyrosine 3-monooxygenase	0.56	Cell cycle, proliferation	-
gi|48675845	IMP cyclohydrolase	0.56	Metabolic enzymes	Wagner et al. 2006
gi|6831527	Dimethylarginine dimethylaminohydrolase 1 (DDAH-1)	0.56	Cell cycle, proliferation	-
gi|71051777	Heat shock protein 8	0.57	Stress response	Wagner et al. 2006
gi|158631214	arginyl-tRNA synthetase	0.57	Translation, Transcriptation	-
gi|6978501	annexin A1	0.57	Metabolic enzymes	Sun et al. 2006
gi|56404680	Protein DJ-1 (Parkinson disease protein 7 homolog)	0.59	Translation	-

gi|62201921	Prothymosin alpha	0.59	Metabolic enzymes	-
gi|91771192	Asparagine synthetase [glutamine-hydrolyzing]	0.59	Protein sythesis	-
gi|33086658	Cc1-6	0.59	Metabolic enzymes	-
gi|149033299	rCG52516	0.59	Protein sythesis	-
gi|47605935	Transforming protein RhoA precursor	0.59	Cell Structure	-
gi|149041736	reticulocalbin 2	0.60	Signaling pathways	-

MetaCore™ was used to put the classified proteins in relation to each other by assigning groups of them to the same regulatory and metabolic networks. According to the MetaCore™ software (GeneGo Inc.), 27 networks were identified in which at least two or more proteins were present. The relation of the proteins to each other in these networks was calculated using Dijkstra's algorithm (Dijkstra, 1959). Furthermore, each of these networks was associated with a Z-score which ranks the networks according to their saturation with the proteins initially identified. A high Z-score indicates that the network is highly saturated with identified proteins. The formula for the Z-score is listed in equation 5.2.

$$Z\text{-}score = \frac{r - n\frac{R}{N}}{\sqrt{n\left(\frac{R}{N}\right)\left(1-\frac{R}{N}\right)\left(1-\frac{n-1}{N-1}\right)}}$$

Where:

N	- total number of nodes in MetaCore database
R	- number of the network's objects corresponding to the genes and proteins in your list
n	- total number of nodes in each small network generated from your list
r	- number of nodes with data in each small network generated from your list

Equation 5.2: Z-score used by MetaCore™ to rank the networks of identified proteins.

33 of the categorised proteins could be found in these 27 networks, but only 4 proteins, grouped in 2 pairs, with direct interactions were identified. The tumour protein, translationally-controlled 1 (TPT1) regulated the eukaryotic translation elongation factor 1 beta 2 (eEF1B), and the heterogeneous nuclear ribonucleoprotein A1 (hnRPA1) influenced the prothymosin alpha (PTMA).

The translationally-controlled tumour protein 1 (TPT1) is believed to be important for cell growth and cell division as it was found considerably up-regulated in cells entering the cell cycle. This conclusion was based on the results from three different studies. Gachet *et al.* (1999) found that overexpression of TPT1 in mammalian cells caused slow growth and a delay in cell cycle progression, a knockdown of TPT1 in *Caenorhabditis elegans* resulted in a slow-growth phenotype (Kamath *et al.*, 2003),

and Li *et al.* (2001) demonstrated that TPT1 can prevent cell death by inhibiting caspase-3-like activity and concluded that the down-regulation of TPT1 was associated with massive cell death. Thus, an antiapoptotic function of TPT1 in human cells has been recognised. Furthermore, Koziol *et al.* (2007) suggested that TPT1 represents a global regulator of pluripotency, and that it might have a general role in establishing an embryonic pattern of gene expression because it controlls the transcription of *oct4* and *nanog*. Baharvand *et al.* (2007) also detected decreasd TPT1 levels in differentiating mouse embryonic stem cells. In MSCs, TPT1 was reported for the first time by Wagner *et al.* (2006), however no details on the involvement in the regulation of the cells was given.

Eukaryotic translation elongation factor 1 beta 2 (eEF1B) is predominantly located in the endoplasmic reticulum (ER) (Sanders *et al.*, 1996). As part of the eEF-1βμδ complex, it catalyses the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on eEF-lα (Iwasaki *et al.*, 1976). eEF-1α is the protein that is responsible for the binding and transportation of aminoacyl-tRNA to the ribosome. Modification of the subunit eEF1B, could affect the formation rate of the eEF-lα-GTP-aminoacyl-tRNA complex, and thereby alter the cellular protein sythesis (Janssen *et al.*, 1988). eEF1B contains a casein kinase II activity. This kinase phosphorylates eEF1B at a specific serine residue and thus influences negatively the nucleotide exchange activity, which in turn has consequences for the regulation of translation (Walter *et al.*, 1984). Sun *et al.* (2006) found eEF1B down-regulated in bone marrow derived MSCs after serial subculturing (3 – 5 passages). They concluded that the down-regulation of passage specific proteins are the reason for their observed decreased in proliferation capacity.

In this study, TPT1 and eEF1B were both down-regulated in MSCs passaged 4 times compared to MSCs passaged only twice. With MetaCore™, a possible direct interaction of TPT1 and eEF1B was found (Figure 5.9). Furthermore, eEF1B and eEF1A are directly connected in this network, indicating that this network may be part of the translation regulation. The observed slight decrease in proliferation of *in vitro* expanded MSCs, and the even more obvious reduction in colony size and in colony forming capacity supports the theory that TPT1 and eEF1B regulate the cell proliferation in MSCs. A list of all networks calculated by MetaCore™ and the

legend for the diagrams in Figures 5.9 and 5.10 are attached in the Appendix B and Appendix C.

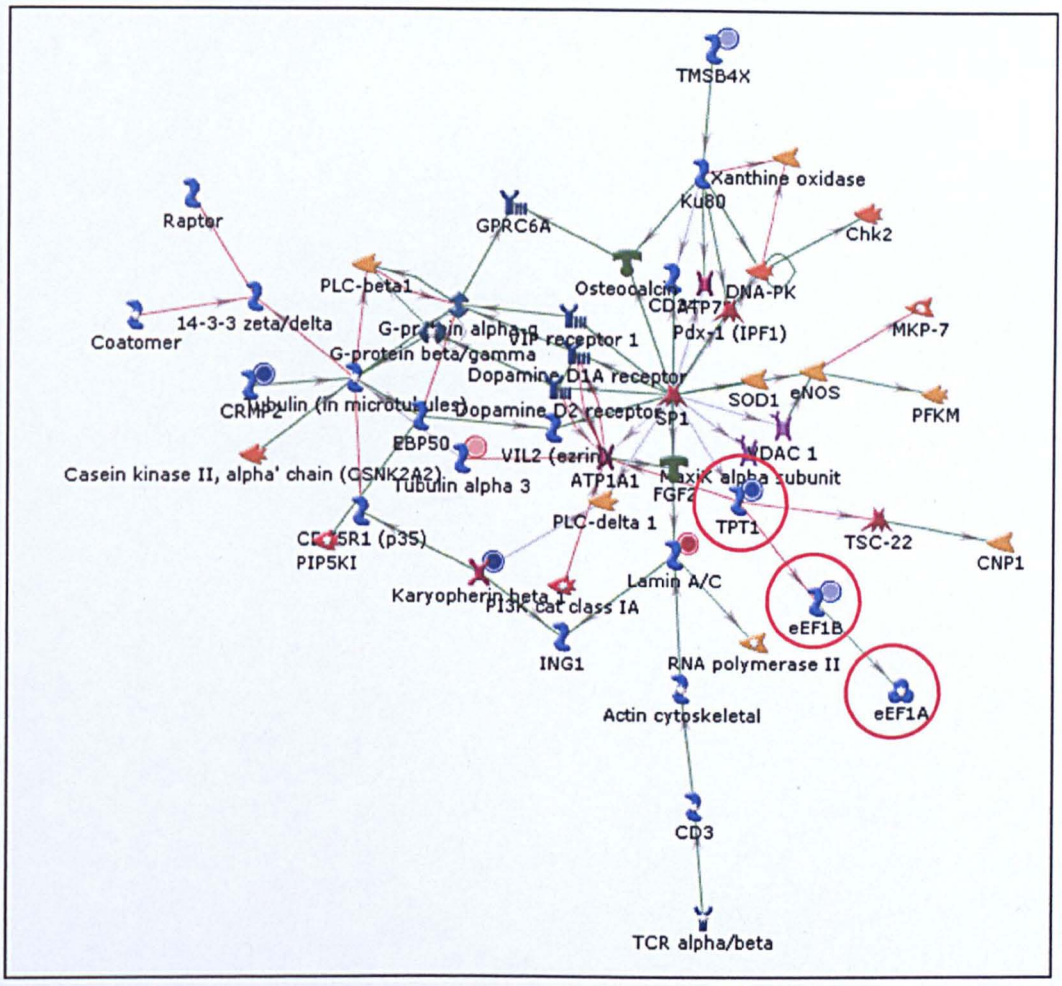

Figure 5.9: Regulatory network calculated by MetaCore™ in which TPT1, eEF1B and eEF1A are in direct interaction. A legend can be found in Appendix C.

In this study, 18 out of the 33 interacting proteins were found expressed in MSCs for the first time. The following paragraphs describe some of these newly discovered proteins. Amongst them were heterogenous nuclear ribonucleoprotein A1 (hnRPA1) and prothymosin alpha (PTMA), which were connected by a direct interaction, according to MetaCore™.

Heterogenous nuclear ribonucleoprotein A1 (hnRPA1) plays an important role in the mRNA metabolism by binding RNA at its N terminus (Dreyfuss *et al.*, 2002) (Krecic *et al.*, 1999). The C-terminal domain also contributes to RNA binding and additionally includes an amino acid sequence, that is involved in hnRPA1 nuclear import and export (Michael *et al.*, 1995) (Weighardt *et al.*, 1995). At steady state, hnRPA1 is predominantly nuclear, but it can shuttle rapidly between the nucleus and the cytoplasm, which is thought to play a role in cell proliferation, cell survival, and differentiation. Two transport receptors of the karyopherin family, Trn1 and Trn2b, mediate the hnRPA1 nuclear import (Pinol-Roma *et al.*, 1992). Another hnRPA1 interacting protein is the E2a-Pbxl oncoprotein, which prevents differentiation and maintains continued cell division. Following inactivation of E2a-Pbxl, hnRPA1 was up to 10 fold down-regulated (Skyes *et al.*, 2003). In addition, the gene encoding testosterone-15-alphahydroxylase (Cyp2A5) was identified as a target of hnRPA1 (Raffalli-Mathieu *et al.*, 2002). By binding to Cyp2A5-RNA, hnRPA1 can prolong the Cyp2A5-RNA half-life (Glisovic *et al.*, 2003).

Prothymosin alpha (PTMA) is a small, highly acidic nuclear protein, which is highly conserved and expressed in a wide variety of cells, tissues, and organisms (Haritios *et al.*, 1984). This suggests that PTMA is required for an essential function in the cell. Numerous studies have indicated that PTMA may be involved in cell growth, and the expression of its gene is generally correlated with cellular proliferation (Haritios *et al.*, 1984). This relationship has been supported by the direct correlation of both PTMA-mRNA and protein levels with levels of proliferation (Eschenfeldt *et al.*, 1989), and the finding that PTMA was phosphorylated in stimulated proliferating cells (Barcia et al., 1992). Additionally, overexpression of PTMA has been shown to accelerate proliferation, and to slow down differentiation in HL-60 promyelocyte (Rodriguez *et al.*, 1998). Moreover, the transcription of the PTMA gene is regulated by the c-myc gene, a key player in the control of proliferation (Eilers *et al.*, 1991). Barbini *et al.* (2006) concluded that high levels of PTMA need to be present in the nucleus for proliferation, whereas programmed cell death requires low levels of PTMA outside of the nucleus. Jiang *et al.* (2003) found that PTMA negatively regulated caspase-9 activation by inhibiting apoptosome formation.

In this study, hnRPA1 was up-regulated, and PTMA was down-regulated although both directly interact with each other (Figure 5.10). The fact that both are related to cell proliferation in several other studies leads to the conclusion that they may be also involved in MSC proliferation

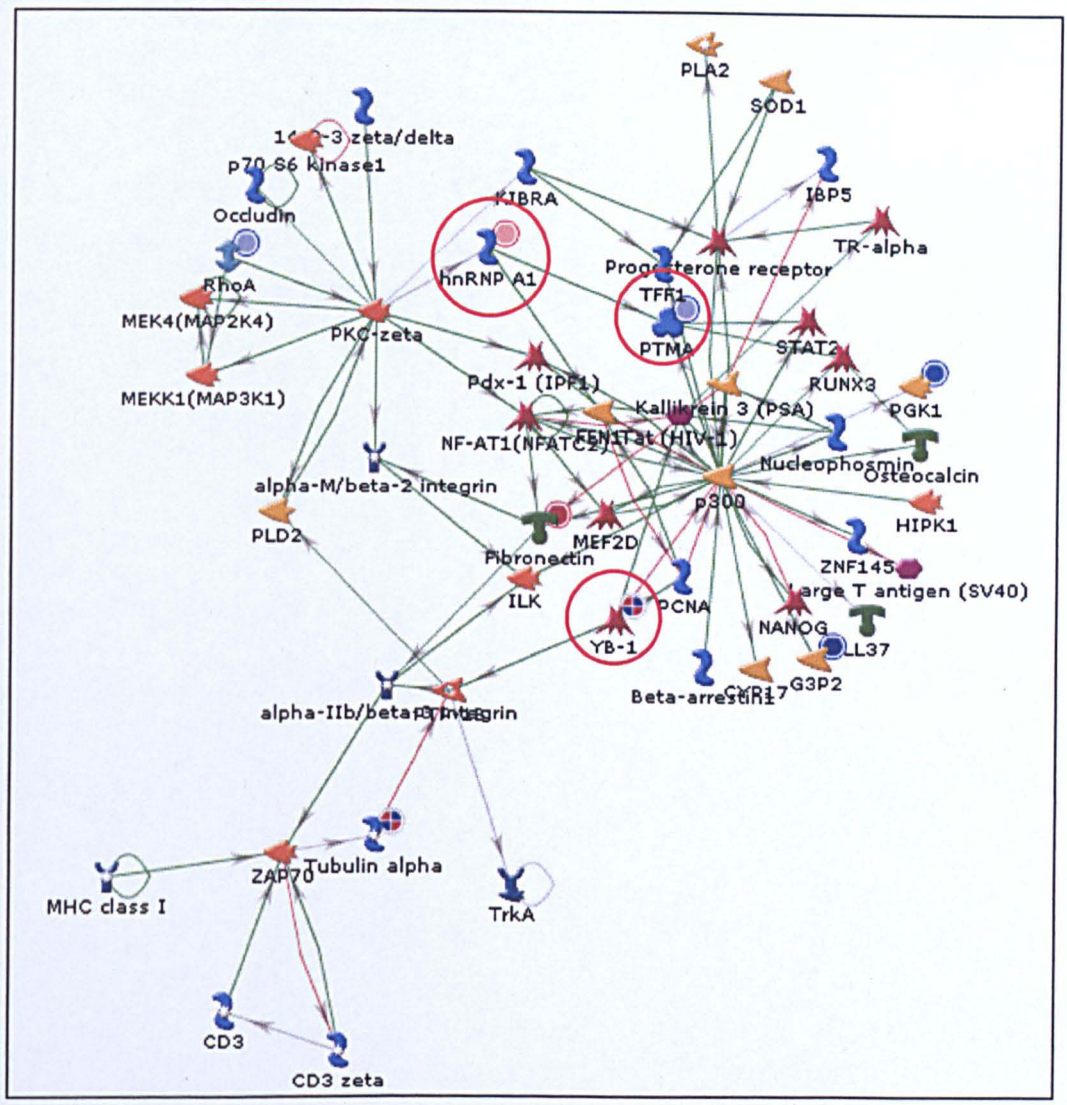

Figure 5.10: Regulatory network calculated by MetaCore™ in which hnRPA1 and PTMA are in direct interaction. A legend can be found in Appendix C.

Another newly described protein present in the same network as hnRPA1 and PTMA is Y box protein1 (YB1) (Figure 5.10), which was up-regulated in subcultured MSCs. YB1 appears to play a critical role in cell proliferation, DNA replication, and drug resistance. It is involved in promoting cell proliferation through the transcriptional regulation of various relevant genes, including proliferating cell

nuclear antigen, p53 protein, epidermal growth factor receptor, DNA topoisomerase IIα, thymidine kinase, and DNA polymerase α.5 (Wolffe *et al.*, 1994) (Okamoto *et al.*, 2000). Furthermore, it activates the transcription of the human multidrug resistance 1 gene. (Ohga *et al.*, 1998). The biological roles of YB1 include modification of chromatin, translational masking of mRNA, participation in the redox signalling pathway, RNA chaperoning, and stress response regulation (Swamynathan *et al.*, 1998). Hence, YB1 appears to be related to both mRNA turnover and translational control.

Further proteins described for the first time are adenylate cyclase-associated protein1 (CAP), dynein light chain1 (DLC1), cysteine and glycine rich protein2 (CRP2), asparagine synthetase (ASNS), ADP-ribosylation factor1 (ARF1) and Sec61. Adenylate cyclase-associated protein1 was down regulated in this study. It is part of the cyclic AMP pathway, and binds to G-actin, thus inhibiting actin polymerisation (Freeman *et al.*, 1995). A CAP homologue of *Drosophila* has been reported to be involved in developmental morphogenesis, probably through its effect on the actin cytoskeleton (Benlali *et al.*, 2000). Moreover, CAP has been associated with the aggressive behaviour of pancreatic cancer cells (Yamazaki *et al.*, 2009) and Moriyama et al. (2002) found that CAP effectively recycles actin and cofilin, thereby allowing a rapid turnover of actin filaments, which is an essential driving force behind cell motility.

Dynein light chain1 (DLC1), also down-regulated in this study, is a component of the cytoplasmic dynein motor complex (Hirokawa *et al.*, 1998). This complex is involved in cytoplasmic organelle transport, mitosis, nuclear migration (Holzbaur *et al.*, 1994) (Vaisber *et al.*, 1993) and promotes normal differentiation of proliferating germ cells (Dorsett *et al.*, 2009). Dorsett *et al.* demonstrated that the dynein motor complex inhibited proliferative cell fate, in part through regulation of METT-10, a conserved putative methyltransferase and thus, dynein can act as an anti-proliferative factor. In addition to being an essential part of the dynein motor function, DLC1 interacts with a number of proteins such as nitric oxide synthase, Pak1, KIBRA and Cip-interacting zinc finger protein 1 (Ciz1), and thereby may regulate numerous biological processes (Rayala *et al.*, 2006) (Hollander *et al.*, 2006). The up-regulation of DLC1 and its interaction with Pak1, KIBRA and Ciz1 is closely related to

stimulated cell growth and regulation of the cell cycle in estrogen receptor positive breast cancer cells. In this context, Rayala *et al.* (2005) discovered that the down-regulation of DLC1 compromised the transactivation function of the estrogen receptor.

Cysteine and glycine rich protein2 (CRP2) is involved in regulatory processes important for cell development and cellular differentiation. In this study, it was found to be up-regulated. CRP2 was shown to be a potent smooth muscle differentiation cofactor triggering the conversion of pluripotent fibroblasts into smooth muscle cells when overexpressed together with serum response factor (SRF) and GATA proteins (Chang *et al.*, 2003). Moreover, a recent report demonstrated that CRP2 can effectively switch on smooth muscle gene activity in adult cardiac myocytes (Chang *et al.*, 2007), suggesting that CRP2 has essential functions in controlling smooth muscle gene activity. Additionally, it was shown that the expression of CRP2 is down-regulated with cellular dedifferentiation induced by oncogenic transformation, injury, or wound healing (Weiskirchen *et al.*, 2001).

Asparagine synthetase (ASNS) was down-regulated in this study, and is classified as a metabolic enzyme. It catalyses the ATP-depended conversion of aspartic acid to asparagines, and thus its transcription is regulated by the availability of the amino acids aspartic acid and asparagines (Gong *et al.*, 1991). Bone marrow mesenchymal stem cells were shown to express high levels of ASNS *in vivo*, leading to high concentrations of asparagine in the bone marrow microenvironment and subsequently protecting the surrounding cell of these niches from the lethal action of asparaginase (Iwamoto *et al.*, 2007).

ADP-ribosylation factor1 (ARF1) is localised in the Golgi apparatus, and has a central role in intra-Golgi transport. It is active during mitosis, and involved in the mitotic fragmentation of the Golgi (Xiang *et al.*, 2007). Furthermore, ARF1 interacts with the AP1, AP3, AP4 and the GA1–3 adaptors of the Golgi (D'Souza *et al.*, 2006) (Robinson *et al.*, 2004), as well as the lipid-modifying enzymes phospholipase D and phosphatidylinositol-specific (PI)4 kinases (Godi *et al.*, 1999). Through these interactions, ARF1 facilitate recruitment of the cytoplasmic coat protein (COP1) to the Golgi membranes, and of the clathrin polymers to the Golgi and endosomal

compartments. Thus, ARF1 might act as a master regulator of coated vesicle formation. Through its regulation of the Golgi, ARF1 plays an important role in post-translational protein modification and trafficking.

Sec61 is an endoplasmic reticulum (ER) membrane protein translocator. It consists of 3 major subunits, and forms a protein-conducting channel through the ER membrane, allowing the translocation of polypeptides into the ER lumen (Rapoport *et al.*, 1996). Furthermore, Sec61 is involved in the destruction of newly synthesised major histocompatibility complex (MHC) class I molecules by dislocating MHC class I molecules from the endoplasmic reticulum to the cytosol and delivering them to the proteasome. This process appears to be a reversal of the reaction by which Sec61 translocates polypeptides into the ER lumen (Wiertz *et al.*, 1996). An additional interaction of Sec61 with a protein complex was discovered by Stockton *et al.* (2003). They used nanospray tandem mass spectrometry to show that this complex was composed of protein disulfide isomerase (PDI), calcium binding protein 1 (CABP1/P5), 72 kDa endoplasmic reticulum protein (ERp72), and BiP (heat shock protein A5/HSPA5), and occlude the cytosolic face of Sec61.

5.4 Conclusions

In this chapter, the effect of serial subculturing on the proteome of rat bone marrow derived MSCs was determined. MSCs were expanded for up to 4 passages in static monolayer cultures. During this time, a small decrease in proliferation rate and a more obvious decline in colony forming potential and colony size were observed. The proteome of MSCs from passage 2 was compared to that of MSCs from passage 4 using iTRAQ. 381 proteins were identified, of which 48 proteins were up-regulated and 57 were found to be down-regulated at passage 4. 79 of the regulated proteins were classified into nine functional categories (cell structure and cytoskeleton, ion transport, proteins synthesis and degradation, metabolic enzymes, translation and transcription, signalling pathways, cell adhesion, cell cycle and proliferation, and stress response) and 49 of these proteins were described in this study for the first time in MSCs. Compared to a study by Celebi and Elcin (2008), in which they also analysed the proteome of sub-cultured bone marrow derived rat MSCs using 2DE

and MALDI-TOF-MS, the iTRAQ approach identified about 10 times more proteins. However, this study did not match any of the identified proteins by Celebi and Elcin (2008). Furthermore, they classified the majority of their proteins as ion-transport (22%), metabolic (17%) and signalling pathway (17%) proteins, whereas the majority of identified proteins in this study were classified as cell structure (25%), metabolic (20%) and proteins synthesis / degradation (14%) proteins. Despite these differences, both studies found that proteins involved in cell proliferation were down-regulated (TPT1, PTMA, DLC1 in this study and Adenylate kinase isoenzyme1, SG2NA in Celebi and Elcin study) and proteins involved in differentiation were up-regulated (YB1, hnRPA1, CRP2 in this study and Mineralocorticoid receptor, NDRG1 in Celebi and Elcin study)

18 of the 49 newly described proteins in this study could be assigned into different regulatory and metabolic networks such as proliferation, differentiation, transcription, translation or translational modification. Most of the proteins related to cell proliferation such as tumour protein, translationally-controlled 1 (TPT1), prothymosin alpha (PTMA) and dynein light chain1 (DLC1) were down-regulated. The proteins Y box protein 1 (YB1), heterogenous nuclear ribonucleoprotein A1 (hnRPA1) and cysteine and glycine rich protein2 (CRP2) were up-regulated. They were identified as regulation factors between proliferation and differentiation, depending on their intracellular concentration and location. Taking all these observations together (the down-regulation of proliferation related proteins and the up-regulation of differentiation related proteins) it can be hypothesized that serial subculturing may stimulate the differentiation of MSCs into early progenitor cells, and thus reduce their proliferation and colony forming capacity. However, to confirm this argument, further studies will be needed.

This experiment was an important addition to the thesis, because it highlights the problem arising from the use of static monolayer cultures for large scale MSC expansion. Furthermore, it was shown that iTRAQ is a useful tool to investigate the proteome of MSCs, and generated results which can be used to complement previous proteomic studies on MSCs. In addition, newly described proteins were presented which regulate MSC proliferation and differentiation. These findings may help to

gain a better understanding of the processes controlling the proliferation and differentiation of MSCs.

Chapter 6

Mesenchymal Stem Cells in Stirred Suspension Cultures

6.1 Introduction

The results in chapter 4 showed that mesenchymal stem cells (MSCs) can be expanded *in vitro* using static monolayer cultures. However, these static culture systems are limited in their effectiveness in generating cell mass due to poor reproducibility, lack of sufficient mixing (leading to gradients for pH, dissolved oxygen, cytokines and metabolites) and limited productivity for a given surface area (Ohgushi *et al.*, 1999). Furthermore, the static culture method also seems to interfere with the ability of the expanded MSCs to keep their colony forming capacity and their full differentiation potential, as demonstrated in chapter 4. Therefore, there is limited scope in using large scale static culture systems. Monolayer cultures are attractive for MSC expansion, since they offer the cells a surface to which they can adhere and then proliferate but studies have shown that MSCs do not necessarily need attachment to grow and proliferate (Zandstra *et al.*, 1994). Hence suspension cultures may be an attractive alternative for *in vitro* MSCs expansion (Baksh *et at.*, 2003). They can generate large numbers of cells and enable easy scale-up, sample collection and process monitoring (Ulloa-Montoya *et al.*, 2005). In addition, no trypsin treatment is required in the expansion process. The chemical stress caused by the trypsin treatment was believed to be a possible reason for the gradual loss of colony forming and differentiation potential of MSCs in static cultures (chapter 4).

So far, two different ways have been described to culture MSCs in suspension, (1) in a roller bottle (Chen *et al.*, 2006) and (2) in a stirred suspension culture (Baksh *et al.*, 2003). The preferred culture medium in both cases was MyeloCult® medium, which has a relatively high serum concentration (25%), compared to the culture medium normally used for MSCs expansion (DMEM or α-MEM, both with 10% serum). The addition of serum to any suspension culture medium has been shown to produce better growth in agitated and aerated cultures, in a dose dependent fashion (Toogood *et al.*, 1980). However, ideally the addition of serum should be avoided, since animal products are unwanted in the treatment of cells for reimplantation into humans. Furthermore, in both cases, the culture medium was supplemented with various cytokines such as human interleukin-3 (hIL-3), stem cell factor (SCF) or human platelet-derived growth factor (rhPDGF-BB). All these growth factors had a positive effect on the proliferation rate of MSCs in suspension cultures (Baksh *et al.*, 2003).

Interleukin-3 (IL-3) is one of the major haematopoietic cytokines that regulates the survival of haematopoietic cells of various lineages by binding to the interleukin-3 receptor (or CD123). CD123 consists of an α-subunit, a transmembrane molecule that specifically binds the ligand, and a β-subunit, a cytoplasmic domain for intracellular signalling (Bazan, 1990). Binding of IL-3 to its receptor initiates rapid tyrosine phosphorylation of Janus kinases (JAKs) and of signal transducer and activator of transcription (STAT) proteins, as well as activation of the phosphatidylinositol-3 kinase (PI-3K)/Akt and Ras/Raf/MAPK kinase (MEK)/mitogen-activated protein kinase (MAPK) pathways (Guthridge *et al.*, 1998, DeGroot *et al.*, 1998, Scott *et al.*, 1999 and Woodcock *et al.*, 1999). The JAK/STAT pathway is used for transmitting extracellular signals through transmembrane receptors directly to promoters of target genes in the nucleus of activated cells. IL-3 receptor binding leads to activation of the receptor-associated JAK2 and JAK1 kinases which further downstream leads in turn to the dimerization of STAT1 and STAT5. Once the STATs dimerize, they translocate to the nucleus and bind to specific enhancer sequences in the promoters of activated genes (Guthridge *et al.*, 1998 and Woodcock *et al.*, 1999). The MAPK pathways include the extracellular signal regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 signalling cascades (Guthridge *et al.*, 1998 and Woodcock *et al.*, 1999). These pathways are vital mediators of a number of cellular responses, including growth, proliferation, and survival. JNK phosphorylates the c-Jun transcription factor, whereas the p38 MAPKs activate the ATF transcription factor. The ERK pathway is involved in preventing apoptosis by up-regulating expression of the genes encoding bcl-2 and bcl-x in response to these cytokines (Kinoshita *et al.*, 1995). In addition to activating survival genes, the ERK pathway prevents apoptosis by inhibiting the pro-apoptotic factor BAD (Scheid *et al.*, 1999). The PI3-K pathway regulates multiple cellular processes such as proliferation, growth, cell size, apoptosis, and cytoskeletal rearrangement (Cantley, 2002 and Vivanco *et al.*, 2002). PI3-K is a heterodimeric lipid kinase composed of a regulatory subunit (p85) and a catalytic subunit (p110). IL-3 stimulation also induces phosphorylation of BAD through the Akt/PKB pathway, resulting in inhibition of apoptosis (del Peso *et al.*, 1997).

Stem Cell Factor exhibits two distinct isoforms, the soluble form and the membrane-bound SCF (Flanagan *et al.*, 1991). Both forms have distinct signalling properties from each other, resulting in varied biological functions mediated by the two isoforms (Massague and Pandiella, 1993). SCF is the ligand of the receptor tyrosine kinase, c-kit, which extracellular domain facilitates the binding of the ligand and the cytoplasmic domain serves to transduce the signal (Schlessinger, 2000). The receptor is widely expressed in haematopoietic stem cells, myeloid progenitor cells, dentric cells, mast cells and pro-B and pro-T cells (Yarden *et al.*, 1987). The binding of SCF induces the homodimerization of the c-kit receptor resulting in the phosphorylation of selective tyrosine residues in c-kit, thereby open docking sites for the Src-homology2 (SH2)-containing signal transducers (Blume-Jensen *et al.*, 1998). c-kit signalling has effects in various biological functions such as spermatogenesis, melanin formation and erythropoiesis (Reber, *et al.*, 2006 and Ali *et al.*, 2007) and can activate PI3-kinase, which is involved in the control of cell proliferation and survival (Blume-Jensen *et al.*, 1998). In addition to PI3-kinase activation, c-kit is an important regulator of interleukin-6 and binding of SCF to the receptor induces activation of multiple additional pathways, including phospholipase C (PLC)-gamma, Src kinase, Janus kinase (JAK) / Signal Transducers and Activators of Transcription (STAT) and mitogen activated protein (MAP) kinase pathways (Reber *et al.*, 2006). Recent studies have demonstrated a role for the intracellular second messenger, cyclic AMP (cAMP), in the expression of both c-kit and SCF in different cell types such as cancer cell lines and dentric cells. Since c-kit plays an important role in the development of various cell types, cAMP may play a dual role in up-regulating c-kit/SCF expression and promoting cell differentiation (Krishnamoorthy *et al.*, 2008 and Shaw *et al.*, 2002).

The downside of both cytokines, IL-3 and SCF, is the fact that they are expensive (£200 / litre culture medium), especially the quantity necessary for large scale suspension cultures (£1000 for a 5 litre culture).

Another additive used to protect freely suspended cells from mechanical stress and damage is Pluronic F-68, which is a synthetic nonionic surfactant used routinely in large-scale mammalian cell culture processes. The function of Pluronic F-68 supplementation is to protect the cells from shear forces and the detrimental effects

of bubble bursting, both which are necessary for oxygenation in large-scale suspension culture (Zhang, S. *et al.*, 1992). The protective effect of pluronic is attributed to its ability to reduce cell-bubble attachment (Michaels *et al.*, 1995), stabilise surface foams (Handa-Corrigan *et al.*, 1989), and increase the cells resistance to hydrodynamic shear forces (Murhammer *et al.*, 1990). It is known, that Pluronic-F68 is able to integrate artificial lipid bilayer due to its surfactant properties (Firestone and Seifert, 2005). Hence there is strong evidence that pluronic interacts with the cell plasma membrane. Already Goldblum *et al.* (1990) hypothesized that Pluronic F-68 can adsorb onto cell membranes and thus protect cells from damage. Ramirez and Mutharasan (1990) used a fluorescence polarization method to measure the plasma membrane fluidity (PMF) of hybridomas throughout a batch culture, and found that Pluronic F-68 decreased the plasma membrane fluidity significantly. They suggested that the protective mechanism of pluronic relies on its ability to decrease the PMF through direct interaction with the plasma membrane. It appears that pluronic can both adsorb onto cell membranes and be incorporated into those membranes (Clarke *et al.*, 1995). Keeping in mind, that the neutral lipid, cholesterol, is the major structural lipid present in the mammalian plasma membrane and is responsible for the mechanical stability and cohesiveness (Yeagle, 1985), a small increase in membrane cholesterol content can result in an increase in membrane rigidity and a decrease in membrane fluidity (Evans and Rawicz, 1990). Conversely, a large increase in plasma membrane cholesterol content results in a large increase in membrane rigidity and a decreased in membrane fluidity (Clarke *et al.*, 1995). Furthermore changes in membrane cholesterol content directly impact transmembrane signalling by altering membrane protein function. These effects have been attributed to bulk lipid loading of the membrane, by direct interaction of cholesterol with the membrane protein resulting in disruption of the ability of the protein to undergo the conformational changes required for signalling, or by a combination of both phenomena (Bastiaanse *et al.*, 1997). Interestingly, the effect of cholesterol enrichment on plasma membrane protein function can be either stimulatory (Bastiaanse *et al.*, 1994) or inhibitory (Bolotina *et al.*, 1989). However, this theory could not be confirmed by the experiments conducted on MSCs, which were expanded in DMEM containing Pluronic F-68 (chapter 4). The study of the interactions between pluronic and cultured cells is limited by the lack of a simple and sensitive assay for Pluronic F-68. Another theory of how pluronic could protect cells

from shear stress was tested by Gigout *et al.* (2008). They synthesized a fluorescent derivative of Pluronic-F68 to quantify and characterise its uptake by CHO cells and chondrocytes. It was demonstrated that labelled pluronic accumulates in the cells in late endosomes and lysosomes but not in the cytoplasm or in the nucleus. The accumulation in the cell membrane could not be distinguished from auto-fluorescence. Thus, shear protection conferred by pluronic may be partly due to alteration and stiffening of cytoplasmic cell mechanical properties by the high pluronic-F68 concentration in intracellular vesicles.

It is an important criterion when expanding mesenchymal stem cell numbers for therapeutic applications to maintain their full differentiation capacity by optimising the culture conditions. Besides the culture medium, there are many parameters such as cell seeding density, agitation, culture vessel and sampling technique which have to be considered as well. Studies on other stem cell types like neural stem cells or endothelial cells from human umbilical veins of newborn babies were conducted to investigate some of the mentioned parameters. Korff and Augustin (1998) found that single endothelial cell in suspension cultures undergo apoptosis if they do not establish cell-cell contact. Hence, a minimal cell seeding density is necessary to enable cell-cell interaction. In another study, the initial cell density decided if large expansion or fast expansion of neural stem cells in stirred suspension cultures was achieved (Kallos and Behie, 1998). Sen *et al.* (2002) used the agitation speed in stirred suspension cultures of neural stem cells to control the formation of aggregates, and thereby regulated the viability of the culture.

Taking these findings into account, it can be inferred that the same culture parameters may be also be of importance for the cultivation of MSCs. The objective of the study presented in this chapter therefore is to develop an optimised protocol for large scale expansion of MSCs in stirred suspension cultures by investigating the effect of the initial cell seeding density, the sampling method, the agitation method and of course the culture medium on the proliferation and differentiation capacity of MSCs. Ideally, the suspension culture method should be kept as simple and as cheap as possible, to allow easy scale-up and the potential use of these cells for medical applications.

6.2 Materials and methods

The methods for general cell culture are given previously in chapter 3. In this chapter, the specific methods for growing and expanding bone marrow derived rat MSCs in a suspension culture system are described. The protocol for these cultures was varied according to the source of MSCs (fresh bone marrow cells or MSCs from static cultures), the sampling or agitation method, and the culture vessel.

6.2.1 Different culture media

The media tested for growing MSCs in a suspension culture were simple variations of the standard DMEM and MyeloCult® medium mentioned in section 3.3. The following variations were used:

(1) DMEM supplemented with 0.05% Pluronic F-68 (about £30 per litre),

(2) DMEM with 0.05% Pluronic F-68 and 10^{-8} M dexamethasone (about £30 per litre),

(3) MyeloCult® supplemented with 0.05% Pluronic F-68 (about £70 per litre),

(4) MyeloCult® with 0.05% Pluronic F-68 and 10^{-8} M dexamethasone (about £70 per litre),

(5) MyeloCult® with 0.05% Pluronic F-68, 10^{-8} M dexamethasone, 2 ng/ml interleukin-3 and 10 ng/ml stem cell factor (about £270 per litre),

(6) MyeloCult® with 0.05% Pluronic F-68, 10^{-8} M dexamethasone, 2 ng/ml interleukin-3, 10 ng/ml stem cell factor and 373 ng/ml heparin sulphate (about £300 per litre),

(7) MyeloCult® with 2.5% gelatine, 10^{-8} M dexamethasone, 2 ng/ml interleukin-3 and 10 ng/ml stem cell factor (about £270 per litre),

(8) MyeloCult® with 10^{-8} M dexamethasone, 2 ng/ml interleukin-3 and 10 ng/ml stem cell factor (about £270 per litre).

6.2.2 Starting suspension cultures with Mesenchymal Stem Cells from different cell sources

Suspension cultures were either started with freshly harvested rat bone marrow cells (BMCs), or with MSCs which had already been expanded in static monolayer cultures for one passage. Rat bone marrow cells were obtained as described in section 3.4. Either 8 ml of BMCs for low initial cell density or 18 ml BMCs for high initial cell density were suspended in 40 ml culture medium and seeded into the suspension culture vessel. This is equivalent to 4 x 10^5 BMCs/ml and 9 x 10^5 BMCs/ml respectively. To start the suspension cultures with pre- expanded MSCs, the rat BMCs were seeded into a T75 flask and incubated as a static monolayer culture, as described in section 4.2.1. The adherent MSCs of the monolayer culture were harvested after 90% confluence, and suspended in 40 ml culture medium to achieve a initial cell concentration in the suspension culture of either 0.5 x 10^5 MSCs/ml, 1.5 x 10^5 MSCs/ml, 2.5 x 10^5 MSCs/ml or 7.5 x 10^5 MSCs/ml.

6.2.3 Maintaining Mesenchymal Stem Cells suspension cultures

Two different agitation methods were applied, either stirred or shaken suspension cultures. The culture vessels used for stirred cultures were either 500 ml glass bottles with screw caps (Scientific Laboratory Supplies Ltd) containing magnetic stirrer bars, or 500 ml spinner flasks (catalogue no. 1965-0010, Bellco) with built in magnetic stirrer paddles. For shaken suspension cultures, the 500 ml glass bottles without the magnetic stirrer bar were used. Prior to every experiment, the culture vessels were cleaned, coated with silicone (Sigmacote from Sigma) to avoid cell adhesion to the glass, dried overnight at 80°C and finally autoclaved. Next, the vessels were rinsed with sterile Phosphate Buffered Saline (PBS) to wash out liquid silicone before fresh BMCs, or trypsin digested MSCs from monolayer cultures were seeded into the culture vessels. For the stirred suspension cultures, both bottles and spinner flasks were then transferred to a magnetic stirrer plate (from Scientific Laboratory Supplies, IKA) within a CO_2 incubator (from Sanyo, MCO – 15AC) and kept at 37°C and 7.5% CO_2 for 1 to 4 weeks. The stirrer speed was about 40 rpm. For the shaken suspension cultures, the glass bottles were transferred into a shaking incubator (Infors HT, Multitron) after being inoculated with the BMCs in suspension in culture medium, and cultivated for up to 4 weeks. The settings for the shaker were

either 20 rpm, 40 rpm or 80 rpm at 37°C in 7.5% CO_2. The medium was changed and samples were taken as explained in section 6.2.4

6.2.4 Sample methods

Two different methods were used for changing the culture medium, and collecting cell samples for further analyses.

(1) The whole suspension culture including all cells was transferred into a 50 ml Falcon tube and centrifuged for 10 minutes at 1200 rpm (900g). Half the supernatant (20 ml) was returned to the culture vessel, which was then replenished with fresh culture medium to a volume of 40 ml. The rest of the supernatant was used to measure the lactate level in the culture medium (chapter 3) before being discarded. The cell pellet was resuspended in 6 ml fresh culture medium, 2 ml of resuspended cells were taken out for further analysis including cell count, CFU-f / differentiation assays or apoptosis assay (all mentioned in chapter 3) and the rest of the cells were put back into the culture vessel to continue the culture.

(2) One third of the suspension culture, including the cells, was taken out of the culture vessel, and replaced with the same volume of fresh medium as described by Baksh *et al.* (2003). To ensure the cells were well mixed in suspension, when the sample was taken, the vessels were shaken shortly just before the medium change. After adding the fresh medium, the suspension cultures were immediately put back into the incubator. The sample taken was transferred into a 50 ml Falcon tube and centrifuged for 10 minutes at 1200 rpm (900g). The supernatant was used to measure the lactate level in the culture medium (chapter 3) before being discarded and the cell pellet was resuspended in 2 to 4 ml medium, according to the pellet size. Resuspended cells were further diluted if necessary and used for the cell count, the CFU-f and differentiation assays or the apoptosis assay as described in chapter 3.

6.3 Results and Discussion

The following results demonstrate the expansion capacity of MSCs in suspension cultures. Different protocols were applied to determine the effect of different culture media (DMEM and MyeloCult®), of different medium additives (pluronic, gelatine, dexamethasone, heparin sulphate, IL-3 and SCF) and of different cultivation strategies (initial cell number, culture vessel, agitation method and sampling strategy) on the proliferation capacity and differentiation potential of harvested MSCs. Each of these effects was tested sequentially in separate experiments, and the best working conditions were then further improved. The total cell number of each suspension culture was monitored over a period of 28 days by analysing the cell samples, which were taken every time the medium was changed, with the methods described in section 3.5. To determine the stem cell character of the expanded MSCs, CFU-f and differentiation assays were carried out with the cells taken from the suspension. The CFU-f assay was used as a quantitative method to obtain the number of colony forming cells in the suspension cultures over time. The differentiation assays on the other hand were used only in a qualitative way to show the existence of MSCs with the ability to differentiate into osteoblasts or adipocytes. The reason for not counting these cells, was the lack of sufficient quantities of cells in the samples taken from the stirred suspension cultures. Therefore, differentiation assays were only conducted with cell samples from suspension cultures, which actually produced enough cells. The information obtained from the CFU-f and the differentiation assays for this chapter is summarised in Table 6.1 in the conclusion section of this chapter.

6.3.1 Establishing a protocol for expansion of Mesenchymal Stem Cells in suspension cultures

Baksh *et al.*, (2003) developed a method which allowed the expansion of human bone marrow derived MSCs in stirred suspension cultures by using spinner flasks and MyeloCult® medium containing 10^{-8} M dexamethasone, 2 ng/ml interleukin-3 (IL-3) and 10 ng/ml stem cell factor (SCF). Applying this method, Baksh *et al.* (2003) reported a 7 fold increase in CFU-f number over a period of three weeks. Hence, the same method was used and later on optimised in this study to expand MSCs in stirred suspension cultures.

Figures 6.1 and 6.2 show the results obtained from the study using the same culture conditions described by Baksh *et al.*, (2003). The total cell number remained unchanged after week one (1.02 fold of the initial cell number). From week two onwards the cell numbers dropped by 88% and by 96% after two and three weeks, respectively (Figure 6.1). The development of the CFU-f number was even more dramatic. Numbers dropped to 73%, 28% and 15% of the initial cell density over the whole culture period (Figure 6.2).

In contrast to the increase in CFU-f number described by Baksh *et al.* (2003), both total cell number and CFU-f number decreased dramatically, indicating something must have been fundamentally wrong with the method used to expand rat MSCs in suspension cultures. The dramatic decline in total cell number after 7 days can be explained by the cell death of BM cells which are not supported by this culture conditions (e.g. haematopoietic cells) and is not necessarily a fact to worry about. However, stress factors, which are caused by parameters such as the culture medium or the cultivation method, may be responsible for the sudden cell death of the colony forming cells in *in vitro* cultures. Using stirred suspension cultures, shear stress is most likely the dominant stress factor, especially for MSCs, which are known to be shear stress sensitive. An easy and fast way to reduce the shear stress caused by the stirrer paddles in the spinner flasks is to change the agitation method and the shape of the stirrer in the culture vessel. Instead of taking spinner flasks with a built in impeller, simple siliconised glass bottles with screw caps and a magnetic stirrer bar were used to stir the culture. Also, some suspension cultures were not stirred at all but put onto a shaking platform in a special incubator, as it is common in many mammalian cell cultures. Furthermore, 0.05% pluronic, which attaches to the cell membrane and thereby helping to protect it from shear stress, was added to the culture medium.

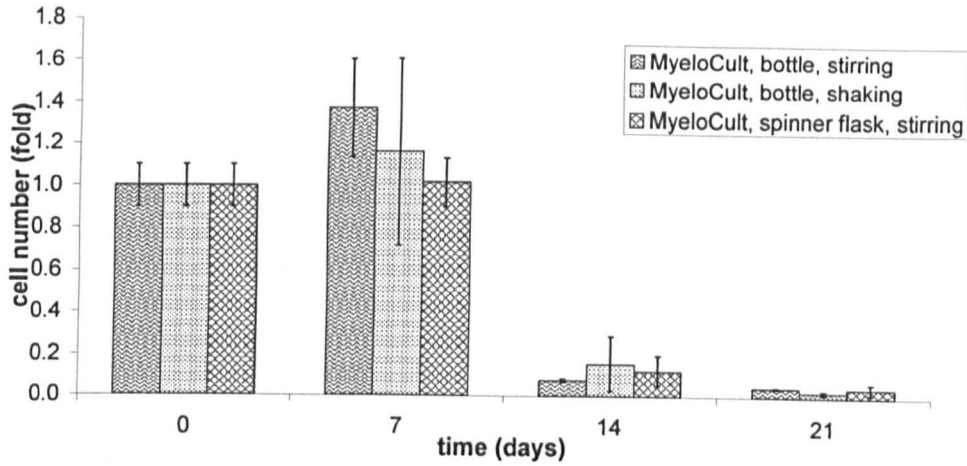

Figure 6.1: Development of total cell number over time in suspension cultures started with 12 x 10^6 rat BMCs in MyeloCult® medium containing 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF. Suspension cultures were either stirred in glass bottles or in spinner flasks or shaken in a special incubator. Results of all conditions are mean +/- S.D. from 3 different suspension cultures. No significant difference in cell number (fold) between the three cultures at any time point.

By changing the culture vessels to glass bottles with magnetic stirrer bars, better results for total cell number and CFU-f number were achieved compared to the spinner flasks. Shaking the MSC suspension cultures did not have a positive effect. Figures 6.1 and 6.2 give a comparison of the best results achieved with stirred suspension cultures in glass bottles, stirred suspension cultures in spinner flasks and suspension cultures shaken in glass bottles. The total cell number for all methods stayed about the same in week one (1.4 fold increase in stirred suspension cultures in glass bottles, 1.2 fold increase in shaken cultures and 1.02 fold increase in stirred cultures in spinner flasks). After that, the numbers decreased continuously and finally dropped by 96 - 98% after three weeks (Figure 6.1). A more obvious difference was revealed in the development of the CFU-f numbers. In the stirred cultures using glass bottles the CFU-f number increased for the first two weeks up to 1.4 fold and finished at 43% of the initial cell density after three weeks, whereas the stirred suspension cultures in spinner flasks dropped to 73%, 28% and 15% of the initial cell density over the culture period (Figure 6.2). The development of the CFU-f numbers in the shaken suspension cultures were about the same as the cultures in the spinner flasks. Thus, the stirred suspension cultures in glass bottles achieved the best result, significantly better than the cultures in the spinner flasks and the shaken cultures in glass bottles ($p = 0.049$).

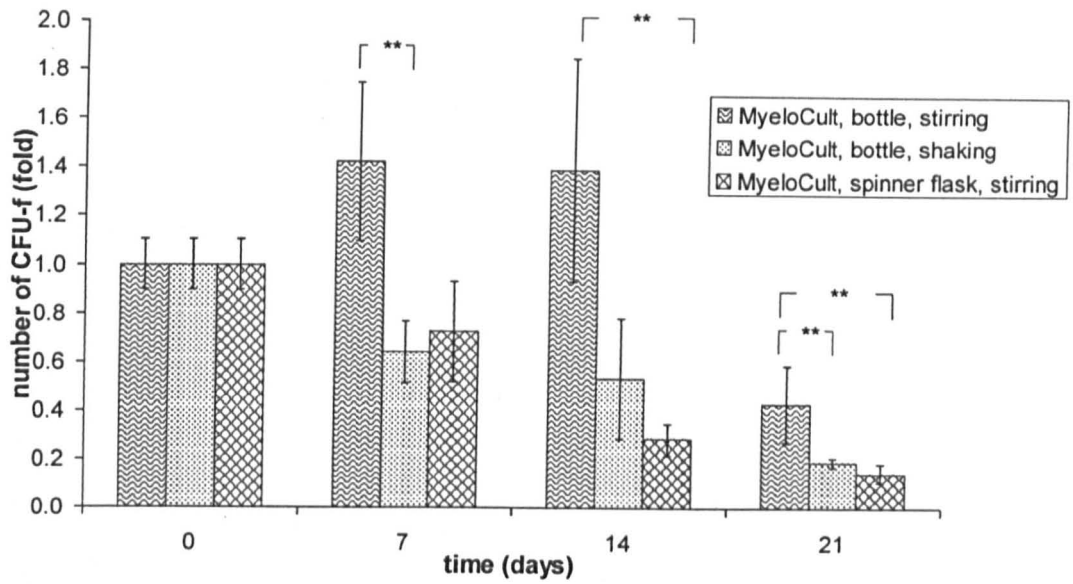

Figure 6.2: Development of CFU-f number over time in suspension cultures started with 12 x 10^6 rat BMCs in MyeloCult® medium containing 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF. Suspension cultures were either stirred in glass bottles or in spinner flasks or shaken in a Infors shaking incubator. Results of all conditions are mean +/- S.D. from 3 different suspension cultures. **p < 0.05, stirred cultures in bottles compared with shaken cultures in bottles and stirred cultures in flasks

The colonies in the CFU-f assays formed from MSCs from stirred spinner flask cultures appeared to be similar in size to the ones from shaken suspension cultures. Both had smaller colonies compared to the colonies from the stirred suspension culture in glass bottles (Table 6.1). Larger colonies are an indicator that either multiple CFU-f cells formed one colony, or that the single MSCs forming a large colony must have had a better proliferation potential than the single MSCs forming a small colony. The round and symmetric shape of the colonies indicated that they were most likely formed by single cells only, starting at one centre. Therefore, the bigger size of a colony is a sign for a higher proliferation rate of the MSCs which in turn is a sign for a healthier cell derived from a suspension culture with lower stress levels.

All three suspension culture systems generated enough cells to run differentiation assays. These assays revealed that MSCs with osteogenic differentiation potential were present in the above mentioned suspension cultures for the complete culture period. Colonies in the osteogenic differentiation assay staining positively for alkaline phosphatase, calcium and collagen could be found on all the plates, indicating that osteoblasts were formed.

The combination of using normal glass bottles (coated with sigmacoat) with magnetic stirrer bars and adding pluronic to the culture medium seemed to reduce the shear stress and resulted in increasing total cell numbers and CFU-f numbers. The magnetic paddle in the spinner flask is a lot larger than the magnetic stirrer bar and moves more liquid with every turn it spins, therefore causing more shear stress. Changing this paddle to a proper cell culture impeller, which is especially designed to mix the liquid by causing the least shear stress possible, could give the spinner flask an advantage over the glass bottle. However, all further experiments to improve the MSC suspension culture system were conducted in coated glass bottles with magnetic stirrer bar.

6.3.2 Shaken Mesenchymal Stem Cells suspension cultures

Shaking the suspension cultures, and thereby trying to lower the shear stress did not increase the number of MSCs in the culture (Figure 6.2). One more study on MSC expansion in shaken suspension cultures was carried out in order to optimise this agitation method. A better cell survival should be obtained by altering the shaker speed from 40 rpm to either 20 rpm or 80 rpm.

Shaking the suspension cultures with 80 rpm resulted in a 3.2 fold increase in total cell number after the first week. Shaker speeds of 20 rpm and 40 rpm achieved only 1.3 fold and 1.2 fold increases respectively. By the end of the culture period, the total cell number decreased at all shaking speeds to 20%, 3% and to 2% of the initial cell number for 80 rpm, 20 rpm and 40 rpm (Figure 6.3).

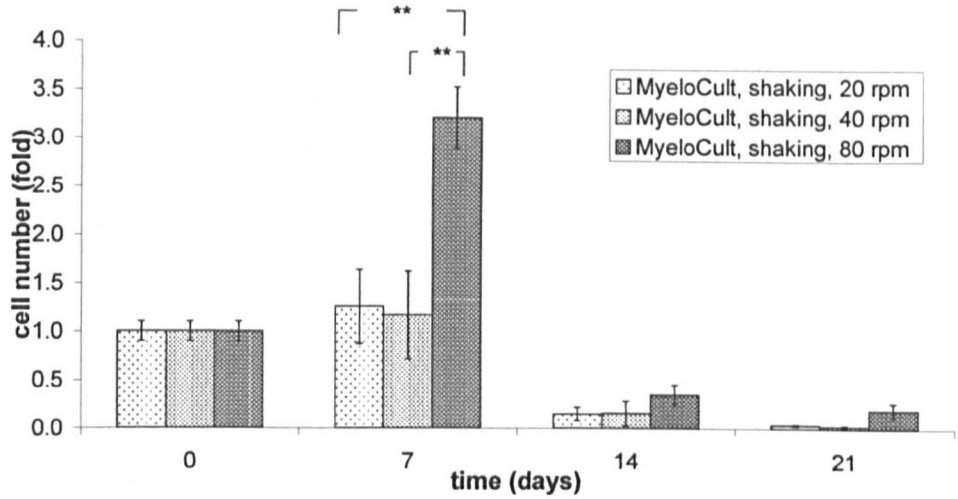

Figure 6.3: Development of total cell number over time in shaken suspension cultures started with 12 x 10^6 rat BMCs in MyeloCult® containing 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF. Results of all three conditions are mean +/- S.D. from 3 different suspension cultures. **$p < 0.05$

For the development of the colony forming cells in the suspension culture no significant effect of the shaking speed was observed. At 40 rpm, the CFU-f numbers dropped only by 36%, 46% and 81% over the whole culture period compared to a decrease of 56% / 79% after week one, 57% / 75% after week two and 86% / 82% after week three for shaking speeds of 20 rpm / 80 rpm (Figure 6.4). However, the probability for the differences between these results is $p = 0.46$ and therefore not significant.

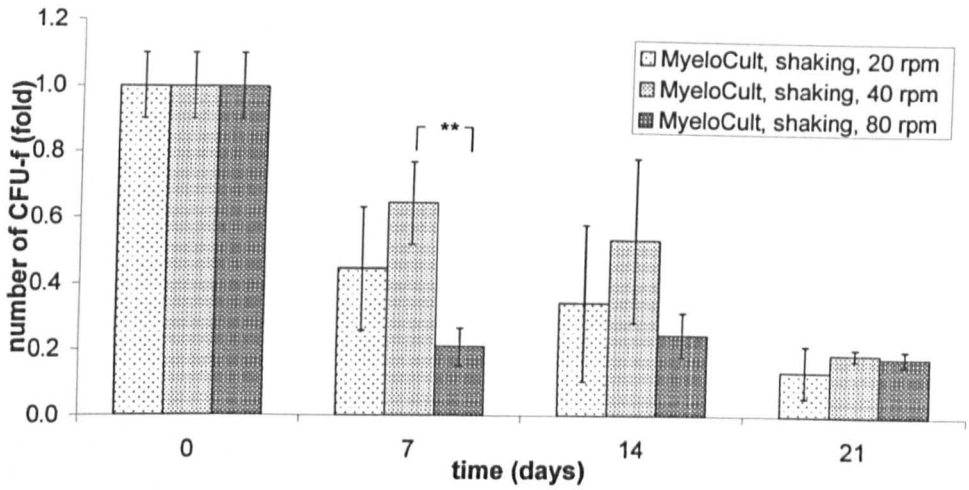

Figure 6.4: Development of CFU-f number over time in shaken suspension cultures started with 12 x 10^6 rat BMCs in MyeloCult® containing 0.05%pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF. Results of all three conditions are mean +/- S.D. from 3 different suspension cultures. **$p < 0.05$

A higher shaking rate should allow better mixing of the solution and a better supply of nutrients to all the cells, which could result in higher cell proliferation. Even though a higher shaking speed assured a better mixing of the culture solution, it also caused more shear stress to the MSCs, which are very sensitive to this sort of stress. Shaking suspension cultures of MSCs at different speeds did not improve the proliferation of colony forming behaviour of the cells in shaken suspension cultures. Only by stirring MSC suspension cultures in glass bottles with magnetic stirrer bars and using culture medium containing 0.05% pluronic, could an actual increase in MSCs be achieved and even this increase (1.4 fold) was lower than the increase (7 fold) reported by Baksh *et al.*, (2003). To obtain a higher proliferation of MSCs in stirred suspension cultures, the culture medium was further modified.

6.3.3 Effect of pluronic, gelatine and heparan sulphate on Mesenchymal Stem Cells in suspension

The improvements focused on the medium additives to help protect the MSCs from shear stress. In the last stirred suspension culture experiments, pluronic was used to attach to the cell membrane and thereby strengthen it but in chapter 4 it has been shown that pluronic also had a negative effect on the proliferation behaviour of MSCs in static monolayer cultures. By removing pluronic from the culture medium the proliferation of MSCs could be increased and a higher MSC number might be achieved. On the other hand the MSCs would not be protected against the shear stress caused from the stirrer. Therefore, a more natural compound, gelatine, was used to replace pluronic. Gelatine is a breakdown product of collagen, which is commonly used to coat culture dishes and to improve the cell adhesion to plastic surfaces (Stark et al., 2008). Furthermore, another compound, heparan sulphate, was added to the culture medium to enhance the positive effect of IL-3 and SCF by mediating these growth factors to the MSCs, but this would further increase the costs of the already expensive culture medium.

The replacement of pluronic with gelatine in the culture medium had a huge effect on the total cell number, which increased by 1.9 fold after one week, then decreased to 80% and finally to 64% of the initial cell number (Figure 6.5). This was a better development for the total cell number, compared to the best result in suspension cultures with pluronic ($p = 0.005$) (1.4 fold increase after week one, then decrease to 7% and to 4% of the initial cell number (Figure 6.7)). As expected, the removal of pluronic from the medium did not result in a higher total cell number (1.05 fold increase then decrease to 15% and to 6% of the initial cell number after one, two and three weeks respectively (Figure 6.5). The addition of heparan sulphate to the medium also had no positive influence the development of the total cell number, and resulted in a decrease of 97% after three weeks in suspension culture (Figure 6.5).

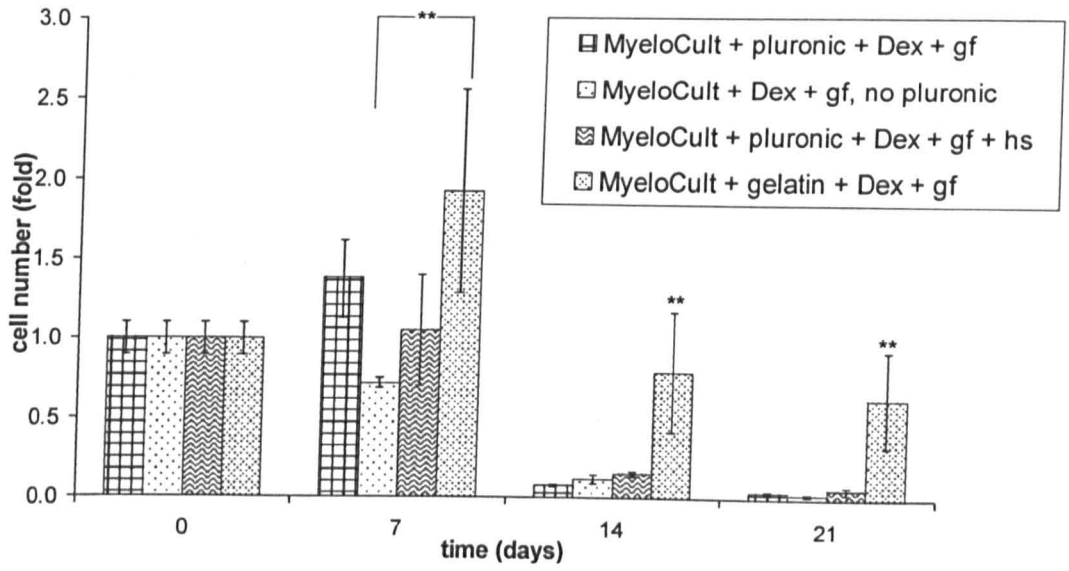

Figure 6.5: Development of total cell number over time in suspension cultures started with 12 x 10^6 rat BMCs in MyeloCult® containing 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF. This culture medium was changed either by removing pluronic, by replacing pluronic with 2.5% gelatine or by adding 232 ng/ml heparin sulphate. Results of all conditions are mean +/- S.D. from 3 different suspension cultures. **$p < 0.05$, cell number in cultures with gelatine compared to cultures without gelatine

None of the changes to the culture medium in this experiment increased the CFU-f numbers. Most noticeably the increase in total cell number in the gelatine cultures was not caused by an increase in colony forming cells. The CFU-f numbers in these cultures decreased over the 3 week period by 66%, 79% and by 89% (Figure 6.6) and in the pluronic free cultures by 29%, 67% and by 88% over the same period of time (Figure 6.6). The development of the CFU-f number in the heparan sulphate culture (decrease by 50%, 73% and by 90% within three weeks) (Figure 6.6) was the most surprising since the control without heparan sulphate actually resulted in higher numbers of colony forming cells ($p = 0.017$). It seemed that the addition of heparan sulphate to the culture medium decreased the number of colony forming cells. This was not expected and needs further investigation to be fully explained.

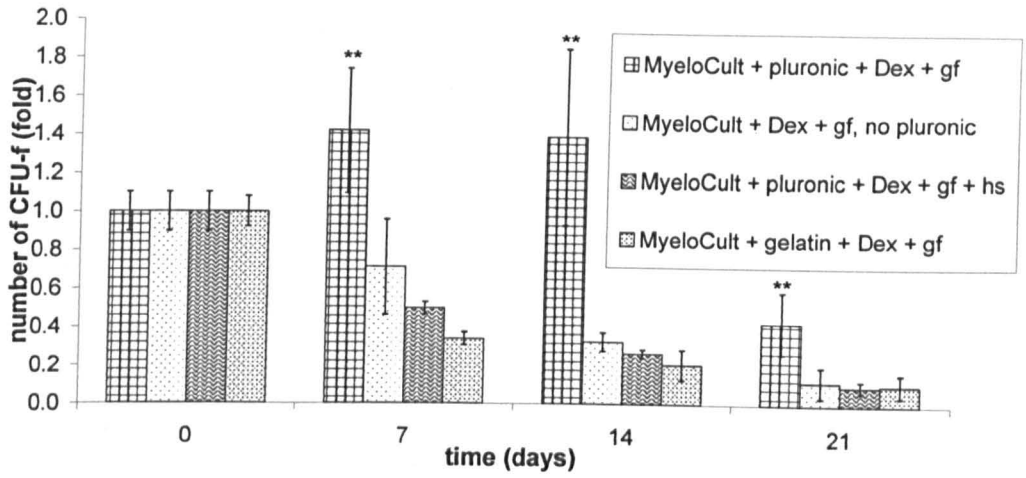

Figure 6.6: Development of CFU-f number over time in suspension cultures started with 12 x 10^6 rat BMCs in MyeloCult® containing 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF. This culture medium was changed either by removing pluronic, by replacing pluronic with 2.5% gelatine or by adding 232 ng/ml heparin sulphate. Results of all conditions are mean +/- S.D. from 3 different suspension cultures. **$p < 0.05$, cultures in MyeloCult® with 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF compared to the other cultures

The colonies formed by MSCs from gelatine and heparan sulphate cultures were all relatively large (Table 6.1). This is surprising since no healthy colony forming cells with good proliferation capacity would be expected to be present in suspension cultures with decreasing CFU-f numbers. The gelatine could cause the MSCs to stick to each other when attaching to the plastic of the plates. Thus several CFU-f cells formed one colony, which would appear larger than colonies formed by single cells. The same reason could apply to heparan sulphate, even so heparan sulphate, in contrast to gelatine, is not known to help MSCs to attach to each other. MSCs derived from suspension cultures without pluronic formed small colonies, and after three weeks they were barely recognisable. Cells from all three suspension cultures formed large colonies in the osteogenic differentiation assay, which could be stained positively for alkaline phosphatase, calcium and collagen. The results from the osteogenic differentiation assay for all medium conditions were very similar and did not reflect the difference in colony shape and size from the CFU-f assay.

The removal of pluronic from the culture medium resulted in a faster decreasing CFU-f number in the suspension cultures compared to the same medium conditions containing pluronic ($p = 0.049$). Despite the decrease in MSCs number, the osteogenic differentiation assay of these cells showed some nice big colonies of osteoblasts, indicating that the proliferation capacity is more affected by the increased shear stress than the differentiation behaviour. The rather surprising drop in colony forming cell number in the heparan sulphate culture and the drop in CFU-f number in the gelatine culture could both be explained by the theory that these medium additives enhanced the cluster forming ability of MSCs, as indicated by the comparable large number of large colonies in the CFU-f assays. If several MSCs stick to each other when forming a colony, then the colony count does not represent the actual MSC number, which could be a multiple of the colony number, depending on how many MSCs formed one colony. Even after trying to optimise the culture medium, the total cell number as well as the CFU-f number did not increase as much as expected, and the differentiation capacity of the MSCs is still limited since no adipocytes were obtained from any suspension culture. Furthermore, the culture conditions mentioned so far all require the use of the most expensive culture medium, MyeloCult® plus growth factors. High costs are of significant disadvantage for future scale-up, therefore further improvement focused on reducing the costs for MSC suspension cultures.

6.3.4 MyeloCult® medium versus DMEM

Studies with MSCs in static monolayer cultures have shown that the use of MyeloCult® medium compared to DMEM did not significantly increase the number of expanded MSCs (Table 4.4). And since DMEM is a less expensive culture medium, suspension culture experiments with bone marrow derived MSCs in DMEM were conducted to compare the MSCs proliferation with the results from suspension cultures in MyeloCult® medium. Experiments were run in stirred suspension cultures started with 12×10^6 BMCs in 40 ml culture medium (either MyeloCult® or DMEM) containing 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF.

With both culture media, a similar development in the total cell number was achieved. In the DMEM cultures, the total cell number increased by 1.4 fold in the first week, but then dropped to 11% (at the second week) and to 3% (at the third week) of the initial cell number (Figure 6.7). In the MyeloCult® cultures, the total cell number also increased by 1.4 fold after the first week and dropped to 7% and to 4% after weeks two and three (Figure 6.7).

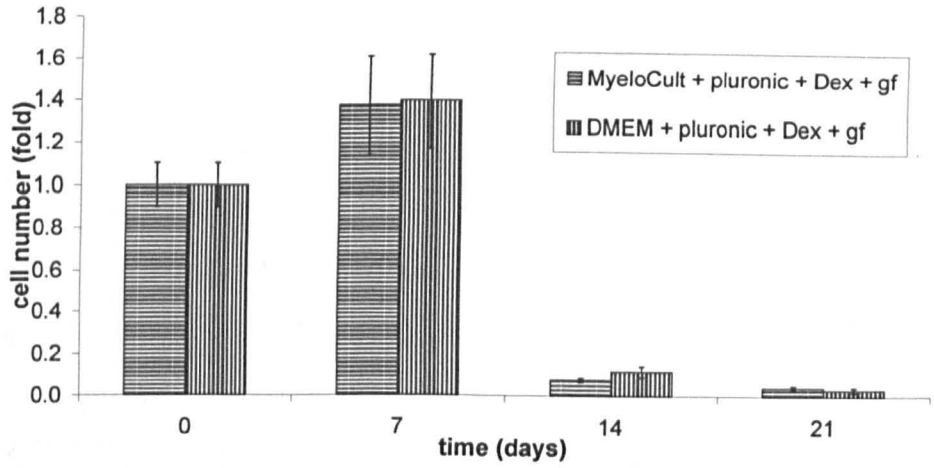

Figure 6.7: Development of total cell number over time in suspension cultures started with 12 x 10^6 rat BMCs either in DMEM with 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF or in MyeloCult® with 0.05% pluronic, 10^{-8} M dexamethasone, IL-3 and SCF. Results of all conditions are mean +/- S.D. from 3 different suspension cultures. No significant difference in cell number (fold) between the DMEM and the MyeloCult® cultures at any time point.

The CFU-f number in suspension cultures with DMEM developed differently than in the MyeloCult® cultures. In the DMEM cultures the number of colony forming cells stayed steady for the first week (1.07 fold increase) and then decreased by 57% after two weeks and by 84% after three weeks (Figure 6.8) whereas in the MyeloCult® cultures the CFU-f numbers actually increased for up to two weeks. A 1.4 fold increase after one week, no change in CFU-f after week two (still a 1.4 fold increase), and a decrease to 43% of the initial cell number at the end of the culture period (Figure 6.8).

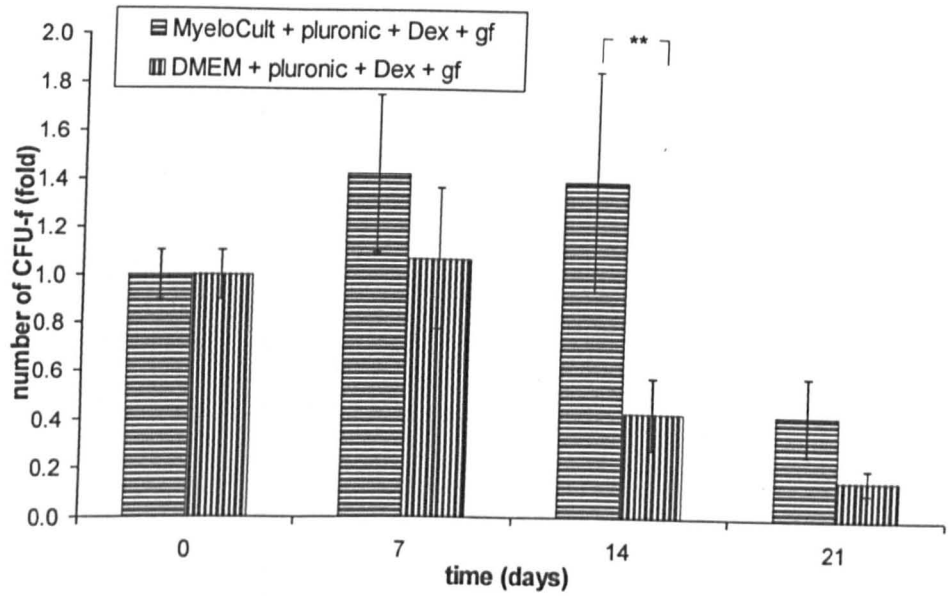

Figure 6.8: Development of CFU-f number over time in suspension cultures started with 12 x 10^6 rat BMCs either in DMEM with 0.05% pluronic, 10^{-8} M dexamethasone, 2 ng/ml IL-3 and 10 ng/ml SCF or in MyeloCult® with 0.05% pluronic, 10^{-8} M dexamethasone, IL-3 and SCF. Results of all conditions are mean +/- S.D. from 3 different suspension cultures. **$p < 0.05$, cultures in MyeloCult® compared to the DMEM culture

Although the numbers of colony forming cells in DMEM and MyeloCult® suspension cultures were different from each other, the size and the shape of the colonies formed on the plates of the CFU-f assays appeared to be similar for both conditions. Some large and numerous small colonies were recognisable, indicating that the health of the CFU-f cells in both suspension cultures were similar (Table 6.1). MSC with osteogenic differentiation potential could be found in both DMEM and MyeloCult® suspension cultures. However, the number of osteoblasts derived from the MyeloCult® suspension culture was obviously higher than from the DMEM cultures.

Suspension cultures in MyeloCult® medium produced more MSCs with better osteogenic differentiation potential than the comparable DMEM cultures. In addition, the proliferation capacity and therefore the health of the colony forming cells in MyeloCult® cultures also seemed to be improved as indicated by a larger colony size in the CUF-f assays. These findings were different to the results from comparable experiments with MSCs in monolayer cultures (chapter 4). Nevertheless, Chen *et al.* (2006) also found that MSCs in suspension cultures with DMEM as culture medium did not grow but when changing to MyeloCult® medium, a significant increase in MSC numbers were achieved. Thus, the usage of the more cost intensive MyeloCult® medium in suspension cultures is necessary in order to achieve increasing numbers of MSCs.

6.3.5 Effect of IL-3 and SCF on Mesenchymal Stem Cells in suspension cultures

An even more cost reducing change to the culture medium is the deletion of the growth factors IL-3 and SCF. Furthermore, dexamethasone was also removed from the medium because studies in MSC monolayer cultures have shown no cell number increasing effect of either the growth factors or dexamethasone (chapter 4). The influence of IL-3, SCF and dexamethasone was tested in DMEM and MyeloCult® medium, both supplemented with 0.05% pluronic.

The total cell number in the MyeloCult® cultures without dexamethasone and without cytokines increased by 2.5 fold in the first week, whereas the total cell number in the comparable DMEM cultures had already decreased by 70% at this stage. After two weeks, the cell number in the MyeloCult® cultures also dropped by 30%, and at the end of the culture period not more than 10% of the initial total cell number was left in the suspension cultures for both media (Figure 6.9).

The colony forming cells in both sets of suspension cultures without dexamethasone and without cytokines decreased from the beginning as shown in Figure 6.10. The number of CFU-f in the MyeloCult® cultures found at each medium change were 50% (at week one), 28% (at week two) and 10 % (at week three) of the initial CFU-f

number. In the DMEM culture, the CFU-f numbers were even lower, 26% (at week one), 18% (at week two) and 5% (at weeks three) of the initial CFU-f number.

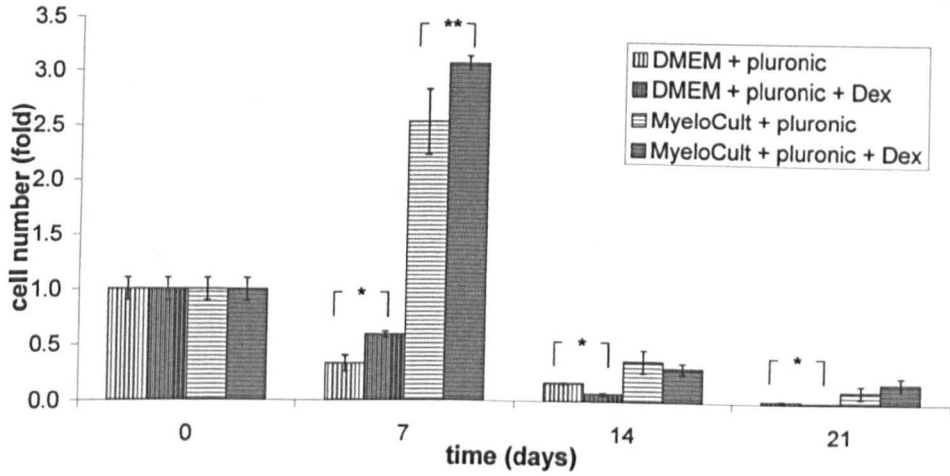

Figure 6.9: Development of total cell number over time in suspension cultures started with 12 x 10^6 rat BMCs in different culture media (DMEM with 0.05% pluronic, DMEM with 0.05% pluronic and 10^{-8} M dexamethasone, MyeloCult® with 0.05% pluronic and MyeloCult® with 0.05% pluronic and 10^{-8} M dexamethasone). Results of all conditions are mean +/- S.D. from 3 different suspension cultures. *$p < 0.01$, cultures in DMEM with pluronic compared to the DMEM cultures with pluronic and dexamethasone, **$p < 0.05$, cultures in MyeloCult® with pluronic compared to the MyeloCult® cultures with pluronic and dexamethasone

In the MyeloCult® cultures with pluronic and dexamethasone only, the total cell number increased by 3 fold after one week, whereas in the DMEM cultures with pluronic and dexamethasone only, the total cell number decreased by 40% (Figure 6.9). For the rest of the culture period, the total cell numbers in cultures with pluronic and dexamethasone only developed similarly to the cultures without dexamethasone and cytokines. For the whole culture period, the CFU-f numbers decreased over time for all conditions. The final value for colony forming cells after three weeks in suspension culture with pluronic and dexamethasone only was 12% for the DMEM cultures and 17% for the MyeloCult® cultures (Figure 6.10).

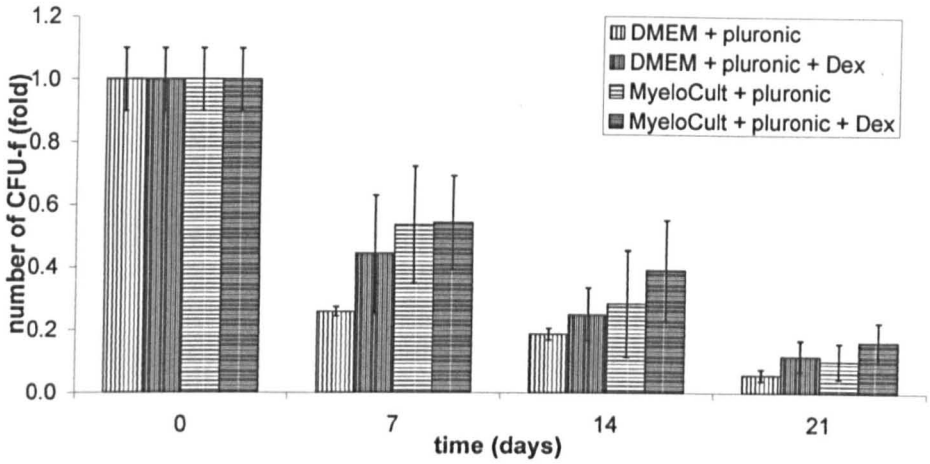

Figure 6.10: Development of CFU-f number over time in suspension cultures started with 12 x 10^6 rat BMCs in different culture media (DMEM with 0.05% pluronic, DMEM with 0.05% pluronic and 10^{-8} M dexamethasone, MyeloCult® with 0.05% pluronic and MyeloCult® with 0.05% pluronic and 10^{-8} M dexamethasone). Results of all conditions are mean +/- S.D. from 3 different suspension cultures. No significant difference in CFU-f numbers (fold) between cultures containing dexamethasone or not for both culture media, DMEM and MyeloCult®

Colonies formed from MSCs taken from dexamethasone cultures appeared to be similar in size and shape to colonies formed by MSCs from the cultures without dexamethasone (Table 6.1). A major difference in colony size is noticeable when comparing the colonies formed from MSCs in DMEM cultures with the colonies formed from MSCs in MyeloCult® cultures. The colonies formed by cells which were derived from MyeloCult® cultures had a larger diameter, which is an indicator for healthier MSCs in the suspension cultures. Only suspension cultures with MyeloCult® medium and with DMEM containing dexamethasone generated enough cells to run differentiation assays. These assays revealed that MSCs with osteogenic differentiation potential were present in the above mentioned suspension cultures.

The most noticeable effect was the deletion of the growth factors from the culture medium. Without IL-3 and SCF, CFU-f numbers decreased from the beginning on for both DMEM and MyeloCult® medium (compare Figure 6.10 with 6.8). By further removing dexamethasone as well from the culture media, the CFU-f numbers decreased even more (Figure 6.10). These finding are little surprising, since IL-3 and SCF are known to increase the MSC number in suspension cultures (Baksh *et al.*, 2003). Dexamethasone is supposed to promote osteogenesis *in vitro* (Chen *et al.*, 1994), effect the osteoblast proliferation and differentiation and induce the expression of osteogenic markers in MSCs (Shur *et al.*, 2001 and Fried *et al.*, 1996). It also inhibits plasminogen activator activity and increases the transcription of genes coding for anti-inflammatory proteins, including lipocortin-1, IL-10, IL-1 receptor antagonist, neutral endopeptidase, IGF-I (Kozawa *et al.*, 1993), IGFBP-3, 4, 5 and (bovine parathyroid hormone) PTH-mediated cAMP (Chen *et al.*, 1991b and Chen and Feldman, 1984). Furthermore, it has been reported to decrease the expression of prostaglandin endoperoxide synthase-2 and the synthesis of PGE2 in osteoblasts (Brum-Fernandes *et al.*, 1994). Dexamethasone interacts and regulates cell function via the glucocorticoid receptors (GR) (Sukhu *et al.*, 1997). The GR plays important roles in both physiological and pathological conditions involving cell growth and differentiation, lipolysis, control of glucose metabolism, immunity, and inflammation (Salvatore *et al.*, 2007). Dexamethasone binds to GR, which resides in the cytosol complexed with a variety of proteins including heat shock protein 90, heat shock protein 70 and the protein FKBP52, that then dimerize and either regulates the transcription rate of target genes by binding to specific DNA sequences named glucocorticoid response elements, or by building a complex with transcription factors such as nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) and prevent them from binding their target genes and hence repress the expression of these genes (Hayashi *et al.*, 2004).

In the present study, it also can be seen that for all culture conditions, the MyeloCult® medium achieved higher CFU-f numbers, which is in agreement with the results from the previous section (6.3.4).

6.3.6 Effect of initial cell source on Mesenchymal Stem Cell proliferation

Instead of reducing the costs of the culture medium, and thereby compromising the expansion potential of the MSCs in suspension, the initial cell source of the suspension cultures was changed in order to simplify the culture protocol and thus save money. So far, the cultures were started with freshly harvested rat BMCs. The alternative was using bone marrow derived rat MSCs, which have already been expanded in static monolayer cultures for one passage (following the method reported in 3.2). By expanding MSCs in static cultures, most of the other cell types from the bone marrow were discarded and the MSCs were already purified by the time they were used to start the suspension cultures. This could be an advantage if the expanded MSCs from the suspension cultures are supposed to be used for therapeutic applications which require a pure stem cell population, e.g. for transplantation.

The development of the total cell number in stirred suspension cultures, started with different initial cell densities of bone marrow derived rat MSCs from static cultures, is displayed in Figure 6.11. The initial cell number was 2×10^6 MSCs (for low cell density), 6×10^6 MSCs (for medium cell density) and 10×10^6 MSCs (for high cell density) per 40 ml suspension culture. Over the complete period of three weeks, the total cell number decreased dramatically for all three initial cell densities. After one week, at the first medium change, the cell numbers dropped by 90% in the low and high cell density cultures and by 96% in the medium density culture. That means not more than 2.3×10^5 MSCs were left in the suspension cultures after one week. This dramatic decrease after so short a time indicated that something was fundamentally wrong with the culture method. At the end of the culture period, 94% of the MSCs in the low density culture and 99% in the medium and high density culture died. The MSCs used for the CFU-f assay did not form any colonies at any time point of the experiment (data not shown).

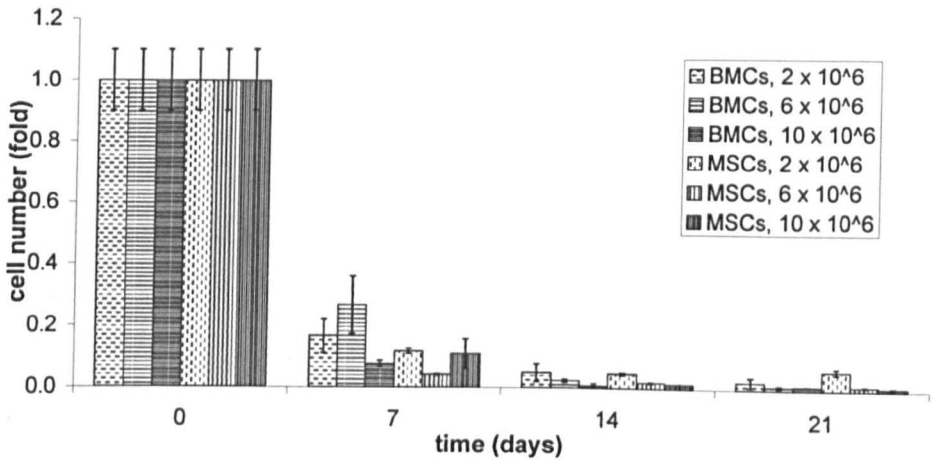

Figure 6.11: Development of total cell number over time in suspension cultures started either with 12 x 10^6, 25 x 10^6 and 50 x 10^6 rat BMCs or with 2 x 10^6, 6 x 10^6 and 10 x 10^6 purified, bone marrow derived rat MSCs. The culture medium was DMEM containing 0.05% pluronic. Results for all 6 cultures conditions are mean +/- S.D. from 3 different suspension cultures.

Whole BMCs in suspension cultures provide a more natural environment for the MSCs present within the bone marrow. This could be a positive influence on the viability of the MSCs, since cell clusters consisting of different cell types can be formed, and cytokines could be secreted by cells present in the bone marrow to support the proliferation of MSCs in suspension (Miao *et al.*, 2004) (Eipers *et al.*, 2000). However, a disadvantage of using BMCs is that the cultures consist of many cell types including haematopoietic stem cells, reticular cells, fibroblasts, endothelial cells, adipocytes and macrophages (see chapter 2, section 2.3), and therefore the cultures would need to be purified before being used for future therapeutic applications, which require pure stem cell samples. This adds complexity and costs.

Experiments with 12 x 10^6 rat BMCs (low cell density culture), 25 x 10^6 rat BMCs (medium cell density culture) and 50 x 10^6 rat BMCs (high cell density culture) in stirred suspension cultures were conducted following the same protocol used for the suspension cultures of already *in vitro* expanded MSCs. As it can be seen in Figure 6.11 the total cell number also decreased dramatically after only one week (drop of 83%, 73% and 92% in low, medium and high density culture, respectively). The final % values of the initial total cell number after three weeks in suspension culture were 2% for the low density culture and 1% for both medium and high density culture.

The total cell number in a suspension culture started with whole BMCs is not as important as the number of colony forming cells. Since BMCs consist of different cell types, a decreasing total cell number does not necessarily mean that the MSC number also decreased. Therefore CFU-f assays were conducted to monitor the development of the MSC number in suspension. Colony forming cells were found in all suspension cultures started with whole BMCs, which was already an improvement compared to the suspension cultures started with purified MSCs only. However, the CFU-f number also decreased over time (Figure 6.12). For all three cell densities the values were similar, after one week in suspension the CFU-f numbers dropped by more than 80%, after two week by 90% and after three weeks by 99%. The CFU-f assays conducted show only some recognisable colonies mainly at the plate from the first week. Thereafter negligible no colonies were formed any more.

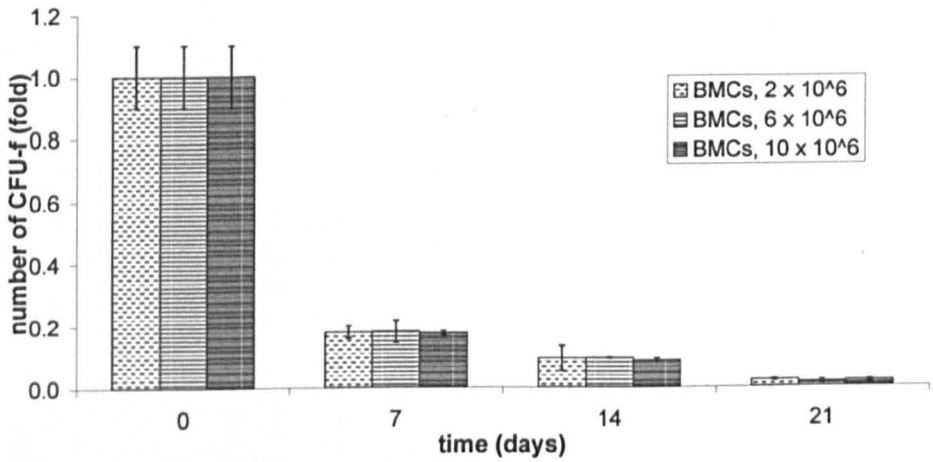

Figure 6.12: Development of CFU-f number over time in suspension cultures started with 12 x 10^6 (low), 25 x 10^6 (medium) and 50 x 10^6 (high) rat BMCs in DMEM containing 0.05% pluronic. Results for all 3 cultures conditions are mean +/- S.D. from 3 different suspension cultures. No significant difference in CFU-f numbers (fold) between the cultures started with different initial cell densities

The cell number and the CFU-f number of suspension cultures started with both BMCs and MSCs decreased dramatically regardless the initial cell density. However, the BMCs cultures contained some colony forming cells and thus seemed to provide a more supportive environment for the MSCs. For future studies, only BMCs rather than MSCs were taken as initial cell source.

In the literature, 1 x 10^6 bone marrow mononuclear cells (MNCs) per millilitre is mostly used to initiate suspension cultures (Chen *et al.*, 2006) (Baksh *et al.*, 2003). As this is even less than the lowest concentration in the experiments shown in Figures 6.34 and 6.35, and no beneficial effect of high cell number could be observed, the lowest cell density of BMCs used for the experiments was taken for all studies on MSC expansion in suspension cultures.

6.3.7 Sampling strategies for Mesenchymal Stem Cell suspension cultures

A further attempt to improve the yield of MSCs from stirred suspension cultures was to change the sampling method. Stirred suspension cultures with 12 x 10^6 BMCs in DMEM containing 0.05% pluronic were used for the following experiments to investigate the effect of two different sampling strategies on MSCs. Both methods are described in section 6.2.4. Method (1) has the advantage that the number of cells taken out as a sample is independent from the number of medium being changed. This can be of benefit if a large quantity of the medium has to be replaced but most of the cells have to remain in the suspension culture. The disadvantage is that the centrifuge step caused additional stress to all cells in culture. The total cell number and CFU-f results for this method are mentioned in the previous section (6.3.1). A comparison of these results with the results obtained from sampling method (2) can be seen in Figures 6.13 and 6.14.

By replacing 1/3 of the culture medium including cells with fresh medium (sampling method 2) no additional stress was caused to the remaining cells in the culture. It also reduced the risk of contamination since less handling was required. This method was successfully applied by Baksh *et al.* (2003) to take samples of mono nuclei cells (MNCs) from stirred suspension cultures.

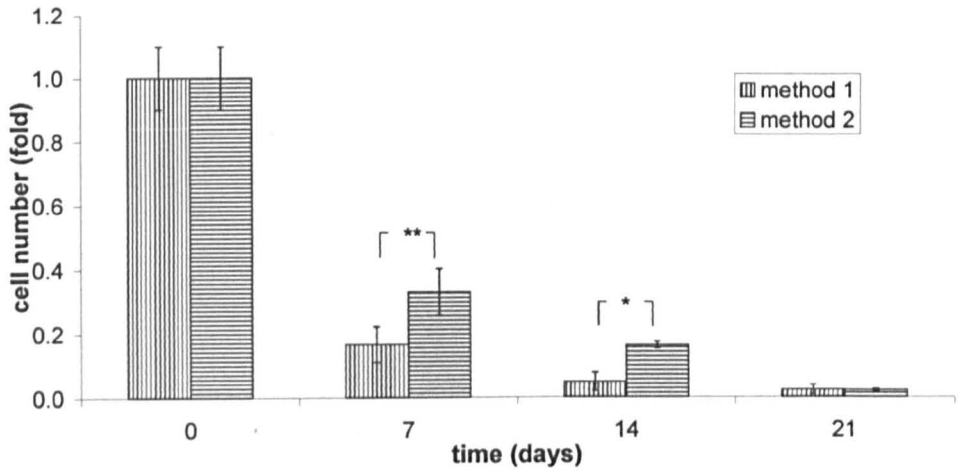

Figure 6.13: Development of total cell number over time in suspension cultures started with 12 x 10^6 rat BMCs. Sampling was performed by either spinning down the whole culture (method 1) or by replacing 1/3 of the medium including cells (method 2). Results are mean +/- S.D. from 3 different suspension cultures. *p < 0.01, **p < 0.05; cultures harvested with method 1 compared to cultures with method 2

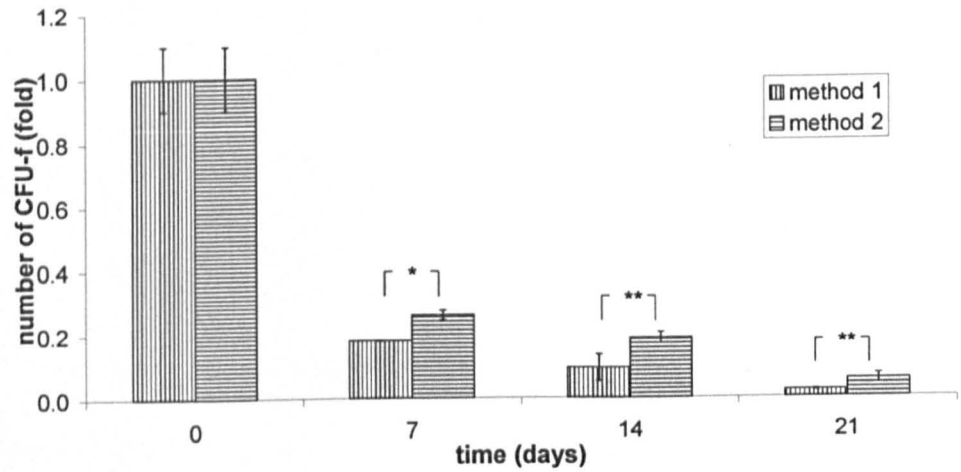

Figure 6.14: Development of CFU-f number over time in suspension cultures started with 12 x 10^6 rat BMCs. Sampling was performed either spinning down the whole culture (method 1) or by replacing 1/3 of the medium including cells (method 2). Results are mean +/- S.D. from 3 different suspension cultures. *p < 0.01, **p < 0.05; cultures harvested with method 1 compared to cultures with method 2

The total cell number in suspension cultures with medium change method (2) dropped over time from 100% (at the start of the suspension culture) to 33% (after one week), 16% (after two weeks) and finally to 2% (after three weeks) (Figure 6.13). The number of colony forming cells in the suspension cultures showed the same pattern of decrease, 100% of the initial CFU-f number at the beginning, 26% at the first medium change, 19% at the second medium change and finally just 5% at the end of the culture period (Figure 6.14). The pictures from the CFU-f assay of both sampling methods were similar but some of the colonies formed from cells harvested with method (2) seemed to be bigger in size. This can be an indicator for healthier colony forming cells in the suspension cultures due to reduced stress levels.

Comparing the two sampling strategies, higher CFU-f numbers at the end of the culture period could be achieved with method 2 ($p = 0.029$) (Figures 6.13 and 6.14). Furthermore this strategy has the advantage that it is less time consuming and the risk of contamination is reduced due to less handling. Therefore, this method of sampling was applied to all suspension cultures.

6.4. Conclusions

In this chapter, studies with bone marrow derived rat MSCs in stirred suspension cultures were conducted to determine the best working culture condition. The aim was to generate large quantities of MSCs with a higher differentiation capacity than MSCs expanded in static monolayer cultures. Furthermore, the cost for this culture system should be kept as low as possible so that the expanded MSCs could be used for medical applications. Different techniques for sample taking and for medium changing, different culture vessels and agitation methods, different culture media and different medium additives were tested in order to develop a useful protocol, which may also be scaled up.

Table 6.1: Summarised information from the pictures of the CFU-f and differentiation assays conducted in the experiments mention in this chapter.

Medium	Culture conditions	Time [days]	Colony formation	Osteogenic differentiation	Adipogenic differentiation
DMEM + pluronic	bottle, stirred	7	medium	-	-
DMEM + pluronic	bottle, stirred	14	medium	-	-
DMEM + pluronic	bottle, stirred	21	medium	-	-
DMEM + pluronic + Dex	bottle, stirred	7	medium	small	-
DMEM + pluronic + Dex	bottle, stirred	14	medium	small	-
DMEM + pluronic + Dex	bottle, stirred	21	small	small	-
DMEM + pluronic + Dex + gf's	bottle, stirred	7	medium	medium	-
DMEM + pluronic + Dex + gf's	bottle, stirred	14	medium	small	-
DMEM + pluronic + Dex + gf's	bottle, stirred	21	small	small	-
MyeloCult + pluronic + Dex + gf's	bottle, stirred	7	big	big	-
MyeloCult + pluronic + Dex + gf's	bottle, stirred	14	medium	big	-
MyeloCult + pluronic + Dex + gf's	bottle, stirred	21	medium	medium	-
MyeloCult + pluronic	bottle, stirred	7	medium	small	-
MyeloCult + pluronic	bottle, stirred	14	big	small	-
MyeloCult + pluronic	bottle, stirred	21	big	small	-
MyeloCult + pluronic + Dex	bottle, stirred	7	big	medium	-
MyeloCult + pluronic + Dex	bottle, stirred	14	big	medium	-
MyeloCult + pluronic + Dex	bottle, stirred	21	big	small	-
MyeloCult + pluronic + Dex + gf's + hs	bottle, stirred	7	big	big	-
MyeloCult + pluronic + Dex + gf's +	bottle, stirred	14	big	big	-

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hs MyeloCult + pluronic + Dex + gf's + hs	bottle, stirred	21	very big	medium	-
MyeloCult + Dex + gf's	bottle, stirred	7	medim	very big	-
MyeloCult + Dex + gf's	bottle, stirred	14	small	medium	-
MyeloCult + Dex + gf's	bottle, stirred	21	barely visible	medium	-
MyeloCult + gelatine + Dex + gf's	bottle, stirred	7	very big	very big	-
MyeloCult + gelatine + Dex + gf's	bottle, stirred	14	big	big	-
MyeloCult + gelatine + Dex + gf's	bottle, stirred	21	medium	big	-
MyeloCult	bottle, stirred	7	medium + big	big	-
MyeloCult	bottle, stirred	14	small	medium	-
MyeloCult	bottle, stirred	21	medium	medium	-
MyeloCult	bottle, shaken	7	medium	very big	-
MyeloCult	bottle, shaken	14	small + medium	big	-
MyeloCult	bottle, shaken	21	small	medium	-
MyeloCult	spinner flask, stirred	7	medium	big	-
MyeloCult	spinner flask, stirred	14	medium	medium	-
MyeloCult	spinner flask, stirred	21	small	big	-

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Since MSCs are sensitive to many forms of stimuli, e.g. shear stress, which can either cause cell death or uncontrolled differentiation into any sort of progenitor cells, suspension cultures were started with whole bone marrow cells (BMCs) to provide an environment similar to the natural niche within the bone marrow. The necessity of the support from the BMCs for the survival of the MSCs in suspension culture was proven by the experiment in section 6.3.6. In suspension cultures started with BMCs, more MSCs with colony forming ability survived than in the suspension cultures started with purified MSCs only.

Another important parameter for the survival of the MSCs was the choice of culture medium. All suspension culture experiments conducted in MyeloCult® medium achieved higher MSCs numbers than in the cultures with DMEM regardless any further medium additives. Moreover, these cells also had better proliferation and differentiation behaviour.

Further medium additives such as dexamethasone, pluronic F-68, interleukin-3 (IL-3) and stem cell factor (SCF) were tested but their positive influence on the proliferation of MSCs were always additive to the beneficial effect of the MyeloCult® medium. Dexamethasone increased the number of MSCs in suspension cultures slightly, and also seemed to improve their differentiation capacity as shown by the experiments with DMEM cultures in which MSCs with osteogenic differentiation potential were found only when dexamethasone was present in the culture medium. Even so, this effect was very small, and could not be shown in static monolayer cultures, dexamethasone was used as a regular medium additive for suspension cultures.

Adding the growth factors IL-3 and SCF to the culture medium led to an increase in MSC number in the suspension cultures. However, this increase was not only dependant on the addition of the growth factors but also on how much shear stress was affecting the proliferation of the MSCs.

Pluronic F-68 played an important role in helping the MSCs to cope with the shear stress and therefore was part of the culture medium. Culturing MSCs in suspension without pluronic resulted in a decreasing MSCs number, regardless of the kind of

culture medium or the presence of growth factors, in it. However, experiments in monolayer cultures have shown that pluronic slows down the proliferation of MSCs, but it seemed that the protective properties were outbalanced by this negative side effect in suspension cultures. Replacing pluronic with gelatine, which was supposed to protect the cells form shear stress without altering their proliferation, reduced the MSCs number in the suspension culture, and caused the cells to aggregate. The addition of heparan sulphate had similar effects, and since both compounds gelatine and heparan sulphate, did not increase the MSC number or their differentiation capacity, none of it was used in further experiments.

The results from all the experiments with different culture media have shown that a combination of MyeloCult® medium supplemented with pluronic, IL-3 and SCF is necessary to increase the MSC number in suspension cultures. Dexamethasone seemed to be helpful in order to improve the osteogenic differentiation, had no negative side effects and was added only in small quantities, so that increase to the total costs of the culture medium was negligible. However, in static monolayer cultures the beneficial effect of the different culture media was not significant, indicating that a more stressful culture system, such as the suspension culture, requires a more supportive culture medium. A summary of the different culture media and their effect on the MSCs expansion in both static and suspension cultures are listed in Table 6.2.

Table 6.2: Summary of the results for the maximum fold increase in CFU-f achieved in static and suspension culture with different culture media. For the suspension cultures, all the media types mentioned included 0.05% pluronic.

Media type	Static cultures	Suspension cultures	Suspension cultures
	CFU-f (fold increase)	CFU-f (fold increase)	Costs (£) / fold increase
DMEM	460	30	0.06
DMEM + Dex	460	40	0.05
MyeloCult®	460	53	0.09
MyeloCult® + Dex	440	54	0.09
MyeloCult® + IL-3 + SCF	470	140	0.13

The need to reduce the shear stress is as important for the expansion of MSCs in suspension cultures as the choice of the right medium. Using spinner flasks with big paddles or shaking the suspension cultures in special shaking incubators increased the shear forces rather then reduced it, as indicated by the decreasing MSC numbers.

Few publications regarding bone marrow derived MSC expansion in stirred suspension cultures can be found. Most noticeable is the publication by Baksh *et al.*, (2003) who successfully expanded human bone marrow derived MSCs over a period of three weeks in 100 ml stirred suspension cultures. But when the experiment was repeated using the same spinner flasks, the same sampling method and the exactly same medium containing the cytokines IL-3, SCF and dexamethasone, the MSC numbers in the cultures actually decreased. The conducted experiments with rat BMCs in suspension culture revealed that a simpler culture vessel, a normal 250 ml glass bottle (coated with silicon) with screw cap and magnetic stirrer, and the additions of a pluronic F-68 were necessary to protect the MSCs from high shear stress and assure an increase in cell number.

The experiments with bone marrow derived rat MSCs in suspension cultures have confirmed that it is possible to expand MSCs in this culture system. However, all attempts to use the cheapest culture conditions failed. In addition, the expansion rates obtained from static cultures were better than in any tested suspension culture (Table 6.2). Furthermore, no MSCs with adipogenic differentiation capacity could be found in either system, implying that the expanded MSCs did not have their full differentiation potential. While in the monolayer cultures the stress from the trypsin treatment may have reduced the differentiation potential, in the suspension cultures, the shear stress could have done exactly the same. Thus both culture systems are not suitable for large scale MSCs expansion.

Chapter 7

Mesenchymal Stem Cells in Pour-off Cultures

7.1 Introduction

In chapters 4 and 6, experiments were conducted to investigate the expansion of mesenchymal stem cells (MSCs) *in vitro* in static monolayer cultures and in stirred suspension cultures respectively. With both culture systems, an increase in MSC number with osteogenic differentiation potential was achieved after a culture period of two to three weeks, indicating that a MSC population with the capacity to form colonies on culture plastic and to differentiate into osteoblasts can be expanded in both adherent culture systems, and non-adherent stirred suspension cultures. The advantages of the static cultures are the simplicity, whereas the stirred suspension cultures offered the advantages of a trypsin-treatment free culture protocol and a potential for automation and scale-up. However, both culture methods exerted either chemical stress or physical stress on the MSCs, and thus restricted their proliferation potential and their full differentiation capacity. This chapter therefore describes a hybrid culture method, named "pour-off culture" which is likely to combine the advantages of static and suspension cultures to overcome the disadvantages of both.

In this study, pour-off cultures were applied to obtain MSCs with high proliferation potential and full differentiation ability. This technique was first described by Oreffo *et al.* (1996), who used pour-off cultures to examine the non-adherent bone marrow stromal fraction from human bone marrow samples. Baksh *et al.* (2007) also used these cultures to characterise human suspension-derived MSCs for therapeutic strategies requiring systemic infusion, and concluded that the bone marrow contains a $CD49e^{low}$ subpopulation of MSCs, which can grow in suspension while maintaining a single and round morphology. Zhang *et al.* (2009) applied the pour-off culture technique to establish the presence of non-adherent bone marrow derived mesenchymal stem cells in adult bone marrow. Zhang *et al.* (2009) found that the adult rat bone marrow harbours pluripotent non-adherent MSCs which can migrate *in vivo* to any organ of the body through the circulation and, in the appropriate microenvironment, can adhere, proliferate and differentiate into specialised cells of the target tissue, and thus function in tissue regeneration.

Pour-off cultures can be seen as static monolayer cultures, which are re-plated regularly without using trypsin to lift off the adherent cells. Thus, only the non-adherent cells are kept in the culture, whereas the adherent cells can be used to analyse the colony forming potential of the non-adherent cells at the time point of re-plating. Pour-off cultures do not have to be stirred, and do not require the use of trypsin. Both were the main stress causing factors in the suspension cultures and the static monolayer cultures, respectively. However, in order to expand MSCs in pour-off cultures successfully, the addition of 1α,25-Dihydroxyvitamin D_3 was necessary. 1α,25-Dihydroxyvitamin D_3 is known to increase the cell proliferation and the osteogenesis of the non-adherent marrow cell fraction (Rickard *et al.*, 1995) and (Zhang *et al.*, 2009). Furthermore, Catherwood (1985) described that 1α,25-Dihydroxyvitamin D_3 reduced the cAMP response to subsequent parathyroid hormone (PTH) in osteoblast-like cells, decreased adenylate cyclase stimulated by PTH and antagonized the positive regulatory effects of cell treatment with glucocorticosteroid on these responses to PTH. Many of the effects of 1α,25-Dihydroxyvitamin D_3 are mediated by its interaction with the vitamin D receptor (VDR), also called calcitriol receptor. The unbound inactive form of the calcitriol receptor resides in the cytoplasm. When 1α,25-Dihydroxyvitamin D_3 binds to the receptor, the ligand-receptor complex translocates to the cell nucleus, where it acts as a transcription factor. Downstream targets of this nuclear hormone receptor are principally involved in mineral metabolism though the receptor regulates a variety of other metabolic pathways, such as those involved in the immune response and cancer. Cells of the osteoblastic lineage have been shown to contain intracellular 1α,25-Dihydroxyvitamin D_3 receptors (Narbaitz *et al.*, 1983). Zhang *et al.* (2009) found that although 1,25-dihydroxyvitamin D3 facilitates the expansion of BM-MSC, the absence of either 1,25-dihydroxyvitamin D3 or its receptor, vitamin D receptor (VDR), does not appear to impede normal tissue and organ growth and development in mice with targeted deletion of the 1α,25-Dihydroxyvitamin D_3 hydroxylase enzyme or VDR, respectively. This suggests multiple mechanisms of action for 1,25-dihydroxyvitamin D_3. Rickard *et al.* (1995) hypothesised that 1α,25-Dihydroxyvitamin D_3 could effect MSCs in two ways: (1) it could render the MSCs more responsive to cytokines through an up-regulation of cytokine receptors, or (2) it could effect the release of mitogenic cytokines from cell types within the non-

adherent marrow cell population, which possess receptors for 1α,25-Dihydroxyvitamin D_3, such as monocytes, macrophages and lymphocytes.

In this chapter, the influence of different parameters (such as initial cell density, culture medium, and pour-off frequency) on the expansion capacity of MSCs and their differentiation potential was investigated. The protocol for culturing MSCs in pour-off cultures from Zhang *et al.* (2009) was changed in order to optimise the proliferation rates and to keep the costs as low as possible. The results from these studies were then compared to the cell numbers obtained from the suspension cultures and static monolayer cultures (chapters 6 and 4).

7.2 Materials and methods

In this chapter, the specific methods for growing and expanding bone marrow derived rat MSCs in pour-off cultures are described. The protocol for these cultures was changed in terms of initial cell number used to start a pour-off culture, the culture medium, and the pour-off frequency. The methods for general cell culture are given in chapter 3. To start a pour-off culture, bone marrow cells were seeded into a culture dish, and after only 24 hours transferred into a new dish (refer to section 7.2.2 for further details about the pour-off culture method). The adherent cells will attach to the culture plastic within the 24 hour period, whilst the non-adherent cells will stay in suspension and may be poured-off into the new dish. The old dish, containing the adherent cells, will be kept for 12 days as colony forming unit fibroblast culture (CFU-f culture) allowing the cells to form colonies which then can be counted. By summing up all counted colonies (each of which originated from a single attached cell) from each pour-off level, the total number of CFU-f cells can be determined.

7.2.1 Different culture media

The media tested for growing MSCs in pour-off cultures were simple variations of the standard Dulbecco's Modified Eagle Medium (DMEM) and MyeloCult® medium mentioned in section 3.3. The following variations were used:

1. Standard DMEM as described in section 3.3,
2. Standard DMEM with 10^{-8} M dexamethasone,
3. Standard DMEM with 10^{-8} M dexamethasone and with 10^{-8} M 1α,25-Dihydroxyvitamin D_3
4. Standard MyeloCult® medium with 10^{-8} M dexamethasone, 10^{-8} M 1α,25-Dihydroxyvitamin D_3, 2 ng/ml interleukin-3 (IL-3) and 10 ng/ml stem cell factor (SCF).

7.2.2 Bone marrow cell pour-off cultures with different initial cell densities

Pour-off cultures were started in triplicate with freshly harvested rat bone marrow cells (BMCs). These were obtained as described in section 3.4. Either 5 ml, 2 ml, 1 ml or 0.5 ml of BMCs were suspended in 20 ml culture medium, seeded into culture dishes and incubated at 37°C in an atmosphere with 7.5% CO_2. The numbers of BMCs used equals a concentration of 5 x 10^5 BMCs/ml, 2 x 10^5 BMCs/ml, 1 x 10^5 BMCs/ml or 0.5 x 10^5 BMCs/ml respectively. After 24 hours, the non-adherent cells, suspended in the culture medium, were removed from the culture dishes with a 10 ml pipette and re-plated into new culture dishes (Figure 7.1). The adherent cells were retained and cultured further either as CFU-f cultures (see section 3.6), or as osteogenic / adipogenic differentiation cultures (section 3.7). This process was repeated daily for 7 to 10 days. Every 3 - 4 days the culture medium in the pour-off cultures was refilled up to 20 ml with fresh medium. The number of colonies formed from the adherent cells in the CFU-f cultures were stained and determined as described in section 3.6.1. The colonies in the osteogenic and in the adipogenic differentiation assay were also stained in order to verify the formation of osteoblasts and adipocytes and thus prove the differentiation capacity of the MSCs. Control cultures were started with 0.3 ml freshly harvested BMCs suspended in 10 ml culture medium (0.6 BMCs/ml) and kept as CFU-f cultures for 14 days (see section 3.6).

These controls were to measure the original number of MSCs present within the rat BM used to start the pour-off cultures.

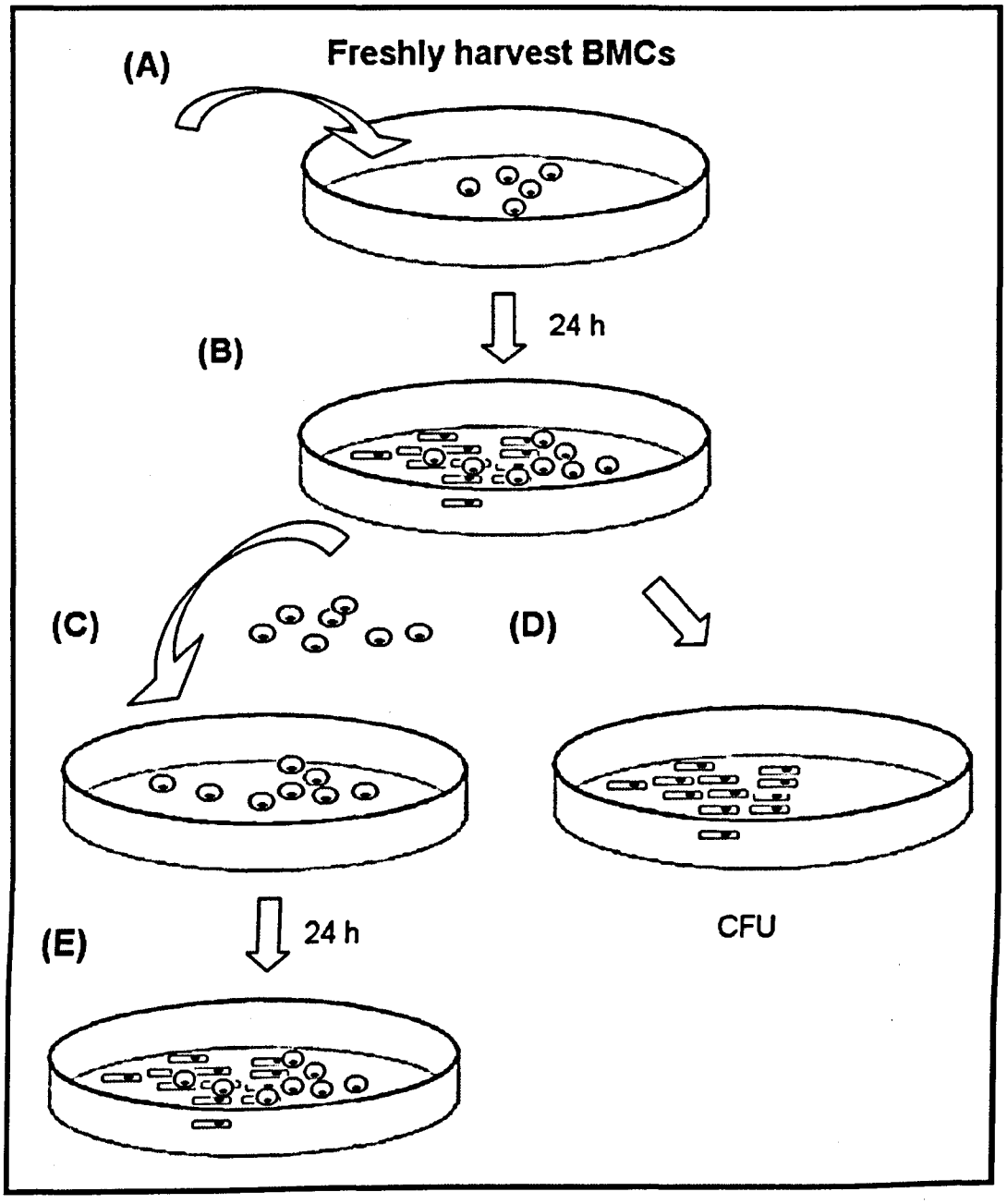

Figure 7.1: The principle of "pour-off cultures". (A) Freshly harvested bone marrow cells are transferred into a cell culture dish with medium and incubated for 24 hours. (B) Some of the BMCs have attached to the plastic surface of the dish, others stay in suspension. (C) The culture medium, containing the non-adherent cells, is transferred to a new culture dish. (D) The adherent cells in the old culture dish are further incubated as CFU-f culture and (E) the re-plated non-adherent cells will be poured-off again after 24 hours.

7.2.3 Varying pour-off frequencies

In this chapter, the effect of the pour-off frequency on the proliferation and differentiation capacity of the MSCs was determined by replating the cells in the pour-off cultures either daily, every 2 days, every 4 days, or every 7 days using otherwise the same culture conditions as described in 7.2.2.

7.3 Results and Discussion

The following results demonstrate the expansion capacity of MSCs in pour-off cultures. The original protocol by Zhang *et al.* (2009) was changed several times in order to determine the effect of different culture media (DMEM and MyeloCult® medium), of different medium additives (1α,25-Dihydroxyvitamin D_3 and dexamethasone) and of different cultivation strategies (initial cell number and pour-off frequency) on the proliferation capacity and the differentiation potential of MSCs. The accumulative CFU-f number, which resembles the total cell number, of each pour-off culture was obtained over a period of 7 to 12 days by summing the counted colonies formed in each culture dish after every re-plating step as described in section 7.2.2. The fold increase for the accumulative CFU-f number was calculated by comparing accumulative CFU-f number with the numbers of MSCs present in the volume of bone marrow used to start the pour-off culture (which was determined by the control CFU-f cultures started with 0.3 ml of bone marrow cells).

$$\text{Fold increase}_{(\text{accumulative CFU-f})} = \frac{\sum \text{CFU-fs}}{\text{initial CFU-f number in BM}} \qquad 7.1$$

Furthermore, the number of colonies at each passage level was recorded. This helps to visualise at what time point the pour-off culture was the most productive. For the development of the CFU-f number per dish the fold increase was calculated by comparing CFU-f number per dish from every day with the CFU-f number on the dish from day 1.

$$\text{Fold increase}_{(\text{CFU-f per dish})} = \frac{\text{CFU-f per dish}}{\text{CFU-f number on dish from day 1}} \qquad 7.2$$

To determine the stem cell character of the expanded MSCs, differentiation assays were started at each pour-off level after the cells were transferred to new dishes. The differentiation assay was used in a qualitative way to prove the existence of MSCs with the ability to differentiate into osteoblasts or adipocytes. The information obtained from the CFU-f -, the differentiation assays and the pour-off culture plates is summarised in Table 7.1 in the conclusion section of this chapter.

7.3.1 The effect of dexamethasone and 1α,25-Dihydroxyvitamin D_3 on Mesenchymal Stem Cells in pour-off cultures

Dexamethasone and 1α,25-Dihydroxyvitamin D_3 have been shown to increase the cell proliferation and the osteogenesis of the non-adherent marrow cell fraction (Rickard *et al.*, 1995) (Akavia *et al.*, 2006). The protocol used to investigate the effect of dexamethasone and 1α,25-dihydroxyvitamin D_3 on MSCs in pour-off cultures was the same as that described by Zhang *et al.* (2009). They expanded bone marrow derived rat MSCs in pour-off cultures by culturing 1 x 10^7 bone marrow cells in a 55cm^2 petri dish with DMEM, containing 10% foetal calf serum, 50 μg/ml ascorbic acid, 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3. The non-adherent cells were re-plated every 24 hours. With this technique, Zhang *et al.* (2009) achieved an increase in MSC number of 2.3 fold after 4 days compared to their initial MSC number at day 1 before the first pour-off.

Figures 7.2 and 7.3 show the results obtained from this study. After one week, accumulative CFU-f numbers in any of the pour-off cultures was approximately the same as the numbers of MSCs present in the volume of bone marrow used to start the cultures. A 1.2 fold increase was observed in the pour-off cultures with DMEM, dexamethasone and 1α,25-Dihydroxyvitamin D_3, and an increase by 1.1 fold in the pour-off cultures with DMEM and dexamethasone after one week. In the pour-off cultures with standard DMEM, a slight decrease of 2% was recorded for the same

period of time (Figure 7.2). However, only the change in the pour-off culture with DMEM, dexamethasone and 1α,25-Dihydroxyvitamin D_3 was significant different to the numbers of MSCs present in the volume of bone marrow used to start the culture ($p = 0.011$) and different to the increase of the other two cultures from day 6 onwards ($p = 0.009$).

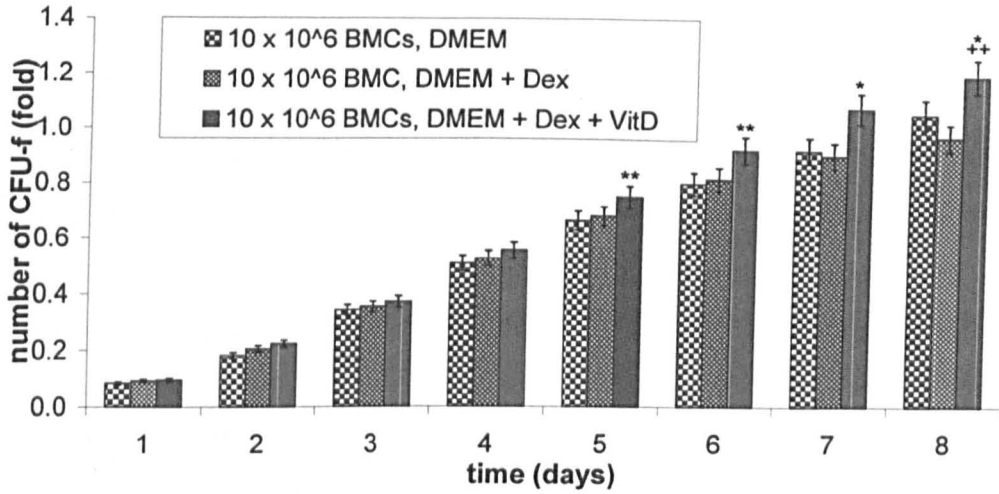

Figure 7.2: Development of accumulative CFU-f number over time in pour-off cultures started with 1 x 10^7 rat BMCs in either standard DMEM, DMEM containing 10^{-8} M dexamethasone, or DMEM containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3. The cultures were re-plated every 24 hours. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. ++$p < 0.05$, compared to the numbers of MSCs present in the volume of bone marrow used to start the culture; *$p < 0.01$, **$p < 0.05$; DMEM, dexamethasone and 1α,25-Dihydroxyvitamin D_3 culture compared to the two other cultures

The highest colony numbers per dish for each pour-off culture was achieved after 5 days in the pour-off culture with DMEM, dexamethasone and 1α,25-Dihydroxyvitamin D_3 (457 colonies = 2.0 fold increase), and after 4 days in the cultures with DMEM containing dexamethasone and with standard DMEM (395 colonies = 1.9 fold increase and 381 colonies = 1.8 fold increase, respectively) (Figure 7.3). After these days, the colony number per dish dropped slowly for the cultures in DMEM with dexamethasone and 1α,25-Dihydroxyvitamin D_3 and in DMEM with dexamethasone. The colony number in the culture with standard DMEM stayed about the same after day 4 for the rest of the culture period.

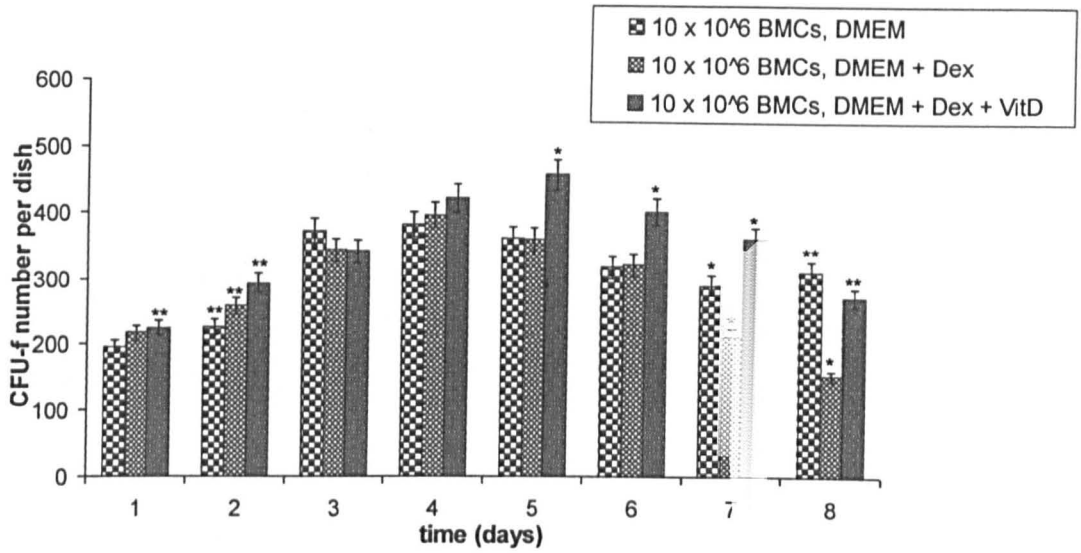

Figure 7.3: Development of CFU-f number per dish in pour-off cultures started with 1 x 10^7 rat BMCs in either standard DMEM, DMEM containing 10^{-8} M dexamethasone, or DMEM containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3. The cultures were re-plated every 24 hours. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. *$p < 0.01$, **$p < 0.05$; the three different pour-off cultures compared to each other

By comparing the result from this study with the result from Zhang *et al.* (2009), it can be seen that a similar maximum increase in colony number per dish was obtained. Zhang *et al.* (2009) achieved an increase of 2.3 fold +/- 0.2, whereas in this study the increase was 2.0 fold +/- 0.1. To determine the stem cell character of these cells, differentiation assays were conducted and the colonies formed in the pour-off cultures were analysed. The majority of the colonies formed by the adherent cells in these pour-off cultures appeared to be similar in shape and size for the whole culture period. This indicates that the cells forming the colonies kept the same proliferation behaviour, which in turn is an indication of constant culture conditions with low stress levels for the cells.

In order to determine the differentiation capacity of the adherent cells on the culture dishes, they were incubated in osteogenic conditions for 14 days, or in adipogenic conditions for 21 days. The adherent cells from the pour-off cultures in standard DMEM and in DMEM with 10^{-8} M dexamethasone did not form any adipocytes or osteoblasts. Consequently, these cells were already too mature and did not have any

differentiation potential left. The colonies formed on the dishes from the pour-off culture in DMEM with 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3 stained positive for alkaline phosphatase, calcium and collagen. Thus, these cells had at least the potential to differentiate into osteoblasts under the right culture conditions.

Finally, the best working culture medium for pour-off cultures was determined by comparing the differentiation and proliferation results from the cultures with DMEM and dexamethasone and with DMEM, dexamethasone and 1α,25-Dihydroxyvitamin D_3 to cultures in standard DMEM. The addition of 10^{-8} M dexamethasone to the DMEM had no positive effect on the proliferation or differentiation potential of the colony forming cells. This finding was also true for MSCs in static monolayer cultures (chapter 4) and in stirred suspension cultures (chapter 6). Dexamethasone is a very controversial medium additive. Depending on the concentration used, it has been reported that it can either induce adipogenesis and reduce cell proliferation and osteogenesis in bone marrow derived MSCs (Yin *et al.*, 2006) or it can have the exact opposite effect, increase cell proliferation and osteogenesis and decrease adipogenesis (Akavia *et al.*, 2006) and (Hong *et al.*, 2009). However, the combined use of 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3 in DMEM seemed to improve the differentiation capacity since cells derived only from this medium condition differentiated into osteoblasts (Figure 7.7). It has been shown before that dexamethasone and 1α,25-Dihydroxyvitamin D_3 can have a combined effect on cells. Chen *et al.* (1991) found that only in combined treatments of 10^{-7} Dexamethasone and 10^{-8} M of 1α,25-Dihydroxyvitamin D_3, primary cultures of rat osteoblast-like cells released insulin-like growth factor-I into the medium.

Although DMEM containing dexamethasone and 1α,25-Dihydroxyvitamin D_3 was found to be the best culture medium for pour-off cultures with regard to the differentiation potential of the cultured MSCs, the highest fold increase in accumulated CFU-f number from this study (1.2 fold) cannot compare to an increase of 4.6 fold for MSCs in static monolayer cultures (chapter 4, Figure 4.5). A hypothesis to improve the expansion rate of MSCs in pour-off cultures is to reduce the quantity of initial bone marrow cells. This will allow the cells to have more space and nutrients to proliferate, and thus will increase the efficiency of this culture

system. Furthermore, the adherent cells will also have more space to form bigger colonies on the dishes.

7.3.2 Different initial cell densities

In order to try to improve the efficiency of the pour-off cultures and the proliferation of colony forming cells, different initial cell densities were used to start the cultures. In section 7.3.1, the pour-off cultures were started with 5 ml bone marrow cell suspension containing 10 x 10^6 BMCs and achieved a maximum increase in accumulated CFU-f number of only 1.2 fold. In this experiment lower initial cell densities of 4 x 10^6 BMCs, 2 x 10^6 BMCs and 1 x 10^6 BMCs (2 ml, 1 ml and 0.5 ml bone marrow cell solution respectively) were used.

The development of the accumulative CFU-f number is displayed in Figure 7.4. For the first 7 days, pour-off cultures with lower initial cell densities had a better fold increase of CFU-f numbers. After 8 days, however, the pour-off culture started with 2 x 10^6 BMCs resulted in the highest increase of accumulated CFU-f (3.8 fold). The pour-off culture started with 1 x 10^6 BMCs achieved a 3.3 fold increase of accumulated CFU-f and only a 1.9 fold increase was observed in the pour-off culture with 4 x 10^6 BMCs.

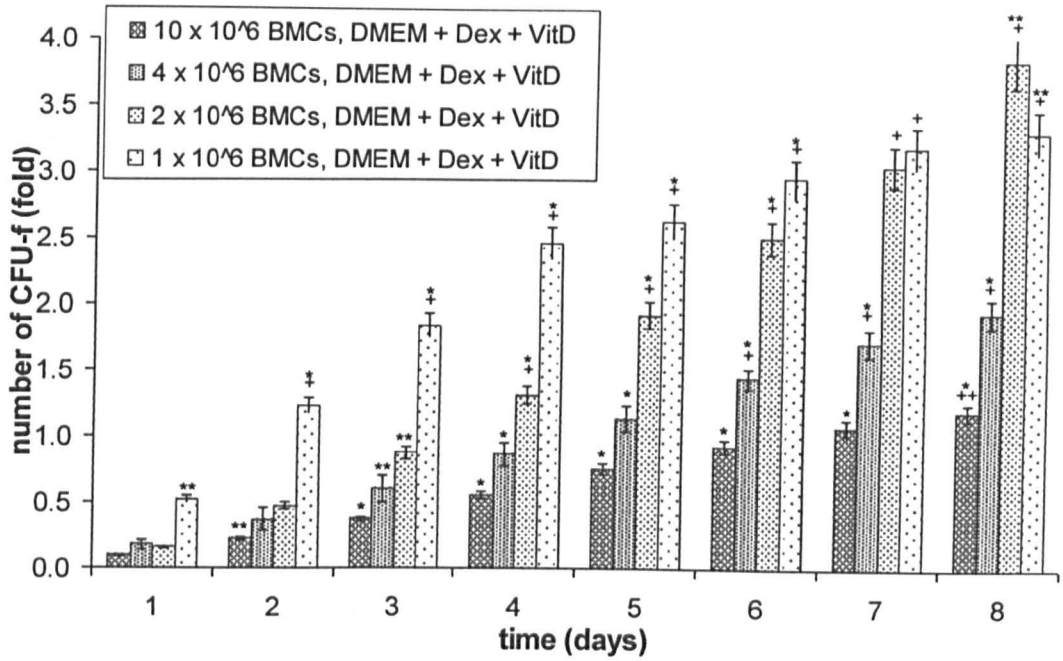

Figure 7.4: Development of accumulative CFU-f number over time in pour-off cultures started with 10 x 10^6, 4 x 10^6, 2 x 10^6 and 1 x 10^6 rat BMCs in DMEM containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3. The cultures were re-plated every 24 hours. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. +$p < 0.01$, ++$p < 0.05$, compared to the numbers of MSCs present in the volume of bone marrow used to start the culture; *$p < 0.01$, **$p < 0.05$; each culture compared to the rest of the other three cultures

The development of the CFU-f number per dish is displayed in Figure 7.5. In the pour-off culture with the highest initial cell density of 10 x 10^6 BMCs, there were 225 colonies on the dish of day 1 and this number increased continuously for 4 days up to 457 colonies per dish (2.0 fold increase) before it decreased to 273 colonies at day 7 (Figure 7.5). The numbers and development in the culture with 4 x 10^6 BMCs were similar, 266 colonies at the first day, 446 colonies at day 5 (1.7 fold increase) and 336 colonies on the dish from day 7 (Figure 7.5). A lower initial cell number of 2 x 10^6 BMCs and of 1 x 10^6 BMCs resulted in fewer colonies on the dishes (58 and 126 colonies on the first dish, respectively). These numbers increased to 222 colonies at day 4 and further to 288 colonies (5.0 fold increase) at day 7 for the pour-off culture with 2 x 10^6 BMCs. In the culture with 1 x 10^6 BMCs, the number of colonies shortly increased to 171 colonies (1.4 fold) at day 2 and then continuously decreased to 28 colonies at day 7 (Figure 7.5).

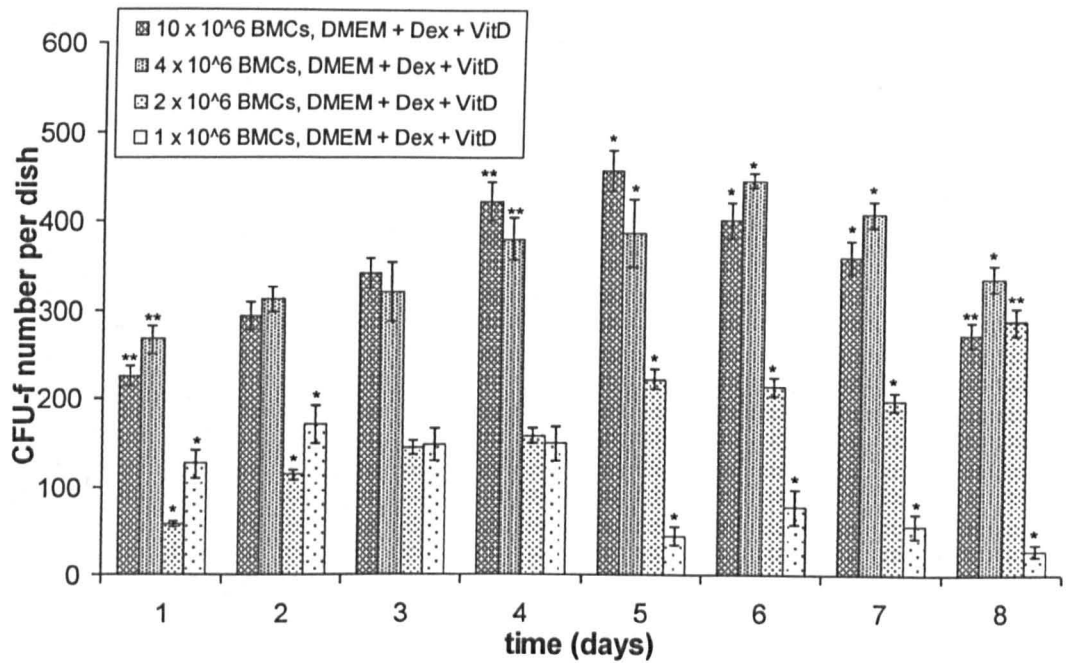

Figure 7.5: Development of CFU-f number per dish in pour-off cultures started 10 x 10^6, 4 x 10^6, 2 x 10^6 and 1 x 10^6 rat BMCs in DMEM containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3. The cultures were re-plated every 24 hours. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. *$p < 0.01$, **$p < 0.05$; each culture compared to the rest of the other three cultures

Both the increase in accumulative CFU-f number (3.8 fold) and in CFU-f number per dish (5.0 fold) was higher in the pour-off cultures with a lower initial cell density of 2 x 10^6 BMCs compared to the cultures started with 10 x 10^6 BMCs (1.2 fold increase in accumulative CFU-f ($p = 0.01$) and 2.0 fold increase in CFU-f number per dish ($p = 0.005$)). With this improved culture method, the result for the CFU-f number per dish was higher than the reported increase from Zhang *et al.* (2009) of 2.3 fold +/- 0.2. In addition, the increase in accumulative CFU-f number (3.8 fold) was in the same range than the expansion rates in the monolayer culture mentioned in chapter 4 (4.6 fold increase).

To further investigate the effect of the initial cell density on the colony size and shape pictures of the stained dishes from the pour-off cultures were taken and analysed. In the pour-off cultures with the highest cell density (10 x 10^6 BMCs), the colonies were quite small for the whole culture period and with increasing colony number, more small colonies seemed to appear. On day 1, the colonies in the pour-

off culture started with 4 x 10^6 BMCs were bigger in size than the colonies in the 10 x 10^6 BMC pour-off culture but more small colonies appeared as well, with increasing colony number. This observation also applied to the development of the colony number and size in the pour-off cultures started with 2 x 10^6 BMCs and with 1 x 10^6 BMCs. A confluent layer of adherent cells, which made accurate counting of the colony number very difficult, was found at day 5 in the culture with 4 x 10^6 BMCs and at day 7 in the culture with 2 x 10^6 BMCs. This was due to a huge number of colonies on the dishes, which merged together as they were growing. In the pour-off culture with the lowest cell density (1 x 10^6 BMCs), the colonies were larger in size than in any of the other pour-off cultures and big colonies could be found on the dishes for up to 5 days. This proved that the decreasing colony size over time in the other pour-off cultures was caused by too many colonies on the dish, where they did not have enough space and hindered each other growth.

Osteogenic and adipogenic differentiation assays were conducted to determine the stem cell character of the MSCs. All colonies formed on the pour-off culture dishes stained for alkaline phosphatase, calcium and collagen after being incubated in osteogenic medium. Thus, MSCs with osteogenic differentiation potential were present throughout the whole culture period. The colonies from the pour-off cultures started with 4 x 10^6 BMCs and with 2 x 10^6 BMCs were so numerous that they formed a monolayer of cells, which stained very well for alkaline phosphatase and calcium but seemed to produce very little collagen. The colonies in the pour-off culture started with 1 x 10^6 BMCs did not merge to a monolayer but stayed as single colonies, which stained very well for all three osteogenic markers. Although it was proven that MSCs could differentiate into osteoblasts after one week in culture, no adipocytes were found in the adipogenic differentiation assays, indicating that the MSCs did not have their full differentiation potential under these culture conditions.

The results obtained from this experiment demonstrated no effect of the initial cell density on the differentiation potential of MSCs in pour-off cultures, but showed that a low starting cell density of 2 x 10^6 BMC per culture resulted in an increased cell proliferation. In general, fewer cells need fewer nutrients and have more space to expand in a given volume. Furthermore, the volume of culture medium changed with the pour-off cultures was small (only 15% every 3 – 4 days) which means that only

few new nutrients were added to the culture and thus a low initial cell density was of advantage in order to achieve a high increase in cell number. In addition, a higher cell density in the culture, and consequently an increased cell-cell interaction, could also have reduced the proliferation potential of the MSCs.

Starting the pour-off cultures with not enough cells (1 x 10^6 BMCs) caused a slowdown of the increase in CFU-f numbers towards the end of the culture period. A possible explanation for this slowdown can be the fact that, with every re-plating, cells will be removed from the cultures and by removing more cells than being reproduced, the culture will run out of cells at some point. Hence, it was important to find the right initial cell density for the culture conditions used with the pour-off technique.

7.3.3 MyeloCult® medium with interleukin-3 and stem cell factor

Experiments with MSCs in stirred suspension cultures (chapter 6) have shown that the use of MyeloCult® medium containing the cytokines interleukin-3 (IL-3) and stem cell factor (SCF) increased the cell proliferation and led to higher cell numbers. However, in static monolayer cultures (chapter 4), the increase of MSCs number was not improved significantly when DMEM was replaced by MyeloCult® medium containing IL-3 and SCF. To assess whether MyeloCult® medium with 2 ng/ml IL-3 and 10 ng/ml SCF will have an effect on the expansion potential of MSCs in pour-off cultures, this culture medium was tested in pour-off cultures with two different cell densities, 4 x 10^6 BMCs per culture and 1 x 10^6 BMCs per culture. By the time this experiment was started, the results from the experiments investigating the effect of the initial cell density were not available. Thus the lower cell density was chosen to offer the cells more space and nutrients for expansion, in case the new medium conditions would actually improve the cell proliferation. The higher cell density was used to be able to obtain results even though the new culture conditions would not improve the cell growth.

By replacing DMEM with MyeloCult® medium containing cytokines, an increase in accumulated CFU-f number was achieved (Figure 7.6). This effect was less obvious

in the pour-off culture with the higher cell density of 4 x 10^6 BMCs (1.9 fold increase after 8 days in the DMEM culture versus 2.6 fold increase in the MyeloCult® culture). In the low cell density pour-off cultures with 1 x 10^6 BMCs, the effect was more noticeable (3.3 fold increase of accumulated CFU-f after 8 days in the DMEM culture versus 8.6 fold increase in the MyeloCult® culture).

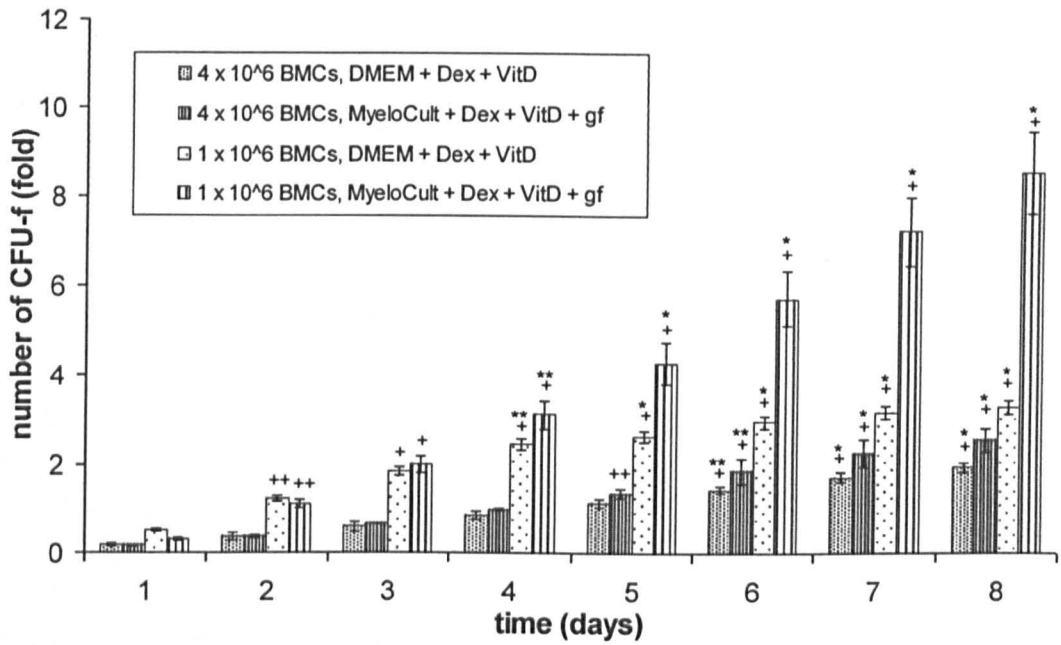

Figure 7.6: Development of accumulative CFU-f number over time in pour-off cultures started with 4 x 10^6 and 1 x 10^6 rat BMCs either in DMEM containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3 or in MyeloCult® medium containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3, 2 ng/ml IL-3 and 10 ng/ml SCF. The cultures were re-plated every 24 hours. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. +$p < 0.01$, ++$p < 0.05$, compared to the numbers of MSCs present in the volume of bone marrow used to start the culture; *$p < 0.01$, **$p < 0.05$; DMEM and MyeloCult® cultures with the same initial cell density compared to each other

The colony numbers per dish also achieved a higher increase in the pour-off cultures with MyeloCult® medium compared to the cultures with DMEM (Figure 7.7). Pour-off cultures in MyeloCult® medium which were started with 4 x 10^6 BMCs had 245 colonies on the dish of day 1 and reached a maximum of 700 colonies per dish at day 5. This equals an increase of 2.9 fold whereas the pour-off cultures in DMEM started

with the same cell density achieved an increase of only 1.7 fold (267 colonies at day 1 and 446 colonies at day 5). The highest increase in colonies per dish (4.8 fold) was achieved with MyeloCult® pour-off cultures having an initial cell number of 1 x 10^6 BMCs (76 colonies at day 1 and 366 colonies at day 6). The maximum fold increase in colonies per dish in DMEM pour-off cultures of the same initial cell density was lower, only 1.4 fold (Figure 7.7). It is also worth noticing that the quantity of colonies formed at day 1 was always higher in the DMEM pour-off cultures than in the MyeloCult® cultures (267 colonies compared to 245 colonies in the high cell density cultures and 126 colonies compared to 76 colonies in the low cell density cultures). However, over time, the colony number per dish increased faster in the MyeloCult® pour-off cultures, indicating that this culture medium supported the proliferation of the non-adherent cells better, which is supported by chapter 6.

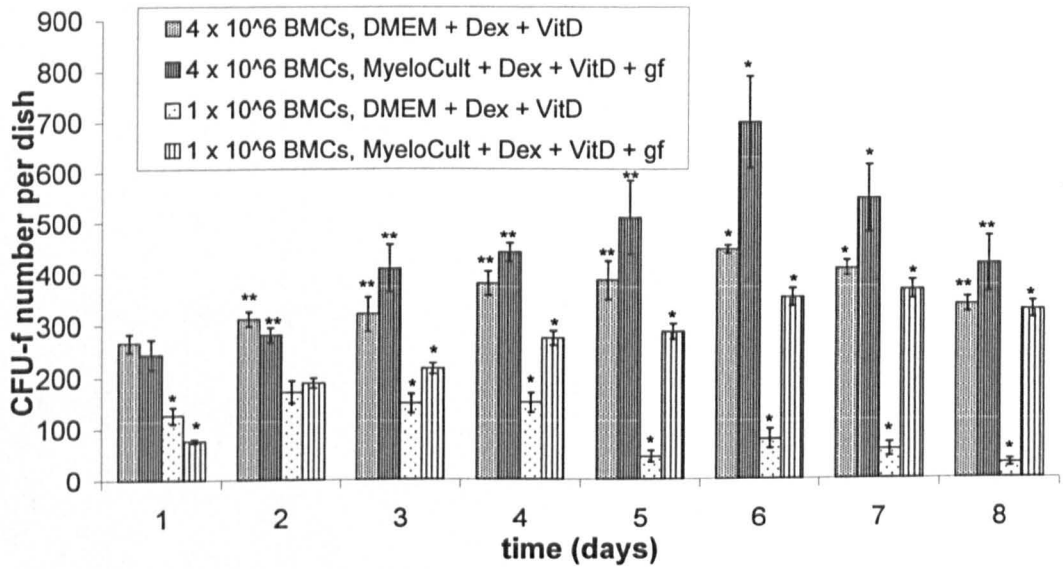

Figure 7.7: Development of CFU-f number per dish in pour-off cultures started with 4 x 106 and 1 x 106 rat BMCs either in DMEM containing 10-8 M dexamethasone and 10-8 M 1α,25-Dihydroxyvitamin D3 or in MyeloCult® medium containing 10-8 M dexamethasone and 10-8 M 1α,25-Dihydroxyvitamin D3, 2 ng/ml IL-3 and 10 ng/ml SCF. The cultures were re-plated every 24 hours. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. *$p < 0.01$, **$p < 0.05$; DMEM and MyeloCult® cultures with the same initial cell density compared to each other

The positive influence of MyeloCult® medium on the proliferation of MCSs in pour-off cultures is recognisable on the number of colonies formed on each culture dish.

The dishes of the MyeloCult® pour-off cultures contained noticeably more colonies than the dishes of the DMEM pour-off cultures with the same initial cell density. The colony forming cells in the high density MyeloCult® pour-off cultures attaching to the plastic surface of the dishes were so numerous that the growing colonies were merging into one monolayer of cells rather than forming individual colonies. Counting single colonies on these plates was very difficult and the values obtained from the image analysis software 'Gene Tools' (Syngene, Cambs.) were not very accurate. For that reason the effect of the MyeloCult® medium containing growth factors on the MSCs expansion was also determined in low cell density pour-off cultures, started with 1 x 10^6 BMCs. The quantity of colony forming cells in these cultures allowed the formation of individual colonies, which could be counted accurately. Furthermore, the colony size in these cultures could be observed. For up to 5 days, the colony size stayed unchanged but with dropping CFU-f number per dish, the colony size, too, declined, indicating a decreasing proliferation potential of the MSCs.

The differentiation capacity of MSCs expanded in MyeloCult® pour-off cultures was determined by culturing the adherent cells in osteogenic and adipogenic differentiation assays. MSCs from all pour-off cultures in MyeloCult® medium were able to differentiate into both osteoblasts and adipocytes. All colonies could be stained for all three osteogenic markers, alkaline phosphatase, calcium and collagen. However, the cultures with low cell density seemed to produce more collagen, noticeable by a better staining of the individual colonies Figure 7.8 illustrate the Oil-Red-O stained adipocytes formed by MSCs, which were re-plated in pour-off cultures for 5 days. During the adipogenic differentiation assay, the adherent MSCs formed a monolayer on the dish (visible in the background of the pictures in Figure 7.8) and only a few cells differentiated into adipocytes.

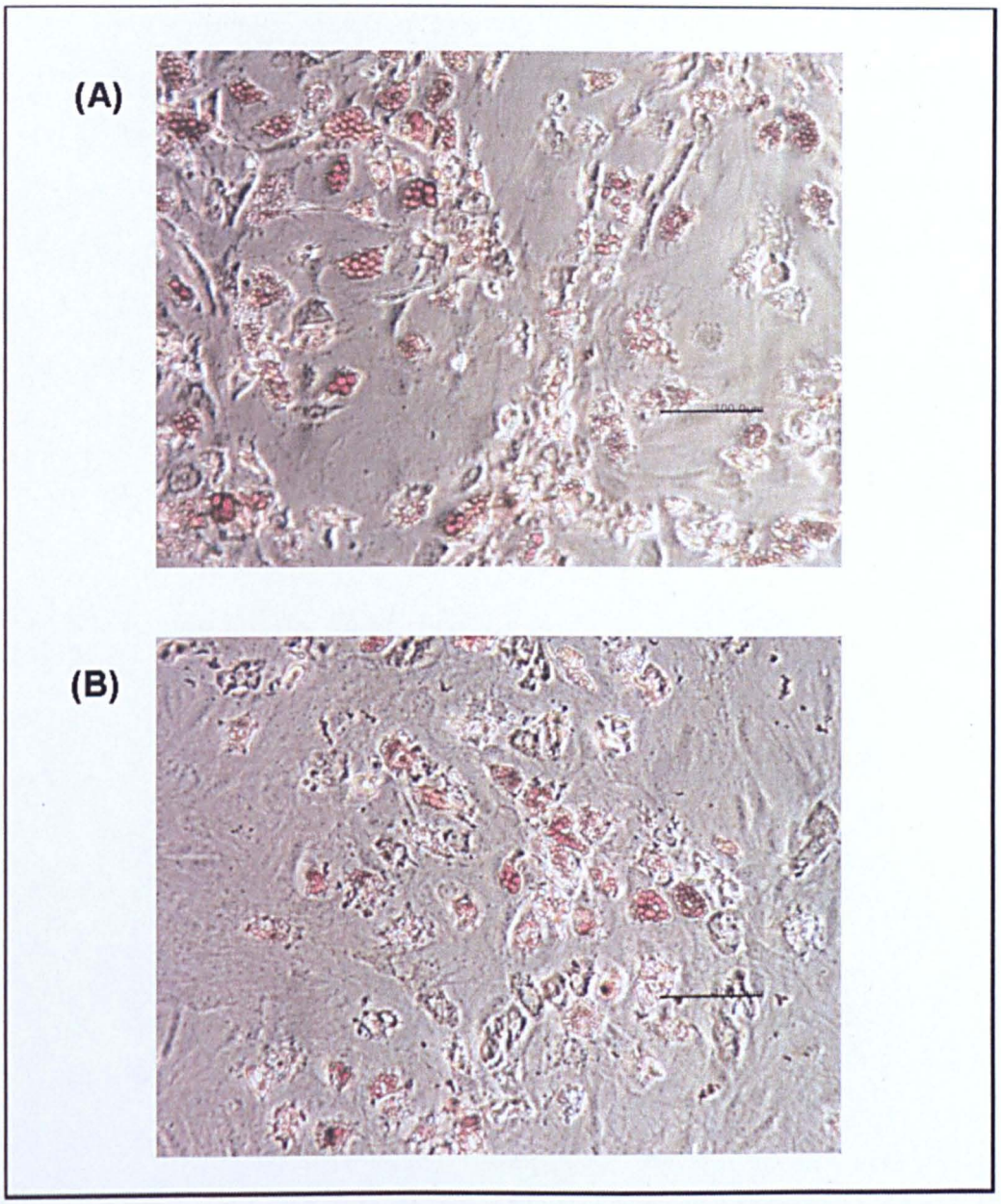

Figure 7.8: Oil Red O stained adipocytes originated from MSCs, which were expanded in MyeloCult® pour-off cultures started with (A) 4 x 10^6 BMCs or (B) 1 x 10^6 BMCs.

In this study, a positive effect of MyeloCult® medium containing IL-3 and SCF on the cell proliferation and therefore also on the colony formation of the MSCs in pour-

off cultures has been shown. The pour-off cultures in MyeloCult® medium started with 1 x 10^6 BMCs achieved the best increase in accumulated CFU-f for all pour-off cultures conducted so far (8.6 fold increase). This is reflected in a high maximum increase of colonies per dish of 4.8 fold in these pour-off cultures. In all MyeloCult® pour-off cultures fewer colonies were present on the dishes of day 1 compared to the control pour-off cultures in DMEM, but the number of colonies increased fast and at day 3, more colonies could be found on the dishes of MyeloCult® pour-off cultures than on the dishes of DMEM pour-off cultures. This indicates that the MyeloCult® medium supported the proliferation of the non-adherent cells in suspension rather than just increased the adherence of these cells to the plastic surface and the colony formation. After an increase in non-adherent cell number, more and more of these cells attached to the surface and formed colonies, hence the delayed increase in CFU-f on the dishes of the MyeloCult® pour-off cultures. This influence of MyeloCult® medium on the proliferation of MSCs in suspension could also explain why MyeloCult® medium had a positive effect on the proliferation of MSCs in stirred suspension cultures (chapter 6), but not on the proliferation of MSCs in static monolayer cultures (chapter 4).

The values for the fold increase of MSCs achieved in the low cell density MyeloCult® pour-off cultures were higher than in both, static monolayer cultures or stirred suspension cultures. This is even more impressive, considering that this result was accomplished in only one week instead of two weeks (static monolayer culture) or three weeks (suspension culture). Furthermore, only MSCs expanded in pour-off cultures had the capacity to differentiate into adipocytes. A lower stress level could be the reason for this increased differentiation potential. Pour-off cultures exert neither chemical stress by using trypsin nor physical stress by using a stirrer system on the MSCs in culture. The main disadvantages of the pour-off cultures are the work intensity and the limited cell culture area of the dishes. Thus, the actual quantity of MSCs harvested from these cultures was lower than the cell number obtained from static monolayer cultures in T-flasks. In order to improve the pour-off culture technique both issues have to be addressed.

7.3.4 Pour-off frequency

One way to reduce the work intensity of the pour-off cultures, and thereby make this culture system more attractive, is to reduce the re-plating frequency. Experiments to determine the effect of a reduced pour-off frequency on the proliferation of MSCs were conducted in MyeloCult® pour-off cultures with 1 x 10^6 BMCs. Instead of re-plating the non-adherent cells every 24 hours, they were transferred into new culture dishes every 2, 4 or 7 days.

Figure 7.9 shows the fold increase of the accumulated CFU-f number after each time the cultures were poured-off. The value "0" for number of pour-off at the x-axes of the graph displays the results from the first dish before the cells were re-plated into a new culture dish. Depending on the pour-off frequency, this value was obtained at day 2, 4 or 7 after starting the pour-off cultures. The best increase in accumulated CFU-f number was achieved by the pour-off cultures being re-plated daily. After 5 pour-offs (6 days), the accumulated CFU-f number increased by 5.7 fold compared to an increase of either 5.2 fold ($p = 0.778$) in the 4 days pour-off cultures after 4 pour-offs (20 days), 3.7 fold ($p = 0.001$) in the 7 days pour-off cultures after 2 pour-offs (21 days) or 2.1 fold ($p = 0.000$) in the 2 days pour-off culture after 3 pour-offs (8 days).

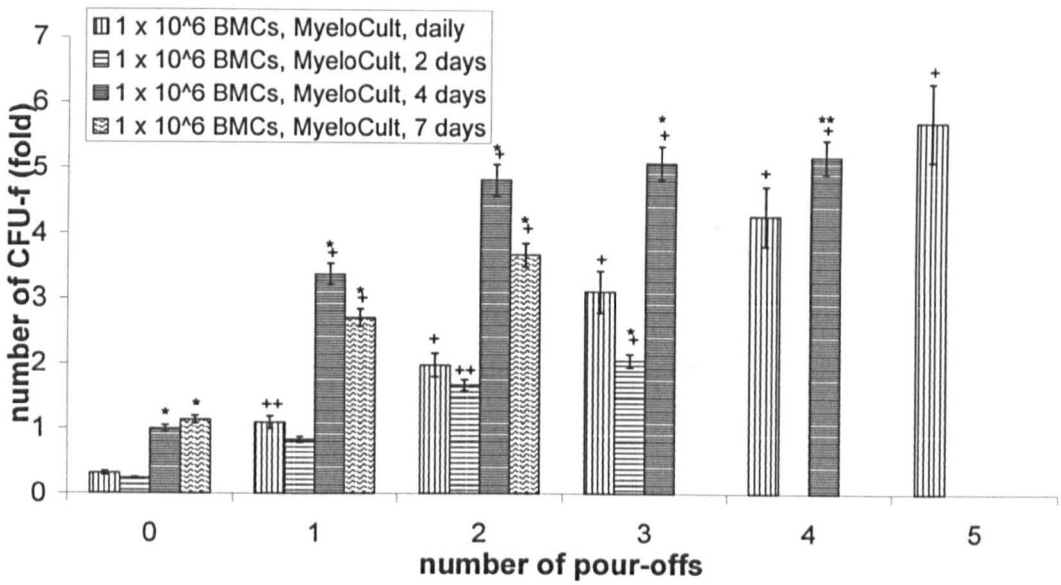

Figure 7.9: Development of accumulative CFU-f number at each time the cells in the pour-off cultures were re-plated. Cultures were started with 1 x 10^6 rat BMCs in MyeloCult® medium containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3, 2 ng/ml IL-3 and 10 ng/ml SCF. The cultures were re-plated either every 24 hours, every 2 days, every 4 days or every 7 days. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. +$p < 0.01$, ++$p < 0.05$, compared to the numbers of MSCs present in the volume of bone marrow used to start the culture; *$p < 0.01$, **$p < 0.05$; any culture compared to the pour-off culture being re-plated daily

The number of colonies per dish after each re-plating is presented in Figure 7.10. Before the first pour-off, 76 colonies were formed in the daily pour-off culture, 206 colonies in the 2 days pour-off culture, 240 colonies in the 4 days pour-off culture and 273 colonies in the 7 days pour-off culture. Due to the different pour-off frequencies, these results were obtained at different time points after the cultures were started. That means, the number of colonies on the first dish would be taken after 1 day for the daily pour-off cultures, after 2 days for the 2 days pour-off cultures and so on. Therefore, it is not surprising that on the first dish of the cultures which were re-plated less often, more colonies were present than on the dish of the cultures which were poured-off more frequently. The colony forming cells in the pour-off cultures with low re-plating frequency had more time to adhere to the surface and form colonies.

An initial increase in colony number per dish for at least 1 pour-off followed by a later decrease in colony number is common for all pour-off strategies (Figure 7.10). For the daily pour-off culture this decrease appeared after 9 pour-offs (results in Appendix D). This indicates that the proliferation potential of the non-adherent colony forming cells was slowing down over time. The maximum increase in colonies per dish observed in this study was achieved by the 2 days pour-off culture after 2 pour-offs (6 days) with an increase from 206 colonies to 713 colonies, that equals a 3.5 fold increase(Figure 7.10).

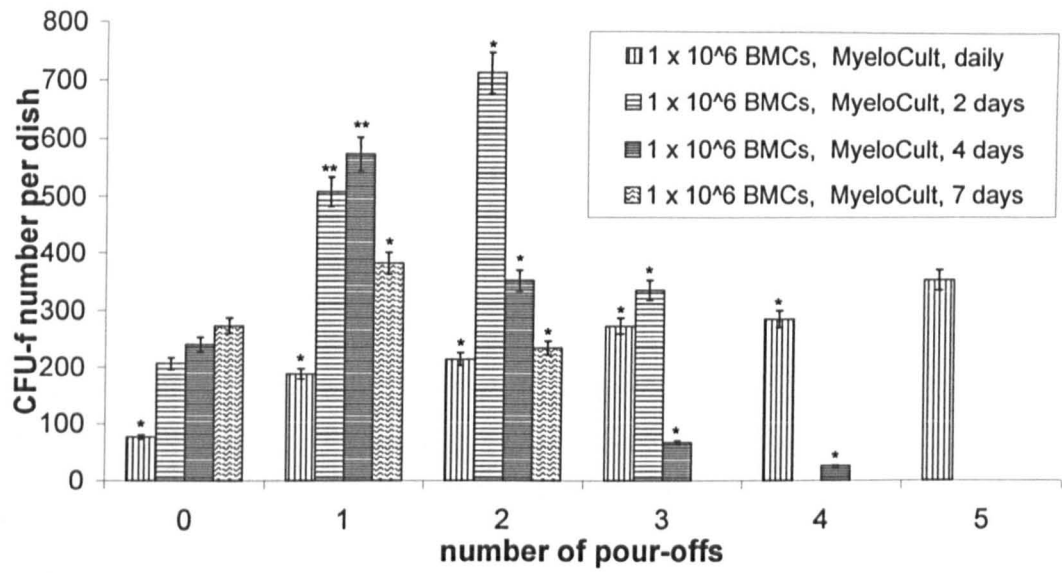

Figure 7.10: Development of the CFU-f number per dish at each time the cells in the pour-off cultures were re-plated. Cultures were started with 1 x 10^6 rat BMCs in MyeloCult® medium containing 10^{-8} M dexamethasone and 10^{-8} M 1α,25-Dihydroxyvitamin D_3, 2 ng/ml IL-3 and 10 ng/ml SCF. The cultures were re-plated either every 24 hours, every 2 days, every 4 days or every 7 days. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures. *$p < 0.01$, **$p < 0.05$; the four different cultures compared to each other

By keeping the BMCs in the same culture dish for more than 24 hours, more of the adherent cells attached to the plastic surface and formed colonies. This resulted in a confluent monolayer of cells in the dishes of the 2 days pour-off culture after 4 days and in the 4 days pour-off culture after 8 days. Counting the colonies on a confluent dish using the image analysis software 'Gene Tools' can result in inaccurate values.

It is likely, that the accurate number of colonies on the confluent dishes was higher than actually determined and that the expansion potential of these pour-off cultures may be higher. In order to determine the full proliferation capacity of cells in these pour-off cultures, bigger culture dishes and an even lower initial cell density have to be used.

After the number of colony forming cells dropped in all pour-off cultures, single colonies could be detected in the 4 days pour-off culture after 16 days. The dishes of the 2 days pour-off culture were covered with a monolayer of cells till the end of the culture period. In the 7 days pour-off culture, single colonies could be detected throughout the whole culture period, although for the first two weeks several colonies merged together.

Adherent cells from the 2 days, the 4 days and the 7 days pour-off cultures were used to run osteogenic and adipogenic differentiation cultures. MSCs from all three pour-off cultures formed colonies, which could be stained positive for alkaline phosphatase, calcium and collagen and thus consisted of osteoblasts. Adipogenic differentiation was only observed for MSCs from the 7 days pour-off culture after 2 weeks in culture (Figure 7.11).

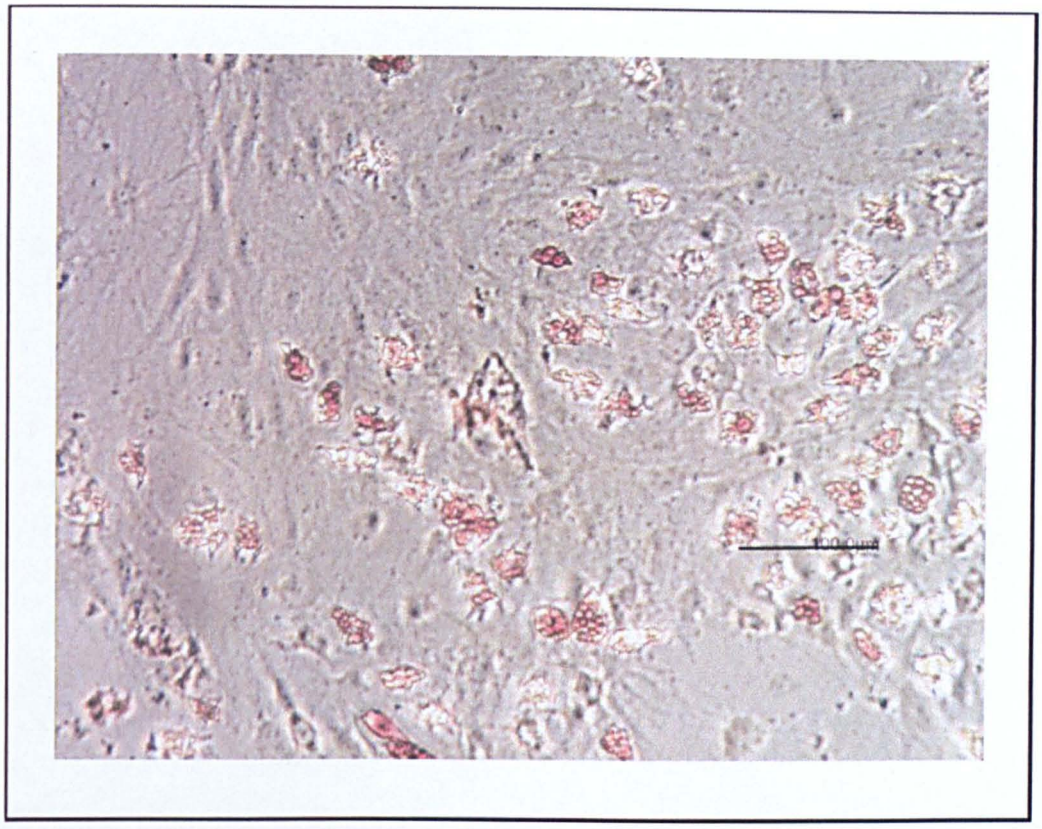

Figure 7.11: Oil Red O stained adipocytes originated from MSCs, which were expanded in MyeloCult® pour-off cultures started 1 x 10^6 BMCs and being re-plated every 7 days.

By comparing the proliferation and differentiation results from this study, the effect of different pour-off frequencies on the expansion capacity of MSCs in pour-off cultures was determined. Increasing accumulated CFU-f numbers were achieved with all pour-off cultures tested in this study. The best expansion was obtained with the daily pour-off culture (8.6 fold increase versus 5.2 fold with the 4 days pour-off culture). It is note worthy that non-adherent cells with colony forming potential were present in all cultures for the first two pour-offs. Regardless of the time these cells spent in the same plastic culture dishes (either 1 day only or up to 14 days), they were still able to form colonies and differentiate into osteoblasts and adipocytes after being transferred into new culture dishes. The high increase in levels of accumulated CFU-f in all pour-off cultures and the increasing colony number per dish after the first pour-offs indicate that the non-adherent MSCs proliferate in suspension.

These findings suggest that there may be a population of cells with colony forming capacity as well as osteogenic and adipogenic differentiation potential present in the non-adherent cell fraction of the bone marrow. These cells have the possibility to adhere to a plastic surface and form colonies or stay in suspension in order to proliferate and adhere at a later time point. Baksh *et al.* (2007) also found, with the same culture system, that the bone marrow must contain MSCs which can be found in the non-adherent cell fraction and which are able to proliferate in suspension while maintaining a single and round morphology. Yet, Baksh *et al.*, (2007) did not observe any colony formation of the adherent cells in pour-off cultures when the suspension cell fraction was removed after 24 hours. Therefore they concluded that the remaining adherent cells either require the present of further non-adherent cell types to initiate their cell cycle or that the adherent cells are a mature cell type which is not able to proliferate and form colonies. However, in the present studies it was shown that the adherent cells grew into colonies although the non-adherent cells were removed every 24 hours in all daily pour-off cultures. Consequently, it seems that the adherent MSCs had their full osteogenic and adipogenic differentiation potential, and did not need the presence of further non-adherent cells to form colonies and differentiate into osteoblasts or adipocytes.

In order to improve the efficiency of the pour-off cultures and establish them as a standard culture system for MSCs expansion, the work intensity has to be reduced and the actual number of MSCs obtained has to be increased. This could be achieved by enlarging the culture volume and the surface area and by automating the re-plating process. The fact that the non-adherent cells in the pour-off cultures did not have to be transferred into new dishes every 24 hours to prevent the complete adherence of all MSCs to the plastic surface will help to automate this culture technique.

7.4 Conclusions

In this chapter, experiments with bone marrow derived rat MSCs in pour-off cultures were conducted to determine the possibility of using this culture system for the *in vitro* expansion of MSCs. The aim was to achieve a large increase in MSCs number with high differentiation capacity over a short period of time. Furthermore, this culture technique should also prove that MSCs can proliferate in suspension as non-adherent cells. Different culture media and pour-off frequencies were tested in order to develop a useful protocol, which allows a possible automation of the whole process.

All experiments conducted with bone marrow cells in pour-off cultures resulted in an increasing accumulated CFU-f number over the whole culture period and in an increasing colony number per dish for a maximum of up to 7 days. Moreover, it has been shown that the non-adherent MSCs fraction is proliferating in suspension, able to adhere to the culture plastic, and has the potential to form colonies and to differentiate into osteoblasts and adipocytes.

In order to obtain an increasing CFU-f number, the addition of 10^{-8} M 1α,25-Dihydroxyvitamin D_3 and 10^{-8} M dexamethasone to the culture medium is required. The addition of dexamethasone alone did not have a significant effect on the proliferation of MSCs.

The type of culture medium was an important factor when expanding MSCs *in vitro*. Pour-off cultures in DMEM generate a maximum increase in CFU-f of 3.8 fold whereas the pour-off cultures in MyeloCult® medium with 2 ng/ml IL-3 and 10 ng/ml SCF achieved an 8.6 fold increase in CFU-f under the same culture conditions. The MyeloCult® medium seemed to support the proliferation of the non-adherent cell fraction in suspension rather than their adherence to the plastic surface. This conclusion is based on the observation that the colony numbers on the culture dishes from day 1 were lower in the MyeloCult® cultures compared to the DMEM cultures. Over time the colony number per dish in the pour-off cultures with MyeloCult® medium increased faster and resulted in higher values.

Table 7.1: Summarised information from the CFU-f -, differentiation assays and pour-off plates conducted in the experiments in this chapter.

Medium	Culture conditions	Time [days]	Colony formation	Osteogenic differentiation	Adipogenic differentiation
DMEM	daily, 10 x 10^6 BMCs	3	medium	-	-
DMEM	daily, 10 x 10^6 BMCs	5	medium	-	-
DMEM	daily, 10 x 10^6 BMCs	7	medium	-	-
DMEM + Dex	daily, 10 x 10^6 BMCs	1	medium	-	-
DMEM + Dex	daily, 10 x 10^6 BMCs	3	medium	-	-
DMEM + Dex	daily, 10 x 10^6 BMCs	5	medium	-	-
DMEM + Dex	daily, 10 x 10^6 BMCs	7	medium	-	-
DMEM + Dex + VitD	daily, 10 x 10^6 BMCs	1	medium	big	-
DMEM + Dex + VitD	daily, 10 x 10^6 BMCs	3	small	big	-
DMEM + Dex + VitD	daily, 10 x 10^6 BMCs	5	medium	medium	-
DMEM + Dex + VitD	daily, 10 x 10^6 BMCs	7	medium	medium	-
DMEM + Dex + VitD	daily, 4 x 10^6 BMCs	1	big	small	-
DMEM + Dex + VitD	daily, 4 x 10^6 BMCs	3	medium	small	-

DMEM + Dex + VitD	daily, 4 x 10^6 BMCs	5	confluent	confluent	-
DMEM + Dex + VitD	daily, 4 x 10^6 BMCs	7	small	confluent	-
DMEM + Dex + VitD	daily, 2 x 10^6 BMCs	1	medium	small	-
DMEM + Dex + VitD	daily, 2 x 10^6 BMCs	3	medium	small	-
DMEM + Dex + VitD	daily, 2 x 10^6 BMCs	5	medium	confluent	-
DMEM + Dex + VitD	daily, 2 x 10^6 BMCs	7	small	confluent	-
DMEM + Dex + VitD	daily, 1 x 10^6 BMCs	1	big	medium	-
DMEM + Dex + VitD	daily, 1 x 10^6 BMCs	3	medium	medium	-
DMEM + Dex + VitD	daily, 1 x 10^6 BMCs	5	big	big	-
DMEM + Dex + VitD	daily, 1 x 10^6 BMCs	7	medium	big	-
MyeloCult +Dex + VitD + gf's	daily, 4 x 10^6 BMCs	1	big	medium	yes
MyeloCult +Dex + VitD + gf's	daily, 4 x 10^6 BMCs	3	medium - confluent	medium	yes
MyeloCult +Dex + VitD + gf's	daily, 4 x 10^6 BMCs	5	confluent	medium	yes
MyeloCult +Dex + VitD + gf's	daily, 4 x 10^6 BMCs	7	confluent	medium	yes
MyeloCult +Dex + VitD +	daily, 1 x 10^6	1	big	big	yes

gf's	BMCs				
MyeloCult +Dex + VitD + gf's	daily, 1 x 10^6 BMCs	3	big	big	yes
MyeloCult +Dex + VitD + gf's	daily, 1 x 10^6 BMCs	5	medium	medium	yes
MyeloCult +Dex + VitD + gf's	daily, 1 x 10^6 BMCs	7	medium – small	medium	yes
MyeloCult +Dex + VitD + gf's	2 days, 1 x 10^6 BMCs	2	big	medium	-
MyeloCult +Dex + VitD + gf's	2 days, 1 x 106 BMCs	4	confluent	big	-
MyeloCult +Dex + VitD + gf's	2 days, 1 x 106 BMCs	6	confluent	big	-
MyeloCult +Dex + VitD + gf's	2 days, 1 x 106 BMCs	8	confluent	big	-
MyeloCult +Dex + VitD + gf's	4 days, 1 x 106 BMCs	4	very big	confluent	-
MyeloCult +Dex + VitD + gf's	4 days, 1 x 106 BMCs	8	confluent	confluent	-
MyeloCult +Dex + VitD + gf's	4 days, 1 x 106 BMCs	12	small	confluent	-
MyeloCult +Dex + VitD + gf's	4 days, 1 x 106 BMCs	16	medium – big	confluent	-
MyeloCult +Dex + VitD + gf's	7 days, 1 x 106 BMCs	7	big – confluent	big	-
MyeloCult +Dex + VitD + gf's	7 days, 1 x 106 BMCs	14	medium	medium	yes
MyeloCult +Dex + VitD + gf's	7 days, 1 x 106 BMCs	21	medium	medium	yes

The cell density appeared to have an influence on the proliferation of MSCs in pour-off cultures. A lower initial cell density led to a higher increase of CFU-f and therefore improved the efficiency of the cultures, whereas a high initial cell density seemed to reduce the proliferation capacity of the non-adherent cells. Furthermore, a smaller number of cells attached to the surface in the low density pour-off cultures, allowing the formation of large colonies without merging into a monolayer of cells.

In order to reduce the inconvenience caused by re-plating the cell every 24 hours, the pour-off frequency was changed. Non-adherent cells were transferred into new culture dishes either every 2 days, 4 days or 7 days. This resulted in a lower fold increase of CFU-fs but it could be shown, that, even after 7 days in the same culture dish, some bone marrow derived cells stayed in suspension and were able to form colonies after being re-plated. This is reflected in the colony numbers per dish, which were increasing in all pour-off cultures for at least two pour-offs.

MSCs expanded in pour-off cultures differentiated into osteoblasts and adipocytes, indicating that they had both osteogenic and adipogenic differentiation capacity. MSCs cultured in static monolayer cultures and in stirred suspension cultures had only osteogenic differentiation potential and did not form adipocytes. Cells in pour-off cultures do not have to be agitated to assure a sufficient supply with nutrients, and they do not have to be treated with trypsin to transfer them into the new cultures dishes, thus less chemical and physical stress is exerted on the MSCs, allowing them to keep a better differentiation and proliferation potential. Table 7.2 compares the best results for MSCs expansion in static monolayer cultures, stirred suspension cultures and pour-off cultures.

Pour-off cultures are very work-, consumable- and time- intensive and require plenty of incubator space. All these disadvantages increase the costs and need to be improved upon before pour-off cultures can be used as an alternative culture system to expand MSCs in a large scale. A cost comparison is given in Table 7.3, however, these numbers are only based on medium consumption and do not take into account the time and other consumables used. The fact that the non-adherent MSCs can stay and proliferate in suspension for up to 7 days, although being exposed to tissue

culture plastic, suggests that systems like perfusion cultures or roller bottles may to be a possible candidate for automating pour-off cultures.

Table 7.2: Summary of the results for the maximum fold increase in CFU-f achieved in static cultures, suspension cultures and pour-off cultures with different culture media. For the suspension cultures, all the media types mentioned include 0.05% pluronic, whereas the medium of all pour-off cultures included dexamethasone and 1α,25-Dihydroxyvitamin D_3.

Media type	Static cultures	Suspension cultures	Pour-off cultures
	CFU-f (fold increase)	CFU-f (fold increase)	CFU-f (fold increase)
DMEM	460	30	-
DMEM + Dex	460	40	380
MyeloCult®	460	53	-
MyeloCult® + Dex	440	54	-
MyeloCult® + IL-3 + SCF	470	140	860

Table 7.3: Comparison of the costs for the achieved fold increase in CFU-f in static cultures, suspension cultures and pour-off cultures with different culture media. For the suspension cultures, all the media types mentioned include 0.05% pluronic, whereas the medium of all pour-off cultures included dexamethasone and 1α,25-Dihydroxyvitamin D_3.

Media type	Static cultures	Suspension cultures	Pour-off cultures
	Costs (£) / fold increase	Costs (£) / fold increase	Costs (£) / fold increase
DMEM	1.3	0.06	-
DMEM + Dex	1.3	0.05	0.02
MyeloCult®	3.0	0.09	-
MyeloCult® + Dex	3.2	0.09	-
MyeloCult® + IL-3 + SCF	3.3	0.13	0.01

Chapter 8

Conclusions and Future Work

In this thesis, culture techniques have been developed to help amplify rat bone marrow derived mesenchymal stem cells (MSCs) using various culture systems. Two different suspension based systems, stirred suspension cultures and pour-off cultures, have been employed to achieve a large scale expansion and different culture conditions were tested for each of the systems in order to optimise the proliferation and differentiation capacity of MSCs. The results obtained from bone marrow MSC expansion in static monolayer cultures were used as a control, against which the findings from the suspension cultures and the pour-off cultures were compared. The static culture system was chosen as a control because it is the traditional and most commonly used method to amplify MSCs *in vitro*. In this chapter, the results from each study are summarised and the findings from the comparison of these are presented. Finally a discussion on future directions in suspension cultures of bone marrow derived MSCs is given.

8.1 Culture systems

The culture systems applied in this thesis to amplify MSC numbers were static monolayer cultures (Chapter 4), stirred suspension cultures (Chapter 6) and pour-off cultures (Chapter 7).

8.1.1 Static monolayer cultures

The results from the static monolayer cultures in chapter 4 were taken as a control against which the findings from the suspension and the pour-off cultures were compared. Furthermore, the static monolayer cultures were used to study how changes of the culture medium would affect the proliferation and differentiation potential of MSCs in a well defined culture system. The effect of pluronic F-68 on the membrane of MSCs, for example, was investigated by Fourier Transform Infrared Spectrometry (FTIR), and by measuring the electrophoretic mobility / net surface charge of MSCs with the zetaPALS from Brookhaven.

Regarding the cell proliferation and the colony forming capacity, the best results in static monolayer cultures were achieved in MyeloCult® medium supplemented with interleukin-3 (IL-3) and stem cell factor (SCF). The final increase in cell number was 5.3 x 10^5 fold after 29 days, and the highest increase in colony numbers (CFU-f) was 4.7 fold after 14 days. The huge discrepancy between increase in total cell number and in CFU-f could be observed in all static monolayer cultures, regardless of the medium conditions. This might be an indication that a MSCs monolayer culture comprising of different progenitor cell types, which have different proliferation and differentiation capacities and that the tested culture conditions could have supported the expansion of early osteogenic precursor cells with limited differentiation and colony forming capacity (Sekiya *et al.*, 2002). This theory is supported by the fact, that all MSCs taken from any static culture at any time point only differentiated into osteoblasts, and did not form adipocytes when cultured in the appropriate differentiation assays.

In addition to these studies, the effect of pluronic F-68 on the MSC membrane was determined using FTIR, and net surface charge measurements. However, with these techniques no pluronic induced difference in the cell membrane could be detected. Furthermore, the implications of serial subculturing on the proteome of bone marrow derived rat MSCs was investigated (chapter 5). During the culture period of 29 days, a small decrease in the proliferation speed and a more obvious decline in the colony forming potential and in the colony size was observed. The proteome of MSCs from passage 2 was compared to that of MSCs from passage 4 using an isobaric tag methology for relative and absolute quantitation (iTRAQ). A total of 105 differentially regulated proteins were identified, of which 33 proteins could be related to each other in different regulator networks as calculated by the MetaCore™ software. 18 of these 33 proteins were described in MSCs for the first time. Most interestingly, the proteins related with cell proliferation such as tumour protein, translationally-controlled 1 (TPT1), prothymosin alpha (PTMA) and dynein light chain1 (DLC1) were down-regulated, whereas proteins identified as regulation factors between proliferation and differentiation such as proteins Y box protein 1 (YB1), heterogenous nuclear ribonucleoprotein A1 (hnRPA1) and cysteine and glycine rich protein2 (CRP2) were up-regulated. These observations suggested that serial subculturing may stimulate the differentiation of MSCs into early progenitor

cells, and thus reduce their proliferation and colony forming capacity. This theory thereby further supports the earlier mentioned idea, that the tested culture conditions could have supported the expansion of early osteogenic precursor cells with limited differentiation and colony forming capacity. This idea was based on the observed discrepancy between increase in total cell number and decrease in colony number.

8.1.2 Stirred suspension cultures

Since it was shown that the static culture system had only a limited productivity for a given surface area, and seemed to interfere with the proliferation and differentiation capacity, as indicated by the results from chapter 4 and 5, stirred suspension cultures were used to achieve a large scale expansion of MSCs (chapter 6). The main problem to overcome in suspension cultures was to reduce the shear forces caused by agitating the culture. The agitation was necessary for the equal distribution of nutrients throughout the culture medium. Furthermore, it helped to keep the MSCs in suspension by stopping them from sinking to the bottom of the culture vessel. However, MSCs are known to be sensitive to physical stimuli including shear stress, which can stimulate uncontrolled differentiation or even damage the cells. In order to improve the survival of undifferentiated MSCs, the protocol for running suspension cultures was changed several times, i.e. using different agitation mechanisms and different culture vessels as well as optimising the culture medium.

The best results for proliferation and colony formation were observed in 40 ml suspension cultures with MyeloCult® medium, containing pluronic F-68, dexamethasone, IL-3 and SCF, which were stirred by a magnetic bar in a 250ml glass bottle. Applying this culture method a maximum increase in MSCs / CFU-f of 1.4 fold after 7 days was obtained. After 3 weeks in stirred suspension, both the total cell number and the CFU-f number decreased dramatically. Hence, the stirred suspension culture is not a useful tool for long-term large scale expansion of MSCs, although the suspension based proliferation of these cells could be proven. Taking into account that the slight increase in cell number was only achieved after adding expensive growth factors to the culture medium, this makes this culture system even less useful for the large scale production of MSCs for medical applications. Furthermore, the surviving MSCs did not have their full differentiation potential. As

with the static monolayer cultures, the harvested MSCs from the stirred suspension cultures only differentiated into osteoblasts, but did not form any adipocytes when cultured in differentiation assays. The lack of the full differentiation potential can be an indication that the stirred suspension cultures, too, cause the MSCs to differentiate into early progenitor cells. The most likely factor responsible for that would be the shear stress caused by the agitation.

8.1.3 Pour-off cultures

The pour-off cultures described in chapter 7 were a combination between static cultures and suspension cultures. They offer some of the cells the possibility to adhere to a surface whilst others stay in suspension. However, only the non-adherent cells were re-plated and therefore kept in culture. Thus, any increase in MSC number / CFU-f must derive from the non-adherent fraction. For that reason, the results from the pour-off cultures can also be seen as evidence for suspension based proliferation of MSCs. In order to establish the best working protocol for this culture system, the culture medium, the initial cell density and the pour-off frequency was optimised.

An increase in CFU-f / MSCs was observed in all pour-off cultures. The highest increase of 8.6 fold after only 8 days was achieved in low density pour-off cultures with 1 x 10^6 rat bone marrow cells per dish, which were re-plated every 24 hours. The culture medium was MyeloCult® containing 1α,25-dihydroxyvitamin D_3, dexamethasone, IL-3 and SCF. Besides obtaining a high increase in cell number, the MSCs harvested from pour-off cultures were also able to form both, osteoblasts and adipocytes, an indication that this culture system exerts low levels of physical or chemical stress, and therefore does not trigger uncontrolled differentiation. The down side of pour-off cultures is the enormous amount of time and work which is required to keep them running. A much needed automation might be possible but would require further adaptations to the protocol.

8.1.4 Comparison of the different culture systems

As mentioned earlier, static monolayer cultures were used as a control in order to compare the findings from the suspension cultures and the pour-off cultures to the results from the standard method for expanding MSCs *in vitro*. The highest MSC numbers obtained from the static monolayer cultures were about 460% of the initial cells. The differentiation capacity of these cells was restricted, so they only formed osteoblasts. In stirred suspension cultures, the increase in MSC number varied for the different culture media. The best result was achieved in MyeloCult® medium with Pluronic F-68, IL-3 and SCF, however, reaching only 140% of the initial cell density, this result was significantly worse than in the control monolayer cultures. Taking into account that the differentiation capacity of MSCs derived from stirred suspension cultures was as good as the differentiation capacity of the control cells in the static cultures, the stirred suspension cultures failed to provide a culture system for large scale expansion of bone marrow derived MSCs. The pour-off cultures supported an increase in MSC number, which resulted in 860% of the initial cell number. In addition, MSCs expanded in this culture system were able to differentiate into ostoblasts and adipocytes. Thus, pour-off cultures worked significantly better than the control and better than the stirred suspension cultures and so far are the most promising culture system for large scale expansion of MSCs with high differentiation potential. A summary of all the proliferation related results described can be found in chapter 7, Table 7.2.

8.2 Culture conditions

Throughout the thesis, the culture conditions for static cultures, stirred suspension cultures and pour-off cultures were changed in order to achieve the best possible increase in cell number. These optimisations mainly focused on the culture medium but, depending on the culture system, also involved changing in the agitation method or the initial cell number. Two different basic culture media were used, Dulbecco's Modified Eagle Medium (DMEM) and MyeloCult® medium (for medium definition see Chapter 3). To both media types various medium additives were added, i.e.

dexamethasone, Pluronic F-68 and a combination of the growth factors IL-3 and SCF.

8.2.1 DMEM vs. MyeloCult® medium

DMEM is commonly used for growing MSCs in static culture systems. It is cheaper than the MyeloCult® medium, which was first mentioned in connection with MSCs by Baksh *et al.* (2003). In static monolayer cultures, the choice of culture medium had no influence on the proliferation and differentiation capacity of MSCs (chapter 4), whereas in stirred suspension cultures the usage of MyeloCult® medium resulted in slightly higher MSC numbers (chapter 6).

8.2.2 Dexamethasone

Dexamethasone is mostly related with enhanced differentiation potential rather than improved proliferation. Nonetheless, Baksh *et al.* (2003) used dexamethasone in their suspension culture studies and reported a successful expansion of bone marrow derived MSCs. The beneficial effect of dexamethasone on the proliferation could not be proven in static cultures (chapter 4) or in suspension cultures (chapter 6) but in the pour-off cultures, a mixture of dexamethasone with 1α,25-dihydroxyvitamin D_3 was essential for the cell growth (chapter 7). All MSCs from cultures containing dexamethasone, however, showed improved osteogenic differentiation potential.

8.2.3 Interleukin-3 and Stem Cell Factor

Baksh *et al.* (2003) found that both cytokines, IL-3 and SCF, were necessary for the expansion of MSCs in stirred suspension cultures. This was confirmed by the suspension culture and pour-off culture studies conducted in this thesis. When a mix of IL-3 and SCF was added to the culture medium of these cultures, a significant increase in MSC number was observed (chapter 6 and 7). In contrast, no positive effect was noticeable in static monolayer cultures (chapter 4). The downside to an IL-3 and SCF mediated increase in proliferation are the extra costs caused by these

cytokines. Large scale up would require vast quantities of growth factors, which increases the production costs of MSCs and thus makes them less attractive for medical applications.

8.2.4 Pluronic F-68

Pluronic F-68 is known to bind to the cell membrane, and thereby strengthen it (Zhang, Z. *et al.*, 1992). Hence, pluronic can be used in agitated culture systems to protect sensitive cells from shear stress. In static monolayer cultures, it could be shown, that the addition of pluronic to the culture medium slowed down the proliferation of MSCs. However, no evidence of altered functional groups in the cells' membrane were seen using FITR and zetaPALS analysis (chapter 4). In suspension cultures, pluronic F-68 was necessary to achieve increasing cell numbers (chapter 6). The different effects of pluronic F-68 on the proliferation potential of MSCs in static and suspension cultures indicated that the protective benefits of pluronic outbalanced the negative side effects only in the more stressful culture conditions.

8.2.5 Further culture parameters

Further culture parameters that have been studied in this thesis and that can influence the proliferation and differentiation capacity of MSCs *in vitro* are the initial cell density, the culture vessel and the medium change frequency. It was found, that in suspension cultures, the best working cell density was 3×10^5 bone marrow cells per ml, whereas in pour-off cultures the optimal cell density was 0.5×10^5 bone marrow cells per ml. In general it can be said that the culture conditions have to be optimised for each culture system accordingly, aiming to provide the most supportive environment, which should be most similar to the *in vivo* niche of the cells.

8.3 Future work

It has been shown that MSCs are able to proliferate in suspension. Nevertheless, stirred suspension bioreactors are not a suitable culture system for large scale expansion of MSCs due to the shear forces caused by agitating the medium. It seems that in such an environment, the addition of growth factors is required to amplify the cells. Future studies on MSC expansion in suspension cultures have to be conducted with a focus on automation and scale-up. These studies should aim to develop a culture system with low physical and chemical stress levels for the cells, guaranteeing MSCs with high proliferation and differentiation capacities. Ideally, the cells should be able to support each other by secreting cytokines, superseding the necessity of adding any additional growth factors. This could reduce the production costs for MSCs.

In this thesis, pour-off cultures were presented as a culture system, which allowed a suspension-based expansion of bone marrow derived MSCs with a high differentiation potential. However, this culture system lacked automation and therefore was very work intensive. Furthermore, experiments with pour-off cultures were conducted in small 20 ml cultures. In order to get large quantities of MSCs for medical applications, future studies would need to find a way to scale-up and automate pour-off cultures. One possibility of how an automated pour-off culture could look like would be a perfusion system, consisting of a culture vessel filled with medium in which the bone marrow cells are seeded. During the expansion period, the medium would be continuously, but very slowly, replaced with fresh medium, so no shear forces are exerted on the cells. This culture system would allow some cells to stay in suspension while others could adhere to the vessel surface and support cell proliferation by secreting cytokines. A scale-up of such a culture system would easily be possible. The main focus when developing this culture system should be on optimising the flow rate and the culture medium, which should be free of additional growth factors.

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Appendix A

Complete list of all identified proteins from the proteomic analysis of mesenchymal stem cells described in chapter 5.

Accession no.	Protein identity	%Cov
gi|157786926	actin related protein 2/3 complex, subunit 3 [Rattus norvegicus]	14.044943
gi|6981326	S100 calcium-binding protein A4 [Rattus norvegicus]	55.445546
gi|54036318	Protein S100-A11 (S100 calcium-binding protein A11) (Calgizzarin)	37.755102
gi|77404363	nucleosome assembly protein 1-like 1 [Rattus norvegicus]	12.276215
gi|109509465	PREDICTED: similar to pyrophosphatase [Rattus norvegicus]	20.049505
gi|81884568	Macrophage-capping protein (Actin regulatory protein CAP-G)	15.472779
gi|62666588	PREDICTED: similar to RNA binding motif protein 3 [Rattus norvegicus]	31.410256
gi|6831527	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	25.964913
gi|54400716	proteasome 26S non-ATPase subunit 12 [Rattus norvegicus]	14.254385
gi|81889864	Tubulin alpha-4A chain (Tubulin alpha-4 chain) (Alpha-tubulin 4)	70.089287
gi|149018217	rCG25732, isoform CRA_d [Rattus norvegicus]	15.486726
gi|56388799	Ckb protein [Rattus norvegicus]	39.460784
gi|57127	unnamed protein product [Rattus norvegicus]	63.636363
gi|70912366	ARP3 actin-related protein 3 homolog [Rattus norvegicus]	16.267942
gi|109502513	PREDICTED: similar to Nuclear transport factor 2 (NTF-2)	21.897811
gi|149040541	eukaryotic translation initiation factor 3, subunit 10 (theta)	24.815361

gi|8393610	karyopherin (importin) beta 1 [Rattus norvegicus]	23.085715
gi|56605666	proteasome (prosome, macropain) 26S subunit, non-ATPase, 3	13.773584
gi|76677911	similar to RIKEN cDNA 2810409H07 [Rattus norvegicus]	13.636364
gi|76779404	Tubulin, beta 2b [Rattus norvegicus]	47.865167
gi|77417616	Ubiquitin-conjugating enzyme E2 N (Ubiquitin-protein ligase N)	29.605263
gi|47605935	Transforming protein RhoA precursor	48.186529
gi|54607147	ras-related C3 botulinum toxin substrate 1	30.208334
gi|396431	rab GDI alpha [Rattus norvegicus]	44.071588
gi|3914232	Transmembrane emp24 domain-containing protein 2 precursor	30.348259
gi|37805239	Enolase 2, gamma [Rattus norvegicus]	28.1106
gi|55977470	Tubulin alpha-1A chain (Tubulin alpha-1 chain) (Alpha-tubulin 1)	74.057651
gi|8515734	trax [Rattus norvegicus]	9.6551724
gi|8394009	peptidylprolyl isomerase A [Rattus norvegicus]	82.317072
gi|157822753	phosphoglucomutase 2 [Rattus norvegicus]	7.5313807
gi|46485440	glucose phosphate isomerase [Rattus norvegicus]	39.605734
gi|74355722	Capping protein (actin filament) muscle Z-line, alpha 2	15.384616
gi|399660	aldehyde reductase [Rattus norvegicus]	36.307693
gi|109495090	PREDICTED: similar to High mobility group protein 1 (HMG-1)	40.927419
gi|157822919	alpha glucosidase 2 alpha neutral subunit [Rattus norvegicus]	20.953576
gi|157819651	chaperonin subunit 7 (eta) [Rattus norvegicus]	21.691176
gi|7242160	nucleophosmin 1 [Rattus norvegicus]	35.958904
gi|149018891	rCG25923, isoform CRA_a [Rattus norvegicus]	49.736148
gi|49065780	26S protease regulatory subunit 4 (Proteasome 26S subunit ATPase 1)	28.863636
gi|59709467	CAP, adenylate cyclase-associated protein 1 [Rattus norvegicus]	31.223628
gi|33086552	Ab2-076 [Rattus norvegicus]	14.330219

gi|55562869	Tumor protein, translationally-controlled 1 [Rattus norvegicus]	31.395349
gi|81910041	WD repeat-containing protein 1	32.178217
gi|81890517	Heat shock protein 105 kDa (Heat shock 110 kDa protein)	8.5081585
gi|81885333	Seryl-tRNA synthetase, cytoplasmic (Seryl-tRNA(Ser/Sec) synthetase)	8.203125
gi|81884348	Cytosol aminopeptidase (Leucine aminopeptidase) (LAP)	21.387284
gi|8392971	branched chain aminotransferase 1, cytosolic [Rattus norvegicus]	23.600973
gi|407164	heat shock protein 70 [Rattus norvegicus]	58.50234
gi|48675845	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP	20.777027
gi|81886881	Heat shock 70 kDa protein 4 (Ischemia responsive 94 kDa protein)	18.452381
gi|109512218	PREDICTED: similar to SH3 domain-binding glutamic acid-rich-like protein	22.162162
gi|149034971	rCG42612, isoform CRA_b [Rattus norvegicus]	21.501014
gi|40254781	GDP dissociation inhibitor 2 [Rattus norvegicus]	53.932583
gi|6978505	annexin A5 [Rattus norvegicus]	60.50157
gi|28933457	glutathione S-transferase, mu 2 [Rattus norvegicus]	24.770643
gi|149039602	rCG57402, isoform CRA_a [Rattus norvegicus]	43.333334
gi|91771192	Asparagine synthetase (Glutamine-dependent asparagine synthetase)	21.033868
gi|81910365	Acidic leucine-rich nuclear phosphoprotein 32 family member E	24.031007
gi|14549433	calcium binding protein NEFA [Rattus norvegicus]	17.857143
gi|149024253	chloride intracellular channel 4, isoform CRA_b [Rattus norvegicus]	56.126481
gi|71051777	Heat shock protein 8 [Rattus norvegicus]	68.421054
gi|37654308	LRRGT00046 [Rattus norvegicus]	14.166667
gi|6978501	annexin A1 [Rattus norvegicus]	70.520234
gi|149029682	rCG42490, isoform CRA_f [Rattus norvegicus]	79.470199
gi|149049470	rCG29914, isoform CRA_b [Rattus norvegicus]	50
gi|32527715	Ac2-048 [Rattus norvegicus]	19.933555

gi|149033381	rCG52521, isoform CRA_a [Rattus norvegicus]	43.40176
gi|56605938	thioredoxin domain containing 4 (endoplasmic reticulum)	30.295566
gi|81885359	Lactoylglutathione lyase (Methylglyoxalase) (Aldoketomutase)	14.673913
gi|149045167	rCG43947 [Rattus norvegicus]	42.925659
gi|38382858	Protein disulfide isomerase associated 3 [Rattus norvegicus]	59.009904
gi|10720174	Hypoxia up-regulated protein1 precursor (150 kDa oxygen-regulated protein)	15.615615
gi|67078526	UDP-glucose pyrophosphorylase 2 [Rattus norvegicus]	11.023622
gi|54673763	Heat shock protein 1, alpha [Rattus norvegicus]	43.929058
gi|149061561	ribonuclease/angiogenin inhibitor 1, isoform CRA_b [Rattus norvegicus]	19.715448
gi|52783577	Dynein light chain 1, cytoplasmic (Dynein light chain LC8-type 1)	32.584271
gi|157786744	dihydropyrimidinase-like 2 [Rattus norvegicus]	27.797204
gi|56754676	Tubulin beta-5 chain	60.585588
gi|81883543	Astrocytic phosphoprotein PEA-15	22.307692
gi|62648070	PREDICTED: similar to Aldose reductase (AR) (Aldehyde reductase)	13.92405
gi|51948402	peptidylprolyl isomerase C [Rattus norvegicus]	47.64151
gi|81885266	Dihydrolipoyl dehydrogenase, mitochondrial precursor	10.805501
gi|56336	unnamed protein product [Rattus norvegicus]	52.380955
gi|58865656	lymphocyte cytosolic protein 1 [Rattus norvegicus]	23.604466
gi|38181546	Macrophage migration inhibitory factor [Rattus norvegicus]	53.043479
gi|62641808	PREDICTED: similar to FK506-binding protein 2 precursor	52.105266
gi|38303969	Heat shock 70kDa protein 5 (glucose-regulated protein) [Rattus norvegicus]	61.009175
gi|8393296	eukaryotic translation elongation factor 2 [Rattus norvegicus]	62.820512
gi|6981324	prolyl 4-hydroxylase, beta polypeptide [Rattus norvegicus]	76.574802
gi|3288594	26S proteasome subunit p112 [Rattus norvegicus]	13.116474
gi|109481425	PREDICTED: similar to MIR-interacting saposin-like protein precursor	43.606558

gi|62665288	PREDICTED: similar to alanyl-tRNA synthetase [Rattus norvegicus]	22.004132
gi|7209586	DA41 [Rattus norvegicus]	21.993127
gi|8394328	superoxide dismutase 1 [Rattus norvegicus]	61.68831
gi|48428789	ADP-ribosylation factor 4	27.222222
gi|55855	precursor (AA -17 to 399) [Rattus norvegicus]	77.644229
gi|8394082	proteasome (prosome, macropain) subunit, beta type 3 [Rattus norvegicus]	8.7804876
gi|62899645	Actin-related protein 2 (Actin-like protein 2)	46.192893
gi|149027310	threonyl-tRNA synthetase, isoform CRA_a [Rattus norvegicus]	21.745153
gi|32363196	Moesin (Membrane-organizing extension spike protein)	53.032929
gi|109504084	PREDICTED: similar to palladin [Rattus norvegicus]	23.899999
gi|109481608	PREDICTED: similar to Calponin-2 (Calponin H2, smooth muscle)	24.626866
gi|34849734	Thioredoxin 1 [Rattus norvegicus]	55.238098
gi|158186672	neural precursor cell expressed, developmentally down-regulated gene 4	25.309336
gi|73532768	coatomer protein complex, subunit gamma [Rattus norvegicus]	26.773456
gi|62078893	ubiquitin-activating enzyme E1, Chr X [Rattus norvegicus]	12.759924
gi|56789700	Peroxiredoxin 1 [Rattus norvegicus]	53.266335
gi|149029720	rCG42522, isoform CRA_a [Rattus norvegicus]	21.875
gi|68085421	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	72.653061
gi|54037164	Guanine nucleotide-binding protein subunit beta-2-like 1	35.646689
gi|21666559	TUC-4b [Rattus norvegicus]	36.749634
gi|4753894	calcyclin [Rattus norvegicus]	83.146065
gi|149025903	calcium/calmodulin-dependent protein kinase II, delta, isoform CRA_c	23.849373
gi|57381	T-plastin [Rattus norvegicus]	32.057416
gi|62646949	PREDICTED: similar to Filamin-C (Gamma-filamin) (Filamin-2)	33.712399
gi|78214352	cysteine and glycine-rich protein 2 [Rattus norvegicus]	17.098446

gi|158081781	hypothetical protein LOC362115 [Rattus norvegicus]	13.119143
gi|8394432	peroxiredoxin 2 [Rattus norvegicus]	33.333334
gi|61556967	eukaryotic translation elongation factor 1 delta [Rattus norvegicus]	28
gi|55583766	Protein phosphatase 1 regulatory subunit 14B	36.05442
gi|81907772	PDZ and LIM domain protein 7 (LIM mineralization protein) (LMP)	25.164112
gi|71051349	Eukaryotic translation elongation factor 1 gamma [Rattus norvegicus]	40.503433
gi|67846074	EH-domain containing 2 [Rattus norvegicus]	17.495395
gi|37194821	Ribosomal protein S27a [Rattus norvegicus]	53.205127
gi|158706420	Protein ARMET precursor (MANF) (Arginine-rich protein)	21.787709
gi|57087	unnamed protein product [Rattus norvegicus]	56.395346
gi|51261175	Peroxiredoxin 5 [Rattus norvegicus]	47.41784
gi|45478134	LRRGT00147 [Rattus norvegicus]	11.157456
gi|55926145	expressed in non-metastatic cells 2 [Rattus norvegicus]	90.789473
gi|50927605	Suppression of tumorigenicity 13 [Rattus norvegicus]	17.663044
gi|8393381	glucose-6-phosphate dehydrogenase [Rattus norvegicus]	29.902911
gi|149066032	myosin, heavy polypeptide 9, non-muscle [Rattus norvegicus]	73.418367
gi|109476367	PREDICTED: similar to ribosomal protein S13 [Rattus norvegicus]	52.903223
gi|149031250	vinculin (predicted), isoform CRA_a [Rattus norvegicus]	76.455027
gi|603877	transgelin [Rattus norvegicus]	84.577113
gi|9798638	glyceraldehyde-3-phosphate dehydrogenase [Rattus norvegicus]	83.783782
gi|157818179	eukaryotic translation elongation factor 1 beta 2 [Rattus norvegicus]	34.666666
gi|109468300	PREDICTED: similar to Alpha-enolase (2-phospho-D-glycerate hydro-lyase)	61.010098
gi|51859516	Heat shock 90kDa protein 1, beta [Rattus norvegicus]	45.856354
gi|6093729	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 precursor	10.989011
gi|8393206	cysteine and glycine-rich protein 1 [Rattus norvegicus]	46.632123

gi|13928824	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	53.725493
gi|8394158	fatty acid synthase [Rattus norvegicus]	14.091817
gi|55742827	Rho GDP dissociation inhibitor (GDI) alpha [Rattus norvegicus]	37.254903
gi|149045751	rCG55135, isoform CRA_a [Rattus norvegicus]	45.789677
gi|68566301	Dynamin-1-like protein (Dynamin-like protein)	21.589404
gi|109487680	PREDICTED: similar to Myosin-11 (SMMHC) [Rattus norvegicus]	49.505979
gi|149036104	6-phosphogluconolactonase (predicted), isoform CRA_a [Rattus norvegicus]	31.099194
gi|50926121	Dncic2 protein [Rattus norvegicus]	13.449366
gi|149029877	filamin, alpha (predicted), isoform CRA_a [Rattus norvegicus]	67.222434
gi|81910761	T-complex protein 1 subunit epsilon (TCP-1-epsilon) (CCT-epsilon)	35.489833
gi|81884451	SUMO-activating enzyme subunit 1 (Ubiquitin-like 1-activating enzyme E1A)	36.389685
gi|149067833	aldolase A, isoform CRA_e [Rattus norvegicus]	60.526317
gi|81861572	Malate dehydrogenase, cytoplasmic (Cytosolic malate dehydrogenase)	19.760479
gi|41350889	Pgam1 protein [Rattus norvegicus]	37.007874
gi|9506501	calponin 3, acidic [Rattus norvegicus]	39.090911
gi|62078969	hypothetical protein LOC360627 [Rattus norvegicus]	7.0567988
gi|70778952	RAD23b homolog [Rattus norvegicus]	19.277108
gi|149036910	actin related protein 2/3 complex, subunit 4 (predicted), isoform CRA_a	33.333334
gi|8393706	lactate dehydrogenase A [Rattus norvegicus]	78.915662
gi|82546819	heterogeneous nuclear ribonucleoprotein F [Rattus norvegicus]	21.204819
gi|8393693	laminin receptor 1 [Rattus norvegicus]	32.881355
gi|81884336	Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin)	21.025641
gi|91207082	Eukaryotic translation initiation factor 5A-1 (eIF-5A-1) (eIF-5A1)	38.961038
gi|51316969	Actin, aortic smooth muscle (Alpha-actin-2)	82.758623

gi|56090273	ribosomal protein S4, X-linked [Rattus norvegicus]	30.79848
gi|38454206	proteasome, 26S, non-ATPase regulatory subunit 6 [Rattus norvegicus]	15.167095
gi|55742755	catenin (cadherin-associated protein), alpha 1, 102kDa [Rattus norvegicus]	18.722467
gi|149033633	nucleoside phosphorylase (mapped), isoform CRA_c [Rattus norvegicus]	33.910036
gi|51859448	Adaptor protein complex AP-2, alpha 2 subunit [Rattus norvegicus]	11.075612
gi|47605563	PDZ and LIM domain protein 5 (Enigma-like PDZ and LIM domains protein)	31.472081
gi|4210985	non-muscle alpha-actinin 1 [Rattus norvegicus]	70.964128
gi|149040761	transgelin 2, isoform CRA_b [Rattus norvegicus]	74.253732
gi|78097100	microtubule-associated protein, RP/EB family, member 1 [Rattus norvegicus]	13.805971
gi|57528425	hypothetical protein LOC494445 [Rattus norvegicus]	40.271494
gi|149041736	reticulocalbin 2 [Rattus norvegicus]	27.179489
gi|149046800	SEC31-like 1 (S. cerevisiae), isoform CRA_c [Rattus norvegicus]	27.097315
gi|81883342	Twinfilin-1	7.9999998
gi|58865400	pleckstrin homology domain containing, family C member 1	16.764706
gi|109489498	PREDICTED: similar to Actin, cytoplasmic 2 (Gamma-actin)	64.899713
gi|8393038	calpain 2 [Rattus norvegicus]	28.857142
gi|81902430	Septin-2 (Vascular endothelial cell specific protein 11)	51.246536
gi|61889115	phosphoserine aminotransferase 1 [Rattus norvegicus]	18.108109
gi|51316981	ADP-ribosylation factor 1	27.624309
gi|83301602	Collagen alpha-1(III) chain precursor	74.231035
gi|109483719	PREDICTED: similar to Collagen alpha-1(XII) chain precursor	35.055822
gi|20138815	Alpha-parvin (Actopaxin)	9.4086021
gi|22024394	fatty acid binding protein 5, epidermal [Rattus norvegicus]	48.888889

gi|57858	ribosomal protein S24 [Rattus norvegicus]	32.330826
gi|61889112	cell division cycle 42 [Rattus norvegicus]	20.418848
gi|5305687	pro-alpha-2(I) collagen [Rattus norvegicus]	78.352767
gi|6911221	CArG-binding factor A [Rattus norvegicus]	24.210526
gi|61557085	spectrin beta 2 [Rattus norvegicus]	17.260391
gi|6981248	nucleolin [Rattus norvegicus]	27.629733
gi|55575	beta-actin [Rattus norvegicus]	90.133333
gi|462690	Nucleoside diphosphate kinase A (NDK A) (NDP kinase A)	83.552629
gi|1729977	Transketolase (TK)	19.743179
gi|62296810	Protein disulfide-isomerase A6 precursor (Protein disulfide isomerase P5)	51.81818
gi|8394168	calmodulin 2 [Rattus norvegicus]	94.630873
gi|62658155	PREDICTED: similar to eukaryotic translation initiation factor 4	20.901126
gi|56090441	ddx5 [Rattus norvegicus]	24.552846
gi|149052999	myosin, heavy polypeptide 10, non-muscle, isoform CRA_b	51.26518
gi|55250051	Txnrd1 protein [Rattus norvegicus]	20.069204
gi|39850096	Rps16 protein [Rattus norvegicus]	39.751554
gi|8394079	proteasome (prosome, macropain) subunit, beta type 2 [Rattus norvegicus]	23.880596
gi|149018456	microtubule-associated protein 4 [Rattus norvegicus]	19.005328
gi|81885353	Basic leucine zipper and W2 domain-containing protein 1	15.274464
gi|44888314	Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) (hnRNP-Q)	40.712947
gi|33086672	Cc2-27 [Rattus norvegicus]	19.191919
gi|61556832	adenine phosphoribosyl transferase (predicted) [Rattus norvegicus]	28.333333
gi|393203	calcium-binding protein [Rattus norvegicus]	18.506998
gi|58865872	enabled homolog [Rattus norvegicus]	40.114069
gi|71122383	Chloride intracellular channel 1 [Rattus norvegicus]	60.580915

gi|51980641	Ribosomal protein L4 [Rattus norvegicus]	21.140142
gi|158711704	collagen, type 1, alpha 1 [Rattus norvegicus]	83.138335
gi|109502869	PREDICTED: similar to RAN binding protein 5 [Rattus norvegicus]	17.86691
gi|61889092	adenylate kinase 1 [Rattus norvegicus]	23.71134
gi|149016579	ribosomal protein S5, isoform CRA_b [Rattus norvegicus]	30.882353
gi|38014694	Valosin-containing protein [Rattus norvegicus]	42.059553
gi|90111077	Prolyl 4-hydroxylase subunit alpha-1 precursor (4-PH alpha-1)	30.149812
gi|149044126	rCG27764, isoform CRA_a [Rattus norvegicus]	25.473729
gi|67460104	Cullin-associated NEDD8-dissociated protein 1	13.170731
gi|53733394	NAD(P)H dehydrogenase, quinone 1 [Rattus norvegicus]	11.313868
gi|38014563	Proteasome (prosome, macropain) subunit, alpha type 5 [Rattus norvegicus]	13.278009
gi|206205	M2 pyruvate kinase [Rattus norvegicus]	72.128057
gi|38181888	Proteasome (prosome, macropain) 26S subunit, ATPase 2	31.408775
gi|9507245	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	59.109312
gi|77539778	alpha actinin 4 [Rattus norvegicus]	71.679473
gi|78000190	tropomyosin 1, alpha isoform b [Rattus norvegicus]	80.281693
gi|157818975	filamin, beta [Rattus norvegicus]	33.66951
gi|61227509	Latexin (Endogenous carboxypeptidase inhibitor) (ECI)	13.901345
gi|157818781	protein kinase C substrate 80K-H [Rattus norvegicus]	22.285715
gi|8394063	proteasome (prosome, macropain) subunit, alpha type 2 [Rattus norvegicus]	26.495728
gi|157817229	actin related protein 2/3 complex, subunit 2 [Rattus norvegicus]	16.666667
gi|763182	fibroblast tropomyosin 4 [Rattus norvegicus]	83.870965
gi|157819579	hypothetical protein LOC363886 [Rattus norvegicus]	11.740891
gi|157786714	SEC23A (S. cerevisiae) (predicted) [Rattus norvegicus]	18.169935
gi|78103212	Thymosin beta-4 (T beta 4)	88.636363

gi|42476181	malate dehydrogenase, mitochondrial [Rattus norvegicus]	60.946745
gi|9506497	clathrin, heavy polypeptide (Hc) [Rattus norvegicus]	28
gi|76253725	chaperonin subunit 6a (zeta) [Rattus norvegicus]	30.32015
gi|6981022	hexokinase 1 [Rattus norvegicus]	12.200436
gi|45478130	LRRGT00145 [Rattus norvegicus]	14.953271
gi|149028892	tropomyosin 1, alpha, isoform CRA_c [Rattus norvegicus]	72.183096
gi|149041248	rCG27551, isoform CRA_a [Rattus norvegicus]	68.586385
gi|75832035	small inducible cytokine subfamily E, member 1 [Rattus norvegicus]	24.444444
gi|66793366	glutamyl-prolyl-tRNA synthetase [Rattus norvegicus]	11.911171
gi|149067319	rCG49111 [Rattus norvegicus]	45.024875
gi|157823877	cytoskeleton-associated protein 4 [Rattus norvegicus]	27.300614
gi|78126139	ribosomal protein S12 [Rattus norvegicus]	31.818181
gi|149037631	procollagen, type VI, alpha 3 (predicted), isoform CRA_c [Rattus norvegicus]	21.977638
gi|2498032	Myosin regulatory light chain 2, smooth muscle isoform	74.269009
gi|56090475	S-phase kinase-associated protein 1A [Rattus norvegicus]	41.717792
gi|56404680	Protein DJ-1 (Parkinson disease protein 7 homolog)	22.751322
gi|149068231	coatomer protein complex, subunit beta 1, isoform CRA_b	27.455121
gi|71153827	Myb-binding protein 1A (PAR-interacting protein) (PIP)	14.806548
gi|149057332	IQ motif containing GTPase activating protein 1 (predicted), isoform CRA_b	37.77912
gi|62648896	PREDICTED: similar to 40S ribosomal protein S20 [Rattus norvegicus]	41.176471
gi|8393910	phosphatidylethanolamine binding protein [Rattus norvegicus]	42.780748
gi|157819753	reticulocalbin 1 [Rattus norvegicus]	22.153845
gi|52001084	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	44.582042
gi|56585024	Phosphoglycerate kinase 1 [Rattus norvegicus]	55.875301

gi|109463545	PREDICTED: similar to AHNAK nucleoprotein isoform 1 isoform 1	56.550646
gi|8393153	LIM protein [Rattus norvegicus]	55.657494
gi|42476045	cathepsin D [Rattus norvegicus]	24.078624
gi|81884182	FK506-binding protein 9 precursor (Peptidyl-prolyl cis-trans isomerase)	22.456141
gi|149066132	rCG59523, isoform CRA_c [Rattus norvegicus]	41.263068
gi|149047669	transmembrane emp24 protein transport domain containing 4 (predicted)	25.991189
gi|149021114	vimentin, isoform CRA_b [Rattus norvegicus]	87.339056
gi|543922	Calnexin precursor	19.458544
gi|56090277	ribosomal protein, large, P1 [Rattus norvegicus]	19.298245
gi|157819755	hypothetical protein LOC364073 [Rattus norvegicus]	20.789075
gi|81910374	T-complex protein 1 subunit beta (TCP-1-beta) (CCT-beta)	21.495327
gi|62906896	Transmembrane emp24 domain-containing protein 10 precursor	30.593607
gi|50403574	60S ribosomal protein L10	20.093457
gi|157787018	adenylosuccinate synthetase, non muscle [Rattus norvegicus]	30.701753
gi|312928	tropomyosin isoform 6 [Rattus norvegicus]	72.983873
gi|31543764	alpha-spectrin 2 [Rattus norvegicus]	28.883496
gi|38197650	Hnrpk protein [Rattus norvegicus]	49.353448
gi|63101555	Sec61 alpha 1 subunit (S. cerevisiae) [Rattus norvegicus]	24.579832
gi|400429	myosin I heavy chain [Rattus norvegicus]	20.719844
gi|149040721	rCG20221, isoform CRA_b [Rattus norvegicus]	19.281046
gi|40786436	eukaryotic translation initiation factor 4A1 [Rattus norvegicus]	28.57143
gi|50403587	60S ribosomal protein L18a	28.977272
gi|55926139	alpha isoform of regulatory subunit A, protein phosphatase 2	21.052632
gi|149032606	vacuolar protein sorting 35 (mapped), isoform CRA_b [Rattus norvegicus]	11.43216
gi|92090591	Glutamate dehydrogenase 1, mitochondrial precursor	18.100359

gi|6981712	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	41.224489
gi|158631214	arginyl-tRNA synthetase [Rattus norvegicus]	17.272727
gi|76096306	vesicle amine transport protein 1 homolog (T californica) [Rattus norvegicus]	33.168316
gi|46397778	Peptidyl-prolyl cis-trans isomerase B precursor (PPIase) (Rotamase)	36.538461
gi|66911068	Pcbp2 protein [Rattus norvegicus]	27.761194
gi|149033299	rCG52516 [Rattus norvegicus]	19.093406
gi|38197632	Ptbp1 protein [Rattus norvegicus]	31.834534
gi|61556835	thrombospondin 1 [Rattus norvegicus]	22.393163
gi|77993298	signal sequence receptor, alpha [Rattus norvegicus]	17.421603
gi|51980308	Heterogeneous nuclear ribonucleoprotein A3 [Rattus norvegicus]	33.773088
gi|77415419	Serpina6 protein [Rattus norvegicus]	16.019417
gi|9910214	eukaryotic translation initiation factor 5 [Rattus norvegicus]	10.02331
gi|57164151	ribosomal protein S3 [Rattus norvegicus]	44.855967
gi|62653926	PREDICTED: similar to 60S ribosomal protein L12 [Rattus norvegicus]	33.939394
gi|149059759	chaperonin subunit 8 (theta) (predicted), isoform CRA_a [Rattus norvegicus]	11.861314
gi|52000745	T-complex protein 1 subunit delta (TCP-1-delta) (CCT-delta)	27.643785
gi|158303324	integrin beta 1 [Rattus norvegicus]	4.3859649
gi|81902084	Citrate synthase, mitochondrial precursor	18.240343
gi|584953	Calponin-1 (Calponin H1, smooth muscle) (Basic calponin)	62.28956
gi|68565369	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	13.661203
gi|6978725	cytochrome c, somatic [Rattus norvegicus]	40.952381
gi|78126159	ribosomal protein S2 [Rattus norvegicus]	52.218431
gi|66730475	hypothetical protein LOC500450 [Rattus norvegicus]	85.915494
gi|76559925	calumenin isoform b [Rattus norvegicus]	62.857145

gi|8393101	cofilin 1 [Rattus norvegicus]	60.240966
gi|61889071	RAB10, member RAS oncogene family [Rattus norvegicus]	16.500001
gi|149016180	aspartyl aminopeptidase, isoform CRA_b [Rattus norvegicus]	20.416667
gi|76364171	ATP-dependent RNA helicase DDX1 (DEAD box protein 1)	14.864865
gi|157817664	hypothetical protein LOC500987 [Rattus norvegicus]	46.153846
gi|149030883	glutathione synthetase, isoform CRA_c [Rattus norvegicus]	17.366412
gi|56346	H2B histone [Rattus norvegicus]	78.399998
gi|154816168	heat shock 70kDa protein 9A [Rattus norvegicus]	22.091311
gi|58865844	fusion (involved in t(12;16) in malignant liposarcoma) (predicted)	30.115831
gi|71795613	ribosomal protein, large P2 [Rattus norvegicus]	71.304345
gi|9845261	lectin, galactose binding, soluble 1 [Rattus norvegicus]	94.074076
gi|38454246	ribosomal protein L3 [Rattus norvegicus]	19.602978
gi|149048132	lamin A, isoform CRA_b [Rattus norvegicus]	72.781956
gi|149016893	rCG23030, isoform CRA_g [Rattus norvegicus]	32.367149
gi|55824765	Serpinh1 protein [Rattus norvegicus]	53.717029
gi|57012987	PRA1 family protein 3 (ARL-6-interacting protein 5)	10.638298
gi|48675371	complement component 1, q subcomponent binding protein	23.655914
gi|34786047	Niemann Pick type C2 [Rattus norvegicus]	17.449665
gi|85057089	Eukaryotic translation elongation factor 1 alpha 1 [Rattus norvegicus]	74.025977
gi|85541036	Myosin-Id (Myosin heavy chain myr 4)	10.337972
gi|149064471	rCG46601, isoform CRA_b [Rattus norvegicus]	20.071685
gi|157821523	septin 6 [Rattus norvegicus]	27.777779
gi|62201921	Prothymosin alpha [Rattus norvegicus]	37.5
gi|56971262	Hnrpa1 protein [Rattus norvegicus]	33.437499
gi|81910340	Protein SEC13 homolog (SEC13-like protein 1)	8.0745339

gi|149015978	fibronectin 1, isoform CRA_a [Rattus norvegicus]	35.849819
gi|109504921	PREDICTED: similar to germinal histone H4 gene [Rattus norvegicus]	72.115386
gi|8393057	serine (or cysteine) proteinase inhibitor, clade H, member 1	53.237408
gi|33086478	Ab1-205 [Rattus norvegicus]	11.59618
gi|62647139	PREDICTED: similar to zyxin [Rattus norvegicus]	16.94153
gi|54261673	Ribosomal protein L18 [Rattus norvegicus]	22.340426
gi|730581	60S acidic ribosomal protein P0 (L10E)	43.217665
gi|58865778	dolichyl-di-phosphooligosaccharide-protein glycotransferase (predicted)	10.657597
gi|149063427	rCG21664, isoform CRA_b [Rattus norvegicus]	38.811189
gi|8394060	proteasome (prosome, macropain) subunit, alpha type 1 [Rattus norvegicus]	9.5057033
gi|54041951	40S ribosomal protein S17	55.555558
gi|149041246	rCG27487, isoform CRA_a [Rattus norvegicus]	18.410042
gi|57129	unnamed protein product [Rattus norvegicus]	47.019866
gi|44888251	Phosphatidylinositol-binding clathrin assembly protein	16.875
gi|6981424	prosaposin [Rattus norvegicus]	33.754513
gi|109510179	PREDICTED: similar to Myristoylated alanine-rich C-kinase substrate	47.75641
gi|109463591	PREDICTED: similar to H3 histone, family 3B isoform 2 [Rattus norvegicus]	47.058824
gi|6981052	heat shock 10 kDa protein 1 [Rattus norvegicus]	16.666667
gi|6981420	pancreatic trypsin 1 [Rattus norvegicus]	13.821138
gi|62638830	PREDICTED: similar to 60S ribosomal protein L17 (L23)	11.413044
gi|157818451	hypothetical protein LOC498234 [Rattus norvegicus]	34.946236
gi|157821467	SH3 domain binding glutamic acid-rich protein-like 3 [Rattus norvegicus]	22.580644
gi|33086566	Ab2-162 [Rattus norvegicus]	20.839694
gi|728810	ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2)	37.919462

gi|145566928	Polymerase I and transcript release factor (Calvin) (cav-p60)	20.408164
gi|6822247	Nogo-A protein [Rattus norvegicus]	21.324162
gi|92373398	Y box protein 1 [Rattus norvegicus]	27.639753
gi|763181	annexin VI [Rattus norvegicus]	41.456166
gi|74474102	unnamed protein product [Rattus norvegicus]	55.743879
gi|730110	Brain acid soluble protein 1 (Neuronal axonal membrane protein NAP-22)	66.818184
gi|33086658	Cc1-6 [Rattus norvegicus]	13.810742
gi|81870810	Myeloid-associated differentiation marker (Myeloid up-regulated protein)	11.63522
gi|157823041	integrin, alpha 11 [Rattus norvegicus]	12.809564
gi|158186676	calumenin isoform a [Rattus norvegicus]	66.984129
gi|9845234	annexin A2 [Rattus norvegicus]	58.702064
gi|66730357	hypothetical protein LOC497813 [Rattus norvegicus]	39.690721
gi|157820787	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	19.23077
gi|23396766	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1	21.633554
gi|62512124	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase precursor	8.3993658
gi|50403620	40S ribosomal protein S18	53.289473
gi|78214309	ribosomal protein L8 [Rattus norvegicus]	36.18677
gi|71681130	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	38.941398
gi|109505801	PREDICTED: similar to Histone H1.2 (H1 VAR.1) (H1c) [Rattus norvegicus]	16.666667
gi|83300587	ATP synthase subunit alpha, mitochondrial precursor	30.198914
gi|77628163	AHNAK 1 [Rattus norvegicus]	29.281768

Appendix B

Complete list of all regulatory and metabolic protein networks as calculated by MetaCore™.

No	Interacting proteins	Processes	Pathways	p-Value	zScore
1	PUR9, NAP1, GDI2, PPase (inorganic), MTAP (Rattus norvegicus)	vitamin catabolic process (5.9%; 1.430e-04), vesicle fusion (5.9%; 4.140e-04), cAMP biosynthetic process (5.9%; 6.009e-04), nucleoside phosphate metabolic process (14.7%; 7.284e-04), nucleotide metabolic process (14.7%; 7.284e-04)	0	4.09E-40	67.76
2	DJ-1, GRP75, ARF1, CRMP2, NQO1 (Rattus norvegicus)	intracellular transport (31.4%; 1.702e-06), generation of neurons (28.6%; 3.041e-06), regulation of apoptosis (31.4%; 4.882e-06), neurogenesis (28.6%; 5.449e-06), regulation of cell death (31.4%; 5.508e-06)	0	1.17E-23	42.35
3	YB-1, RhoA, ACTR3, hnRNP A1, HSC70 (Rattus norvegicus)	regulation of developmental process (54.3%; 6.237e-13), regulation of biological quality (54.3%; 1.714e-12), regulation of localization (37.0%; 1.675e-11), regulation of cell migration (23.9%; 2.175e-11), regulation of signal transduction (39.1%; 2.416e-11)	0	9.17E-23	39.24

4	G3P2, CapG, Lamin A/C, DLC1 (Dynein LC8a), TMSB4X (Rattus norvegicus)	negative regulation of biological process (50.0%; 3.190e-08), negative regulation of cellular process (47.5%; 5.121e-08), regulation of biological quality (45.0%; 1.048e-07), regulation of cell death (32.5%; 4.991e-07), regulation of programmed cell death (32.5%; 4.991e-07)	0	2.66E-21	38.8
5	PTMA, hnRNP A1, TPT1, Fibronectin, Prostasin (Rattus norvegicus)	positive regulation of cellular process (45.8%; 3.326e-08), positive regulation of biological process (47.9%; 3.762e-08), regulation of cell proliferation (31.2%; 2.089e-07), regulation of developmental process (39.6%; 2.707e-07), regulation of biological quality (39.6%; 5.450e-07)	0	1.71E-20	35.94
6	Fibronectin, Annexin V, TPT1, DJ-1, eEF1B (Rattus norvegicus)	regulation of biological quality (65.9%; 1.867e-17), wound healing (34.1%; 6.804e-17), blood coagulation (27.3%; 3.039e-16), coagulation (27.3%; 3.039e-16), hemostasis (27.3%; 5.947e-16)	5	4.08E-16	29.35
7	PUR9, ASNS, DLC1 (Dynein LC8a), GC1QBP, TCP1-eta (Rattus norvegicus)	multicellular organismal process (69.0%; 2.350e-05), collagen biosynthetic process (6.9%; 4.333e-05), multicellular organismal macromolecule metabolic process (10.3%; 6.760e-05), multicellular organismal metabolic process (10.3%; 1.426e-04), positive regulation of biological process (41.4%; 4.004e-04)	0	2.07E-17	34.21
8	CRP2, GC1QBP, Fibronectin, Annexin I, MTS1 (S100A4) (Rattus norvegicus)	anatomical structure development (57.4%; 9.712e-09), actomyosin structure organization (12.8%; 1.031e-08), lipoprotein catabolic process (8.5%; 1.134e-08), lipoprotein particle clearance (8.5%; 9.704e-08), organ morphogenesis (29.8%; 2.691e-07)	1	2.82E-18	32.65

9	ENPP1, NQO1, Lamin A/C, Fibronectin, HSP47 (Rattus norvegicus)	homeostatic process (37.0%; 3.572e-06), ossification (18.5%; 8.143e-06), bone development (18.5%; 1.011e-05), regulation of biological quality (44.4%; 1.688e-05), multicellular organismal process (70.4%; 2.419e-05)	0	7.77E-16	33.23
10	MDH2, SURF1, ATP5A, PLOD1, ITGA11 (Rattus norvegicus)	aerobic respiration (16.7%; 1.542e-05), ribonucleoside diphosphate biosynthetic process (11.1%; 3.049e-05), ADP biosynthetic process (11.1%; 3.049e-05), purine ribonucleoside diphosphate biosynthetic process (11.1%; 3.049e-05), oxygen homeostasis (11.1%; 3.049e-05)	0	2.77E-14	32.4
11	CAP1, hnRNP A1, PTMA, G3P2, DLC1 (Dynein LC8a) (Rattus norvegicus)	cAMP biosynthetic process (21.7%; 3.717e-22), cAMP metabolic process (21.7%; 1.665e-19), cyclic nucleotide biosynthetic process (21.7%; 3.862e-18), cyclic nucleotide metabolic process (21.7%; 3.398e-16), activation of adenylate cyclase activity (21.7%; 2.331e-14)	0	3.35E-16	29.66
12	Fibronectin, DJ-1, AHNAK, CapG, CAP-G (Rattus norvegicus)	regulation of developmental process (58.0%; 8.286e-16), positive regulation of cellular process (58.0%; 9.852e-14), cell communication (78.0%; 1.175e-13), positive regulation of biological process (60.0%; 1.496e-13), signal transduction (74.0%; 1.964e-13)	0	4.08E-16	29.35
13	Lamin A/C, PTMA, Reticulon 4, Annexin I, GRP75 (Rattus norvegicus)	regulation of developmental process (62.1%; 5.274e-11), regulation of cell differentiation (34.5%; 1.499e-07), positive regulation of developmental process (37.9%; 1.625e-07), negative regulation of developmental process (34.5%; 4.236e-07), regulation of apoptosis (37.9%; 5.701e-07)	0	1.50E-13	29.05

14	RhoA, ARF1, PKNbeta, PTMA, Lamin A/C (Rattus norvegicus)	cell differentiation (52.2%; 1.390e-10), intracellular signaling cascade (45.7%; 3.570e-10), cellular developmental process (52.2%; 5.083e-10), positive regulation of developmental process (34.8%; 1.093e-09), system development (56.5%; 5.629e-09)	0	4.78E-12	23
15	DJ-1, hnRNP A1, Annexin II, Karyopherin beta 1, Annexin V (Rattus norvegicus)	positive regulation of biological process (47.7%; 1.603e-07), response to steroid hormone stimulus (20.5%; 2.766e-07), regulation of multicellular organismal process (34.1%; 4.309e-07), generation of neurons (27.3%; 5.438e-07), positive regulation of cellular process (43.2%; 8.989e-07)	0	4.78E-12	23
16	YB-1, HSP47, PRNP, HSP10 (mitochondrial), NQO1 (Rattus norvegicus)	positive regulation of cellular process (69.4%; 1.901e-19), positive regulation of biological process (71.4%; 3.193e-19), regulation of cell proliferation (53.1%; 1.491e-18), regulation of developmental process (63.3%; 2.316e-18), negative regulation of biological process (65.3%; 6.752e-17)	0	5.55E-12	22.76
17	Fibronectin, Lamin A/C, Karyopherin beta 1, NQO1, MTS1 (S100A4) (Rattus norvegicus)	regulation of cell proliferation (47.9%; 2.694e-15), positive regulation of biological process (62.5%; 3.128e-14), regulation of apoptosis (43.8%; 1.423e-13), regulation of cell death (43.8%; 1.818e-13), regulation of programmed cell death (43.8%; 1.818e-13)	0	5.55E-12	22.76
18	TPT1, HSC70, DLC1 (Dynein LC8a), Reticulon 4, PPase (inorganic) (Rattus norvegicus)	positive regulation of biological process (66.0%; 1.444e-16), regulation of developmental process (58.0%; 8.286e-16), positive regulation of cellular process (60.0%; 1.024e-14), negative regulation of biological process (60.0%; 1.842e-14), negative regulation of cellular process (56.0%; 1.810e-13)	0	5.55E-12	22.76

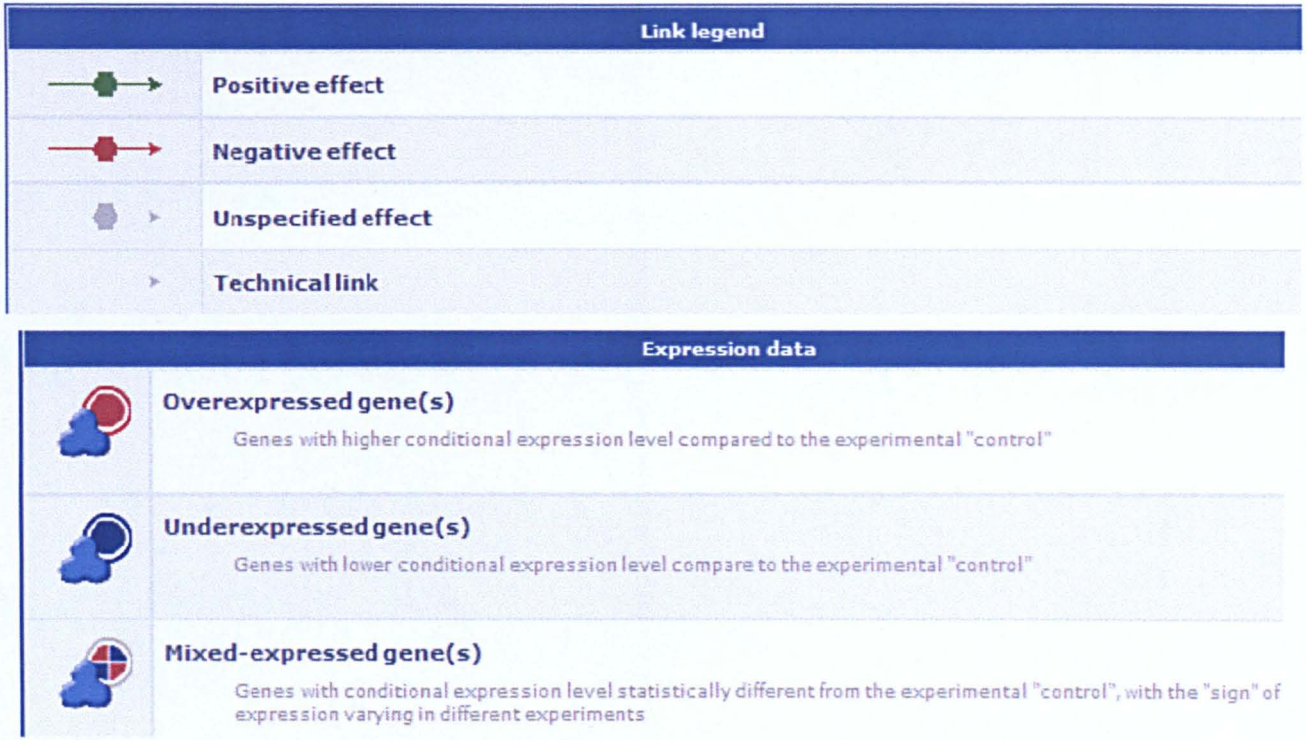
19	RhoA, Annexin II, Annexin VI, Annexin V, AHNAK (Rattus norvegicus)	intracellular signaling cascade (62.5%; 5.324e-19), regulation of multicellular organismal process (54.2%; 3.502e-17), regulation of developmental process (58.3%; 2.127e-15), positive regulation of biological process (64.6%; 3.026e-15), negative regulation of biological process (62.5%; 3.761e-15)	0	5.55E-12	22.76
20	Prostasin, PGK1, SEC61 alpha, G3P1, PPase (inorganic) (Rattus norvegicus)	glucose metabolic process (26.1%; 5.919e-08), hexose metabolic process (26.1%; 2.825e-07), monosaccharide metabolic process (26.1%; 3.730e-07), cellular carbohydrate metabolic process (26.1%; 8.768e-06), response to hypoxia (21.7%; 1.065e-05)	0	3.76E-09	20.35
21	ARF1, GDI2, PDIA3, Rab-1A, RNP24 (Rattus norvegicus)	protein transport (62.2%; 8.840e-25), establishment of protein localization (62.2%; 1.094e-24), vesicle-mediated transport (57.8%; 1.472e-24), protein localization (64.4%; 2.698e-24), macromolecule localization (64.4%; 1.427e-23)	0	3.13E-08	16.52
22	Gapdh, FKBP6 (Rattus norvegicus)	peptidyl-proline modification (50.0%; 7.171e-04), pachytene (50.0%; 9.561e-04), meiotic prophase I (50.0%; 2.270e-03), prophase (50.0%; 2.389e-03), glycolysis (50.0%; 6.445e-03)	0	3.69E-03	16.41
23	ATP5B, PRNP, Caveolin-1, gamma-ENaC, Fyn (Rattus norvegicus)	localization of cell (62.5%; 4.917e-06), cell motion (62.5%; 4.917e-06), chemical homeostasis (62.5%; 7.266e-06), protein kinase cascade (50.0%; 4.859e-05), homeostatic process (62.5%; 5.182e-05)	0	9.39E-05	16.35

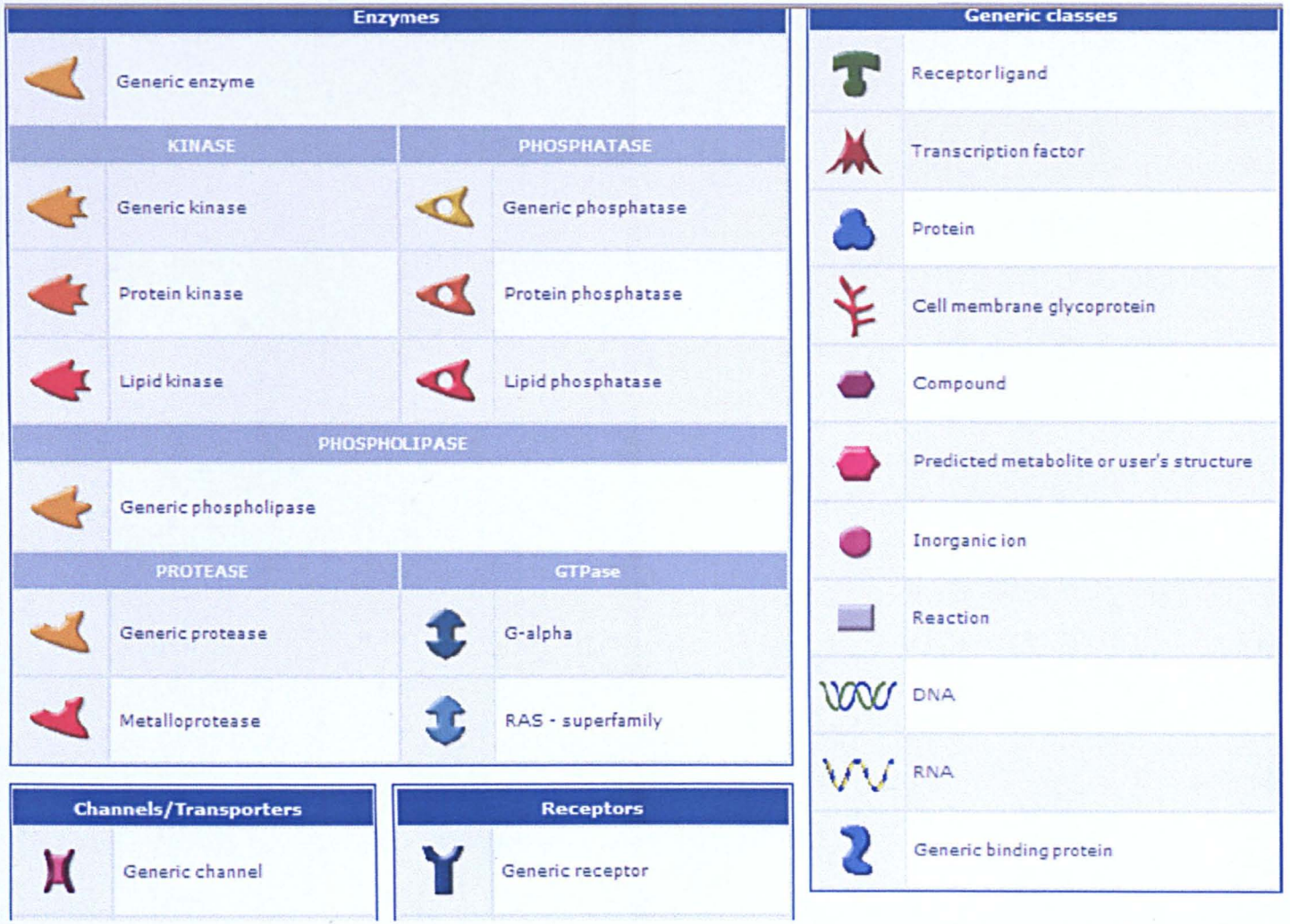
24	YB-1, hnRNP A1, 14-3-3 theta, PRNP, RPS7 (Rattus norvegicus)	positive regulation of cellular process (82.0%; 4.173e-28), positive regulation of biological process (82.0%; 2.174e-26), regulation of developmental process (74.0%; 1.963e-25), positive regulation of macromolecule biosynthetic process (56.0%; 1.272e-23), positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (54.0%; 8.177e-23)	0	3.86E-08	16.17
25	Lamin A/C, PTMA, YB-1, ARF1, MTS1 (S100A4) (Rattus norvegicus)	positive regulation of cellular process (70.8%; 6.658e-20), positive regulation of biological process (72.9%; 1.060e-19), regulation of cell proliferation (52.1%; 1.242e-17), positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (45.8%; 7.750e-17), positive regulation of transcription (43.8%; 1.686e-16)	0	3.86E-08	16.17
26	PRNP, Annexin II, PDIA3, AHNAK, SP1 (Rattus norvegicus)	regulation of immune system process (40.9%; 2.486e-15), positive regulation of biological process (65.9%; 1.073e-14), positive regulation of immune system process (31.8%; 1.739e-13), regulation of response to stimulus (36.4%; 1.744e-13), interspecies interaction between organisms (31.8%; 3.554e-13)	0	1.84E-06	13.3
27	YB-1, RhoA, Karyopherin beta 1, HSC70, EGFR (Rattus norvegicus)	positive regulation of biological process (83.7%; 4.548e-27), positive regulation of cellular process (77.6%; 1.704e-24), organ development (71.4%; 5.915e-20), response to chemical stimulus (65.3%; 1.846e-17), regulation of developmental process (61.2%; 3.129e-17)	0	2.36E-06	12.88

28	HSC70, G3P2, Lamin A/C, Annexin VI, Fyn (Rattus norvegicus)	immune response-activating cell surface receptor signaling pathway (26.5%; 1.933e-21), immune response-regulating cell surface receptor signaling pathway (26.5%; 5.734e-21), immune response-activating signal transduction (26.5%; 3.950e-20), immune response-regulating signal transduction (26.5%; 1.534e-19), antigen receptor-mediated signaling pathway (22.4%; 1.778e-18)	0	2.36E-06	12.88
29	PGMU, alpha-D-Glucose-6-phosphate = alpha-D-Glucose 1-phosphate, alpha-D-Glucose 1-phosphate = alpha-D-Glucose-6-phosphate, alpha-D-Glucose 1-phosphate, alpha-D-Glucose-6-phosphate (Rattus norvegicus)	glucose metabolic process (100.0%; 9.503e-03), hexose metabolic process (100.0%; 1.237e-02), monosaccharide metabolic process (100.0%; 1.297e-02), response to radiation (100.0%; 1.680e-02), cellular calcium ion homeostasis (100.0%; 1.745e-02)	0	1.00E+00	-0.06

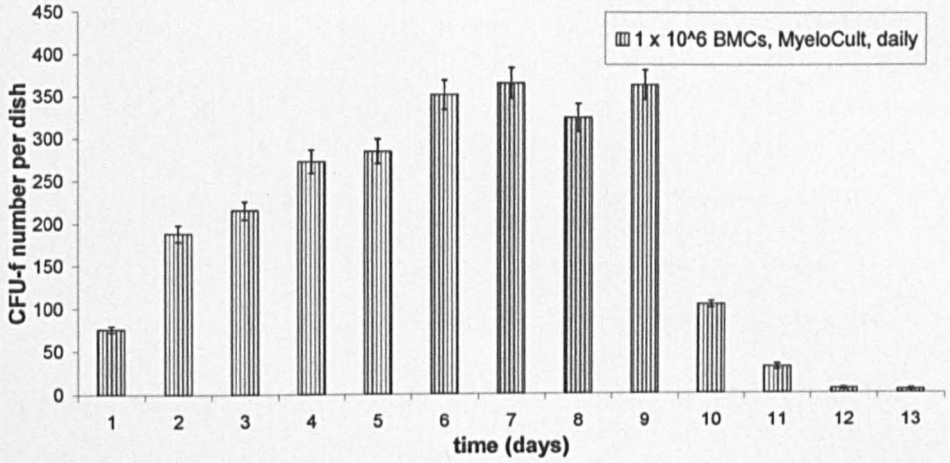
Appendix C

Legend for the diagrams in Figures 5.9 and 5.10 of the proteins networks calculated by MetaCore™.




Enzymes
Generic enzyme
KINASE
PHOSPHATASE
Generic kinase
Generic phosphatase
Protein kinase
Protein phosphatase
Lipid kinase
Lipid phosphatase
PHOSPHOLIPASE
Generic phospholipase
PROTEASE
GTPase
Generic protease
G-alpha
Metalloprotease
RAS - superfamily
Channels/Transporters
Generic channel
Receptors
Generic receptor
Generic classes
Receptor ligand
Transcription factor
Protein
Cell membrane glycoprotein
Compound
Predicted metabolite or user's structure
Inorganic ion
Reaction
DNA
RNA
Generic binding protein

Appendix D



Development of CFU-f number per dish in pour-off cultures started with 1 x 10^6 rat BMCs in MyeloCult® medium containing 10^{-8} M dexamethasone, 10^{-8} M 1α,25-Dihydroxyvitamin D3, 2 ng/ml IL-3 and 10 ng/ml SCF. The cultures were re-plated every 24 hours for up to 13 days. Results of all conditions are mean +/- S.D. from 3 different pour-off cultures.