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A Step Towards Identifying the True Adult Murine Mesenchymal Stem Cell

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"...no one tells me how to live my life,
it is a set-up until you're fed up
it's no good when you are misunderstood
so why should I care what the world thinks of me
don't leave a stranger give you a social disease ..."

Abstract

Research spanning over a few decades has contributed to the discovery and understanding of a population of cells that are precursors of connective tissue cell-types. These cells currently referred as Mesenchymal Stem Cells (MSCs), have also been shown by some researchers to have the capacity to give rise to neuron- and muscle-like cells *in vitro*, making them very attractive as cellular source for clinical application in regenerative medicine. Despite the intense focus on therapeutic research on going in the MSC field, the biology of these cells remains elusive, especially at its hierarchical organisation. Although some data suggest that MSCs are composed of different sub-populations of progenitors or cells that are prompted to differentiate preferentially into one or few different cell-types *in vitro*, there is no defined hierarchy proposed yet, especially at the most primitive level.

The initial aim of this project was to characterise the adult murine MSC (muMSCs) compartment since most of the studies performed to date were conducted with human cells. During the process of such, a new cell type was found. Under a conventional isolation/culture system a minor sub-population of muMSCs was identified and then characterised (based on the expression of SSEA-1 antigen; Stage Specific Embryonic Antigen-1). The data presented strongly suggest that this new sub-population is not only the founder of the MSC compartment but also they are multipotent both *in vitro* and *in vivo*. Moreover, this new cell-type can be directly identified from fresh bone marrow thus confirming its true existence *in vivo*. Detailed study on SSEA-1^{pos}-MSCs revealed that these cells present several common features with Embryonic Stem Cells (ES) and therefore suggests that a group of cells with embryonic features might persist throughout adult life.

The identification/characterisation of these adult murine SSEA-1^{pos} cells should facilitate the identification of a similar cell-type in the human MSC compartment. Furthermore, this study opens new questions on the developmental origin and importance of adult tissue-specific stem cells in the maintenance of tissue homeostasis.

Abbreviations

AFP: alpha-fetoprotein

AGPR: asialoglycoprotein receptor

AP: Alkaline Phosphatse

aP2: acid binding protein 2

ASM: acid sphingomyelin

α-sma: α-smooth muscle actin

bFGF: basic Fabroblast Growth Factor

BHA: butylated hydroxyanisole

BLM: bleomycin

BM: bone marrow

BMMNCs: Bone Marrow Mononuclear Cells

BMPs: Bone Morphogenetic Proteins

BrdUrd: 5-bromo-2-deoxyuridine

BSA: Bovine Serum Albumin

BSPII: Bone Sialoprotein II

BST: butylated hydroxytoluene

C/EBPs: CAAT/enhancer binding protein

$CaCl_2$: calcium chloride

CFU-F: Colony Forming Unit-Fibroblast

CK18: cytokeratin18

CK19: cytokeratin19

CRUK: Cancer Research UK

DAPI: 4'-6-diamidino-2-phenylindole

dex: dexamethasone

dH_20: distilled water

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethylsufoxide

EGF: Epidermal Growth Factor

ES: Embryonic Stem

ETBP: Extra Thick Blotting Paper

FACS: Fluorescence Activated Cell Sorter

$FeCl_3$: ferric chloride

FGF-4: Fibroblast Growth Factor-4

FISH: Fluorescence In Situ Hybridisation

FTM: Fast-Twitch Myosin

GADPH: glyceraldehydes-3-phosphatase

G-CSF: Granulocyte-Colony Stimulating Factor

GDNF: glial cell line-derived neurotrophic factor

GFAP: Glial Fibrillary Acidic Protein

eGFP: Green Fluorescent Protein

GLAST: Glutamate/Aspartate Transporter

GS: Glutamine Synthetase

H_2O_2: hydrogen peroxide

HCl: hydrogen chloride

HGF: Hepatocyte Growth Factor

HSC: Haematopoietic Stem Cell

hMSC: human Mesenchymal Stem Cell

HNF: Hepatocyte Nuclear Factor

HS: Horse Serum

Hst: Hoechst

i.v: intravenous

IGF-1: Insulin-like Growth Factor-1

IL-: interleukin-

IMBX: 1-methyl-3-isobutylxanthine

K^+: potassium ion

KDR (or Flk-1 or VEGF-R2): Vascular Endothelial Growth Factor Receptor2

LDL: Low Density Lipoprotein

Lin: lineage

M.O.I: multiplicity of infection

MAPC: Multipotent Adult Progenitor Cell

mdx: Duchenne Muscular Dystrophy

MFI: Mean Fluorescent Intensity

MHC-I: Major Histocompatibility Class I

mLIF: murine Leukemia Inhibitor Factor

MPC: Mesenchymal Progenitor Cell

MSC: Mesenchymal Stem Cell

muMSC; murine Mesenchymal Stem Cell

Na^+: sodium ion

NaOH: sodium hydroxide

NBF: Neutral Buffered Formaldehyde

NCID: notch-1 intracellular domain

NGF: Nerve Growth Factor

NGFR (or Trk): Neuronal Growth Factor Receptor

NH_4Cl: ammonium chloride

NOD/SCID: non-obese diabetic severe combine immunodeficiency

NPD: Niemann-Pick

NSE: Neuron Specific Enolase

OI: Osteogenesis Imperfecta

ON: over night

PAS: Perodic acid-Schiff

PBS: Phosphate Buffered Solution

PBS-2%: Phosphate Buffered Solution with 2% of FBS

PCR: Polymerase Chain Reaction

PDGF: Platelet Derived Growth Factor

PEI: polyethyleneimine

Pen/strep: penicillin/streptomycin

PFA: paraformaldehyde

PK: Proteinase K

PPARγ- Peroxixome Proliferating Activated Receptor-γ

PROD: Pentoxyresorufin O-Dealkylase

Px: purifiedx

PY: Pyronin Y

RA: Retinoic Acid

RT: room temperature

RT-PCR: Reverse Transcription-Polymerase Chain Reaction

SCF: Stem Cell Factor

SDS: sodium dedecylsulphate

SFFV: Spleen Focus Foaming Virus

SSEA-1: Stage Specific Embryonic Antigen-1

Stro-1: stromal precursor cell marker-1

TGFβ: Transformin Growth Factor-β

UnpPx: unpurifiedx

VEGF: Vascular Endothelial Growth Factor

VSVG: Vesicular Stomatitis Virus G

vWF: von Willebrand Factor

WB: western-blot

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Chapter 1 - General and Concise Introduction

The bone marrow mesenchymal compartment contains stem/progenitors of skeletal tissue components such as bone, cartilage, the haematopoiesis-supporting stroma and adipocytes. These cells are currently denominated by mesenchymal stem or progenitor cells (MPCs). In addition, they may be experimentally induced to undergo unconventional differentiation, possibly forming neural-, myogenic-, endothelial- and visceral mesoderm-like cells. As such, they represent an important concept of post-natal non-haematopoietic stem cells and an easy source for potential therapeutic use. Despite having been studied for more than three decades and currently being used in different clinical settings, their biology remains elusive. Based on the aim of this project, the main focus of this introductory chapter will be an overview of the basic *in vitro* and *in vivo* biology of these cells, followed by a more detailed discussion on the hierarchical organisation of the bone marrow mesenchymal compartment. As such, other areas in which MSCs were used as vehicles for gene therapy, models to study the molecular mechanisms of adipogenesis, osteogenesis, or other differentiation pathways and also characterisation of their immunomodulatory properties will not be discussed here.

1.1 - What is a mesenchymal stem cell?

The adult bone marrow has generally been seen as an organ composed of two main and distinct lineages: the haematopoietic tissue and a group of mesenchymal-derived cells. Within this last group exists a putative subset of cells referred to as mesenchymal stem or mesenchymal progenitor cells. These cells can be *ex vivo* expanded and induced *in vitro* to differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, myoblasts, astrocyte/neuronal-like cells and haematopoietic-supporting stroma (Dennis *et al.*, 1998; Dexter, 1982; Majumdar *et al.*, 1998; Pittenger *et al.*, 1999). The multipotentiality of these cells, their relatively easy isolation

and culture (especially from humans), as well as their high *ex vivo* expansion potential, renders these cells an attractive therapeutic tool.

Despite increasing experimental and clinical interest in recent years, the understanding of MSC biology remains elusive. In its most basic definition, a stem cell has the capacity for self-renewal and the ability to give rise to one or more types of differentiated progeny (Morisson *et al.*, 1997; Prockop, 1999). Within that context, and based on its *in vitro* behaviour, it would seem that the MSC should be considered as a stem cell. It has been demonstrated that low-density plated MSCs can proliferate *in vitro* and expand more than a few hundred to a thousand-fold in a short period of time (Colter *et al.*, 2000; Digirolamo *et al.*, 1999; Javazon *et al.*, 2001) and they are able to give rise to different cell-types as mentioned above. However, characteristics of MSCs differ among species and according to the methods of isolation/expansion applied, and there is no specific marker or combination of markers that identify MSCs either *in vivo* or *in vitro*. Therefore, MSCs are currently defined by a combination of morphologic, phenotypic, and functional properties, many of which could be the result of *in vitro* manipulation.

Despite the terminology commonly used, whether MSC qualify as stem cell remains a valid question. MSCs have clearly not been proven to be stem cells (Jordan *et al.*, 1990; Morisson *et al.*, 1994, 1995 and 1997). Firstly, MSCs have not been demonstrated *in vivo* to be capable of regeneration or maintenance of a tissue compartment at a single cell level. Secondly, MSCs have not been transplanted, isolated, and serially transplanted. Because of the inability to prospectively isolate MSCs and to characterise them and study their biologic properties in a relatively unmanipulated state, the concept of an MSC remains an assumption based on extrapolations from *in vitro* studies.

1.2 - Nomenclature used to refer to MSC or MPC

The first contribution to the understanding of mesenchymal precursor cells was made by Friedenstein and colleagues (Friedenstein *et al.*, 1969 and 1970). With the objective to identify cells in bone marrow responsible for bone formations in ectopic transplant studies, Friedenstein *et al.* demonstrated the growth of single cell-derived colonies of cells morphologically resembling fibroblasts (Friedenstein *et al.*, 1969 and 1970). The clonogenic mesenchymal precursor cells responsible for colony growth under these conditions were originally termed as fibroblast colony-forming cells (CFU-F). This original term has been gradually abandoned and replaced by diverse names like marrow stromal cells (MsCs) (Prockop, 1997), MSCs (Caplan, 1994) or MPCs (Conget and Minguell, 1999). As mentioned above, such denominations reflect a semantic rather than a functional issue, which applies not only to the putative stem cell *per se*, but to a vast repertoire of committed progenitors exhibiting at least one or more differentiation potentials *in vitro*.

CFU-Fs were described as rapidly adherent, non-phagocytic clonogenic cells capable of extended proliferation *in vitro*. However, *in vivo* CFU-Fs are essentially in a non-cycling state as demonstrated by ^{3}H-thymidine labelling studies (Epichina and Latzinik, 1976; Keiliss *et al.*, 1971). CFU-Fs have been detected in the bone marrow of essentially all species examined including cats, dogs, sheep, rabbits, non-human primates, and humans. The CFU-Fs plating efficiency is highly dependent on the culture conditions, and there is an immense variability in the requirements from one species to another. In rodents, irradiated marrow feeder cells are essential in addition to selected lots of serum, in order to obtain the maximum number of CFU-Fs, whereas in humans they are feeder cell-independent (Kuznetsov *et al.*, 1997a). The mitogenic factors that are required to stimulate the proliferation of CFU-F are not completely known at this present time, but at least includes platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-ß

(TGF-ß), and insulin-like growth factor-1 (IGF-1) (Gronthos *et al.*, 1995; Kuznetsov *et al.*, 1997b).

It is necessary to mention that the nomenclature of marrow stromal cells has also been utilised to designate haematopoietic-sustaining monolayers of long-term marrow stroma or Dexter-type cultures (Dexter, 1982; Gartner *et al.*, 1980). However, culture conditions, evolving phenotypes, differentiation potential and secretion products of the above cells are not similar and, in fact, are quite distinct to that of MPCs or mesenchymal stem cells (Chichester *et al.*, 1993; Majumdar *et al.*, 1998).

Indeed, the adherent layer derived from long-term *in vitro* bone marrow culture consists of fibroblastic cells, macrophages, adipocytes, endothelial cells, and smooth muscle cells (Dexter, 1982). The term of MSC used in this study refers to mesenchymal stem cells rather than mesenchymal stromal cells. Additionally, most of the understanding of MSC biology has been performed in humans, thus the majority of the examples referred to here were from studies performed on human cells, especially bone marrow derived MSCs (hMSCs).

1.3 - *In vitro* characteristics of MSCs/MPCs

1.3.1 - General *in vitro* features of MSCs/MPCs

MSCs can be isolated from different sources, which include adult bone marrow (BM), peripheral blood, adipose tissue, cord blood and different foetal tissues (Campagnoli *et al.*, 2001; Charbord *et al.*, 2002; Gronthos *et al.*, 2001; Kuznetsov *et al.*, 2001; Zuk *et al.*, 2002). Most MSC populations have been isolated via methodology originally described by Friedenstein and later modified by Caplan utilising the physical property of plastic adherence (Friedenstein *et al.*, 1992; Haynesworth *et al.*, 1996). However, macrophages, endothelial cells, lymphocytes, and smooth muscle cells also adhere to plastic and contaminate early BM preparations, especially from mouse BM preparations (Clark *et al.*, 1995; Deans and Moseley, 2000).

Enrichment for MSCs can be achieved by expansion and passing in relatively low-serum containing medium, ultimately eliminating contamination (Colter *et al.*, 2001; Digirolamo *et al.*, 1999; Javazon *et al.*, 2001). In mouse-derived cultures, the resulting adherent fractions remain morphologically heterogeneous, containing cells ranging from narrow spindle shaped to large polygonal and, in some confluent cultures, tightly packed, slightly cuboidal cells. Although in human-derived adherent fractions, the cultures display a rather homogeneous "look" of fibroblast-like cells, cell cycle studies revealed that they are rather heterogeneous. In fact, while a small fraction of MSC/MPC are actively engaged in proliferation, the vast majority of cells are standing at the G0/G1 phase of the cell cycle (Conget and Minguell, 1999). Moreover, the G0/G1 population of MSC includes a minor and variable subset of resting quiescent cells, as evidenced by RNA and DNA content (Conget and Minguell, 1999).

After subcultivation, hMSC have highly variable expansive potential. In some cases, some preparations can be expanded over more than 15 cell doublings, others cease replicating after just a few cell doublings (Bruden *et al.*, 1997; Digirolamo *et al.*, 1999; Phinney *et al.*, 1999a) This may arise from several factors, amongst them the procedure used to harvest the marrow (Bruden *et al.*, 1997; Blazsek et al., 1999; Digirolamo *et al.*, 1999; Phinney *et al.*, 1999a), the low frequency of MSCs in marrow harvests (Koc *et al.*, 1999), and the age or condition of the donor from which MSCs were originated (D'Ippolito *et al.*, 1999; Galotto *et al.*, 1999; Phinney *et al.*, 1999b). In moderate subcultivation situations, MSCs maintain their normal karyotype and telomerase activity (Pittenger *et al.*, 1999). However, extensive subcultivation impairs cell function by the onset of evident signs of senescence (Digirolamo *et al.*, 1999; Rubio *et al.*, 2005) and/or apoptosis (Conget and Minguell, 1999).

1.3.2 - Immunophenotype and production of cytokines/matrix molecules

Panels of surface antigens have been reported to be expressed on MSC derived from different cellular sources (Barry *et al.*, 2001; Bianco *et al.*, 2001; Deans and Moseley, 2000; Deschaseaux *et al.*, 2003; Filshie *et al.*, 1998; Gronthos *et al.*, 1995; Lodie *et al.*, 2002) although there is no agreement on the phenotypical characterisation in the published literature. Variation in the isolation techniques and culture media used to grow MSCs has led to variable findings regarding the phenotype of these cells in different laboratories. Nevertheless, MSCs display a repertoire of expression of different surface antigens, which includes numerous receptors important for cell adhesion with haematopoietic cells. Some of the surface antigens reported to be on human bone marrow derived MSCs are Stro-1 (Gronthos *et al.*, 1999), CD13, CD71 (transferring receptor), CD73, CD90 (Thy-1), CD105 (endoglin), CD106 (VCAM-1) and CD166 (ALCAM). In the murine system, these cells express Sca-1 and not CD90 (Peister *et al.*, 2003). In general, however, MSCs uniformly lack antigens such as CD14 and CD45 that typically identify haematopoietic cells.

It is important to mention the expression of different types of α-integrins and hyaluronic acid on MSCs. This implies a dynamic participation of these cells in the marrow microenvironment, especially in the organisation of the extracellular matrix in the marrow or in the bone. This is further strengthened by data showing they produce a vast array of matrix molecules, including fibronectin, laminin, collagen, and proteoglycans (Chichester *et al.*, 1993, Pittenger *et al.*, 1999; Prockop, 1997). Indeed, *in vitro* cell adhesion studies using different human $CD34^{pos}$ haematopoietic cell lines showed that adherence of these cells to stroma could be partially inhibited by divalent cation chelation or RGD peptide competition, implicating integrin-dependent adhesion (Ghaffari *et al.*, 1997). Additionally, the attachment was sensitive to digestion by chondroitinase ABC, suggesting CD44-dependent binding interactions (Ghaffari *et al.*, 1997).

MSCs have been shown to constitutively secrete several cytokines (Cheng *et al.*, 2000; Dormady et al., 2001; Haynesworth et al., 1996; Mbalaviele *et al.*, 1999; Majumdar *et al.*, 1998). Many of them are relevant in HSC (haematopoietic stem cell) proliferation and differentiation such as: IL-6 (interleukin-6), Flt-3 ligand, SCF (stem cell factor), G-CSF (granulocyte-colony stimulating factor). MSC can support human LTC-IC (long-term culture-initiating cells), so they can provide a feeder layer for cultured HSC (Cheng *et al.*, 2000; Kadereit *et al.*, 2002), without additional cytokines. Moreover, MSCs can enhance hHSC engraftment in xenotransplant experiments when co-injected with haematopoietic cells (Angelopoulo *et al.*, 2003; Noort *et al.*, 2002). The therapeutic potential of hMSCs supporting HSC engraftment in patients undergoing bone marrow transplantation has been studied in several settings. As an example, patients in remission following treatment for breast cancer showed rapid haematopoietic recovery after being infused with autologous cultured/expanded MSCs (Koc *et al.*, 2000).

MSCs also secrete cytokines with immunomodulatory effects such as IL-1, IL-11, IL12, (interleukin-1, -11, -12). Most of these and other immunomodulatory cytokines are produced by MSC in response to IL-1 (Dormady et al., 2001; Haynesworth et al., 1996; Mbalaviele *et al.*, 1999; Majumdar *et al.*, 1998). The immune phenotype of cultured hMSCs is widely described as MHC-I^{pos}, MHC-II^{neg}, CD40^{neg}, CD80^{neg} and CD86^{neg} (Tse *et al.*, 2003). It has been shown that treatment with interferon can up-regulate MHC-I and induce MCH-II expressions, but it does not change the co-stimulatory molecule expression (Tse *et al.*, 2003). This non-immunogenic status of MSCs suggests that these cells might be effective in inducing tolerance. For example, hMSCs have been shown to inhibit T-cell responses not only by direct contact but also by IL-10 secretion in a dose-dependent manner (Aggarwal and Pittenger 2005; Beyth *et al.*, 2005).

1.4 - *In vitro* differentiation capacities of MSCs

Being mesenchymal in origin, it is not surprising that MSCs can terminally differentiate into several mesenchymal cell-types upon appropriate stimuli *in vitro*. These differentiation assays have been commonly used to determine the multipotentiality of MSCs. However, in recent years several groups have reported that MSCs can also adopt the cell fates of neuroectoderm and endoderm origin (Black and Woodbury, 2001; Deng *et al.*, 2001; Jiang *et al.*, 2003; Kohyama *et al.*, 2001; Lee *et al.*, 2004; Sanchez-Ramos *et. al.*, 2000).

After more than two decades, the original formulations of cytokines/chemicals used for differentiating MSCs *in vitro* have evolved and might differ from one laboratory to another. Nevertheless, some of the basic compounds used for each lineage specification will be concisely discussed here.

The osteogenic lineage differentiation is normally achieved by treatment with dexamethasone (dex), β-glycerolphosphate and ascorbate (Bruder *et al.*, 1997). The responsiveness of the cells to such chemicals is measured by the increase in calcium mineralization of the extracellular matrix and in alkaline phosphatase (AP) activity. Usually, after 1 week, calcium accumulation is observed and AP activity is seen. Also the addition of other factors other than glucocorticoids, such as bone morphogenetic proteins (BMPs), increases the number of bone nodules in cell cultures (Gori *et al.*, 1999). The exact sequence of differentiation events is still not very clear; however, alkaline phosphatase increases then decreases when mineralization is well progressed, with osteopontin expression appearing prior to certain other matrix proteins, including BSPII (Bone Sialoprotein II) and osteocalcin. BSPII is first detected in differentiated osteoblasts forming bone and osteocalcin appearing with mineralization (Aubin, 1998; Liu *et al.* 2003; Owen *et al.* 1991).

Adipogenic differentiation can be induced with a combination of different glucocorticoid family members, like 1-methyl-3-isobutylxanthine (IBMX), dex, indomethacin and insulin (Nakamura *et al.*, 2003; Ryden *et al.*,

2003; Pittenger *et al.*, 1999). Induced cultures will accumulate lipid vacuoles as well as the lipogenic enzymes glycerol-3-phosphatase dehydrogenase, lipoprotein lipase and fatty acid binding protein (aP2). Cells containing lipid vacuoles are observed following staining with Oil Red O solution after 2-3 weeks of induction. These adipocytes usually remain healthy in culture for a few months. In general, MSCs can be differentiated into the adipocyte lineage in a similar fashion as pre-adipocyte cell-lines, such as 3T3-L1 and 3T3-F442A, when stimulated with these adipogenic hormones (Nakamura *et al.*, 2003; Ryden *et al.*, 2003). However, phenotypic changes, such as lipid vesicles, can be observed at a later stage in comparison to the already pre-committed cell-lines (Nakamura *et al.*, 2003; Ryden *et al.*, 2003). Nevertheless, similar key regulatory genes are necessary and sufficient for this conversion, which involves the CCAAT/ enhancer binding proteins (C/EBPs) α, β, δ, as well as the peroxisome proliferators-activated receptor (PPARγ; Nakamura *et al.*, 2003; Ryden *et al.*, 2003; Suzawa *et al.*, 2003).

Chondrogenic differentiation is observed when cells are exposed to the influence of TGF-β_3 (Mackay *et al.*, 1997). Cultured MSCs are placed in suspension in a polypropylene tube and gently spun in a centrifuge to form a pelleted micromass that develops in a multilayered matrix. After culturing in serum-free medium for 2-3 weeks containing TGF-β_3, the cells express an extensive matrix rich in cartilaginous proteoglycans. These chondrocytes express type-II collagen and chondrocyte-like lacunae can be observed in histological sections (Mackay *et al.*, 1997).

Wakitani *et al.* demonstrated that MSC can also differentiate into myoblasts and myotubes by treatment with 5'-azacytidine. Although the exact mechanisms by which 5'-azacytidine induces myogenic differentiation on MSCs are not known, it has been suggested that this cytosine analogue may activate some silent genes by inhibiting DNA methylation (Chiu and Blau, 1985; Creusot *et al.*, 1982; Jones and Taylor, 1980) or induce changes in some specific genes to trigger their response to exogenous regulators (Clough *et al.*, 1982). Indeed, myogenic differentiation was achieved when

MSC cultures were also treated with horse serum (HS) and hydrocortisone, followed by 5'-azacytidine treatment (Gang *et al.*, 2004; Reyes *et al.*, 2001). Induced MSCs will gradually form myotubes and acquire expression of different myogenic proteins such as: desmin, myogenin, α-actinin, etc (Gang *et al.*, 2004; Muguruma *et al.*, 2003; Reyes *et al.*, 2001; Santa-Maria *et al.*, 2004). New methodologies for myogenic differentiation are available nowadays, which includes induction with IGF-1 and co-culture with myocytes (Lee *et al.*, 2005; Reyes *et al.*, 2001; Santa-Maria *et al.*, 2004).

Surprisingly, MSC seem to be able to differentiate into different tissues of embryonic neuroectodermal origin. Several reports have suggested that different agents, especially cytokines, growth factors, neurotrophins and retinoic acid (RA), could promote neural cell induction from MSCs *in vitro* (Black and Woodbury, 2001; Deng *et al.,* 2001; Kohyama *et al.*, 2001; Jiang *et al.*, 2002 and 2003; Sanchez-Ramos *et. al.*, 2000; Woodbury *et al.*, 2000 and 2002).

One of the methodologies applied by many groups implies that both rodent and human MSCs could be rapidly (minutes to a few hours) induced to differentiate exclusively into more than 70% of neurons *in vitro* by using simple chemical means (Black and Woodbury, 2001: Woodbury *et al.*, 2000 and 2002). The majority of induced MSCs not only exhibited a neuronal morphology, but also expressed several neuronal markers. Treatment with β-mercaptoethanol in serum-free medium induced changes in cell morphology as early as 60 min, formation of neuronal perikary and processes within hours and expression of the neuronal marker neuron-specific enolase (NSE; Black and Woodbury, 2001: Woodbury *et al.*, 2000 and 2002). Similar results were observed with dimethylsulfoxide (DMSO) in combination with butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT). Differentiated MSCs appeared to express the neuronal markers NSE, neurofilament-M, tau, and NeuN (Black and Woodbury, 2001: Woodbury *et al.*, 2000 and 2002). However, these findings turned out to be artifactual as demonstrated by a recent study performed by Tuszynski's group. Findings of this study indicated that chemical neuronal induction was a result of cellular stress leading to the

physical contraction of cells into a neuronlike morphology. In addition, similar morphological changes can also be observed in other primary and transformed cell lines and by other deleterious chemical agents (Lu *et al.*, 2004). These results suggest that "neuronal induction" of MSCs by simple chemical means does not constitute a differentiation process but rather is a cellular response to environmental stress.

However, neuroectodermal differentiation can be achieved by stimulation with bFGF and EGF. This simple cytokine cocktail usually promotes towards a more astroglia phenotype (Jiang *et al.*, 2002 and 2003; personal observations). However, some Tau and NeuN positive cells can also be obtained using such induction methodology (Jiang *et al.*, 2002 and 2003; personal observations). For a more appropriate neuronal differentiation several groups reported the use of this simple cytokine combination with additional factors such as, neuronal growth factor (NGF), RA, and others (Jiang *et al.,* 2003; Kohyama *et al.*, 2001; Sanchez-Ramos *et al.*, 1998 and 2000)

In one study the authors used a demethylating agent, as well as the neural inducer Noggin, along with neurotrophins, to induce neural cell differentiation from rodent MSCs (Kohyama *et al.*, 2001). Furthermore, small molecules such as dibutyryl cyclic AMP and IMBX that increase intracellular cyclic AMP, have been used for the *in vitro* differentiation of MSCs into neural cells (Deng *et al.*, 2001). After induction, it usually takes several days or even weeks for MSCs to differentiate into neural cells, which is similar to the time needed for the neuronal differentiation of embryonic and neural stem cells (Kohyama *et al.*, 2001; Jiang *et al.*, 2002 and 2003 Sanchez-Ramos *et. al.*, 2000). Furthermore, only a small proportion of pluripotent stem cells within the adult mesenchymal stem cell pool can be induced to differentiate into neurons with appropriate morphological and electrophysiological characteristics (Jiang *et al.*, 2003; Kohyama *et al.*, 2001).

More recently, some reports have shown that MSCs can also be induced to adopt the cell fates of visceral endoderm (hepatocyte-like cells) and endothelial-like cells *in* vitro (Cao *et al.*, 2005; Lee *et al.*, 2004; Reyes *et al.,* 2001 and 2002). When hMSCs were stimulated with hepatocyte growth

factor (HGF) and fibroblast growth factor-4 (FGF-4) for more than 3 weeks, cells started to acquire hepatocyte-like features with expression of specific markers like albumin and cytokeratin-18 (CK18) and adopt some functional features like albumin and urea production (Lee *et al.*, 2004). Simply treating with vascular endothelial growth factor (VEGF) induced MSCs to undergo an endothelial differentiation pathway (Cao *et al.*, 2005; Reyes *et al.,* 2001 and 2002). A few studies reported that induced cells could acquire endothelial cell features by expressing the von Willebrand factor (vWF) and CD31 and could form "vascular tubes" in the angiogenesis assay in vitro (Cao *et al.*, 2005; Reyes *et al.,* 2001 and 2002).

1.5 - *In vivo* capacities of MSCs

In comparison to *in vitro* characterisation, there has been minimal data on the *in vivo* behaviour of MSCs. The data currently available can be separated into observations following site-directed, mostly via injury models, or systemic administration. Few studies have assessed the efficacy of the *in vivo* contribution of MSCs into different tissue cell-types or have examined whether donor cells engraft and differentiate into participatory cells or if the presence of donor cells is due to fusion events. Even less defined are the events after systemic administration. It seems that after systemic infusion, large numbers of the cells lodge in the pulmonary vascular bed and engraftment in other tissues (if at all) occurs with low frequency. Most of the studies used polymerase chain reaction (PCR) to access the presence of donor cells in different recipient's tissues (Devine *et al.*, 2001 and 2003; Liechty *et al.*, 2000; Mahmud *et al.*, 2004). Such sensitive methodology can detect the persistence of small numbers of cells and thus, could overestimate immensely the levels of engraftment (if it could be considered as such) of MSCs *in vivo*. In reality, the evidence supports non-specific lodging of MSCs in multiple vascular beds with persistence of very small numbers of donor cells in various tissues.

1.5.1 - MSCs into bone and cartilage

The most successful *in vivo* application of MSCs has been for the repair of bone or cartilage. Evidence that MSCs are able to engraft in skeletal tissues came from early transplantation studies using gene-tagged (osteonectin and collagen-1A1 genes) bone marrow cultures (containing MSCs) with demonstration of donor-derived osteoblasts/osteocytes in different areas of bone tissue (Hou *et al.*, 1999; Pereira *et al.*, 1998). These studies opened up the possibility to use MSC for treatment of genetic bone disorders, such as osteogenesis imperfecta (OI; a disorder in which osteoblasts produce defective type-I collagen, leading to osteopenia, multiple fractures and severe bone deformities). Indeed, allogeneic MSCs were used to treat children with OI. In these studies, patients that were infused with MSCs showed osteoblast engraftment (although at low frequency) and new dense bone formation (Horwitz *et al.*, 1999 and 2002). Most patients had increased total bone mineral content in association with increased in growth velocity and reduced frequencies of bone fractures (Horwitz *et al.*, 1999 and 2002).

Such experiments are useful to show the potential of unaltered MSC. Other approaches are available where MSC were transduced with a virus vector carrying a gene, which may influence the lineage restriction of the cells or correct a specific genetic defect. In a recent study, Chamberlain *et al.* showed that a gene construct targeting exon 1 of the gene for collagen-IA1 can efficiently correct mutated collagen-IA1 production in MSCs (Chamberlain *et al.*, 2004). The results obtained with these genetically corrected MSCs from two patients with osteogenesis imperfecta were extremely encouraging. In all cultures, genetically corrected MSCs were able to produce an adequate amount of wild-type collagen (Chamberlain *et al.*, 2004). Most importantly, the quality of bone synthesised by the altered MSCs was improved, as evidenced by the results of a standard assay in which the MSCs were infused into ceramic cubes and then implanted into immunodeficient mice (Chamberlain *et al.*, 2004).

In another study, Moutsatos and colleagues used MSC expressing an

inducible BMP-2, and showed that these cells could both form and regenerate bone tissue *in vivo* and *in vitro* (Moutsatsos *et al.*, 2001).

Other advances include the ability to grow MSC in a mineral matrix containing ground coral exoskeleton and graft them into defective long bones in a sheep model (Kon *et al.*, 2000). The purpose for using a matrix is that it already has an open porosity similar to bone and is biologically compatible and resorbable. The combination of MSC plus matrix resulted in bone union with lamellar cortical medullary canal morphogenesis in some of the animals tested (Petite *et al.*, 2000). In other studies, MSCs implanted on a hydroxyapatite/tricalcium phosphate matrix or alone, have hastened bone formation when sited in craniofacial and long-bone defects (Mankani *et al.*, 2001; Tsuchida *et al.*, 2003).

MSCs were also used to decelerate (if not reverse) degeneration of intervertebral discs (Sakai *et al.,* 2003). Autologous rabbit MSCs grown in a type-II collagenous matrix were used to replace degenerated discs in these animals. Treated discs showed little alteration from non-operated/normal discs, with intense extracellular matrix staining using Safranin O for extracellular proteoglycans, while untreated/degenerating discs had progressive vacuolation of nucleus pulposus cells over time, inner annulus fibrosus collapse and extensive fibrosis and disc flattening (Sakai *et al.*, 2003).

1.5.2 - MSCs into cardiomyocytes

Myocardium has been another tissue, which has been an active experimental target for systemic or site-directed MSCs cellular therapy. MSCs appear to possess unique properties that may allow for convenient and highly effective cell therapy. Indeed, these cells have been shown to improve myocardial function in animal models of acute myocardial infarction (Orlic *et al.*, 2001; Shake *et al.*, 2002; Toma *et al.*, 2002). Although MSCs can be used allogeneically and delivered systemically only very few studies have convincingly demonstrated cardiomyocyte differentiation and functional integration of these cells into infracted regions. The differentiation into a

cardiomyocyte-like phenotype when implanted into healthy myocardium was only observed in a few studies (Orlic *et al.*, 2001; Toma *et al.*, 2002).

Martin and his colleagues (Shake *et al.*, 2002) have demonstrated that after injection into infarcted myocardium, engrafted MSCs could differentiate toward a myogenic lineage, as evidenced by expression of muscle-specific proteins including: α-actinin, troponin-T, tropomyosin, myosin heavy chain–MHC, phospholamban, and other muscle-specific proteins (Shake *et al.*, 2002). Additionally, the presence of connexin-43, a protein responsible for intracellular connection and electrical coupling between cells, was also observed in those studies, further suggesting cardiomyocyte differentiation. However, engrafted MSCs in the infarct areas did not show a complete myogenic differentiation with mature sarcomeric organisation, intercalated discs, etc. This is in contrast to the observations seen when hMSC were injected into viable, adult, non-infarcted murine myocardium (Toma *et al.*, 2002). The lack of extensive myocytic differentiation in infarcted heart may be due to the different local extracellular milieu in driving differentiation of MSC.

MSCs can also be readily transduced by a variety of vectors and maintain transgene expression after *in vivo* differentiation. Transgene expression by MSCs may ultimately be used to enhance cell engraftment or the extent of differentiation. Mangi *et al.* used a retroviral vector to overexpress the prosurvival gene Akt in MSCs before implantation into infarcted rat myocardium (Mangi *et al.*, 2003). The overexpression of Akt increased MSC survival and prevented pathologic remodeling by reducing intramyocardial inflammation, collagen deposition and cardiac myocyte hypertrophy with improvement in cardiac output (Mangi *et al.*, 2003).

Accumulative data suggest that several mechanisms other than cardiomyocyte replacement might contribute to minimise or even reverse the post-myocardial infarction tissue. These include: induction of angiogenesis, decrease in apoptosis and increase in collagen production. Recent data from Kinnaird *et al.* suggests that the mechanism of MSC-mediated improvements may reside in their ability to secrete a variety of angiogenic cytokines (FGF,

VEGF, matrix metalloproteinases, IL-1, angiopoietin, etc), many of which are upregulated under hypoxic conditions (Kinnaird *et al.*, 2004). In another report, the authors demonstrated that implanted porcine MSCs express vWF, VEGF and other proteins indicative of angiogenesis, in addition to smooth muscle actin (sma) (Davani *et al.*, 2003; Tomita *et al.*, 1999).

1.5.3 - MSCs into skeletal myocytes

Much less is known about the capacity of MSCs in contributing to skeletal muscle in comparison to myocardial muscle *in vivo.* Luyten's group conducted the first demonstration of skeletal muscle repair by hMSCs. In this case the authors used synovial membrane derived MSCs and showed that these cells have myogenic differentiation capacity in the nude and Duchenne muscular dystrophy (mdx) mouse models. When these cells were implanted into a regenerating nude mouse after cardiotoxin administration, they contributed to myofibres and to long-term persisting functional satellite cells (De Bari *et al.*, 2003). In this study no evidence of nuclear fusion hybrids was observed between donor human cells and host mouse muscle cells (De Bari *et al.*, 2003). Also, when administrated into immunosuppressed mdx mice, hMSCs were able to rescue some of the pathophysiological features of mdx mice muscles (De Bari *et al.*, 2003). A recent report, Dezewa *et al.* reported that myogenic differentiation from human and rat MSCs was achieved by Notch1 intracellular domain gene transfer and administration of certain trophic factors (Dezawa *et al.*, 2005). The methods employed in this study could systematically and efficiently induce muscle lineage cells with high purity from a large population of MSC. The induced population effectively differentiated into mature myotubes with some persisting satellite cells that continued to function in host muscle to restore degenerating muscles in the absence of repeated transplantation (Dezawa *et al.*, 2005).

1.5.4 - MSCs into neuroectodermal cells

Only a few studies have shown beneficial effects of MSCs in central nervous system and spinal cord injury (Hofstetter *et al.*, 2002; Lu *et al.*, 2001; Wu *et al.*, 2003). One of the first demonstrations of MSCs contribution to neuroectodermal cells *in vivo* was a study performed by Kopen *et al.* in which 5-bromo-2-deoxyuridine (BrdUrd) labelled muMSCs were injected directly in neonatal mouse brains. Injected cells were able to give rise to astrocytes (co-expressing BrdUrd and GFAP, glial fibrillary acidic protein) as soon as two weeks after transplantation (Kopen *et al.*, 1999). In a more recent study by Schuchman and his colleagues they performed direct intracerebral transplantation of MSCs in a progression of neurological disease mouse models (Jin *et al.*, 2002). The mice (knockout mouse model of types A and B Niemann-Pick disease, NPD; ASMKO mice) have a lysosomal storage disorder resulting from loss of acid sphingomyelinase (ASM) activity. Syngenic MSCs were transduced with a retroviral vector overexpressing ASM and were injected into the hippocampus and cerebellum of 3 weeks old ASMKO pups. The authors showed that transplanted cells were able to migrate away from the injection sites and survived at least 6 months after transplantation (Jin *et al.*, 2002). The most important finding of their study was the observation that most of the mice survived for more than 7 months, while the untreated animals did not. Although overall ASM activity detected in brain homogenates was low, surviving Purkinje cells contained the retrovirally expressed human enzyme and transplanted animals showed a reduction in cerebral sphingomyelin (Jin *et al.*, 2002). A follow-up study from the same group suggests that the degenerative microenvironment of Purkinje neurons in the NPD cerebellum modulates the cell fate switch of BM-MSCs via cell fusion (Bae *et al.*, 2005). Nevertheless, these results reveal for the first time the potential of treating neurodegenerative lysosomal storage disorders by intracerebral transplantation of bone marrow-derived MSCs.

In another study Dewaza *et al.* showed a highly efficient and specific induction of cells with neuronal characteristics, without glial differentiation, from both rat and human MSCs using gene transfection with Notch1

intracellular domain (NCID) and subsequent treatment with bFGF, forskolin and ciliary neurotrophic factor. MSCs expressed markers related to neural stem cells after transfection with NICD, and subsequent trophic factor administration induced neuronal cells. Some of them showed voltage-gated fast sodium and delayed rectifier potassium currents and action potentials compatible with characteristics of functional neurons (Dezawa *et al.*, 2004). Further treatment of the induced neuronal cells with glial cell line-derived neurotrophic factor (GDNF) increased the proportion of tyrosine hydroxylase-positive and dopamine-producing cells (Dezawa *et al.*, 2004). Moreover, the authors also showed that after transplantation of these GDNF-treated cells into a rat model of Parkinsons disease, there was an improvement in apomorphine-induced rotational behaviour and adjusting step and paw-reaching tests of the animals tested (Dezawa *et al.*, 2004).

Two reports have described the use of MSC transplantation in models of peripheral nerve injury. Dezawa *et al.* described the *in vitro* expression of the glial-cell markers p75, S100, GFAP and O4 by rat MSCs following exposure to a cocktail of growth factors and integration of such cells in the regenerating growth of the rat sciatic nerve. Myelination of regenerated fibres was recognised using confocal and immunoelectron microscopy on the transplanted animals (Dezawa *et al.*, 2001). Also, Cuevas *et al.* described the migration and differentiation of MSCs following injection of cultured undifferentiated cells into the site of a sciatic nerve axotomy repair (Cuevas *et al.*, 2002).

Finally, in the rat model of spinal cord injury, site-directed application of MSCs has been shown to improve the neurological outcome in two studies (Chopp et al., 2000; Deans and Moseley, 2000). In both studies, rare donor cells expressing neural markers can be identified, but the more likely effect is related to the production of trophic factors by the MSCs. A second interesting observation and possible mechanism is the formation of "guiding strands" that bridge the epicenter of the injury and promote directed growth of new axons (Hofstetter *et al.*, 2002).

1.5.5 - MSCs into hepatocytes and pneumocytes

It is important to mention that MSCs have also been shown to contribute to different types of epithelia *in vivo*. Although only less than a handful of studies have been reported to date, those that are available in the literature have shown quite convincingly that MSC produce such unorthodox potentials.

Ortiz *et al.* showed that after systemic administration of MSCs from male bleomycin (BLM)-resistant mice into female BLM-sensitive recipients, engrafted male cells were found to be localised in areas of BLM-induced injury and exhibited an epithelium-like morphology. Moreover, purification of type-II epithelial cells from the lungs of transplant recipients resulted in a 3-fold enrichment of donor-derived cells as compared with whole lung tissue (Ortiz *et al.*, 2003). The authors also showed that MSC administration immediately after exposure to the BLM also significantly reduced the degree of BLM-induced inflammation and collagen deposition within lung tissue (Ortiz *et al.*, 2003). Collectively, these studies demonstrate that murine MSCs home to the lung in response to injury, adopt an epithelium-like phenotype, and reduce inflammation and collagen deposition in lung tissue of mice challenged with BLM.

Hepatic contribution of MSCs was demonstrated in a xenotransplant setting. hMSCs were administrated directly to allylalcohol treated rat livers and hepatocyte-like cells were detected by immunostaining for human-specific alpha-fetoprotein (AFP), albumin (Alb), cytokeratin 19 (CK19), CK18 and asialoglycoprotein receptor (AGPR) and also confirmed by reverse transcription-polymerase chain reaction (RT-PCR) for expression of AFP (alpha-foetal protein) and Alb mRNA (Sato *et al.*, 2005). In this study, cell fusion was not likely to be involved since the authors showed that both human and rat chromosomes were independently identified by fluorescence *in situ* hybridisation (FISH) (Sato *et al.*, 2005). The differentiation appeared to follow the process of hepatic ontogeny, reprogramming of gene expression in

the genome of MSCs, as evidenced by expression of the AFP gene at an early stage and the albumin gene at a later stage (Sato *et al.*, 2005).

1.6 - Hierarchical organisation of MSC compartment

Some of the examples given previously suggest a non-linear relationship between the putative mesenchymal stem cell and its end-stage mature phenotypes. The concept of proliferative hierarchy has been developed to explain structured cell populations in a tissue, involving stem, committed and mature cells (Booth and Potten, 2000; Niemann and Watt, 2002; Weissman *et al.*, 2001). This concept, which (if) is applicable to the vast repertoire of bone marrow-derived mesenchymal stem/progenitors, is based on the assumption that proliferation, differentiation, and maturation are, in principle, independent. However, populations of committed cells can also divide and mature, showing intermediate properties between stem cells and functional mature cells. Therefore, the notion of stemness of what is currently defined as a mesenchymal stem/progenitor population is not a property of a particular cell-type, but a spectrum of capabilities of different cell-types within a population. A few reports have attempted to draw a scheme for a proliferative hierarchy in mesenchymal stem/progenitors, in which most of them have proposed a hierarchy for osteoprogenitors, involved in bone cell development (Gronthos *et al.*, 1999; Herbertson *et al.*, 1995; Richard et al., 1996), as discussed below.

The bone marrow MSC population shares some properties with fibroblastic cells such as expression of matrix proteins and, interestingly, some markers of myofibroblastic cells (by expressing smooth muscle actin; SMA) and some characteristics of endothelial cells such as endoglin and MUC-18 (Filshie *et al.*, 1998). Therefore, some authors claimed that the true "mesenchymal stem cell" can be isolated using rather standard procedures and characterised using a long list of indeterminate markers as just mentioned (Barry *et al.*, 2001; Deschaseaux *et al.*, 2003; Filshie *et al.*, 1998; Gronthos et al., 1995; Lodie et al., 2002). However, despite this putative "purification" and extensive characterisation, the resulting population was no

more "pure" than multi-colony-derived strains isolated by simple, short-term adherence to plastic (Barry *et al.*, 2001; Deschaseaux *et al.*, 2003; Caplan *et al.*, 1994; Filshie *et al.*, 1998; Gronthos *et al.*, 1995; Lodie *et al.*, 2002).

In summury, all data reported to date have strengthened the concept/speculation that cultures of bone marrow-derived MSCs are not homogenous, but consist of a mixture of uncommitted and committed progenitors exhibiting divergent differentiation properties. However, the ability to isolate the subset of marrow mesenchymal cells with the most extensive replication and differentiation potential would naturally be of utmost importance for both theoretical and applicative reasons.

1.6.1 - Uncommitted stem/progenitors in MSC cultures

When examining cultures of bone marrow-derived MSCs on the basis of cellular proliferative status, they appear to be non-homogenous. The work performed by Colter *et al.* has shown that in stationary cultures of bone marrow, MSCs contain a minor population of small and agranular cells (RS-1 cells) with a low capacity to generate colonies and are negative for the expression of the cell cycle-specific antigen Ki-67 (Colter *et al.*, 2000). Quiescent RS-1 cells express an antigenic profile that is different from that displayed by the most abundant, fast-growing and committed precursors found in expanded cultures of MSCs (matureMSCs). By studying a precursor-product relationship between RS-1 and other cell-types, the authors came to the conclusion that the high expansive capacity of matureMSCs depends on the presence of RS-1 cells (Colter *et al.*, 2000). Although it has not been proven, the authors suggested that RS-1 cells might cycle under stimulation of factors secreted by the most mature mesenchymal progenitor cells. Thus, it seems that RS-1 cells may represent an *ex vivo* subset of recycling uncommitted cells (Colter *et al.*, 2000).

Additionally cells in the quiescent state seem to represent a population of uncommitted mesenchymal cells, since they do not express the osteogenic and adipogenic commitment markers (Colter *et al.*, 2000); Pittenger *et al.*, 1999) Cbfa-1 and PPAR-γ, respectively. Also, after prolonged

exposure to foetal bovine serum (FBS), the slow-proliferating quiescent cells give rise to committed precursors that grow quickly and terminally differentiate (Pittenger *et al.*, 1999).

Additional evidence came from studies using isolated cell populations derived from foetal rat calvaria. The isolated clonally derived population, clone RCJ 3.1, can differentiate in a time-dependent sequence into four mesenchymal phenotypes. This progression, which was elicited by stimulating the cells only with osteogenic differentiation medium, gave rise to multinucleated muscle cells at day-9, adipocytes at day-12, chondrocytes at day-16, and mineralised bone nodules at day-21 (Grigoriadis *et al.*,1990). Together, it has been shown that a population of cells from foetal rat periosteum, isolated on the basis of granularity (S cells), exhibit various properties of uncommitted mesenchymal progenitors. Thus, S cells are slow cycling, do not express differentiation-associated markers, and when grown in culture, generate cartilage, adipose, smooth muscle, and bone phenotypes (Ghilzon *et al.*, 1999; Zohar *et al.*, 1997). Altogether, these findings demonstrate that despite *ex vivo* manipulation and subcultivation, cultures of so-called "MSCs" contain a rare subset of uncommitted cells displaying features of true progenitor or stem cell features.

1.6.2 - Committed progenitors in MSC cultures

In addition to uncommitted mesenchymal progenitors, several groups of committed progenitors can be found in cultures of bone marrow-derived MSCs. Muraglia *et al.* investigated the differentiation potential of different clonally derived committed progenitor populations and demonstrated that approximately 30% of all clones exhibit a tri-lineage (osteogenic/chondrogenic/adipogenic) differentiation potential, while the rest exhibit either a bi-lineage (osteogenic/chondrogenic) or a pure osteogenic potential (Muraglia *et al.*, 2000). Clones with a differentiation potential limited to the osteogenic/adipogenic or to the chondrogenic/adipogenic phenotypes, as well as pure chondrogenic and adipogenic clones, were not seen

(Muraglia *et al.*, 2000). These observations have been extended by other studies showing the existence of a quadripotential mesenchymal progenitor (clone BMC9), which under appropriate conditions differentiates into osteoblasts, chondrocytes, adipocytes and haematopoietic-supporting stroma (Dennis *et al.*, 1999).

Other experimental approaches have been followed to gain insight into the characteristics and differentiation potential of bone-resident mesenchymal progenitors. In one of these studies, four cellular subsets were sorted from primary cultures of normal human bone, according to the differential pattern of expression of the stromal precursor cell marker Stro-1 and the osteoblastic marker alkaline phosphatase (Gronthos *et al.,* 1999). The Stro-1^{pos}/AP^{neg} subset exhibited a pre-osteoblastic phenotype, as evidenced by a reduced ability to form a mineralised bone matrix and by the lack of expression of bone sialoprotein, osteopontin and parathyroid hormone receptor (Gronthos *et al.,* 1999). The other subsets correspond to intermediate and fully differentiated osteoblasts. As expected, after sorting and re-culturing, only cells in the Stro-1^{pos}/AP^{neg} subpopulation were able to give rise to all of the four subsets of Stro-1/AP cells present in the primary culture (Gronthos *et al.,* 1999). Thus, these results have demonstrated that cultures of human bone are not homogenous, but on the contrary, they include committed osteoprogenitors as well as end-stage differentiated osteoblasts.

1.6.3 - Multipotent Progenitor Cells in BM

Despite the valuable efforts made by many groups to identify and isolate the "true" multipotent stem cell from adult BM, to date the putative MSC with the capacity of differentiating into different cell-types both *in vitro* and *in vivo* (not only to the conventional mesenchymal lineages but also to visceral mesoderm, endothelial and neuroectoderm cell-types) is yet to be found *in vivo*. From all the studies reported, it seems that the closest candidate is the RS-1 cells described by Prockop's group. However, the

multipotent capacity of these cells both *in vitro* and *in vivo* into different cell-types of the three germ-line layers, remains to be shown.

Verfaillie's group has recently described a cell-type that has not only multi- but also pluripotent features. These cells, termed multipotent adult progenitor cells (MAPCs), express markers of ES cells such as Rex-1, Oct-4 and SSEA-1. These cells were obtained by initial co-purification with MSCs and grow as adherent cells *in vitro* from postnatal marrow (and other organs) of mice, rats and humans (Jiang *et al.*, 2002a and 2002b). However, unlike MSCs, MAPCs can be cultured for more than 120 population doublings *in vitro* in a relatively nutrient-poor medium (Jiang *et al.*, 2002a) with expression of telomerase and maintenance of telomere length (Reyes *et al.*, 2001).

Clonal populations of MAPCs were established and could be differentiated into endothelium by culturing with VEGF with the acquisition of functional features *in vitro* (Reyes *et al.*, 2002). Endodermal cell-types induced by FGF-4 and HGF showed functional characteristics of hepatocytes. These differentiated cells expressed the early hepatocytic markers HNF-1, HNF-3 and GATA-4 after 4 days and the late hepatic markers CK18 and albumin after 14 days post-induction (Schwartz *et al.*, 2002). A convincing demonstration of the functionality of the cells was provided by different assays such as urea and albumin productions, cytochrome activity, LDL (low density lipoprotein) uptake and gluconeogenesis (Schwartz *et al.*, 2002). When murine MAPCs were cultured with a series of bFGF, EGF and other neurotrophic factors, different mature neuronal phenotypes could be observed (Jiang *et al.,* 2003). Thorough analysis revealed that some of the cells expressed markers of dopaminergic neurons, others expressed markers of serotonergic and GABAnergic neurons. Co-culture of these cells with primary mouse astrocytes further matured the cells and led to a more elaborate array of axons. In a series of patch-clamp recordings the authors showed occurrence of spiking behaviour that could be attributed to voltage-gated sodium channels and also suggested the occurrence of synaptic events (Jiang *et al.,* 2003).

MAPCs also display their broad differentiation potential *in vivo*. For these assays, MAPCs were derived from ROSA 26 mice, which express

galactosidase under a ubiquitous promoter, so that cells from these animals could be tracked. ROSA-26-derived MAPCs injected into murine blastocysts resulted in chimeric mice with ROSA-26 cells contributing to nearly all somatic tissues, including brain, lung, myocardium, liver, intestine and kidney (Jiang *et al.,* 2002a). After intravenous administration into a sublethally irradiated immunodeficient mouse, MAPCs differentiated to varying degrees into haematopoietic cells in the marrow, blood and spleen and into epithelial cells in the liver, lung, and intestine. However, it is not yet clear whether MAPCs are a distinct, rare subpopulation of MSCs normally present or whether their *in vivo* and *in vitro* potential are a phenomenon developed under unique *in vitro* cell culture conditions.

Cell populations with comparable potentials have been isolated/expanded from cord-blood cells by Kogler *et al.* and BM cells under hypoxic culture-conditions by D'Ippolito *et al.* In the first case, the authors not only showed that these cord blood derived multipotent cells have differentiation capacities *in vitro*, but also *in vivo*, including haematopoietic reconstitution (Kogler *et al*, 2004). However, in both studies, cells were not prospectively identified/isolated and therefore their true existence remains to be proven (D'Ippolito *et al.*, 2003; Kogler *et al*, 2004).

1.7 - Aims of the thesis

All the studies reported and described here have shown that a putative stem, or very primitive progenitor, might exist in the bone marrow derived adherent cultures (and most possibly *in vivo*), but it is still yet to be identified and characterised. The aim of this thesis was to further examine the murine mesenchymal cultures with the objective to identify this "true" stem cell and provide a detailed characterisation of it.

Chapter 2 - Materials and Methods

2.1 - Isolation, enrichment and expansion of muMSCs

Bone marrow cells were collected by flushing the femurs, tibias and iliac crests from 6 to 12 weeks old of Non-Obese-Diabetic/Severe-Combine-Immunodeficient (NOD/SCID), NOD/SCID-β_2^{null} and C57BL6/J mice with phosphate-buffered saline (PBS; CRUK central cell services) supplemented with 2% of foetal bovine serum (FBS; Gibco, Paisley, UK; PBS-2%). Bone marrow cell suspensions were submitted to red cell-lysis using 3x the initial volume of ammonium chloride solution (NH_4Cl; Stem Cell Technologies, Vancouver, Canada) for 10 min on ice. After this time, FBS was added to the suspensions to neutralize the NH_4Cl. Red cell-depleted bone marrow mononuclear cells (BMMNCs) were plated at a density of 10^6 cells/cm^2 in Murine Mesenchymal Medium with Murine Mesenchymal Supplements (Stem Cell Technologies), further supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (pen/strep, Gibco). For a 25-cm^2 tissue culture flask, 5 ml of medium was used and the volume of medium was changed proportionally according to the area of the flasks/plates used. Non-adherent cells were eliminated by a half medium change at day 3 and the whole medium was replaced weekly with fresh medium.

The cells were grown for 2 to 3 weeks until attaining almost confluency. The whole adherent fraction was then detached by trypsinization using a 0.25% trypsin/versine solution (CRUK, central cell services) for 5-10 min at 37 °C. Once detached, FBS was added to the cell suspensions to neutralize the reaction and cells were collected, spun down at 1300 rpm for 7 min and the supernatant was discarded (unless stated, these centrifugation conditions were applied for all methodologies concerning "cell washing and passing"). Cells were then re-plated using a 1:3 dilution factor. Subsequent passing and seeding of the cells was performed at a density of 5000 cells/cm^2. The passage number of cultured (unpurified, Px or enriched, purPx) MSCs refers to the number of times that the cells have been

trypsinized. To enrich MSCs, adherent cells from passage 2 (P2) and 3 (P3) were stained with rat anti-mouse CD45-CyChrome and CD11b-PE or a combination of CD45 and biotin-conjugated Lineage (Lin) Cocktail Antibodies followed by streptavidin-PE (see in the Appendix III section for information on the antibodies used). To eliminate the hematopoietic contaminants, the negative fraction from both cell surface antigens was sorted using the fluorescence activated cell sorter Vantage (FACS; Becton-Dickinson, Oxford, UK).

2.2 - Isolation and expansion of SSEA-1^{pos}-MSCs

Six weeks old NOD/SCIDs were used to isolate SSEA-1^{pos} -MSCs in a similar fashion as described above. SSEA-1^{pos} cells were isolated from cultures already devoid of hematopoietic contaminants. Isolation of SSEA-1^{pos}-MSCs cells was performed using FACS sorting after the cells had been labelled with SSEA-1 antibody followed by anti-mouse IgM-PE antibody. For SSEA-1^{pos}-MSCs enrichment, anti-PE immunomagnetic beads were used according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). For clonal analysis, single SSEA-1^{pos} cells were sorted from the CD45/CD11b^{neg} fraction of passage 1 (P1) of adherent cultures into single wells of a 96-well plate. To determine whether SSEA-1^{pos}-MSCs could be isolated prospectively from mouse bone marrow, lineage negative cells were obtained first from BMMNCs using the Murine Lineage Cocktail from Stem Cell Technologies according to the manufacturer's instructions. Lineage (Lin) negative cells were further stained with anti-CD31, anti-CD45 and anti-SSEA-1 and cells were analysed based on Lin^{neg}/CD45^{neg}/CD31^{neg} and SSEA-1 ^{pos} expressions.

Culturing SSEA-1^{pos}-MSCs in MAPC conditions was performed as described by others (Jiang et al., 2002 and 2003). Briefly, SSEA-1^{pos}-MSCs were cultured on 1 $\mu g/cm^2$ of fibronectin (Sigma, Dorset, UK) and cells were maintained at a density of 500 to 2500 cells/cm^2. For a 25-cm^2 tissue culture flask, 5 ml of medium was used and the volume of medium was changed proportionally according to the area of the flasks/plates used. MAPC medium

consisted of 60% DMEM-Low Glucose (Dulbecco's modified Eagle medium, Gibco) and 40% MCDB-201 containing: 1x insulin-transferrin-selenium (ITS), 1x linoleic-acid-bovine-serum-albumin (LA-BSA), 10^{-9} M dex, 10^{-4} M ascorbic acid 2-phosphate (all from Sigma), 2% FBS, 1000 units/mL of mLIF (murine Leukemia Inhibitory Factor; Chemicon, Temecula, CA, USA), 10 ng/mL of hPDGF-BB and 10 ng/mL of mEGF (both from Peprotech, London, UK).

2.3 - Immunostaining of cell surface and intracellular antigens for flow cytometry analysis

For cell surface antigens, aliquots of different cell suspensions (50 µl containing 10^4 to 10^6 cells) were stained with antibodies (see Appendix III for antibody concentrations used) at 4°C for 30 min. After this period, cells were washed with 2 ml of PBS-2% (unless stated, a similar volume of washing solution was applied for all methods concerning "cell washing"). When secondary and tertiary incubation steps were required, cells were stained in a similar fashion with two washes in between steps. Finally, cells were resuspended in 300-500 µl of PBS-2% containing DAPI (4',6-diamidino-2-phenylindole; Molecular Probes, Eugene, Oregon, US) at the final concentration of 10 ng/ml, for exclusion of dead cells.

For intracellular antigens, aliquots of different cell suspensions (50 µl containing 10^5 to 10^6 cells) were fixed by adding 500-1000 µl (depending on the cell suspension concentration) of a 2% PFA (paraformaldehyde; Sigma) solution for 10 min at RT (room temperature) then permealized with PBS containing 0.1% Triton-X-100 (using same volume as fixation step; Sigma) for 10 min at RT. Cells were washed twice between steps. Each antibody incubation step was performed at RT for 30 min or 1 h for cytoplasmic or nuclear antigens respectively. Cells were then washed twice and resuspended in 300-500 µl of PBS-2% containing DAPI (at the final concentration of 10 ng/ml), to visualise the cell populations and exclusion of debris. The analysis was performed using an LSR (Becton-Dickinson).

2.4 - Colony Forming Units-Fibroblast (CFU-F) assay

Red-cell depleted BMMNCs from NOD/SCID, NOD/SCID-β_2^{null} and C57B6/J mice were plated at a density of $5x10^5$ cells/cm^2 and cultures were maintained as described in Chapter-2.1. On day 14, the medium was removed from the wells, washed twice with PBS and fixed in methanol for 10 min at RT. Giemsa stain (CRUK, central cell services) was applied to the cultures at RT for 5 min, rinsed with distilled water (dH_20) and colonies were counted microscopically.

2.5 - *In vitro* lentivirus-mediated gene (eGFP) transfer into muMSCs

2.5.1 - General description of lentivirus used

Transduction of MSCs was performed with an HIV-1-based self-inactivating (SIN) lentiviral vector (pHRSINcPPT-SEW), which carries the eGFP (green fluorescent protein) reporter gene under the control of the spleen focus-forming virus (SFFV) LTR (gift from Prof. A. Thrasher, Institute Child of Health, London-UK). Research Fellow Dr Elena Siapati performed all plasmid preparations used in these studies. Briefly, three types of plasmids were used to transiently transfect a producer cell line (293T). The plasmids consisted of: the first expressing the core proteins and enzymes essential for viral replication, reverse transcription and integration (pHRSINcPPT-SEW), the second encoding the pseudotype envelope protein (VSVG; vesicular stomatitis Virus G envelope, pMDG), and the third containing the *cis*-acting sequences required for optimal packaging, reverse transcription, and integration of the transgene (p8.91).

2.5.2 - Brief description of plasmid preparation

L-ampicillin-agar plates (CRUK, central cell services) with transformed bacteria (by caesium chloride method) containing the different types of plasmids, already available in the laboratory, were used for DNA plasmid preparation. A single colony from each plate was removed with the aid of a loop and inoculated in 2 ml of fresh starter culture medium (L-Broth; CRUK central cell services) containing the selective antibiotic (L-ampicillin at concentration of 50 μg/ml) and incubated for overnight (ON) at 37 °C in a 300 rpm shaker. Next the whole amount of inoculum was diluted with a litre of culture medium (a 1:500 dilution) and then left to grow for ON using the same conditions. At the end of the incubation time, the bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4 °C and the pellet was used for DNA plasmid extraction. All plasmid extraction was performed using the Qiagen Plasmid Mega kit according to the manufacturer's instructions.

2.5.3 - Brief description of lentivirus preparation

A total of 10^7 293T cells were seeded in one 150-cm^2 flask ON prior to transfection. Cells were cultured in DMEM with 10% FBS, pen/strep, and the medium was changed 2 h prior to transfection. A total of 100 μg of plasmid DNA was used for the transfection of one flask: 17.5 μg of pMDG, 32.5 μg of p8.91 and 50 μg of transfer vector plasmid were added to 5 ml of OptiMEM (Gibco) and filtered through a 0.2 μm filter. 2.5 μl of 1M PEI (polyethyleneimine, Sigma) was added in 5 ml of OptiMEM and filtered through a 0.2 μm filter and the two 5 ml solutions were added together and left at RT for 20 min.

The cells were washed once in OptiMEM and the DNA plus PEI complexes were then added to them. After 4 h of incubation, the medium was replaced by fresh complete medium. At 24 h post-transfection, the medium was replaced sufficiently enough to cover the cells. On the following day, medium was collected and centrifuged at 2500 rpm for 10 min at 4 °C

and filtered using a 0.45 μm filter. Vector particles were then concentrated 20- to 100-fold by ultracentrifugation at 23000 rpm for 2-3 h at 4°C. The supernatant was carefully decanted off and the pellet was resuspended in 50 μl serum-free X-VIVO-10 medium (BioWhittakerEurope, Verviers, Belgium) and kept at -80°C until use. Titration of the virus was carried out using HeLa cell line. Briefly, 50000 cells were seeded per well of a 12-well plate in 1 ml of complete DMEM and allowed 4 h to adhere. An aliquot of 30 μl of virus was added to a final volume of 1.5 ml of complete DMEM. From this 1:50 starting dilution, virus particles were diluted until 1:50000 dilution. To the first well of the 12-well plate, 1 ml of unconcentrated virus was added (1:2 dilution) and in the second well 0.2 ml of unconcentrated virus was added with 0.8 ml of medium (1:10 dilution). To the second row of the plate, 1 ml of each serial dilution of virus was added (1:100, 1000, 10000 and 100000). Two wells without medium were used as a negative control. The plates were placed in the incubator for two days before the cells were detached and analysed by flow cytometry. The titre of the virus was determined as follows:
(% of positive cells) x (dilution) x (n° of cells infected)/100

2.5.4 - Lentivirus transduction into muMSC

For transduction, $1x10^4$ purified MSCs from passage 4 (purP4) were seeded into individual wells of a 12-well plate. On the following day, virus particles were added at multiplicity of infection (M.O.I) of 5, 10, 30 or 50 and transduction was performed for 20 h. Cells were harvested on day 1, 3 and 5 after virus removal and analysed for eGFP expression by flow cytometry.

2.6 - Cell cycle analysis with Hoechst/PyroninY (Hst/PY)

Different cell-types were resuspended at a density of 10^6 cells/ml in Hst buffer containing 5 nmol/ml of Hst (Hoechst33342; Sigma). After incubation at 37 °C for 45 min, pyronin-Y (PY) (Sigma) was added to a final concentration of 2.5 μg/ml for an additional 45 min. Cells were washed once,

resuspended in Hst buffer and then analysed. In some cases, antibody staining was performed after the Hst/PY step and was carried out in Hst buffer containing the two dyes. In this case, cell suspensions were washed with Hst buffer. Pulse processing was used in order to exclude any unstained, apoptotic or clumped cells and analysis was performed using the LSR.

2.7 - *In vitro* differentiation culture conditions

MSCs were plated on cover slips at a density of 3000 to 5000 in mesenchymal medium for 24h. In studies using enriched SSEA-1^{pos}-MSCs and clonally derived SSEA-1^{pos}-MSC, cells were plated at a density of 5000 to 10000 cells/cm^2 in MAPC expansion medium for 24h. With the exception of chondrogenic differentiation, in all other cases, after attachment the cells were washed once with basic differentiation medium (consisting of DMEM-low glucose supplemented with 2% FBS and pen/strep) and then replaced with basic differentiation medium containing specific differentiation supplements. Each specific differentiation medium was changed every 2 to 3 days and cultures were kept for 14 days with the exception of chondrogenic differentiation, in which cells were incubated for 3 weeks. Each specific differentiation condition was as follows (all chemicals and cytokines were purchased from Sigma and Peprotech respectively):

-<u>osteogenic:</u> 50 mM ascorbic acid 2-phosphate, 100 nM Dex and 10 mM b-glycerophosphate;

-<u>adipogenic:</u> 1μM Dex, 50 μM indomethacin, 500 nM 3-isobutyl-1-methylxanthine and 5 ug/ml of insulin or 5% Horse Serum (HS);

-<u>chondrogenic</u>: 10^5 to 10^6 cells were pelleted in a 15 ml-conical polypropylene tube, supernatant discarded and medium was added slowly to the pellet to avoid disturbance. Serum free medium consisted of: DMEM-high glucose, 6.25 ug/ml of insulin, 6.25 ug/ml of transferrin, 6.25 ug/ml of

selenite, 5.33 ug/ml of linolite, 1.25 mg/ml of BSA, 100 nM Dex, 50 ug/ml of ascorbic acid 2-phosphate and 10 ng/ml of $TGF\beta_3$ (transforming growth factor-β3);

-myogenic: 10 μM 5'-azacytidine, 50 μM hydrocortisone and 5% HS for an initial 24 h period. Cultures were then maintained in the same conditions without 5'-azacytidine;

-visceral mesoderm (hepatocyte-like cells): 10 ng/ml of HGF and FGF-4
-endothelium: 10 ng/ml of VEGF;

-neuroectoderm (astrocyte/neuronal-like cells): 50 ng/ml of bFGF and 20 ng/ml of mEGF.

2.8 - Assessment of *in vitro* differentiation potential of different populations of MSCs

2.8.1 - Immunostaining

Cells from different populations of MSC were first washed twice with PBS and then fixed in PBS with 4% PFA at RT for 10 min or in ice-cold methanol (for structural cytoplasmic antigens) for 2 min. After this time, cells were permeabilized with PBS containing 0.1% Triton-X-100 for 10 min. Fixed cultures were blocked with PBS with 10% serum (rat and/or goat) for 30 min at RT and sequentially stained with primary antibodies at 4°C ON, followed by appropriate secondary antibodies for 1h at RT. In-between all steps, cells were washed twice with PBS containing 2% of bovine serum albumin (BSA; Sigma). The amount of each reagent and washing solution added per well (for a 12-well plate) was approximately 500 μl and 2 ml respectively. When both extracellular and intracellular antigen detection was required at the same time, cells were firstly fixed as above, and then stained for the cell surface antigen for 1h at RT for each antibody-staining step. Cultures were

then permeabilized and stained for intracellular proteins in a similar fashion as described. Finally, cover slips were mounted with Fluorescence Mounting Medium (Dako, Cambridgeshire, UK) containing DAPI for nuclear staining.

2.8.2 - Immunocytochemistry

2.8.2.1 - Alkaline Phosphatase detection

Alkaline phosphatase activity was determined as recommended by the manufacturer's instructions contained in Sigma kit #85.

2.8.2.2 - Oil Red O staining

Firstly, medium was aspirated off and cultures were washed twice with PBS and fixed in 10% Neutral Buffered Formaldehyde (NBF) for 30 min at RT. Formalin solution was then removed and cultures were rinsed with dH_20 and then 60% isopropanol was added for 5 min. Isopropanol was then poured off and Oil Red O (Sigma) working solution (3 parts of Oil Red stock solution with 2 parts of dH_20) was added to the fixed cultures for 10 min. After this time, cultures were rinsed with tap water until the water was clear, counterstained with Mayer's hematoxylin (Sigma) for 1 min, rinsed with dH_2O and mounted with Glycergel Mounting Medium (Dako).

2.8.2.3 - Chondrogenic pellet processing and staining

Cell pellet was fixed in 10% NBF for 1-24h at RT then immersed into 70% ethanol until embedded. The technical staff from the CRUK Histopathology Unit performed pellet embedding and sectioning. Each embedded pellet was cut into 10 μm thick sections. Different types of staining were used for chondrogenic differentiation assessment: Alcian Blue, Alizarin Red S and Safranin O (all solutions were prepared by the staff of the Histopathology Unit). Firstly, sections were de-paraffinized by immersing in

2x xylene for 10 min, followed by sequential hydrating steps in 4 grades of ethanol (100%, 95%, 80%, 70% and 50%) for 2 min each.

For Alcian Blue staining, sections were hydrated to water, stained with Alcian Blue solution for 5 min, rinsed with water and counterstained with 1% neutral red solution for 1 min. At the end, slides were blot dried, differentiated in 100% ethanol, cleared in xylene and mounted with DePex Mounting Medium (BDH, Leicestershire, UK).

For Alizarin Red S staining, staining solution was applied for 30 sec to 5 min, blot dried, differentiated in acetone (20 dips), cleared in xylene and mounted with DePex.

For Safranin O, slides were sequentially immersed in a 0.02% Fast Green solution for 3 min, 1% acetic acid solution for 30 sec and 0.1% Safranin O solution for 5 min. Slides were dehydrated in sequential grades of ethanol (70%, 90% and 100%), cleared in xylene and mounted with DePex.

2.9 - Assessment of *in vitro* functionality of MCSs

2.9.1 - Alkaline phosphatase activity assay

Cells were collected by trypsinization, washed once with PBS and pelleted. Cell pellets were lysed in 100 μl of AP lysis buffer for 10 min at RT. Lysates were spun down for 15 min at 1500 rpm and supernatants were collected. Enzyme activity was assayed by adding 200 μl of the working solution from the Red Microwell Kit (Sigma) to 10 μl of each sample, for 30 min at 37 °C, in a single well of a flat 96-well plate. Each supernatant was assayed in triplicate and absorbance was measured at 490 nm. AP activity was determined by assuming that 1 O.D is equivalent to 64 nmol of p-nitrophenol (Phinney *et al.*, 1999). Protein content was determined in aliquots of each lysate using the conventional Bradford assay.

2.9.2 - Calcium quantification assay

Calcium extraction was done by scraping the culture plates with 0.5 M of HCl (choric acid), then shaking for 4h at 4 °C (500 μl for one well of a 12-well plate). Lysates were then spun down at 3500 rpm for 5 min and supernatants were collected. Calcium quantification was assayed by adding 200 μl of the working solution from the Calcium Calometric Kit (Sigma) to 10 μl of each sample, for 15 min at RT, in a single well of a flat 96-well plate. Each supernatant was assayed in triplicate and absorbance was measured at 575 nm. A standard curve with known concentrations of calcium chloride ($CaCl_2$) was used to plot the absorbance values. Protein content was determined in aliquots of each lysate using the Bradford assay.

2.9.3 - IL-1α treatment

Cells were incubated with 100 U/ml of IL-1α (interleukine-1α, Peprotech) in serum-free medium for 6h. Cells were then detached from the plates, collected and stained with antibodies against MHC-I, ICAM-1, VCAM-1 and P-selectin for flow cytometry analysis.

2.9.4 - *In vitro* angiogenesis assay

Preparation of the extracellular matrix was done using a flat 96-well plate according to the manufacturer's instructions (Chemicon). Cells were first detached from their original plates by trypsinization and resuspended in serum-free medium containing 10 ng/ml of VEGF at the final concentration of $2x10^6$ cell/ml. 50 μl from each cell suspension was added to a single well and cells were incubated for 6-8h at 37°C.

2.9.5 - Uptake of Low-Density Lipoprotein

Dil-Ac-LDL staining kit was purchased from Biomedical Technologies (Stoughton, MA, US) and the assay was performed according to the manufacturer's instructions.

2.9.6 - Periodic acid-Schiff (PAS) assay

PAS assay was used to determine glycogen deposition in cells. Cultures were washed twice with PBS, oxidized in 1% of periodic acid solution (BDH) for 5 min and rinsed thrice with dH_2O. Afterwards, slides were treated with Schiff's reagent (BDH) for 15 min, rinsed with dH2O for 5-10 min, stained with hematoxylin for 1 min, rinsed with dH_2O and mounted with Glycergel Mounting Medium.

2.6.7 - Pentoxyresorufin O-Dealkylase (PROD) assay for Cytochome-P450 activity

Different cell-types were maintained in their respective conditions in the presence or absence of 1 mM of Phenobarbital (Sigma) for 48 h followed by treatment with 10 μM of pentoxyresorufin (Sigma) for 48h. After that period cells were washed twice with PBS and assessed using a fluorescence microscope.

2.9.8 - Measurement of cytoplasmic Ca^{2+} increase

Cells were assayed for their response to different neurotransmitters by measuring cytoplasmic calcium fluxes. Indo-1 acetoxymethyl ester (Indo-1 AM, Molecular Probes) stock solution was prepared in DMSO (Sigma) to a concentration of 1 mM. Aliquots of the solution were added to the cell suspensions (un- and differentiated cells; 10^6 cells/ml) to a final

concentration of 1 μM and incubated at 37°C for 1h. After loading, cells were washed twice in serum-free PBS, resuspended at 10^6 cells/ml and analysed on the LSR. Specific substances (50 mM of potassium, K^+; 50 mM of histamine; 50 mM of glutamate; all from Sigma) were added ~30 sec after fluorescence reading. The basal values were taken from the mean fluorescence levels before drug addition. The filter set-up on the LSR for Indo-1 (UV excitation only) is for calcium-bound Indo-1 violet FL-5 424/44 nm BF filter and unbound Indo-1 green FL-4 530/30nm BF filter. Calcium flux was measured as a ratio between calcium bound Indo-1 and unbound or FL-5/FL-4 versus time. Full-scale deflection of the calcium flux was measured by the addition of Ionomycin (10 μg/ml; Molecular Probes).

2.9.9 - ^{3}H-glutamate uptake

^{3}H-glutamate uptake was determined as described by others (Duan *et al.*, 1999; Stanimirovic *et al.*, 1999). Briefly, cultures grown in 24-well plates were washed twice with PBS and then equilibrated in Kreb's buffer (or similar buffer with same molarity of choline-Cl as sodium substitute, Na^+) for 20 min at 37°C. 1 μCi/ml of L-[G-^{3}H] glutamic acid (Amersham Biosciences, Buckinghamshire, UK) (at final concentration of 1 mM) was then added to each well and cells were incubated at 37°C for 1h. The reaction was stopped by rapid washing twice with ice-cold PBS. Cells were then lysed with 125 μl of 0.1% Triton-X-100 solution plus sodium hydroxide (NaOH; at final concentration of 0.1 M), for a few minutes then neutralized with HCl. The radioactivity present in cell lysates was measured by adding 100 μl of each lysate into 5 ml of the Betaplate Scint scintillation fluid (Wallac, Milton Keynes, UK) and scintillation was counted using the Beckman LS6500 counter (Beckman Coulter, High Wycombe, Buckinghamshire, UK). Aliquots of the lysates were also assayed for protein content.

2.9.10 - Glutamine synthetase (GS) activity-colorimetric assay

This assay is based upon the formation of γ-glutamylhydroxamate from glutamine and hydroxylamine. A coloured complex is then formed with ferric chloride, which can be measured colometrically. This is a measure of glutamyltransferase which as been shown to reflect GS activity (Duan *et al.*, 1999; Stanimirovic *et al.*, 1999). Cell suspensions were washed twice in cold PBS and resuspended in GS extraction buffer. Then cells were disrupted by sonication (4x 15 s burst at an amplitude of 7-8 nm peak-to-peak) with cooling intervals of 1 min. After centrifugation at 7600 rpm for 5 min, supernatants were assayed for protein content or for enzyme activity. Cell extracts (100 μl) were incubated at 37°C for 30 min with 400 μl of GS solution. The reaction was terminated by adding 1 μl of ferric chloride ($FeCl_3$) reagent and absorbance was measured at 535 nm. A standard curve with known concentrations of γ-glutamylhydroxamate (Sigma) was used to plot the absorbance values.

2.10 - Transplantation of eGFP-MSCs and clonally derived SSEA-1^{pos}-MSCs

A total of 41 NOD/SCID mice aged between 8 to 12 weeks old were used in two independent *in vivo* studies. All animal procedures were performed in accordance with the CRUK institutional and UK Home Office guidelines. eGFP-MSCs ($2x10^6$), obtained few days post-viral removal to minimize further expansion, were delivered intravenously (i.v.) by tail vein injection into each sublethally irradiated mouse (375 cGy using a ^{137}Cs source). Mice were then sacrificed on different days and bone marrow and specific tissues/organs were collected. In most of the cases, half of each tissue was fixed in 10% NBF ON at 4 ºC. On the following day, tissues were washed and equilibrated in 50% ethanol for 1h and kept in 75% ethanol at 4 ºC until being embedded). Other halves were kept at -20 ºC for further RNA/DNA extraction. In some cases, small samples of each tissue were also

cryo-embedded by snap freezing into dry-ice- or liquid nitrogen- cooled 2-methylbutane (Sigma) and kept in -80 °C until being processed.

For experiments concerning clonally derived cells, undifferentiated SSEA-1^{pos} clone-3 cells ($2x10^5$; at 28 doublings) were injected intravenously (facial vein) into five 2 days old NOD-SCID-β_2^{null} mice. Animals were maintained for 8 weeks and then sacrificed and bone marrow and specific tissues/organs were collected by snap freezing.

2.11 - Detection of donor-derived cells upon transplantation

2.11.1 - Detection of eGFP transgene by PCR

Genomic DNA was isolated from each tissue by lysing in buffer containing SDS (sodium dedecylsulphate) and Proteinase K (PK; all from Sigma) followed by phenol-chloroform extraction. After being thawed from RNAlater a small piece of each tissue, (~20 mg) was dissected and washed twice with 1 ml of PBS. Supernatant was removed and 600 μl of SNET Lysis Buffer with PK at a final concentration of 500 mg/ml was added and tissues were digested ON at 55 °C with mild shaking (900 rpm). On the following day, digested tissue was allowed to cool down to RT, before 4 μl of 100 μg/ml Rnase (Qiagen) was added and incubated for 30 min at 37 °C. An equal volume of phenol:chloroform:isopentane (25:24:1; Sigma) was added and vortexed for 20 sec. 550 μl of the supernatant was transferred to a new tube and an equal volume of chloroform was added. The mixture was then vortexed for 10 sec, followed by centrifugation at maximum speed (~14000 rpm; max) for 10 min at RT. Supernatant was collected and transferred to a new tube with 0.3 volumes of 3 M of sodium acetate (pH 5.2) and 2 volumes of ethanol. This was quickly inverted for 5 min for DNA precipitation and then centrifuged at RT at max speed. After this time, the supernatant was removed and DNA was washed with 1 ml of cold 70% ethanol and centrifuged for 5-10 min at max speed. Ethanol was then removed and the

pellet was let to dry for 15 min before DNA was dissolved in 100 µl of dH_20 on a rotating shaker.

For each 50 µl PCR reaction, 250 ng of genomic DNA was used. Amplification was performed using the Taq PCR Core Kit (Qiagen) according to the manufacturer's instructions. Set of primers and their respective annealing temperatures and number of amplification cycles information can be found in the Appendix II section. Murine glyceraldehyde-3-phosphate dehydrogenase (muGAPDH) was used as an internal PCR control.

2.11.2 - Tissue Processing and Immunohistochemistry

All tissues were embedded and sectioned by the technical staff from the CRUK Histopathology Unit. Each embedded tissue was sectioned between 10 to 15 levels with a 70 to 100 µm gap between each one. Each level was serially sectioned at least 4 times. Paraffin sections (4 µm thick) were first de-paraffinized by immersing in Histoclear (RA Lamb, East Sussex, UK) twice for 10 min each, followed by sequential hydrating steps in four decreasing grades of ethanol for 2 min. Tissue sections were immersed in water before submitting to antigen retrieval by microwaving for 10 min in Antigen Unmasking working solution (Vector Laboratories, Peterborough, UK; 3.75 ml in 400 ml dH_20). After this time, tissue sections were washed twice with dH_2O and quenched for endogenous peroxidase for 5 min (PBS with 2% H_2O_2; hydrogen peroxide) and when necessary, for endogenous alkaline phosphatase also for 5 min (methanol with 20% acetic acid). Non-specific staining was blocked with PBS with 10% serum (rat and/or goat) for 30 min RT and when biotin-labeled secondary antibodies were used, endogenous biotin was blocked with the Biotin Blocking System Kit (Dako). Staining with primary antibodies was performed at 4°C ON, followed by appropriate secondary antibodies for 1h at RT. When biotin-labelled antibodies were used, an additional incubation step with streptavidin or avidin-conjugated reagent was performed at RT for 30 min. In-between all steps, tissue sections were washed twice with PBS with 2% of BSA. Immunoreactivity was detected using different peroxidase-based systems or

alkaline phosphatase-based systems, or a combination of both according to the manufacturer's instructions (Vector Laboratories).

Sections were then washed twice in water for 3 min, counterstained with hematoxylin for 2 min, followed by an additional two washes with water. Finally, sections were dehydrated twice for 2 min in 95% ethanol, twice for 2 min in 100% ethanol and twice for 10 min in Histoclear. Slides were mounted with DePex Mounting Medium.

For frozen tissues, cryosections (6 μm thick) were fixed in cold acetone for 10 min, rinse twice with PBS and blocked with serum and endogenous biotin in a similar fashion as above. All staining procedures were the same as described but using fluorescent conjugated secondary antibodies or tertiary reagents instead. Sections were mounted with Fluorescence Mounting Medium (Dako) containing DAPI and visualised using a fluorescent microscope (Zeiss AxioVision-2, Zeiss, Welwyn Garden City, UK).

2.12 - RT-PCR and quantitative Real-Time RT-PCR

RNA extraction was performed using the RNeasy mini and micro Kits (Qiagen) depending on the amount of cells available, according to the manufacturer's instructions. mRNA was reverse transcribed using OmniScript (Quiagen) and when low amounts of RNA were obtained SensiScript (Qiagen) was used instead. All instructions can be found in the manufacture's booklets. For each 25 μl PCR reaction, 100 ng of equivalent cDNA was used. Amplification was performed using the Taq PCR Core Kit (Qiagen) according to the manufacturer's instructions. Murine β-actin was used as an internal PCR control. PCR products were verified by running on a 2% agarose gel.

For quantitative real-time PCR, 25 ng of cDNA was used per PCR reaction (25 ml), using SybrGreen Master Mix reagent (Applied Biosystems, Cheshire, UK) according to the manufacturer's instructions. Amplification was performed using the ABI Prism 7700 sequence detection system (Applied

Biosystems). The specificity of the PCR products was verified by running on a 2% agarose gel and also using the dissociation curve V1.0 software (Applied Biosystems). Set of primers and their respective annealing temperatures and number of amplification cycles information can be found in the Appendix II section.

2.13 - Western Blot (WB)

2.13.1 - Preparation of cell lysates

Cells were collected by trypsinization, washed once with PBS and spun down. Pellet was lysed in 100 µl of WB lysis buffer on ice for 10 min. Lysates were spun down at 1400 rpm for 15 min at 4°C. Supernatant was then transferred to a new tube and an aliquot was used to determine protein concentration. Supernatant was mixed with an equal volume of 2x WB sample buffer, boiled for 5 min and cooled down for 5 min at RT.

2.13.2 - Preparation and running the gel

Firstly, resolving gel (10%) and stacking gel were prepared and glass plate and spaces were assembled. The running gel was poured to approximately 1 cm below the wells of the comb and sealed with the stacking gel. When the stacking gel had set, the assemble was placed in the gel rig and immersed in running buffer. Prior to running the gel, wells were flushed out thoroughly with running buffer. Approximately 15 µl of each sample (containing around 15 µg of protein) was loaded into each well and ran with constant current (35 - 37 mA with voltage set at >300 V) for 2.5 hr.

2.13.2 - Membrane transfer (for BDH semi-dry electroblotter)

For one gel, 6 pieces of extra thick blotting paper (ETBP) and a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK) of the exact size of the gel were cut, wet in 1x blotting buffer and assembled as follows: 3x ETBP - Blotting Membrane - gel - 3x ETBP. The transfer was done for ~1 hr at 0.8 amp/cm^2 (8x10 cm gel should run with 64 mA for 1-2 hours). When finished, the membrane was immersed in blocking buffer ON. On the following day, incubation of primary antibody (diluted in blocking buffer) was carried out for 60 min at RT. Membrane was washed 3x 10 min with PBS containing 0.05% Tween-20 (Sigma). Secondary antibody incubation step was done at RT for 45 min (antibody also diluted in blocking buffer). After this time, the membrane was washed thrice for 10 min with PBS containing 0.05% Tween-20 (Sigma). Detection was carried out using Amersham ECL Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

*Formulation for other solutions used in this study can be found in the Appendix I section.

Chapter 3 - *In vitro* and *in vivo* characterisation of muMSC

3.1 - Brief introduction and description of the study

As described in the general introduction section, MSCs have the capacity to give rise to fat, bone and cartilage as well as to neuronal progenitors (Woodbury *et al.*, 2000) and muscle (Wakitani *et al.*, 1995) cells *in vitro* making them a potential cellular source for clinical applications in regenerative medicine. *In vivo* studies involving a variety of animal models have shown that MSCs may be useful in the repair or regeneration of damaged or mutated bone, cartilage, or myocardial tissues (Bianco *et al.*, 2000; Pereira *et al.*, 1998; Toma *et al.*, 2002). They may also provide a useful tool for gene therapy strategies involving genes encoding secreted proteins (Bartholomew *et al.*, 2001; Pereira *et al.*, 1995; Duan *et al.*, 2003). Although the *in vitro* differentiation capacities of MSCs have been well documented, the same potentials in contributing to different tissue cell-types *in vivo* remain elusive.

So far, most *in vivo* studies have been performed by targeting a specific tissue/organ of interest, normally with the use of an injury model (Hofstetter *et al.*, 2002; Kopen *et al.*, 1999; Ortiz *et al.*, 2003). Systemic delivery of MSCs is an attractive non-invasive strategy that allows repeated administration of large numbers of cells with great potential clinical applications (Bianco *et al.*, 2001; Koc *et al.*, 2002). However, (when this study started) limited data were available regarding the ultimate fate of systemically infused MSCs. In a baboon model Devine *et al.* suggested that MSCs home to a variety of non-haematopoietic organs and may possess the capacity to proliferate within these tissues (Devine *et al.*, 2003). hMSCs have been shown to be capable of differentiation into multiple cell types following peritoneal implantation in fetal sheep (Liechty *et al.*, 2000). Using a partially purified muMSCs population Pereira *et al.* suggested that these cells follow a broad initial distribution and their presence could be detected in different

tissues of the recipient animals few months after transplantation without, however, exploring their *in vivo* differentiation potential (Pereira *et al.*, 1995).

Therefore, the main aim of the first part of this project was to evaluate the tissue distribution of muMSCs after intravenous administration and their "natural" *in vivo* differentiation capacities in recipient animals not forced to undergo substantial organ degeneration first.

Most of the understanding of MSC biology has been acquired from studies on hMSCs. This is one of the uncommon situations where a cell-type is better characterised in human than in mice. muMSCs are indeed poorly understood, in terms of their phenotype, cell isolation and expansion. Although mouse and human MSCs are generally similar in terms of overall phenotype and *in vitro* differentiation capacities, this study showed that isolated and expanded muMSCs express different adhesion molecules and integrins compared to their human counterparts. Such discrepancies may account for and explain potential differences between human and mouse MSCs in homing and their *in vivo* capacity to contribute to specific cell-types (Liechty *et al.*, 2002; Devine *et al.*, 2003). Therefore, it was important to characterise these cells, reporting detailed and useful information about isolation, expansion and phenotype. *Ex vivo*-expanded muMSCs were then tagged by transduction with a lentiviral construct carrying the green fluorescent protein (eGFP) and, at various time points after infusion, multiple tissues were analysed for the presence of the reporter gene by polymerase chain reaction (PCR). Immunohistochemistry was used to screen and determine any tissue-specific incorporation of donor-derived cells in the recipient animals.

3.2 - Isolation and characterization of muMSCs

Since the ease of isolation and expansion of muMSCs is strain-dependent (Phinney *et al.*, 1999; Peister *et al.*, 2004), initially three different mouse strains (NOD/SCID, NOD/SCID-β_2^{null} and wild-type C57BL6/J) were used (mouse strains that were easily available in the lab) to determine the

frequency of these cells in the bone marrow. muMSCs express a variety of cell surface antigens which are shared with different bone marrow-derived cell populations. As there is no specific antigen that can be used to directly define muMSCs in the bone marrow, the analysis was based on previous studies (Phinney *et al.*, 1999; Peister *et al.*, 2004). The murine mesenchymal compartment in the bone marrow can be defined as $CD45^{neg}Lin^{neg}CD31^{neg}$ or $CD45^{neg}CD11b^{neg}TER119^{neg}CD31^{neg}$, which is the cellular fraction devoid of hematopoietic and endothelial cells (**Figure 1**). By using this criterion no differences in the frequency of MSCs in the bone marrow of these mouse strains were found (**Figure 1**, with 0.16%, 0.16% and 0.1% respectively). Moreover, we did not see significant differences in the number of CFU-Fs with 1.66±0.38, 1.83±1.51 and 1.83±0.88 colonies/cm^2 obtained from NOD/SCID, NOD/SCID-β_2^{null} and C57B6/J BMMNCs respectively (**Figure 2**).

However, MSCs from the immunodeficient strains exhibited more independence from the haematopoietic contaminants for its survival in early passages. muMSCs from the immunodeficient strains exhibited higher cell expansion and survival potential after removal of the haematopoietic cells than those from the wild-type C57Bl/6J strain. Thus, all subsequent experiments were conducted using NOD/SCID bone marrow. As previously reported (Phinney *et al.*, 1999), we observed that MSC cultures were contaminated with haematopoietic cells co-expressing CD45 and CD11b, which persisted for many passages (**Figure 3**). Contaminating cells consisted of 0.5-2% of B ($CD19^{pos}$), T ($CD3^{pos}$), NK ($NK1.1^{pos}$) cells and granulocytes ($Ly\text{-}6Gr^{pos}$) (from 3 to 5 different extractions) but interestingly, the majority of the persisting adherent $CD45^{pos}/11b^{pos}$ cells had a more progenitor/stem cell phenotype expressing a combination of stem cell antigens such as Sca-1, CD34 and CD90. No $Ter119^{pos}$ or $CD31^{pos}$ cells were found after the first passage, suggesting that CD45 and CD11b markers could be safely used as a routine method for purifying MSCs.

In addition, Sca-1 expression on MSCs was found to increase from 42±11% (P0), to 44±10.9% (P1), 81.7±12.9% (P2) and finally to >98.5% in passage 3, indicating a possible acquisition of this antigen through time in

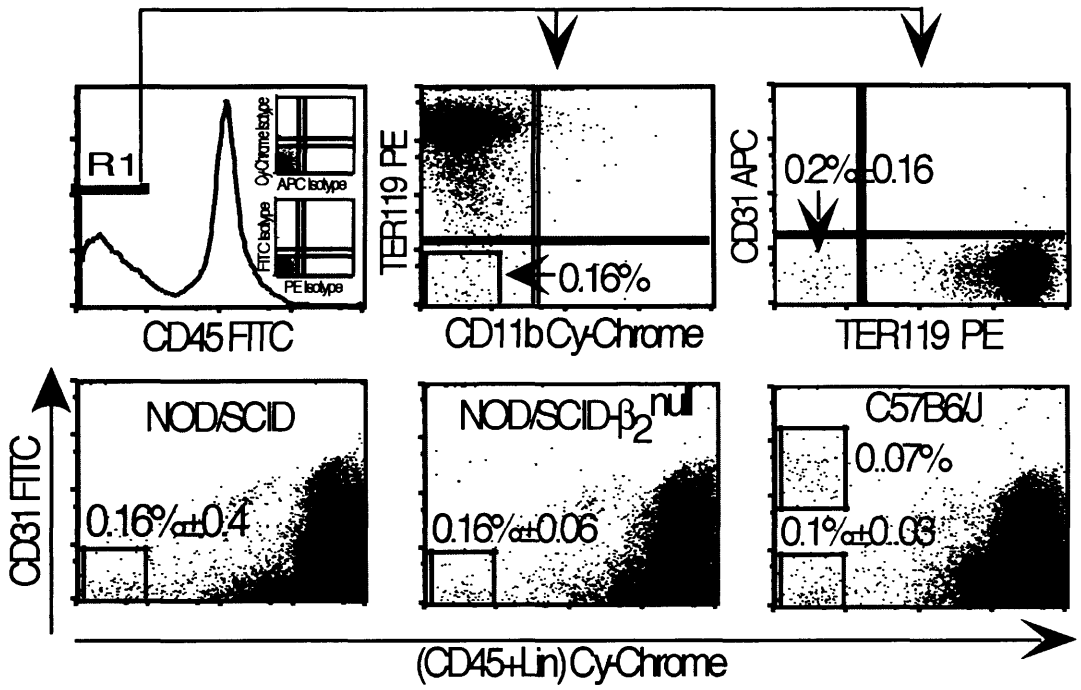


Figure 1. Determination of murine mesenchymal compartment frequency in the bone marrow

Representative FACS plots analysis of NOD/SCID BMMNCs (top plots) stained with CD45/CD11b/TER119/CD31 antibodies; the frequency of mesenchymal compartment in the bone marrow can be considered as the negative fraction for CD45 (R1) with the combination of the other cell surface antigens used here. Alternatively, same frequencies can also be determined by using the following antibody combination: CD45/Lin/CD31 (bottom plots). By using this criterion no differences in the frequency was found between the three mouse strains studied.

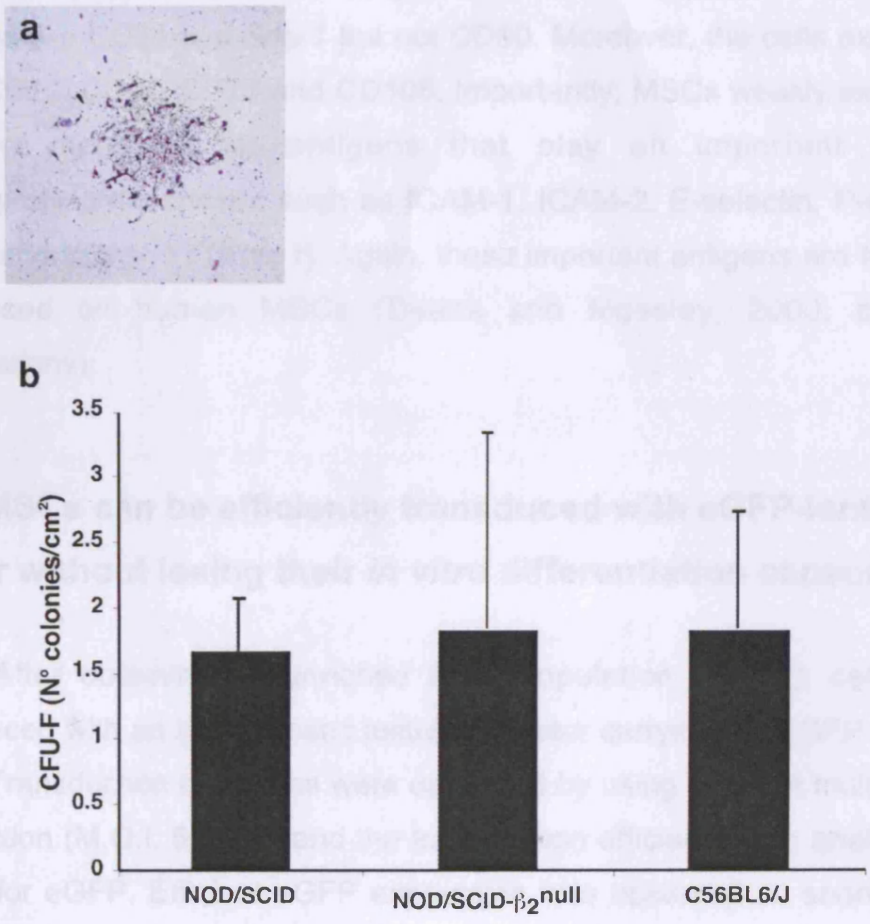


Figure 2. CFU-F frequency between different mouse strains

(**a**) Representative picture of a 14 days-old NOD/SCID derived CFU-F colony. (**b**). CFU-F frequency between different mouse strains used in this study. Results shown are mean ±s.d from two independent experiments and each one performed in triplicate.

culture. Alternatively, Sca-1^{pos} cells may have a selective advantage in the culture conditions used.

After purification (>99.8% purity by FACS), MSCs were immunophenotyped by FACS analysis to reveal the expression of stem cell antigens like CD34 and Sca-1 but not CD90. Moreover, the cells expressed CD9, CD29, CD44, CD73 and CD105. Importantly, MSCs weakly expressed or were negative for antigens that play an important role in homing/rolling/attachment such as ICAM-1, ICAM-2, E-selectin, P-selectin, VLA-5 and VCAM-1 (**Table I**). Again, these important antigens are found be expressed on human MSCs (Deans and Moseley, 2000; personal observations).

3.3 - MSCs can be efficiently transduced with eGFP-lentivirus vector without losing their *in vitro* differentiation capacities

After obtaining an enriched MSC population (purP4), cells were transduced with an HIV-1-based lentivirus vector carrying the eGFP reporter gene. Transduction conditions were optimised by using different multiplicities of infection (M.O.I: 5 to 50) and the transduction efficiency was analysed by FACS for eGFP. Efficient eGFP expression was observed as soon as one day after removal of the virus supernatant (**Figure 4a**). The highest transduction efficiency (>98%) was achieved when an M.O.I 50 was used and this expression was maintained days after virus removal (**Figure 4b**: 5 days post-virus removal). The percentage of eGFP-expressing cells remained virtually unchanged (>96.5%) in subsequent passages (purP4 to purP10), which correlated to 35 to 40 days post-transduction. This also indicated that the SFFV promoter was not subject to reporter gene silencing in these cells. To evaluate whether lentiviral transduction altered the differentiation properties of MSCs *in vitro,* transduced cells were stimulated to differentiate into several cell-types. *In vitro* differentiation studies showed that eGFP-MSCs could give rise to different lineages (**Figure 5**) such as

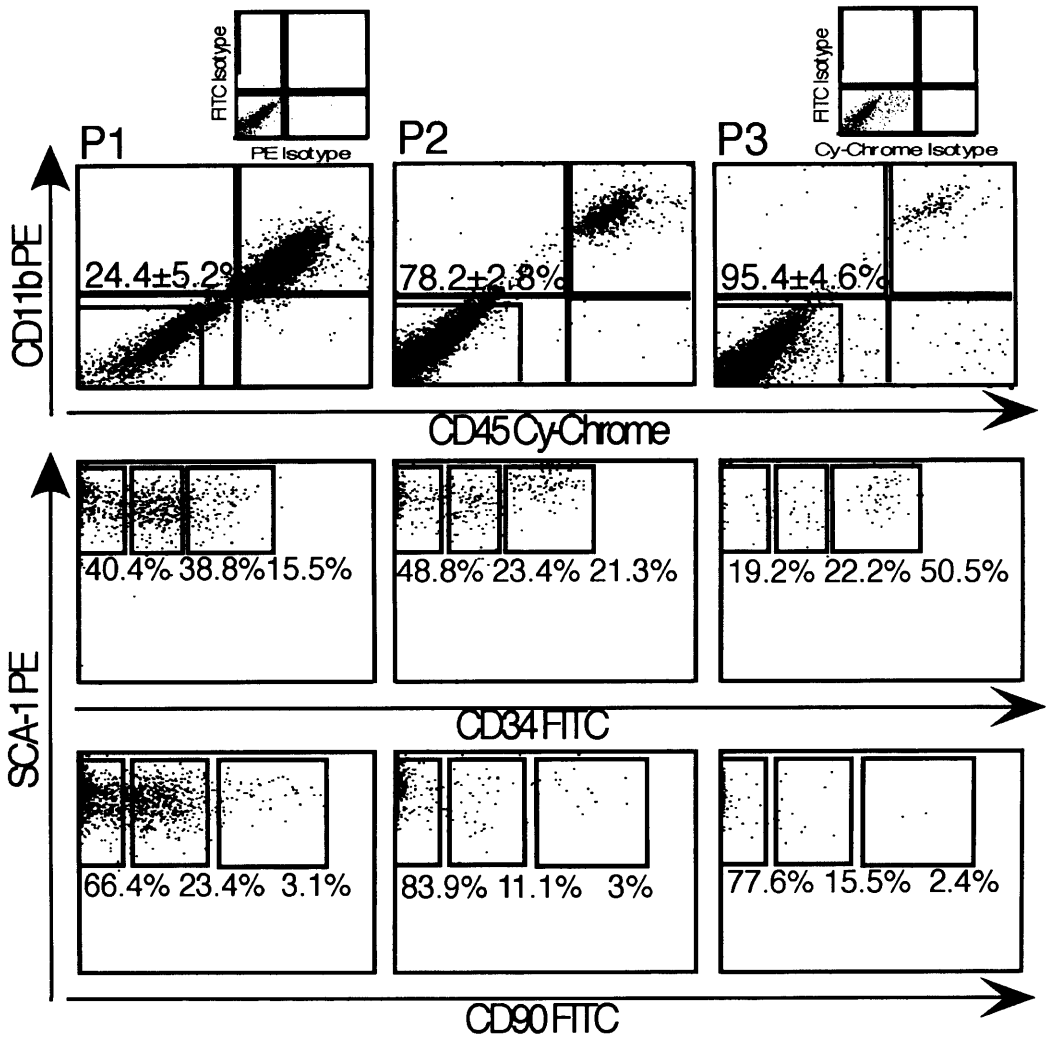


Figure 3. Persistence of haematopoietic cell contamination in MSC cultures

The presence of CD45/CD11b^{pos} cells in different passages (P1-P3) of MSC cultures is shown (top plots). Percentages indicate the CD45/CD11b^{neg} populations, which were considered as the MSC fraction. The CD45/11b^{pos} haematopoietic cell population expressed Sca-1 and different levels of CD34 and CD90 (middle and bottom plots, respectively). Results shown are mean ±s.d from three independent experiments.

Table I. Immunophenotype of muMSCs

Common Name	CD	Detection
	Hematopoietic Antigens	
LFA-3L	CD2	Neg
CD3 Complex	CD3ε	Neg
T4 Cell	CD4	Neg
Ly-1	CD5	Neg
T8 Cell	CD8	Neg
LSP-R	CD14	Neg
B Cell Antigen	CD19	Neg
	CD34	Pos (++)
Leukosialin	CD43	Neg
Leukocyte Common Antigen	CD45	Neg
	CD45/B220	Neg
NK Cell Antigen	NK1.1	Neg
Erythroid Antigen	TER119	Neg
Granulocyte Antigen	Ly-6G	Neg
Thy-1	CD90	Neg
Stem Cell Antigen	Sca-1	Pos (+++)
Early B Lineage Antigen	AA4.1	Neg
	Adhesion Molecules	
ICAM-1	CD54	(+/-)
ICAM-2	CD102	Pos (+)
L-selectin	CD62L	Pos (+)
E-selectin	CD62E	Neg
P-selectin	CD62P	Neg
PECAM-1	CD31	Neg
HCAM	CD44	Pos (+++)
VCAM-1	CD106	Pos (+)
MadCAM-1	MadCAM-1	(+/-)
	Integrins	
LFA-1 α Chain	CD11a	Neg
Mac1	CD11b	Neg
CR4 α Chain	CD11c	Pos (+)
VLA-4	CD49e	Neg
VLA-5	CD49e	Pos (+)
VLA-β1 Chain	CD29	Pos (++)
Integrin αIEL Chain	CD103	Neg
Integrin β7 Chain	β7	Neg
LPAM-1	α4β7	Neg
	Cytokine Receptors	
IL2-R	CD25	Neg
IL7-R	CD127	Pos (+/-)
c-Kit	CD117	Neg
Flk-2/Flk-3	CD135	Neg
	Others	
Ecto-5'-nucleotidase	CD73	Pos (++)
Endoglin	CD105	Pos (+)
Tetraspan	CD9	Pos (+++)
MHC I	H-2K^{q}	Pos (+++)
MHC II	I-Ag^{7}	Pos (+)

muMSCs were analysed by FACS with a panel of antibodies using isotype controls to set up the initial gates. Depending on the mean fluorescence intensity levels observed, samples were scored as Negative (Neg), Positive (Pos) borderline (+/-), low positive (+), fairly positive (++) or highly positive (+++).

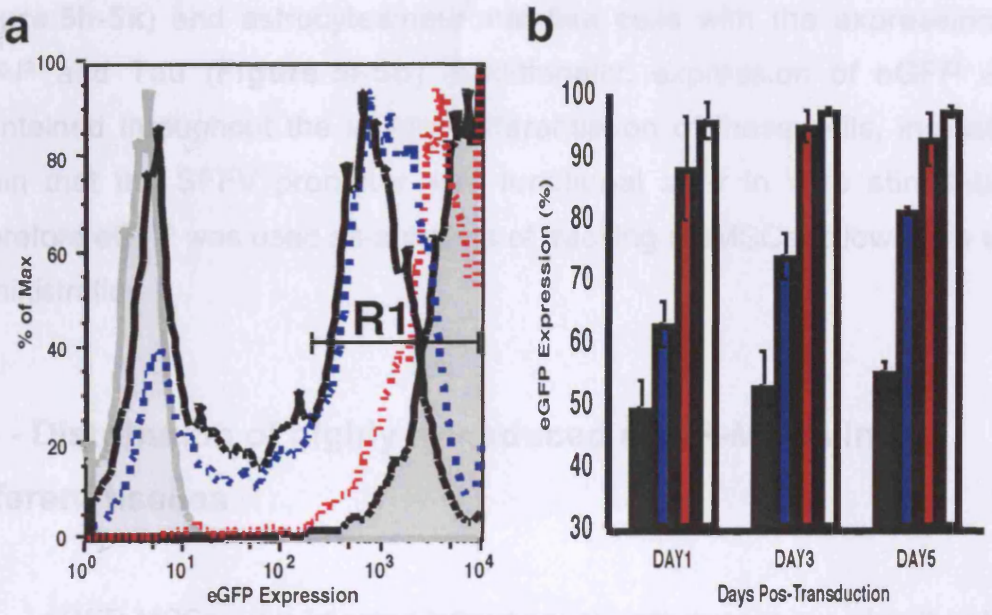


Figure 4. *In vitro* eGFP expression by muMSCs following lentiviral transduction

A highly enriched muMSC population (purP4) was transduced with lentivirus for 20h. Analysis for eGFP expression was performed 1, 3 and 5 days post-transduction. (**a**) A representative overlay FACS plot from day 1 with M.O.I: 5 (filled line), 10 (dotted line), 30 (dashed line), 50 (grey area) and untransduced control MSCs (light grey area) is shown. Cells gated in R1 expressing high levels of eGFP from different time points of analysis were plotted in the graph (**b**). Results shown are mean ±s.d from two independent experiments, and each time point was performed in duplicate.

adipocytes which were visualised with Oil Red O staining (**Figure 5b, 5c**). Chondrogenic capacity was confirmed with Alizarin-red, Alcian-blue (data not shown) and Safranin O (**Figure 5e**) staining indicating the presence of sulphated proteoglycans in the micro-pellet formation. The differentiation into osteocytes was revealed using staining for alkaline phosphatase and calcium production (**Figures 5f, 5g**). eGFP-MSCs were also able to give rise to myocytes with the expression of dystrophin, and fast-twitch myosin (FTM; **Figure 5h-5k**) and astrocytes/neuronal–like cells with the expression of GFAP and Tau (**Figure 5l-5o**). Additionally, expression of eGFP was maintained throughout the *in vitro* differentiation of these cells, indicating again that the SFFV promoter was functional after in vitro stimulation. Therefore eGFP was used as a means of tracking muMSCs following *in vivo* administration.

3.4 - Distribution of highly transduced eGFP-MSCs into different tissues

eGFP-MSCs were injected intravenously into syngeneic mice that had been sub-lethally irradiated to prime the bone marrow for homing of infused cells. However, sub-lethal irradiation (at the dose of 375 cGy) is a non-invasive method known to also cause minor damage to the gut and lungs. PCR analysis for eGFP was used to qualitatively evaluate the distribution of the transduced donor-derived cells in different tissues of the recipient animals (**Table II**). With the exception of peripheral blood and bone marrow, each tissue was subjected to at least three independent genomic DNA extractions (from three different parts of the tissue) and three PCR analysis respectively. Only when at least two out of the three valid PCR reactions were positive for eGFP, was the tissue considered harboring infused cells.

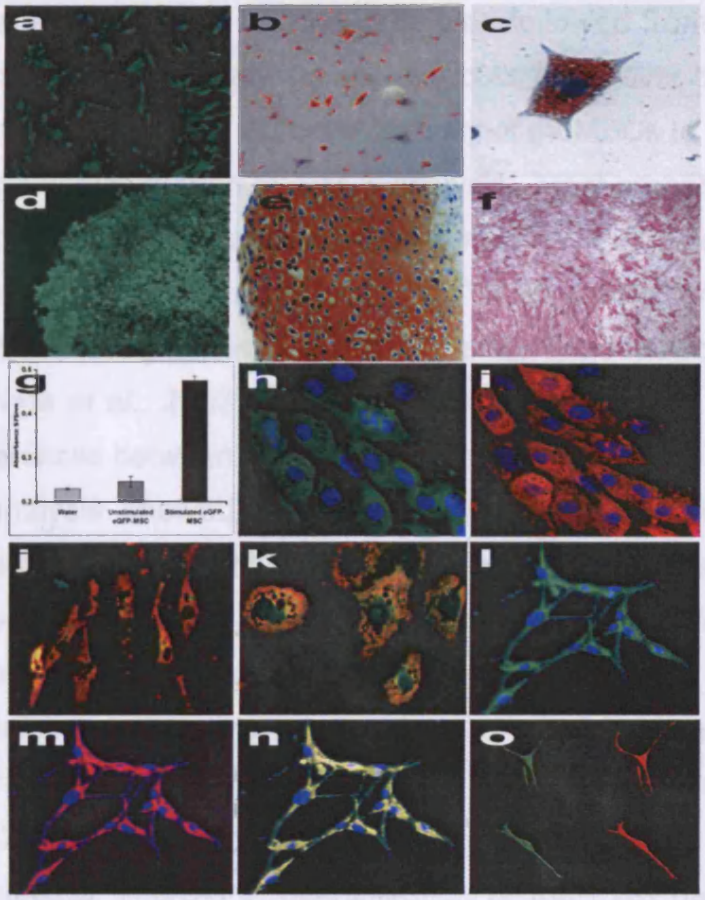


Figure 5. *In vitro* differentiation of eGFP-MSCs

Unstimulated eGFP-MSCs were viewed under UV light (**a**). After 14 days of induction, cells were fixed and stained with Oil Red O (**b**, **c**). Chondrogenic differentiation was revealed with Safranin O staining (**e**) with a representative section of the micropellet viewed under the fluorescent microscope before the staining (**d**). The osteogenic potential of MSCs was determined by staining for alkaline-phosphatase (**f**) and calcium production (**g**): stimulated eGFP-MSCs (black bar), non-stimulated eGFP-MSCs (grey bar), control-water (light grey bar). Results shown are mean ±s.d from two independent experiments, each one performed in triplicate. eGFP expression alone on myocyte-like cells (**h**). Myogenic differentiation was confirmed by staining with dystrophin-Cy3 (**i**, **j**) and FTM-Cy3 (**k**). eGFP expression alone on astrocyte- (**l**) and neuronal- (**o**) like cells. Neuronal differentiation was confirmed by staining with GFAP-Cy3 (**m**, **n**) and Tau-TRITC (**o**). Overlay of eGFP and dystrophin (**j**), eGFP and FTM (**k**) and eGFP with GFAP (**n**). Cells were counter-stained either with haematoxylin (**b**, **c**, **f**) or DAPI (**h-j**, **l-n**) or methyl green (**e**). Magnifications: x100 (**a**, **b**, **f**), x200 (**d**, **j**, **l-n**) and x400 (**c**, **e**, **h**, **i**, **k**, **o**).

The distribution of the infused cells was followed from day 1 to 28 post-transplantation. Surprisingly, no $eGFP^{pos}$ cells could ever be detected in the bone marrow, indicating a deficient capacity of muMSCs to home to their tissue of origin (also confirmed by FACS analysis). Most probably the lack of appropriate adhesion molecules and integrins may have impeded these cells to home and seed the bone marrow properly. These data differ from what is known about the homing capacity of hMSCs to the bone marrow (Liechty *et al.*, 2000; Devine *et al.*, 2003) suggesting that there may be some *in vivo* functional differences between mouse and human MSCs.

PCR analysis showed that the lungs, liver and kidney were the primary resident organs of muMSCs, as eGFP could be detected as early as one day post-transplantation. Distribution to the muscle, heart, brain and spleen did not occur until day 7 after infusion. The fact that eGFP-MSCs were found circulating in the peripheral blood at all of the time-points, may explain why the reporter gene was able to be detected abundantly in many tissues by PCR, while by immunohistochemistry low or no eGFP positive cells were detected. Therefore, quantification of the PCR data would not necessarily correlate with muMSC differentiation events but would most likely represent the extent of circulating cells in the respective tissues.

3.5 - Extensive trapping of muMSCs especially in the lung

An adverse effect observed in the majority of the experimental animals transplanted with eGFP-MSCs was retention of these cells in the lung alveoli. Aggregates of MSCs were observed in the lung as early as one day post-transplantation and in many cases persisted thereafter to result in tissue damage with development of fibrotic tissue (with cysts containing collagen deposition) as well as impaired organ function (**Figure 6f**). These animals exhibited distress, with gradual weight loss and breathing difficulties, and had therefore to be sacrificed. Immunohistochemistry was performed on tissues where trapped eGFP-MSCs had been observed (e.g. spleen, lungs, and gut), and revealed that these cells maintained their MSC phenotype by expressing laminin and endoglin (data not shown). Lungs extensively infiltrated with

Table II. Percentage of mice that were positive for eGFP in different tissues detected by PCR

GUT	17% (1/6)	0% (0/6)	43% (3/7)	17% (1/6)	62% (8/13)
BRAIN	0% (0/6)	0% (0/7)	14% (1/7)	20% (1/5)	8% (1/13)
LIVER	57% (4/7)	17% (1/7)	43% (3/7)	33% (2/6)	69% (9/13)
LUNG	83% (5/6)	17% (1/7)	17% (1/7)	60% (3/5)	54% (7/13)
KIDNEY	43% (3/7)	17% (1/7)	43% (3/7)	43% (3/7)	50% (6/12)
SKIN	29% (2/7)	0% (0/7)	0% (0/5)	25% (1/5)	54% (7/13)
MUSCLE	0% (0/6)	0% (0/6)	17% (1/7)	17% (1/6)	8% (1/13)
HEART	0% (0/7)	0% (0/7)	29% (2/7)	33% (2/6)	8% (1/13)
SPLEEN	0% (0/6)	0% (0/6)	40% (2/5)	43% (3/7)	46% (6/13)
BONE MARROW	0% (0/3)	0% (0/1)	-	0% (0/4)	0% (0/5)
PERIPHERAL BLOOD	25% (1/4)	33% (1/3)	25% (1/4)	60% (3/5)	60% (6/10)
Days post-infusion	1	2	7	14	28

Different tissues/organs were collected at various time points (day 1, 2, 7, 14 and 28) after infusion of eGFP-MSCs into sub-lethally irradiated recipients. DNA was extracted from these tissues and analysed for the presence of the reporter gene by PCR. Numbers shown indicate mice positive for eGFP over total number of mice analyzed. (-) Not performed.

infused cells did not show reliable staining for lung-specific antigens such as lectin *Lycopersicon esculentum* (**Figure 6d**), pan-CK or Surfactant Protein C (data not shown), due to the extent of tissue damage.

3.6 - Contribution of eGFP-MSCs to different tissue cell-types at low frequency

Tissues were subsequently screened for the presence of eGFP-MSCs, focusing on the last time point (day 28) for the detection of differentiation events by immunohistochemistry. In order to avoid any false positive results, a positive event was only considered when $eGFP^{pos}$ cells could be detected in the adjacent serial sections and when more than one $eGFP^{pos}$ cell could be found on different levels of sections within a particular tissue. Isotype control antibody for eGFP was also used to exclude non-specific staining.

In two animals in which no lung damage was observed, some $eGFP^{pos}$ MSCs (**Figure 7c, 7d**) were detected, which had given rise to cells with respiratory bronchiolar epithelial morphology, staining positive with pan-CK antibody (**Figure 7c**).

eGFP-expressing cells with hepatocyte morphology were detected in the liver of 3 out of 13 recipient animals. This was a low frequency event and in most cases only 1 to 3 positive cells were seen per section, suggesting that no or little cell expansion had occurred after the cells reached the liver. Interestingly, all positive cells were found near or not far from the blood vessels. These $eGFP^{pos}$ cells had not only acquired proper hepatocyte morphology (**Figure 7e**, **7f**, **7h**) but also stained positive for specific antigens such as albumin (**Figure 7f**) and CK18 (**Figure 7h**).

In addition, specific $eGFP^{pos}$ cells were observed in the kidneys of 2 out of 13 mice analysed. These cells had renal epethelial cell morphology and were seen at a frequency of 5-10 cells per transverse-section as well as on different levels of the tissue (**Figure 8b**, **8c**, **8e**).

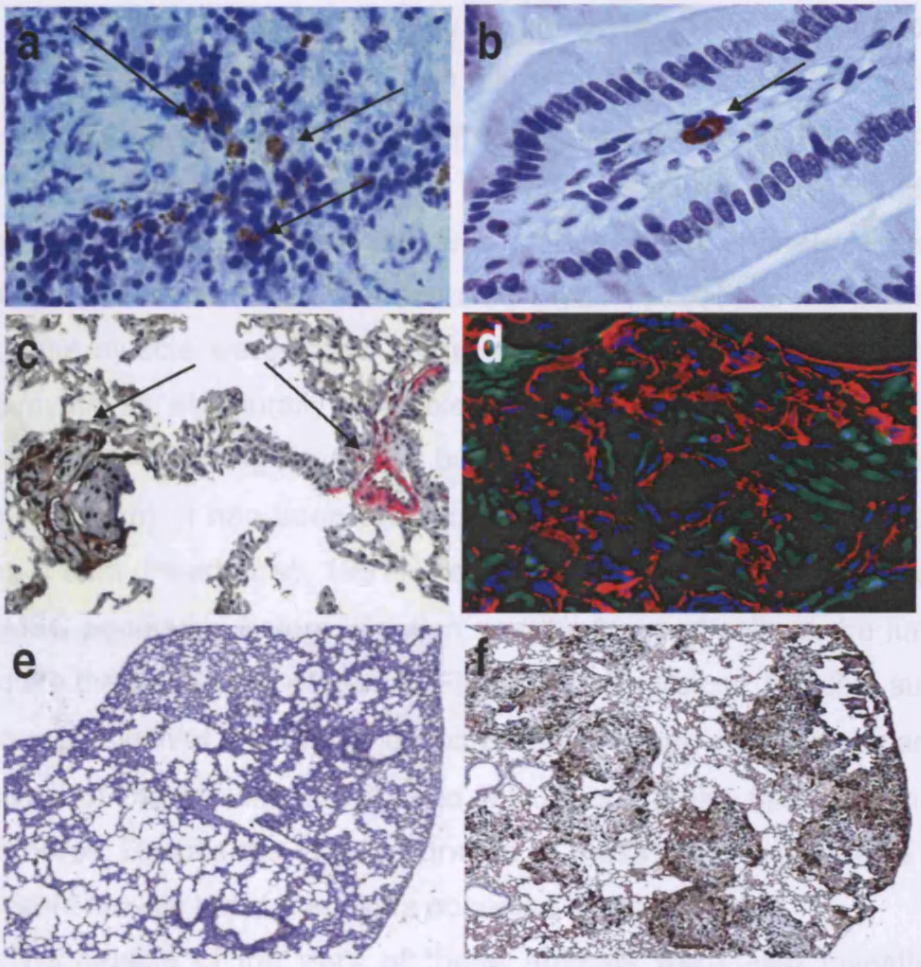


Figure 6. Transplanted eGFP-MSCs are circulating and/or trapped in the different tissues

Immnunohistochemistry revealed the presence of infused eGFP-MSCs in different tissues. Circulating or trapped (arrows to brown cells) MSCs were found in the spleen (**a**), gut (**b**) and lung (**c**). In the lung, trapped cells stained neither for α-sma [(red-pink) indicated by the right-side arrow] (**c**) nor for the lectin Lycopersicon esculentum-Cy3 (**d**, overlay with eGFP). Trapped eGFP-MSCs have caused severe lung damage in some of the mice. A representative section of an undamaged lung (**e**) and severely damaged lung (**f**) from two different mice that were injected with eGFP-MSCs (brown cells). Sections were counterstained with haematoxylin (**a-c**, **e**, **f**) or DAPI (**d**). Magnifications: x25 (**e**, **f**) x100 (**c**), x200 (**d**) and x400 (**a**, **b**).

Immunohistochemistry on serial sections was performed to confirm expression of kidney-specific antigens by these eGFP-expressing cells. Indeed, these donor-derived cells stained positive for lectins such as *Ricinus communis* or *Lotus tetragonolobus* (**Figure 8b**, **8d** respectively).

Since muMSCs were previously shown to have the capacity to differentiate into skeletal muscle in an injury model (De Bari *et al.*, 2003), a more detailed screening was performed at this particular tissue for the presence of donor-derived cells. No $eGFP^{pos}$ myofibres were found from skeletal muscle isolated from the femur of recipient animals. However, infiltrating $eGFP^{pos}$ cells that had acquired myofibroblast-like morphology in the skeletal muscle were found (**Figure 9a**, **9d**). These cells were found among myofibres, structurally resembled connective tissue and had acquired expression of α-sma (**Figure 9e**, **9f**) but not desmin (**Figure 9b**, **9c**) or FTM (data not shown). It has been previously reported that some stromal cells express α-sma (Peled *et al.*, 1991). However, this antigen was undetectable in the MSC population before injection and the trapped cells in the lung did not acquire the expression of α-sma (**Figure 6c**). Therefore, the data suggest that the expression of α-sma was an acquisition through a possible change in phenotype of the infused MSCs into a supporting-like tissue within the muscle fibres. Despite the large infiltration of these myofibroblast-like cells, no apparent muscle impairment was observed in these mice.

The muscle of the ears of these animals were also investigated $eGFP^{pos}$ cells were found in 2 out of 13 mice (**Figure 10b**, **10c**). These cells exhibited a myofibre morphology and expressed desmin (**Figure 10d**, **10e**) and FTM (**Figure 10f**, **10g**) indicating an acquisition of a myofibre-like phenotype. Interestingly, these $eGFP^{pos}$ cells formed clusters of 3-5 cells each suggesting a possible expansion of donor-derived cells. However, it cannot rule out the possibility that eGFP-MSCs fused with existing myofibres. The difference observed here among the two skeletal muscle regions is in agreement with recent reports indicating significantly different incorporation of bone marrow-derived cells among skeletal muscles (Brazelton *et al.*, 2003).

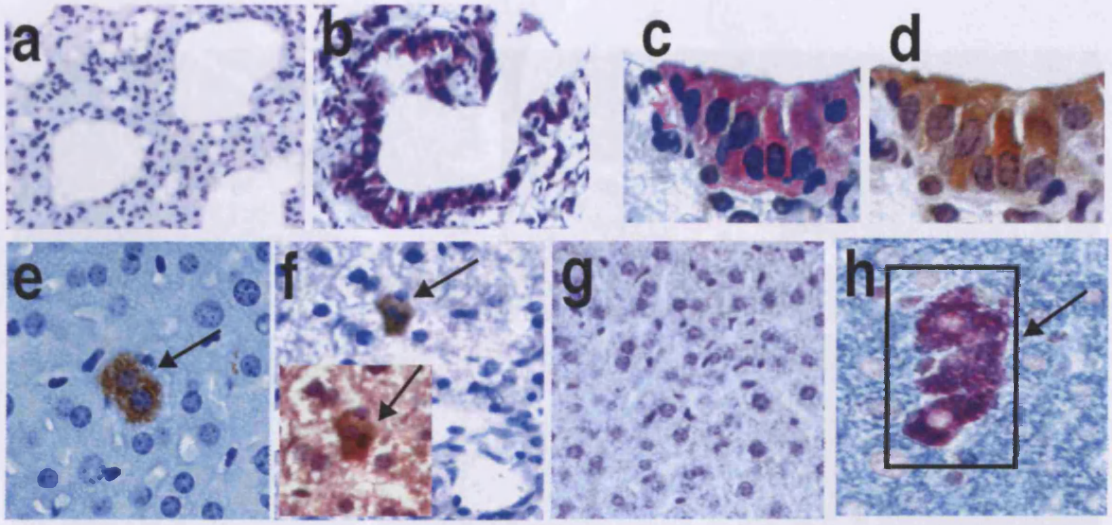


Figure 7. *In vivo* contribution of eGFP-MSCs into bronchiolar epithelial cells and hepatocytes

Representative paraffin sections of lung stained either with rabbit immunoglobulins (isotype control) (**a**) or with rabbit anti-pan-CK antibodies (**b**). Serial sections (**c**, **d**) show that a group of pan-CK positive cells (red) (**c**) also stained positive for eGFP (**d**). Representative liver sections with hepatocytes staining positive for eGFP (**e**, **f**; brown). eGFP expression coincided with albumin (red) giving a brown-purple staining (**f**, small bottom-left figure). Serial paraffin sections from another liver showing purple cells with hepatocyte morphology, as a result of co-expression of eGFP (red) and CK18 (blue) (**h**). Isotype control sections shown in **g**. All sections were counterstained with hematoxylin (except for **h**). Magnifications: x100 (**a**, **b**) and x400 (**c**-**h**).

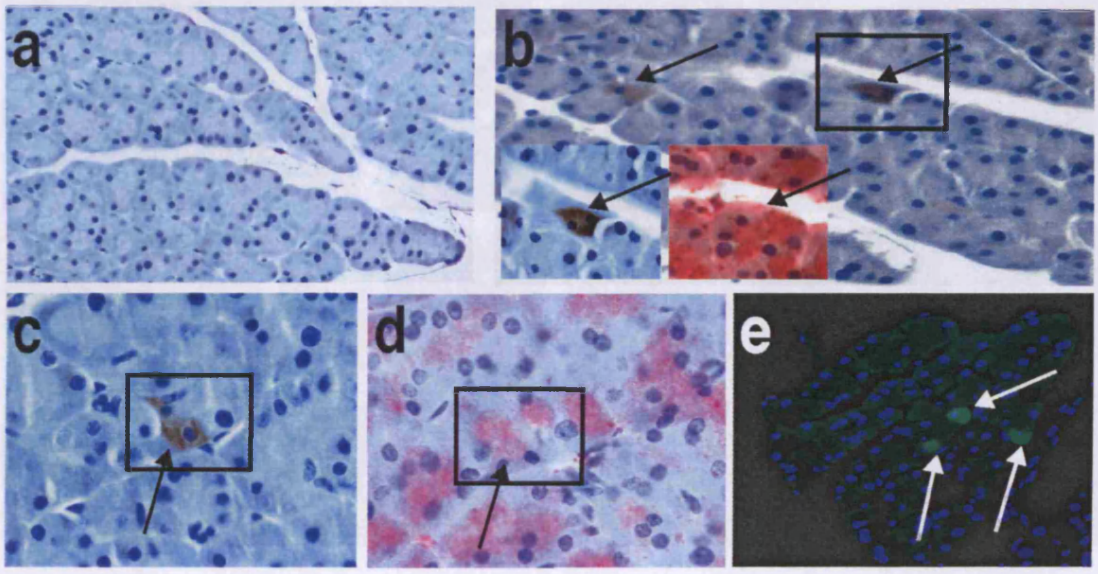


Figure 8. Contribution of eGFP-MSCs into renal epithelial cells

Paraffin-embedded transverse-sections stained with isotype control antibodies show no background staining (**a**). Arrows indicate $eGFP^{pos}$ cells after staining with rabbit anti-eGFP antibody (**b**, **c**) co-expressing both lectins *Ricinus communis* (**b**, small bottom-centre figure; red staining) and *Lotus tetragonolobus* (**d**; pink-red staining) as shown in serial sections. A frozen section of a kidney demonstrating $eGFP^{pos}$ cells by direct visualisation using a fluorescent microscope (**e**). All sections were counterstained with haematoxylin or DAPI **e**). Magnifications: x200 (**a**, **b**, **e**) and x400 (**c**, **d**).

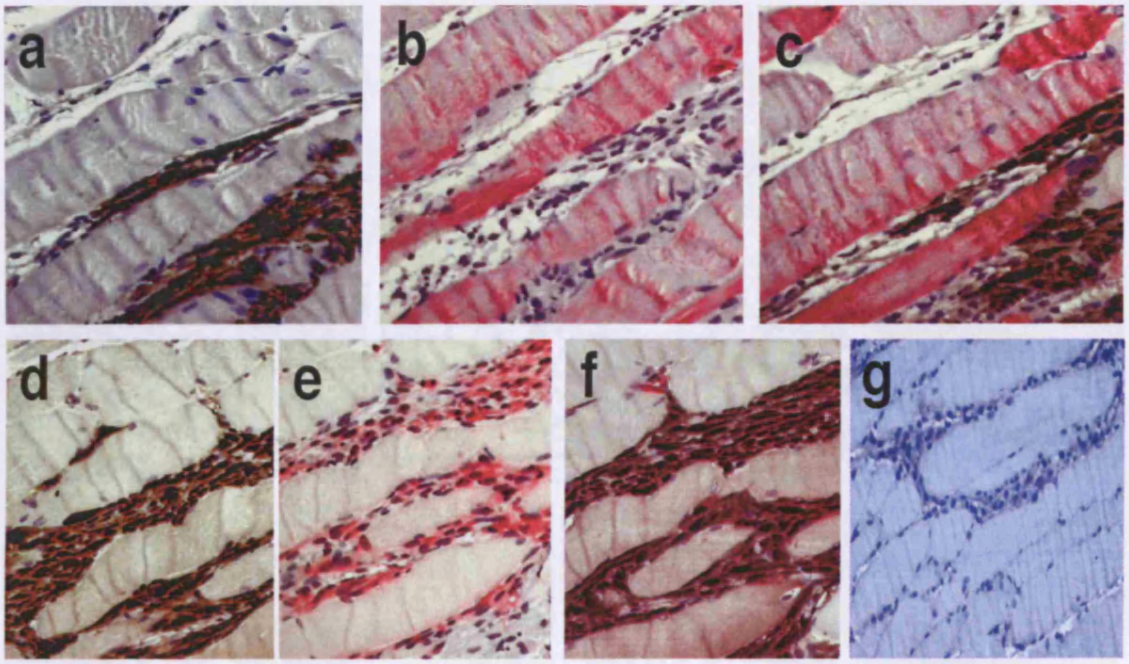


Figure 9. Contribution of eGFP-MSCs into myofibroblasts *in vivo*

eGFP positive cells with myofibroblast morphology were located among skeletal muscle fibres (**a**, **d**; brown). No expression of desmin was observed (**b**; red staining) showing mutual exclusion of expression when double staining for desmin and eGFP was performed (**c**). These same $eGFP^{pos}$ cells (brown) stained positive for α-sma (red) in serial sections (**e**) resulting in a red-brown color. (**f**). Same section stained for both rabbit and mouse immunoglubulins (isotype control) (**g**). Sections were counterstained with haematoxylin. Magnifications: x200 (**g**) and x400 (**a-f**).

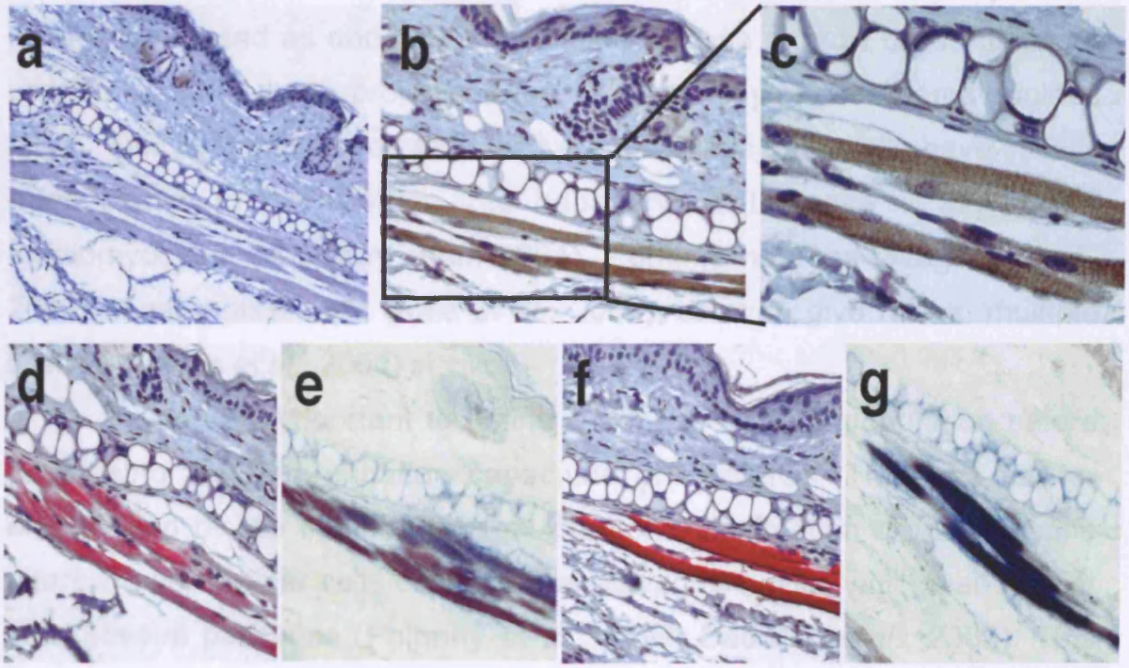


Figure 10. Contribution of eGFP-MSCs into myofibre-like cells *in vivo*
Representative section of the skin from the ear with myofibres staining positive for eGFP (**b**, **c**; brown cells). Same group of myofibres demonstrating expression of desmin (**d**, **e**; pink-red staining) and FTM (**f**, **g**; red) alone or in combination with an eGFP antibody (blue) giving a purple color (**e**, **g**). Sections were counterstained with haematoxylin (exception for **e**, **g**). Magnifications: x200 (**a**, **b**, **d-e**) and x400 (**c**).

In conclusion, multiple incorporation events of muMSCs into the recipient tissues, following by systemic infusion under substantial tissue damage conditions were seen.

3.7 - Discussion

Reports in the literature have so far given a rather confusing concept about the *in vivo* contribution potential of MSCs. Firstly, the majority of the studies have used an undefined population of bone marrow cells, making it unclear whether these properties are of mesenchymal or haematopoietic origin. This is of vital importance as haematopoietic stem cells have initially been shown to differentiate into hepatocytes (Lagasse *et al.*, 2000), cardiomyocytes (Jackson *et al.*, 2001), Purkinje neurons (Wagers *et al.*, 2002), renal tubular cells (Kale *et al.*, 2003), or even give rise to multiple tissues (Krause *et al.*, 2001) *in vivo*.

It was very important to define an enriched MSC population before addressing the differentiation capacities of muMSCs. This was further emphasised by the observation that the adherent fraction of mouse bone marrow mononuclear cells contains haematopoietic cells, which can persist after several passages (Phinney *et al.*, 1999; Baddoo *et al.*, 2003). This finding was confirmed here, and further indicated that a high proportion of these contaminating haematopoietic cells have a progenitor/stem cell phenotype. By using CD45 and CD11b it was possible to define and sort a highly enriched population of muMSCs that were devoid of haematopoietic cell contamination and could be efficiently transduced with a lentivirus vector. Moreover, the SFFV promoter in this construct retained the reporter gene expression through *in vitro* differentiation of muMSCs, indicating that it is functional in different cell lineages and could be utilised in gene therapy applications using MSCs.

At the time when these studies were conducted, there were no conclusive reports illustrating the *in vivo* contribution of muMSCs to multiple cell-types upon intravenous delivery without severe tissue damage being applied to the recipient animals. Only when injury models (Gojo *et al.*, 2003)

were used and MSCs were locally delivered, the differentiation potentials of these cells were observed. Nevertheless, muMSCs can specifically differentiate into astrocytes (Kopen *et al.*,1999) and type II pneumocytes (Ortiz *et al.*, 2003) when injected directly into the spinal cord, brain or delivered into the lungs, respectively. Multilineage differentiation of murine MSCs has been observed only in the case of murine "Multipotent Adult Progenitor Cells" (MAPCs). These cells were shown to differentiate into haematopoietic cells in the marrow, blood, spleen, as well as into various tissue cell types after i.v administration (Jiang *et al.*, 2002). However, it remains to be determined whether MAPCs exist as such *in vivo* or are a consequence of de-differentiation of an MSC-like cell, which might have emerged from a specific and selective culture system, giving rise to a unique *in vitro* phenotype.

This study demonstrates that a highly enriched muMSC population can be incorporated into several tissues, after systemic infusion into recipient animals that only received sub-lethal irradiation. The data indicate that infused MSCs can give rise to hepatocytes, renal tubular cells, bronchiolar epithelial cells, myofibres and myofibroblast-like cells, although at a low frequency. Different explanations could be proposed for the rare occurrence of these events. It was chosen to inject MSCs into recipient animals not forced to undergo severe organ degeneration and/or repair first. It is possible that injured tissues express specific receptors or ligands to facilitate trafficking, adhesion and infiltration of MSCs to the site of injury. Indeed, a recent report showed that muMSCs could differentiate into type II pneumocytes after systemic infusion in bleomycin-treated mice (Ortiz *et al.*, 2003). The difference in the type of lung cells that MSCs differentiated into compared with this study, can be explained either by the importance of specific tissue damage in promoting/enhancing this type of phenomena, or by the different experimental design used here. Although sub-lethal irradiation could incur some damage to the lungs, the physical retention of MSCs in this organ might have resulted from the large number of cells transplanted, or the fact that they weakly express appropriate adhesion molecules. As a consequence, this retention may have drastically reduced the number of potential MSCs capable of homing to other organs. These

findings are in accordance with previous reports (Gao *et al.*, 2001; Barbash *et al.*, 2003). However, there are no reports on the potential detrimental effect that this retention of MSCs in the lung may cause on lung function. Thus, it can be suggested that systemic infusion might not be the best delivery route for this type of stem cell. In order to drive or enhance the homing and differentiation ability of the transplanted MSCs for tissue repair, specific or selective pressure may be required.

Again, the lack or weak expression of integrins and other adhesion molecules that play a vital role in cell migration and/or attachment to different tissues may also account for the inability of muMSCs to home back to the bone marrow. As reported recently, this loss and/or change in homing/attachment capacity of these cells could be due to the culture conditions used (Rombouts *et al.*, 2003). Based on this fact, better understanding of the signalling mechanisms that attract bone marrow cells to specific tissues will be necessary in order to enhance the prospects of systemic delivery of MSCs as a therapeutic method for tissue repair. Local infusion of MSCs to a tissue of interest might be an attractive alternative route of delivery.

Another possible explanation for the low frequency of the differentiation events seen here is that the MSC compartment is very heterogeneous and although an enriched MSC population was used, this may not have included the most primitive and possibly most multipotent cells. As described in the following chapters, MSCs contain a minor population of quiescent cells sharing some MAPC features (Jiang *et al.*, 2002). Interestingly, despite the high transduction efficiency obtained with the lentivirus vector, it was observed that the majority of this subpopulation of cells lay in the untransduced fraction (<2%). Therefore, cells with higher differentiation capacity might have unexpectedly been excluded from these experiments.

Finally, it is still unclear what mechanism is responsible for the observed differentiation phenomena shown in this study. Recent reports on the differentiation capacity of haematopoietic cells have identified fusion as the primary mechanism responsible for the observed phenomena (Terada *et al.*, 2002; Ying *et al.*, 2002; Alvarez-Dolado *et al.*, 2003; Wang *et al.*, 2003;

Camargo *et al.*, 2004; Willenbring *et al.*, 2004). Although there are other mechanisms apart from fusion that have been shown to occur (Harris *et al.*, 2004; Jang *et al.*, 2004). The contribution of MSCs into different cell types reported here could have arisen from trans-differentiation, de-differentiation, fusion with resident cells or even a combination of these possible mechanisms. Although fusion is not the mechanism responsible for the *in vitro* differentiation capacity of muMSCs described here, we could not rule it out as a phenomenon occurring *in vivo* and *in vitro*, as recently described for haematopoietic cells (Alvarez-Dolado *et al.*, 2003). It is possible that eGFP-MSCs that have been incorporated into the liver and the skin (myofibres) could have resulted from fusion. The main aim of this study was to address the "natural" distribution of MSCs into different organs after systemic delivery, and possible *in vivo* contribution capacity of these cells. Due to the experimental design of this study it was unable to address the mechanism of these differentiation events.

In summary, a defined and highly enriched muMSC population was capable of incorporating into various cell-types when administered systemically under minimal damage conditions. Despite the low frequency of such phenomena, this observation demonstrates for the first time that muMSCs have multipotent capacities *in vivo*.

Chapter 4 - Newly identified SSEA-1^{pos} cells as the most primitive fraction of muMSC compartment

4.1 - Brief introduction and description of the study

Since the first description of MSC in early 70's, researchers have failed to isolate the most primitive cell-type from the bulk cultures of mesenchymal cells. Through indirect methods and accumulative observations throughout the years, it has created in the scientific community the notion/speculation that *ex-vivo* expanded MSCs are very heterogeneous and composed mainly by mesenchymal cells (without any stem cell property) and some progenitors/stem cells. The lack of an appropriate way to isolate and expand the "true" mesenchymal stem cell from a very heterogeneous population had led to much discouraging data concerning their *in vivo* function because in most (if not all) of these studies undefined bulk cultures of MSC were used. Indeed, results just presented in chapter-3 confirmed this speculation. Firstly, it was observed that upon *in vitro* stimulation only a fraction of the cells was capable of differentiating into its respective lineage. Moreover, the *in vivo* contribution capacity of the cells was somewhat rare. This is also the reason why it has been called "enriched or bulk MSC cultures" instead of "MSC cultures" throughout this manuscript.

In fact, as described in the general introduction section, much of the mesenchymal stem cell biology remains elusive, especially its hierarchical organisation. Although some data suggest that MSCs are composed of different subpopulations of progenitors or cells that are prompted to differentiate preferentially into one or few different cell-types *in vitro* (Bianco *et al.*, 2001; Gronthos *et al.*, 1999 and 2003; Muraglia *et al.*, 2000; Zannettino *et al.*, 2003; Wistel-Gendebien *et al.*, 2003), there is no defined hierarchy proposed yet, especially at the most primitive level. Several groups have reported the use of different cell surface antigens to isolate/enrich different

MSC-progenitors (Gronthos *et al.*, 1999 and 2003; Wistel-Gendebien *et al.*, 2003). However, there is no defined universal stem/progenitor cell marker yet, which could be used to isolate/enrich the putative most primitive MSC population capable of highly differentiating into different cell-types both *in vitro* and *in vivo*, ultimately demonstrating the true multipotency of MSCs.

Recently, murine multipotential adult progenitor cells (Jiang *et al.*, 2002) with features of embryonic stem cells have been described. In fact when injected into early blastocysts, a single MAPC can contribute to most, if not all, somatic cell types. Similarly, when using culture conditions mimicking the microenvironment of the marrow niche it is possible to isolate multipotent cells from human bone marrow (D'Ippolito *et al.*, 2004). Nevertheless, like MAPCs, no prospective isolation of these multipotent cells exists yet, making their existence as such *in vivo* still uncertain.

As such, there was a necessity to identify (or attempt to) the putative stem cell within the bulk MSC cultures. Based on studies performed by D. Prockop's and C. Verfaillie's groups a new subpopulation of MSCs was identified. These cells have a peculiar phenotype by expressing the SSEA-1 antigen. Optimisation of protocols for SSEA-1^{pos} cell isolation from bulk cultures was first conducted. Then, SSEA-1^{pos}-MSCs were immunophenotyped and differentiation capacity studies were carried out. Some molecular phenotyping and *in situ* localisation of this newly identified cell-type directly in the bone marrow was also performed.

4.2 - muMSCs differentiate into cell-types of the three-germline layers *in vitro*

In order to minimize potential culture artefacts that could alter any cell fate, a simple method to enrich and expand muMSC based on their ability to adhere to plastic followed by negative selection using CD45 and CD11b was performed as described previously. After enrichment, the *in vitro* differentiation capacities of MSCs were verified. Similarly to eGFP-MSCs, un-transduced cells upon appropriate culture conditions were able to give rise to

adipocytes, osteoblasts, myoblasts and astrocyte/neuronal-like cells determined by immunocytochemistry staining (**Figure 11**). Furthermore under endothelial culture conditions some of the MSCs acquired the expression of vWF and CD31 (data not shown). Similarly, under hepatocyte differentiation conditions albumin, HNF (Hepatocyte Nuclear Factor), and CK-18 positive cells could also be detected (**Figure 11**). Therefore, bulk cultures of muMSCs were capable of differentiating into different cell-types of the three-germline layers *in vitro.* As mentioned above, during the course of the experiments, it was observed that upon MSC differentiation down a specific pathway, only a fraction of the cells were capable of doing so. With the exception of astrocyte/neuronal and endothelial differentiation, where a higher proportion of cells expressed the respective lineage-specific antigens, in all other *in vitro* conditions only clusters of differentiated cells could be observed. Data shown were further supported by FACS analysis performed on the same or other lineage-specific antigens used in immunocytochemistry studies (see below).

4.3 - Identification of SSEA-1^{pos} cells in the muMSCs compartment

The *in vitro* results are in accordance with the concept/speculation that the MSC compartment is heterogeneous and composed of different types of stem/progenitor cells, which are capable of differentiating into one or a few lineages under the appropriate *in vitro* conditions (Bianco *et al.*, 2001; Deans and Moseley, 2000; Gronthos *et al.*, 1999 and 2003; Javazon *et al.*, 2001; Muraglia *et al.*, 2000; Zannettino *et al.*, 2003; Wistel-Gendebien *et al.*, 2003).

Firstly, immunophenotyping studies were conducted by using several markers reported to be expressed on MSCs from different species (Deans and Moseley, 2000; Javazon *et al.*, 2001). As shown by others (Colter *et al.*, 2000; Deans and Moseley, 2000; Muraglia *et al.*, 2000), no subpopulation could be defined by using such antigens (**Figure 12**).

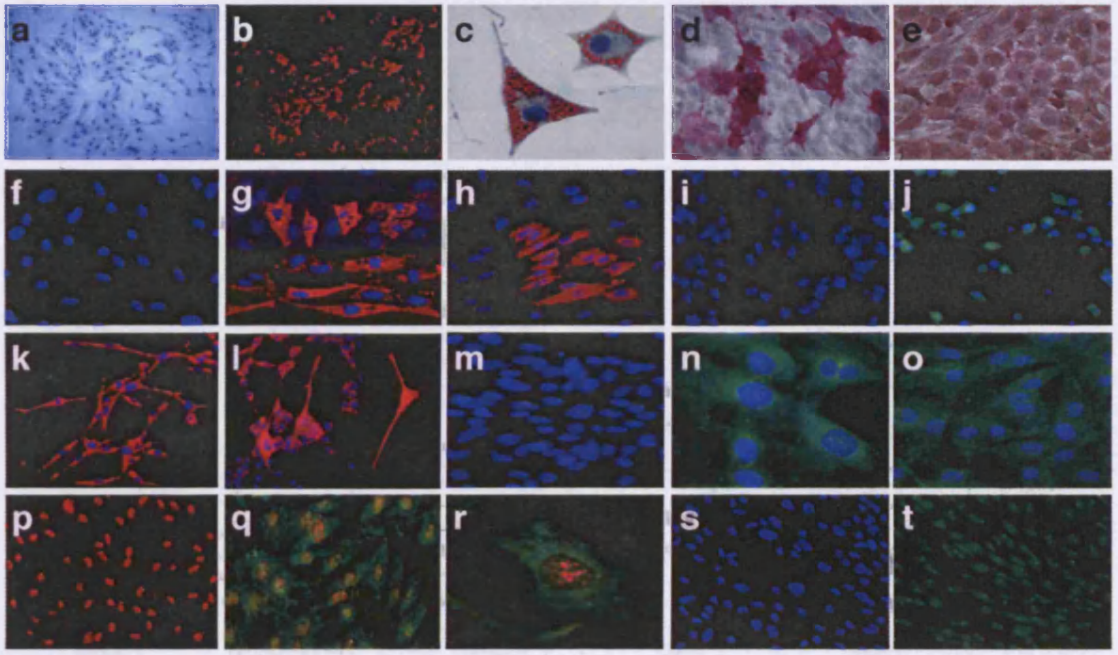


Figure 11. *In vitro* differentiation of muMSCs into different cell-types of the three-germline layers

Representative pictures from several different experiments (n=3 to 6). Unstimulated MSCs were viewed under light microscope (**a**). After 14 days of adipogenic induction, cells were fixed and stained with Oil Red O (**b**, **c**; **b**-was viewed under black-phase contrast). Osteogenic potential was determined by alkaline-phosphatase staining (**d**, **e**). An area of osteoblast-like cluster formation is also shown (**e**). Staining for specific antigens for myogenic (**f-h**), astrocyte/neuronal (**i-l**), hepatocyte (**m-r**) and endothelial (**s** , **t**) lineages were performed by immunofluorescence. Myogenic differentiation was confirmed with single staining for dystrophin-TRITC (**g**) and desmin-Cy3 (**h**). Specific staining for astrocyte/neuronal antigens was performed with NeuN-FITC (**j**), GFAP-Cy3 (**k**) and Tau-TRITC (**l**). Hepatocyte and endothelial-like cells were confirmed with staining for CK18-FITC (**n**, **r**), albumin-FITC (**o**, **q**), HNF-TRITC (**p**, **q**, **r**) and vWF-FITC (**t**) respectively. Cells were counter-stained either with haematoxylin (**a-e**) or DAPI (**f-k**, **m-o**, **s**). Respective isotype controls conjugated with FITC and Cy3/TRITC were also performed for each individual lineage (**f**, **i**, **m**, **s**). Original magnifications of X100 (**a**, **b**), X200 (**d**, **f-m**, **o-q**, **s**, **t**), X400 (**c**, **e**, **n**, **r**).

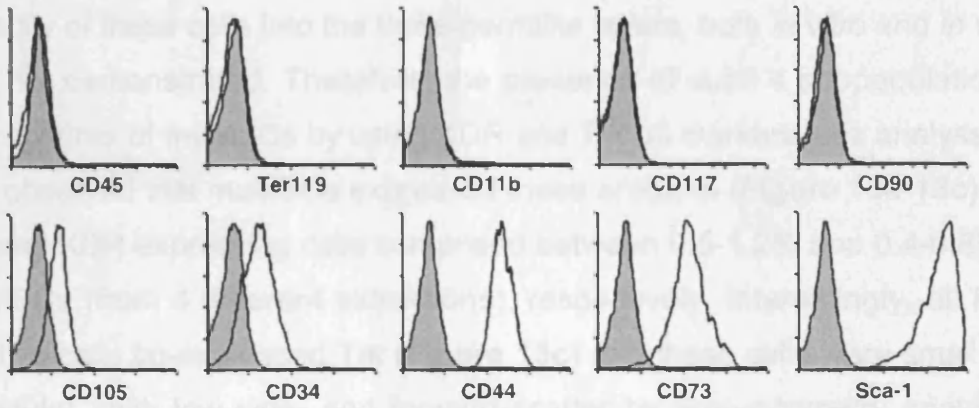


Figure 12. Immunophenotype profile of enriched MSC cultures

Examples of FACS histograms showing that enriched MSCs cultures after depletion of CD45/CD11b^{pos} cells no longer contain haematopoietic contaminants (CD45/ Ter119/CD11b/CD117 expressing cells). Moreover, using different markers that have been used to define mouse, rat and human MSCs, such as CD90, CD105, CD34, CD44, CD73 and Sca-1, failed to reveal any distinct subpopulation within the bulk cultures. All FACS plots shown: grey and white areas are the isotype control and specific antibody staining respectively.

Therefore, the existence of a primitive subpopulation previously reported for human and rat MSCs (Colter *et al.*, 2000 and 2001; Javazon *et al.*, 2001) was investigated. The small and quiescent subpopulation, referred to as RS1 cells, displays a phenotype (e.g. expressing CD71; Trk or NGF, nerve growth factor; KDR or Flk-1 or VEGF-R2, vascular endothelial growth factor receptor-2), which is different from the most abundant, fast growing and committed precursors (Colter *et al.*, 2000). However, the full differentiation capacity of these cells into the three-germline layers, both *in vitro* and *in vivo,* was not demonstrated. Therefore, the presence of such a subpopulation in bulk cultures of muMSCs by using KDR and Trk as markers was analysed. It was observed that muMSCs expressed these antigens (**Figure 13a-13c**) and Trk and KDR expressing cells comprised between 0.5-1.2% and 0.4-0.8% of muMSCs (from 4 different extractions), respectively. Interestingly, all KDR positive cells co-expressed Trk (**Figure 13c**) and these cells were small and agranular (with low side- and forward-scatter by flow-cytometry analysis). Thus, these data indicate that muMSCs do effectively contain RS1 cells.

Bulk cultures of muMSCs were then analysed for the presence of SSEA-1 expressing cells, an antigen described to be expressed on MAPCs. FACS analysis revealed the existence of a subpopulation expressing SSEA-1 in MSCs cultures (**Figure 13d**). The SSEA-1 positive subpopulation, comprised 0.45-0.97% of all muMSCs (n=4) and, interestingly, this proportion remained constant throughout further passages (data not shown). Further analysis of muMSCs also revealed that the majority of the quiescent cells (in confluent cultures) residing at G0 were composed mainly of SSEA-1^{pos} cells (**Figure 13f**, **13g**).

These findings led to speculation that SSEA-1^{pos}-MSCs might be similar to, or at least part of, the RS1 subpopulation. SSEA-1^{pos}-MSC population is still heterogeneous and it does not only include the RS1 cells (that are KDR^{pos} and Trk^{pos}) but other cell-types as well. The SSEA-1^{pos}/KDR^{pos}/Trk^{pos} fraction comprised around 70-80% of the SSEA-1^{pos} cells, while the second major fraction was negative for KDR and Trk (**Figure 13e**) and comprised <25% (n=4) of this subpopulation. A third small fraction could be occasionally identified, expressing only Trk.

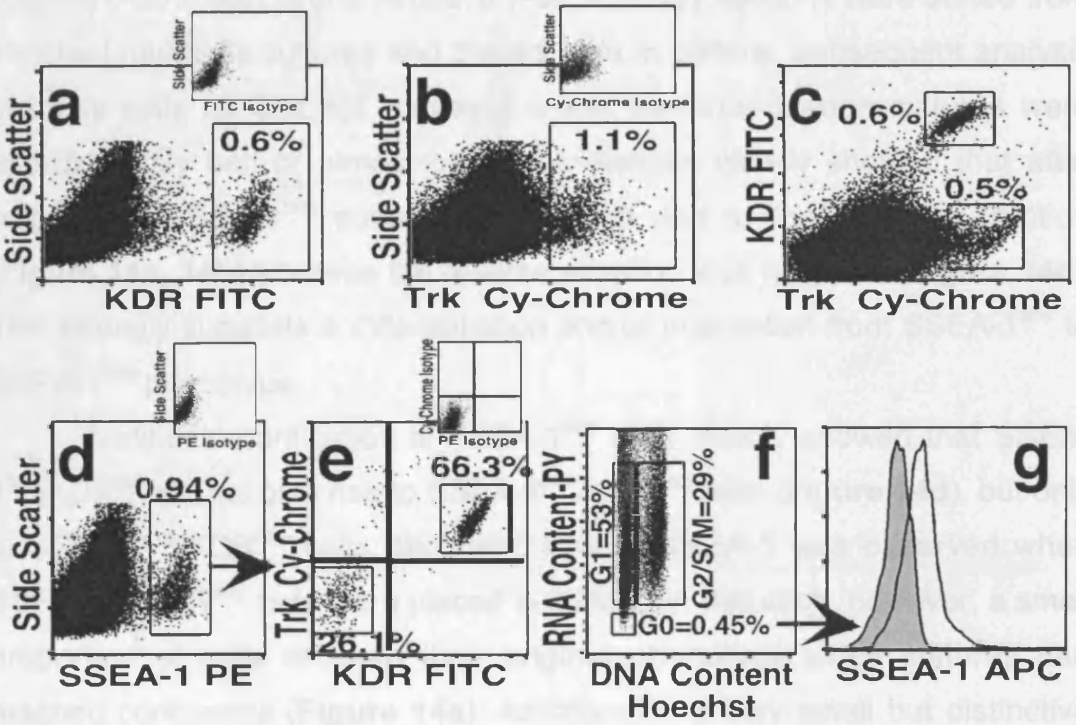


Figure 13. Revealing the hierarchical organization of muMSC

Representative flow cytometry plots of MSCs from passage 5. FACS analysis showing that MSCs contain RS1-like cells expressing KDR (**a**, **c**) and Trk (**b**, **c**). SSEA-1 is expressed on MSCs (**d**) and this population is still heterogeneous for KDR and Trk expression (**e**). Hst/PY staining (**f**) reveals that the quiescent (G0) MSC subpopulation is highly enriched for SSEA-1 expressing cells (**g**; grey and white areas are the isotype control and SSEA-1 antibodies staining respectively). Insert FACS plots represent isotype control staining.

4.4 - SSEA-1^{pos}-MSCs are at the apex of the muMSCs hierarchy and multipotent *in vitro*

All the data presented support the hypothesis that SSEA-1^{pos} cells are at the apex of the muMSCs hierarchy. In order to test this, both SSEA-1 positive (>90% purity) and negative (>98% purity) fractions were sorted from enriched muMSCs cultures and placed back in culture. Subsequent analysis of these cells for SSEA-1 expression was performed when cultures were approximately half or almost confluent. Results clearly showed that after culture the SSEA-1^{pos} subpopulation gave rise to the negative fraction (**Figure 14a, 14b**) whereas the reverse situation was not seen (**Figure 14c**). This strongly suggests a differentiation and/or maturation from SSEA-1^{pos} to SSEA-1^{neg} phenotype.

Additional purification of SSEA-1^{pos} cells clearly showed that SSEA-1^{pos}/KDR^{pos} did not give rise to SSEA-1^{pos}/KDR^{neg} cells (**Figure 14d**), but only to SSEA-1^{neg}/KDR^{neg} cells. Similarly, loss of SSEA-1 was observed when SSEA-1^{pos}/KDR^{neg} cells were placed in culture. In this case, however, a small proportion of cells retained their original phenotype when cultures had reached confluence (**Figure 14e**). Additionally, a very small but distinctive fraction of SSEA-1^{pos}/KDR^{pos} cells was also found suggesting that SSEA-1^{pos}/KDR^{neg} cells could give rise to SSEA-1^{pos}/KDR^{pos} cells (RS1 cells).

To further examine that SSEA-1^{pos}-MSCs are the most primitive cells, the *in vitro* differentiation capacities of these cells were determined. Different methods were applied for isolating these rare cells from bulk cultures. Based on the fact they usually comprise <1% of the whole MSC fraction, direct sorting resulted in a very poor viability of the cells due to long sorting hours required to obtain reasonable amounts of cells for subsequent experiments. Immunolabelling followed by separation using Dynabeads^{TM}, StemSep^{TM} and MACS^{TM} cell purification systems were tested and two rounds of column separations by the MACS system gave the best purity, viability and yield of the cells. As such, all subsequent isolations of SSEA-1^{pos} cells were performed using MACS.

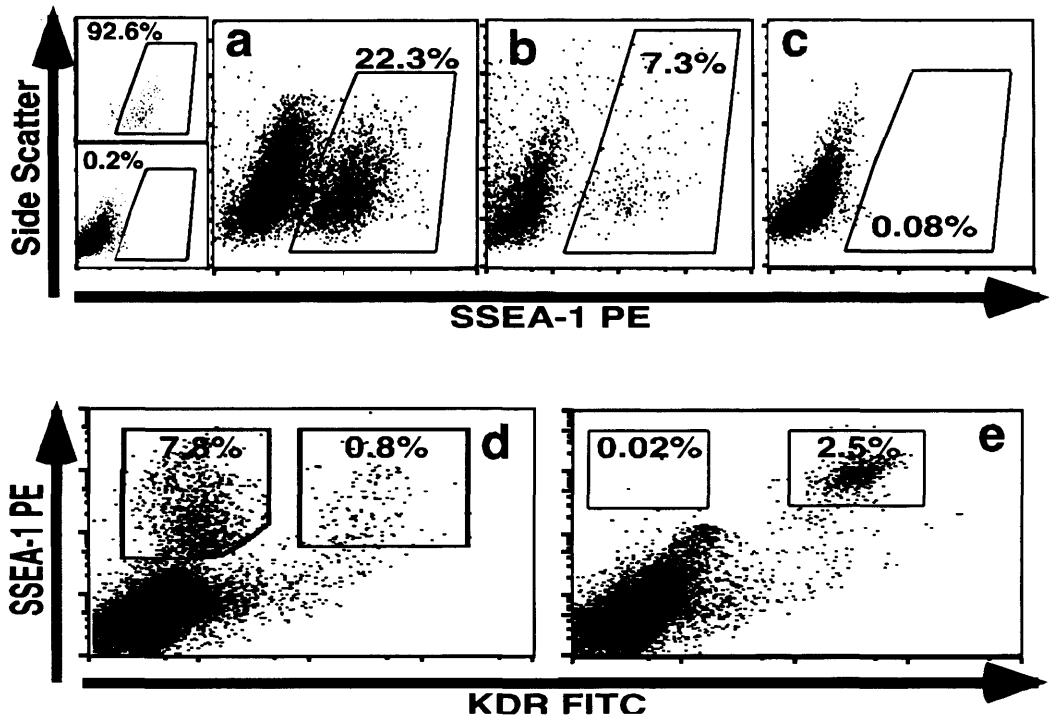


Figure 14. SSEA-1^{pos}-MSC at the top of murine MSC hierarchy

Hierarchical studies performed with different subpopulations of MSCs. Purified SSEA-1^{pos} (**a** – upper left plot) and SSEA-1^{neg} (**a** – bottom left plot) cells were plated and analysed few days later. Expression of SSEA-1 from the SSEA-1^{pos} cultures after attaining half confluency (**a** – right plot, n=2) and almost confluency (**b**, n=3). Cells from the negative fraction were also stained for the same antigen when cultures attained half confluency (**c**, n=3). Similar studies were also performed with the two main subpopulations of SSEA-1^{pos} cells. SSEA-1^{pos}/KDR^{neg} (**d**, n=3) and SSEA-1^{pos}/KDR^{pos} (**e**, n=7) fractions were re-analysed for the expression of the same antigens when cultures attained almost confluency.

Enriched SSEA-1^{pos}-MSCs (~60-70% purity) were compared to unfractionated muMSCs. FACS results clearly showed higher expression of different antigens for each specific lineage when the enriched SSEA-1^{pos} fraction was used, compared to bulk cultures (**Figure 15**). The expression of these antigens was not detected on unstimulated cells (**Figure 15**). For the neuroectodermal lineage, all the studies were focussed on the astrocytic lineage since most of cells acquired GFAP and GLAST, although some Tau and NeuN positive cells were also detected in the induced cultures.

Based on the fact that SSEA-1^{pos} cells comprise a very small fraction from the bulk MSC cultures and they are very difficult to isolate, western-blot analysis for the expression of all the specific antigens was not possible. Thus, FACS analysis was used instead. To validate the FACS data, the specificity of all the antibodies was confirmed by western-blot first and this was performed using bulk MSC cultures (before and after *in vitro* stimulation). Moreover, appropriate antibody-isotype controls were also used, which were selected based on the closest match between their respective M.F.I (mean fluorescent intensity) values with the ones obtained from an antibody which recognises an antigen that is absent in the cells (e.g. anti-eGFP, since the cells were not tagged with eGFP).

4.5 - SSEA-1^{pos}-MSCs share similarities with MAPCs and exist *in vivo*

This higher differentiation capacity of SSEA-1^{pos} cells led to the investigation of whether SSEA-1^{pos} cells share similar features with MAPCs, as this is the only cell-type known to have high differentiation capacities. Therefore SSEA-1^{pos} and SSEA-1^{neg} cells were placed in MAPC culture conditions and allowed to expand for ~10 doublings (n=3) before they were further analysed for the expression of antigens, previously used to characterise MAPCs. Both positive and negative cells were capable of expanding under those culture conditions but only the positive fraction

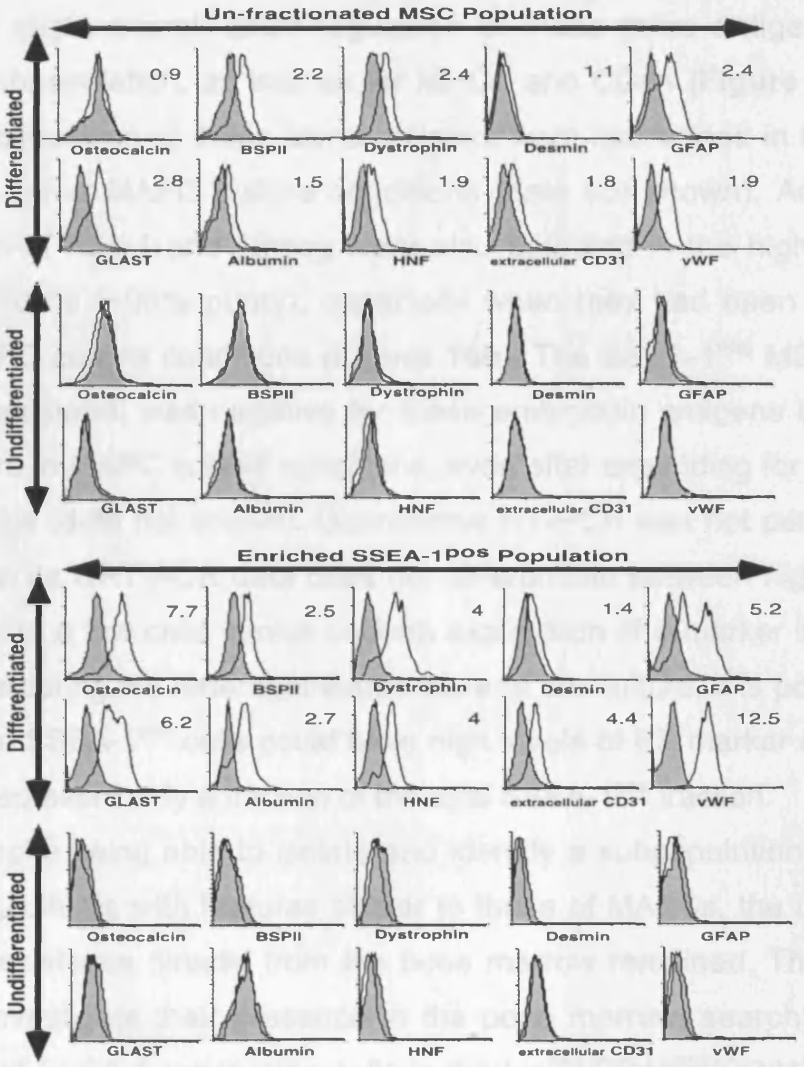


Figure 15. Comparative study of the *in vitro* differentiation capacities of un-fractionated MCSc and enriched SSEA-1^{pos}-MSC populations

FACS histograms show levels of expression of specific antigens for MSC differentiation into osteogenic (osteocalcin and Bone Sialoprotein II, BSP II), myogenic (dystrophin and desmin), astrocyte (GFAP, GLAST), hepatocyte (albumin and HNF), and endothelial (extracellular CD31 and vWF) lineages. Grey and white areas are the isotype control and specific antibody staining respectively. The numbers shown indicate the levels of expression for each antigen that were calculated using the M.F.I (mean fluorescent intensity) values obtained from each specific staining over the values from their respective isotype controls.

maintained the expression of SSEA-1, KDR and Trk (**Figure 16a**). Results showed a slight overall down-regulation of these three antigens on the positive subpopulation, as well as for MHC-I and CD44 (**Figure 16a**). The levels of expression of these same antigens were maintained in further cell doublings under MAPC culture conditions (data not shown). Additionally, expression of Rex-1 and Nanog were also detected in the highly purified SSEA-1^{pos} cells (~90% purity), especially when they had been expanded under MAPC culture conditions (**Figure 16b**). The SSEA-1^{neg} MSC fraction on the other hand, was negative for these embryonic antigens before and after culture in MAPC culture conditions, even after expanding for more than 30 doublings (data not shown). Quantitative RT-PCR was not performed on these cells, as QRT-PCR data does not differentiate between high levels of expression in a few cells versus uniform expression of a marker in all of the cells. Considering the heterogeneous nature of the culture, it is possible that a subset of SSEA-1^{pos} cells could have high levels of ES marker expression that may represent only a fraction of the total SSEA-1^{pos} fraction.

Despite being able to isolate and identify a subpopulation of cells in bulk MSC cultures with features similar to those of MAPCs, the question of their true existence directly from the bone marrow remained. Therefore, in order to investigate their presence in the bone marrow, searching for the presence of SSEA-1 expressing cells in the Lin^{neg}/CD45^{neg}/CD31^{neg} fraction (Anjos-Afonso *et al.*, 2004) of bone marrow cells was performed. As such, an SSEA-1^{pos} population in the MSC compartment (R1 from **Figure 17a**) was identified. This population comprised ~0.04% of the Lin^{neg}/CD45^{neg}/CD31^{neg} fraction in an adult mouse (aged >12 weeks old). Interestingly, the same population in 2 weeks old mice was 30-fold enriched, with SSEA-1^{pos} cells reaching a frequency of 1.5%. This SSEA-1^{pos} cell population decreased with age (**Figure 17b**) showing a variation during mouse ontogeny. Also, these SSEA-1^{pos} cells expressed Sca-1 (**Figure 17a**). Additionally, using multicolour immunofluorescence staining it was possible to identify the *in situ* bone marrow location of these cells, which is in close vicinity to CD45/CD11b/TER119/CD31^{pos} cells and slightly enriched below the endochondral-bone region in a developing bone (**Figure 19a-g**).

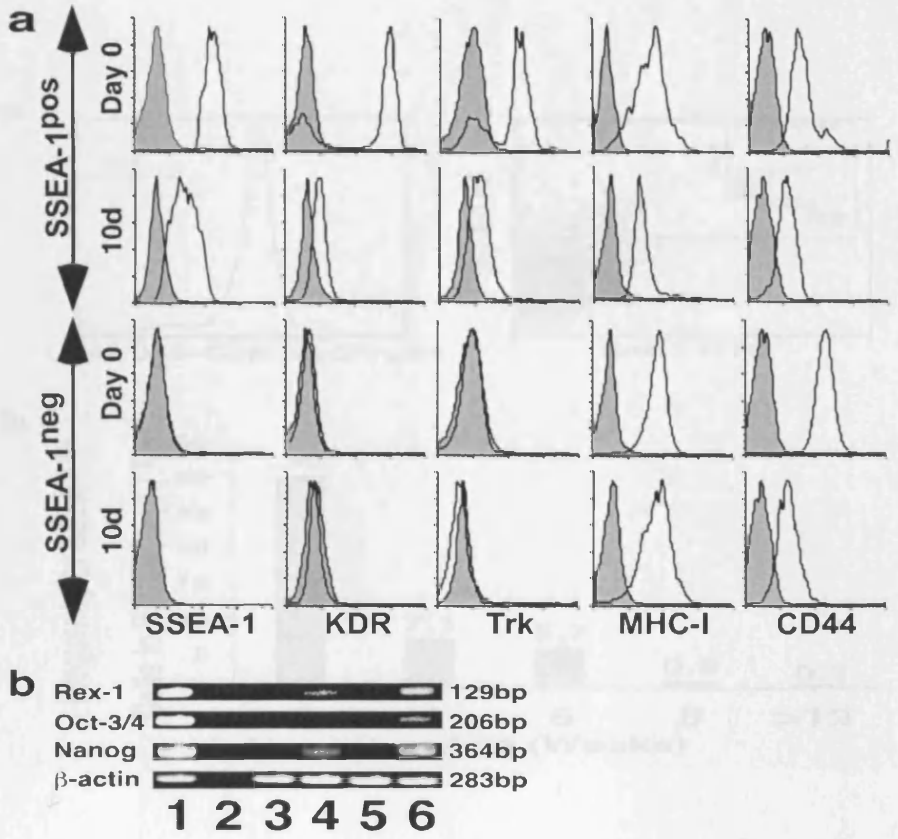


Figure 16. SSEA-1^{pos}-MSCs share similarities with MAPCs

(**a**) Both FACS sorted SSEA-1^{pos} and SSEA-1^{neg} cells were expanded for ~10 doublings in MAPC culture system and then analysed for characteristic surface antigens (n=3) previously used to define MAPCs. As a comparison, the same surface antigens were used to immunophenotype these two fractions before expansion (day 0). Flow histograms: grey and white areas represent isotype control and specific antibody staining respectively. (**b**) Expression of Oct-3/4, Rex-1 and Nanog by RT-PCR on different MSC subpopulations; representative gel electrophoresis with β-actin as the internal PCR control; lane 1- ES cells, lane 2- water, lane 3- SSEA-1^{neg} -MSCs, lane 4- MACS enriched SSEA-1^{pos}-MSCs, lane 5- FACS purified and expanded SSEA-1^{neg}-MSCs in MAPC conditions, lane 6- FACS purified and expanded SSEA-1^{pos}-MSCs in MAPC conditions.

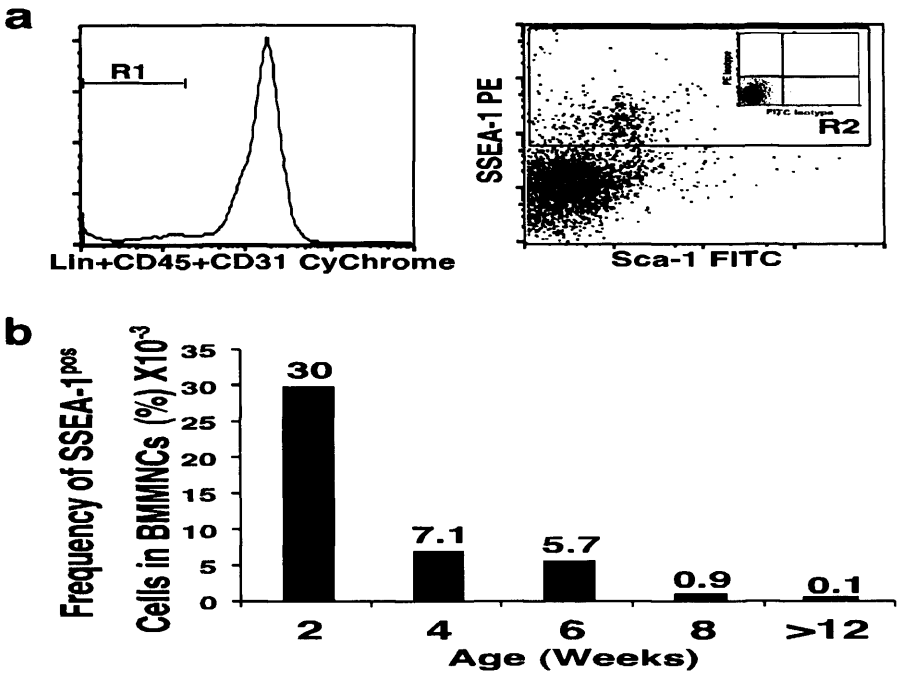


Figure 17. SSEA-1^{pos}-MSCs exist *in vivo*

Identification of SSEA-1 expressing cells directly from BMMNCs. Lineage depleted BMMNCs were stained with Lin/CD45/CD31, SSEA-1 and Sca-1. Representative FACS staining performed on cells derived from 2 weeks old mice. The negative fraction for the expression of Lin/CD45/CD31 (**a**) was gated (R1) and plotted for the expression of SSEA-1 and Sca-1 (**a**). Variation of the frequency of the SSEA-1^{pos} cells during the mouse ontogeny (**b**). The frequency was calculated by multiplying the percentage of MSCs in BMMNCs (R1) with that of SSEA-1^{pos} cells in the MSC population (R2) obtained for each group of mice. The analysis was performed on pooled samples of BMMNCs from 8 (2 weeks old), 10 (4 weeks old), 6 (6 weeks old) and 3 (>12 weeks old; n=2) mice respectively for each group.

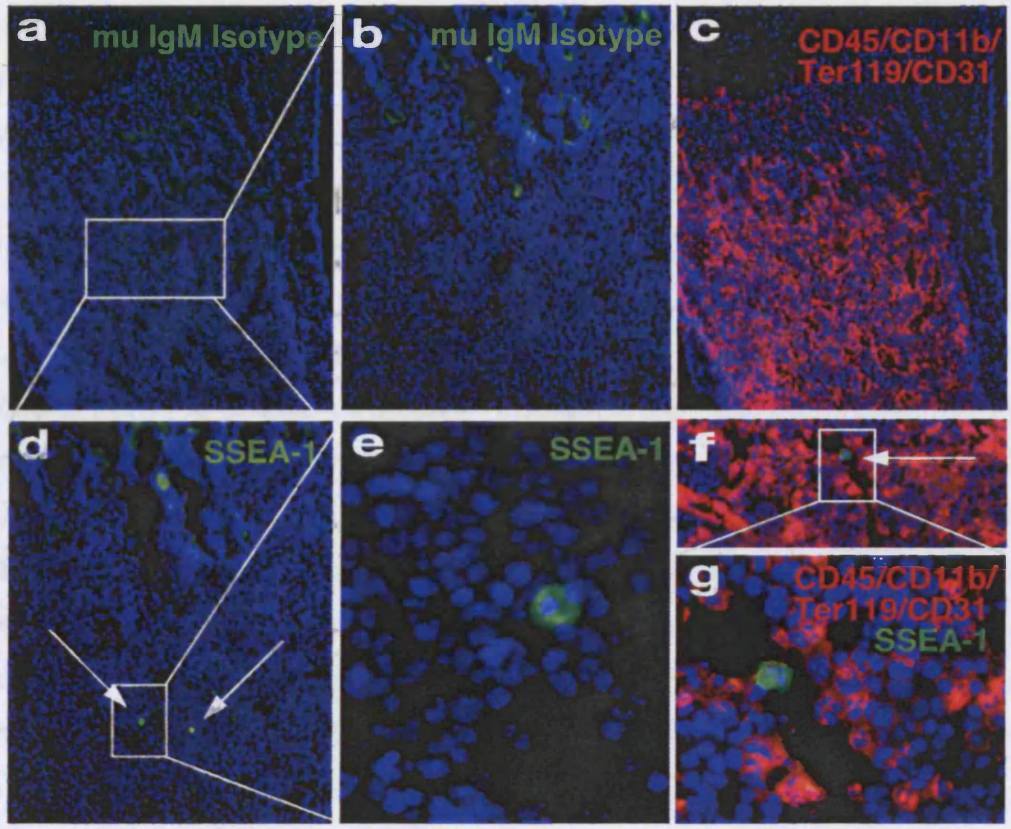


Figure 18. ***In situ*** **identification of SSEA-1^{pos} cells**

Bone sections were stained either with mouse IgM immunoglobulins (isotype control) (**a, b**) or with SSEA-1 antibody (**d-g**). Serial sections (**a, d**) show specific staining for SSEA-1 (green cells indicated by the arrows) in the bone marrow. SSEA-1^{pos} cells can be found throughout the marrow but they are slightly enriched below the endochondral–bone area (highlighted area of **d**) in a developing bone. Representative bone sections with marrow stained positive for CD45/CD11b/Ter119/CD31 (**c, f, g**, red). SSEA-1^{pos} cells are located in close vicinity to CD45/CD11b/Ter119/CD31^{pos} cells but they did not stain positively for either hematopoietic or endothelial antigens. As an example, a SSEA-1 expressing cell (green cell indicated by the arrow, **f**) shows no expression of CD45/CD11b/Ter119/CD31. All sections were counterstained with DAPI. Magnifications: x100 (**a, c**) x200 (**b, d**), x400 (**f**) and x1000 (**e, g**).

Although the above results clearly demonstrate that SSEA-1^{pos} can give rise to SSEA-1^{neg} cells and are therefore suggesting that SSEA-1 expressing cells are the most primitive cell-type, this hypothesis is based on the assumption that these two groups of cells are developmentally related. It is very possible that some or even most of the non SSEA-1 expressing cells in the bulk MSC cultures have derived from a different multipotent stem/progenitor that lacks SSEA-1 expression. As such, it was tested whether SSEA-1^{neg} cells directly isolated from the bone marrow could establish MSC cultures. For this experiment, both SSEA-1^{pos} and SSEA-1^{neg} fractions from the Lin/CD45/CD31^{neg} fraction were purified directly from the bone marrow by FACS and cultured independently (**Figure 19a**). Since the initiation of muMSC cultures is dependent on the presence of haematopoietic cells, Lin/CD45/CD31^{pos} cells were added to each purified SSEA-1 fraction. The number of cells used for each fraction was proportional to their frequency in the bone marrow. When purified SSEA-1^{pos} cells were used, although no delineate CFU-F colonies were formed and the time required for the cells to reach confluency was longer, MSC cultures were obtained in all of the attempts tested (3 out of 3 independent cultures; **Figure 19b**). This is probably due to the significant lower number of adherent haematopoietic cells found in these cultures in comparison to the conventional method of initiating MSC cultures (**Figure 19c**). However, no MSC cultures were obtained when SSEA-1^{neg} cells were used (0 out of 5 independent cultures), demonstrating that establishing muMSC cultures is dependent on the presence of SSEA-1 expressing cells. Also, when BMMNCs from old mice were used (> 15 weeks old NOD/SCIDs; as less SSEA-1^{pos} cells are present) the adherent fractions did not go beyond P2, as most of the cultures did not harbour a significant amount of CD45/CD11b^{neg} cells (**Figure 19d**).

4.6 - Discussion

In this chapter is reported the identification, prospective isolation from cultures and characterisation of a new subpopulation of mouse MSCs, based on the expression of the SSEA-1. These cells can be found not only after *in*

vitro culture but also directly from mouse bone marrow, therefore proving the existence of a subpopulation of MSCs with some embryonic features that reside in adult life. Although the *in situ* localisation of these cells in the bone marrow was determined, due to their rarity and weak avidity of the SSEA-1 antibody, the exact distribution of these primitive cells in the marrow could be different from the data presented. A better marker such as Oct-4, using Oct-4-eGFP mice, might be very useful to further determine the distribution of these cells *in vivo*.

Despite the heterogeneity of the SSEA-1^{pos} fraction, the hierarchical studies performed strongly suggest that this subpopulation is the founder of MSC compartment, thus providing the evidence for a hierarchical organisation. Although the relationship and differences between the two main subpopulations of SSEA-1^{pos} cells (SSEA-1^{pos}/KDR^{pos} and SSEA-1^{pos}/KDR^{neg}) were not completely explored, SSEA-1 seems to mark the most primitive fraction described so far. Based on the data shown and personal observations made, it appears that KDR and Trk expressions (the acquisition of high expression) seen were due to *ex-vivo* expansion conditions applied. Firstly, KDR and Trk expressions within the mesenchymal compartment directly from bone marrow (defined as Lin/CD45/$CD31^{neg}$) and in early adherent cultures were dim and the two subpopulations could not be delineated as clearly as in later passages when cultures were already devoid of haematopoietic contaminants. Secondly, when isolating SSEA-1^{pos} in general from bulk cultures (which includes the two main fractions) and expanded in the MAPC culture system, the two sub-fractions seem to converge to a common phenotype KDR/Trk^{dimpos}.

All the observations made suggest that *ex-vivo* expansion using conventional mesenchymal medium might have driven the cells to a possible more mature phenotype or one with more confined differentiation potentials.

Nevertheless, it does not mean that it is less important to understand the biology of each sub-fraction. On the contrary, defining expansion conditions for the maintenance of self-renewal and properties for SSEA-1^{pos}/KDR^{pos} may be very interesting, because this sub-fraction could be more useful for *in vivo* vascular and nervous system repair studies, as they already

express the appropriate receptors they could be more prone to differentiate into endothelial and astrocyte/neuronal-like cells.

Finally, it was also demonstrated that SSEA-1^{pos}-MSCs have a much higher capacity to differentiate into several cell-types of the three germline-layers than their negative counterparts *in vitro* and also required to initiate MSC cultures. Thus, all the data presented strongly suggest that SSEA-1^{pos} cells are at the apex of the murine mesenchymal stem cell compartment (**Figure 20**).

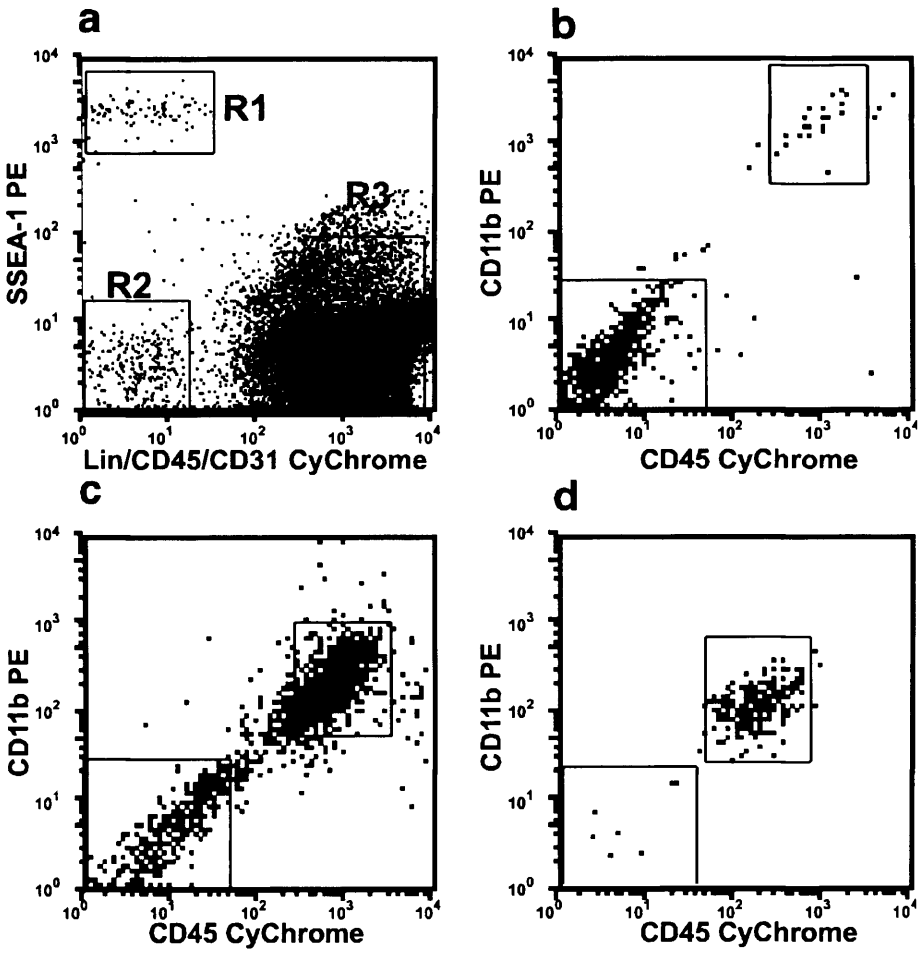


Figure 19: SSEA-1^{pos} cells are required to establish muMSC cultures

(**a**) Both SSEA-1^{pos} (R1) and SSEA-1^{neg} (R2) fractions were purified from lineage depleted BMMNCs and cultured independently in the presence of Lin/CD45/CD31^{pos} (R3) cells. (**b**) Only when the SSEA-1^{pos} fraction was used, were bulk MSC cultures obtained (n=3). Representative FACS plot of P1 adherent culture initiated by SSEA-1^{pos} cells showing little haematopoietic contamination (CD45/CD11b^{pos} cells) in comparison to P1 adherent culture initiated with un-sorted BMMNCs (**c**). (**d**) When older mice were used (>15 weeks old) adherent fractions could not be passaged beyond P2 due to the lack of mesenchymal cells in culture (CD45/CD11b^{neg} subset).

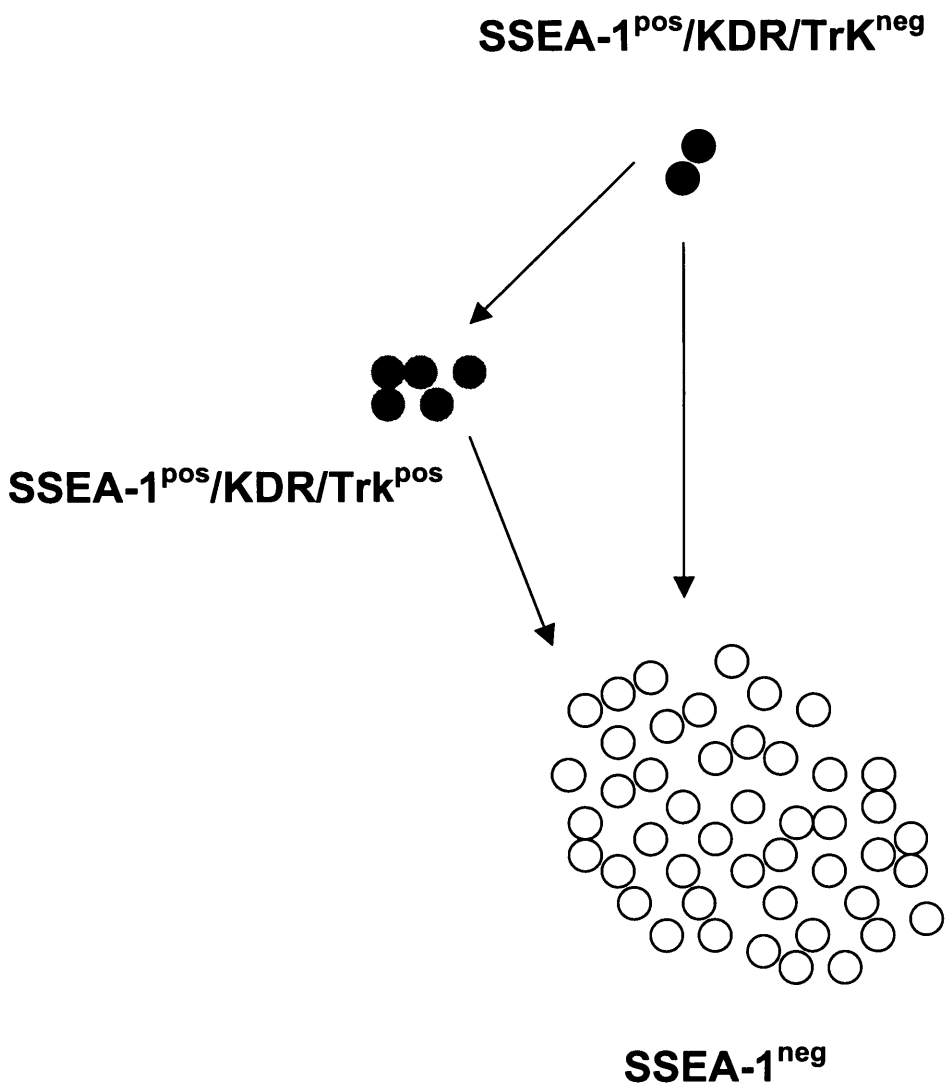


Figure 20: Hierarchical model proposed for the primitive bone marrow derived muMSC compartment.

Chapter 5 - *In vitro* and *in vitro* multipotency of clonally derived SSEA-1^{pos}-MSCs

5.1 - Brief introduction and description of the study

In order to fit into the conventional definition of a stem cell (which should be clonally self-renewing and multipotent) a single stem cell must be isolated and demonstrate its multipotentiality.

From the results presented so far, it is still unclear whether the high multilineage differentiation potential seen was uniform for all SSEA-1^{pos} cells or restricted to different SSEA-1^{pos} fractions with more confined differentiation capacities. Also, it is unclear whether the expression of Oct-4, Nanog and Rex-1 is homogeneous for all the cells.

So far all the data presented were conducted using either enriched or purified SSEA-1^{pos} fractions. Indeed, as shown above, the SSEA-1^{pos} cells are still heterogeneous in regards to KDR and/or Trk expression. As such, it was attemped to isolate and expand single SSEA-1^{pos} cell-derived populations. Many attempts were made and finally three SSEA-1^{pos} clones were obtained. These clones were characterised at the cellular and molecular level in a similar fashion as previously demonstrated in Chapter-4. Due to the high maintenance required for expanding these cells (seeding/maintenance at very low density and constant replenishment of fresh cytokines), one clone (clone-3) was randomly selected for a more detailed description. The multilineage differentiation into the three germline-layers *in vitro* was tested and the functional aspects of the different cell derivates were also assayed. Finally, in order to prove that this newly identified cell-type had multipotent features *in vivo*, transplantation studies were performed and different tissues were screened for the possible contribution of these SSEA-1^{pos} cells into different cell-types.

5.2 - Characterisation of clonally derived SSEA-1^{pos} cells

Due to the peculiar nature of murine mesenchymal cells it was not possible to isolate/expand any mesenchymal cell-type directly from the bone marrow (cells defined as Lin/CD45/CD31^{neg}). Indeed, for the isolation of muMSC in general, it seems that certain factors and/or direct contact with haematopoietic cells is essential to establish early cultures. Establishing cultures of these cells directly after isolation from the bone marrow was completely unsuccessful despite many attempts. Future studies are required to determine why direct isolation of MSC (or SSEA-1^{pos} cells) from murine bone marrow was not achievable. As such, a single SSEA-1^{pos} cell was sorted from the earliest adherent cultures to minimize the *ex-vivo* expansion time required (**Figure 21a**) and expanded under MAPC culture system, shown previously to be able to maintain the phenotype and features of SSEA-1^{pos} cells. Under this stringent culture system, three SSEA-1^{pos} clones were obtained. Preliminary studies using low number of cell doublings revealed that all three clones showed similar cell expansion kinetics, phenotype (**Figure 21b**, **21c**) and *in vitro* differentiation capacities (data not shown). Thus, clone-3 was randomly selected for more detailed characterisation. Clone-3 cells were able to expand linearly over time (**Figure 21b**) with the maintenance of expression of SSEA-1 (high expression), MHC-I and CD44 and low levels of KDR and Trk at different time points (**Figure 21c**), suggesting that these cells have self-renewal features *in vitro*. Interestingly, high levels of expression of Oct-3/4, Rex-1 and Nanog at the RNA level were detected (**Figure 22**).

Thus, it was investigated whether this high RNA expression of these embryonic transcription factors could also be reflected at the protein level. It was observed that the expression of such factors at the protein level was not only maintained throughout the expansion period but also comparable to ES cells (determined by FACS analysis, **Figure 23a**). To support the FACS data nuclear localisation was performed using immunocytochemistry staining (**Figure 23b**). Also, the SSEA-1^{neg} fraction did not stain for the antibodies used, further confirming the specificity of the results presented.

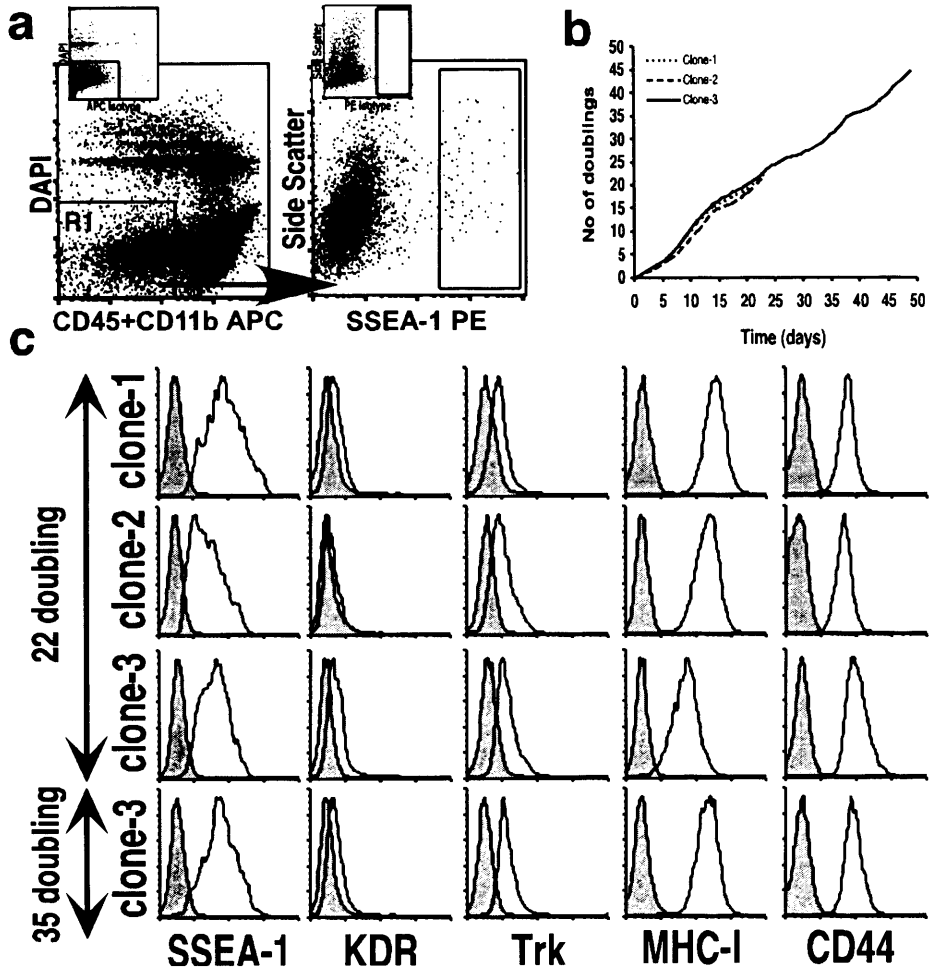


Figure 21. Isolation and characterisation of clonally derived SSEA-1^{pos}-MSCs

A single SSEA-1^{pos} cell was sorted from adherent cultures from passage 1, which is composed mainly of CD45/CD11b^{pos} cells (**a**). (**b**) Representative graph of growth kinetics from the three surviving clones. (**c**) Expressions of SSEA-1, KDR, Trk, MHC-I, CD44 on different clonally derived populations and the maintenance of phenotype of clone-3 cells throughout time.

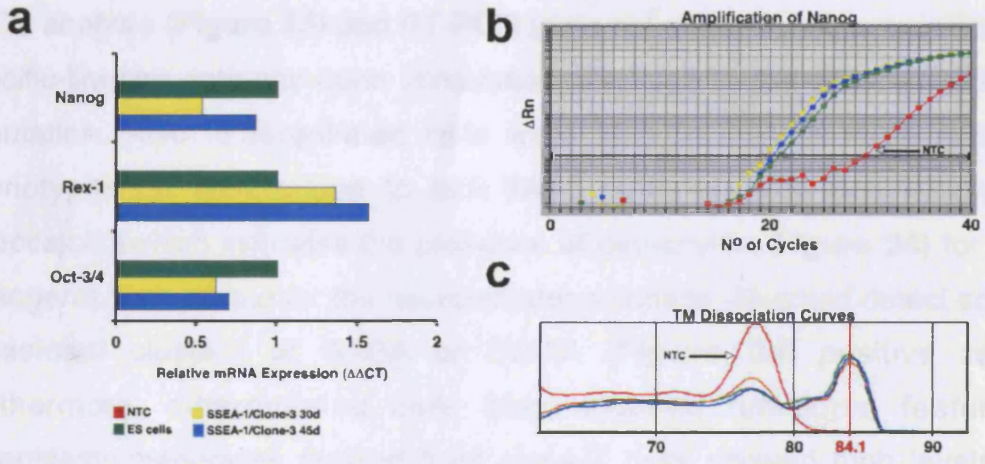


Figure 22. Clonally derived SSEA-1^{pos} cells express high levels of ES transcriptional factors at RNA level

(a) Real-time analysis of Nanog, Rex-1 and Oct-3/4 expressions in ES cells (green lines and bars), clone-3 cells at 30 doublings (yellow lines and bars) and at 45 doublings (blue lines and bars) respectively. Relative mRNA expressions (ΔΔCT) were obtained after normalising the CT values from each gene with the internal PCR control (β-actin) then using the ΔCT values from ES cells as reference. All quantitative results shown are the mean values, each one performed in triplicate. As an example, amplification plot of Nanog (**b**) and its respective dissociation curve (**c**), to verify the specificity of the product (in this case the specific TM is 84.1 ºC), are also shown. NTC: non-template control.

5.3 - Clonally derived SSEA-1^{pos} cells are multipotent *in vitro*

Similarly to what has been shown in Chapter-4, clone-3 SSEA-1^{pos} cells were able to give rise to osteoblast/osteocyte, endothelial-, hepatocyte-, astrocyte/neuronal-like cells, which were confirmed by detecting specific antigen expressions for each lineage using immunostaining (**Figure 24**), FACS analysis (**Figure 25**) and RT-PCR (data not shown). The acquisition of specific-lineage antigens upon stimulation was high in the clonally derived population. Also, differentiated cells were able to acquire more mature phenotypes (in comparison to bulk MSC cultures) with acquisition of osteocalcin (which indicates the presence of osteocytes; **Figure 24**) for the osteogenic lineage and for the neuroectoderm lineage we could detect some occasional clusters of GABA or DOPA (**Figure 24**) positive cells. Furthermore, differentiated cells also acquired functional features. Osteoblasts/osteocytes derived from clone-3 cells showed high levels of alkaline phosphatase activity (**Figure 26a**) and were able to deposit calcium (**Figure 26b**).

Four different assays were used to determine the functional aspects of hepatocyte-like cells derived from the clone-3 cells. Initially, assays were used for the determination of levels of albumin and urea. However, the commercial available kits (mouse specific albumin ELISA kit and Urea calometric kit) were not sensitive enough to correctly quantify the low amounts of albumin and urea, respectively, produced by the cells. Hepatocyte-like cells were shown to deposit glycogen and to have cytochrome P450 activity instead. Indeed, differentiated cells were able to deposit a considerable amount of glycogen, which was revealed by the PAS assay (**Figure 27a**), and showed cytochrome P450 activity even before induction with phenobarbital (**Figure 27b**).

After stimulation with VEGF, clone-3 cells did not only acquire expression of CD31 and Tie-2 but also Weibel-Palade bodies containing vWF. One characteristic of an endothelial cell is that they can take up LDL (Steinberg *et al.*, 1985) and therefore this was tested.

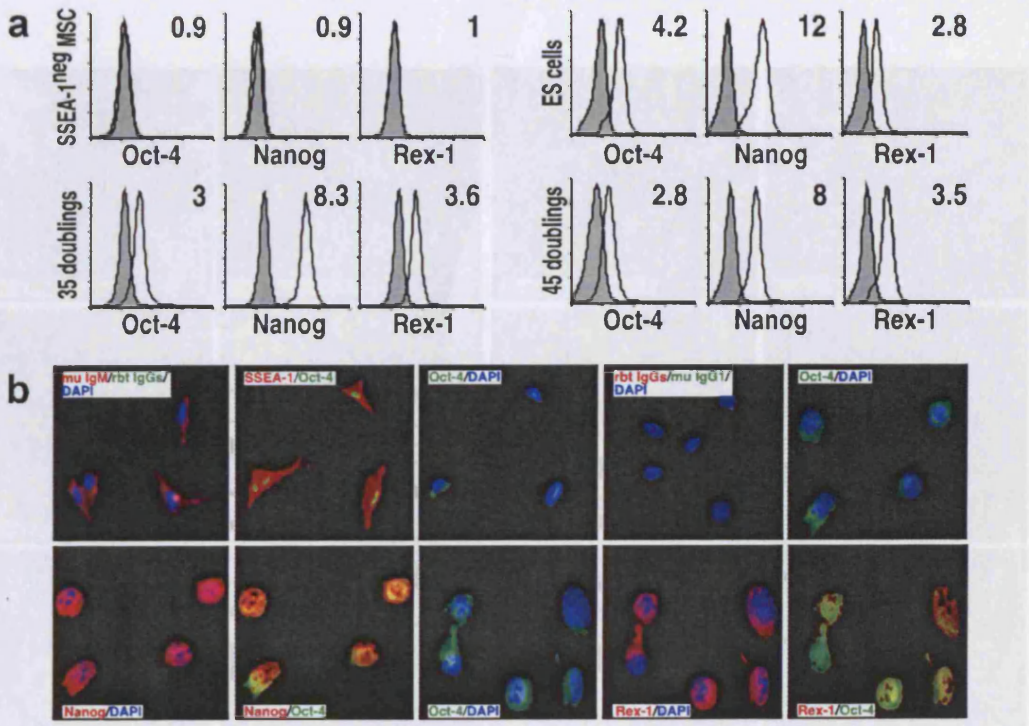


Figure 23. Clonally derived SSEA-1^{pos} cells express high levels of ES transcriptional factors at protein level

(a) Flow histograms showing levels of expression of Oct-4, Nanog and Rex-1 in the clonally derived population. SSEA-1^{neg}-MSCs and ES cells were used as negative and positive controls respectively. For all flow histograms: grey and white areas are the isotype control and specific antibody staining respectively. (**b**) Multicolour immunostaining performed on clone-3 cells from 45 doublings showing that Nanog (red; Alexa594) and Rex-1 (red; Alexa594) are more homogeneously expressed in the nucleus, whereas Oct-4 (green, Alexa488) expression seems to be more concentrated in some areas surrounding the nucleus. An example of SSEA-1 (red; TexasRed) with Oct-4 staining is also shown. Appropriate rabbit IgGs, mouse IgM and mouse IgG1 isotype controls were also used. Magnifications: X200 (first top three pictures of **b)** and X400 (remaining pictures of **b**).

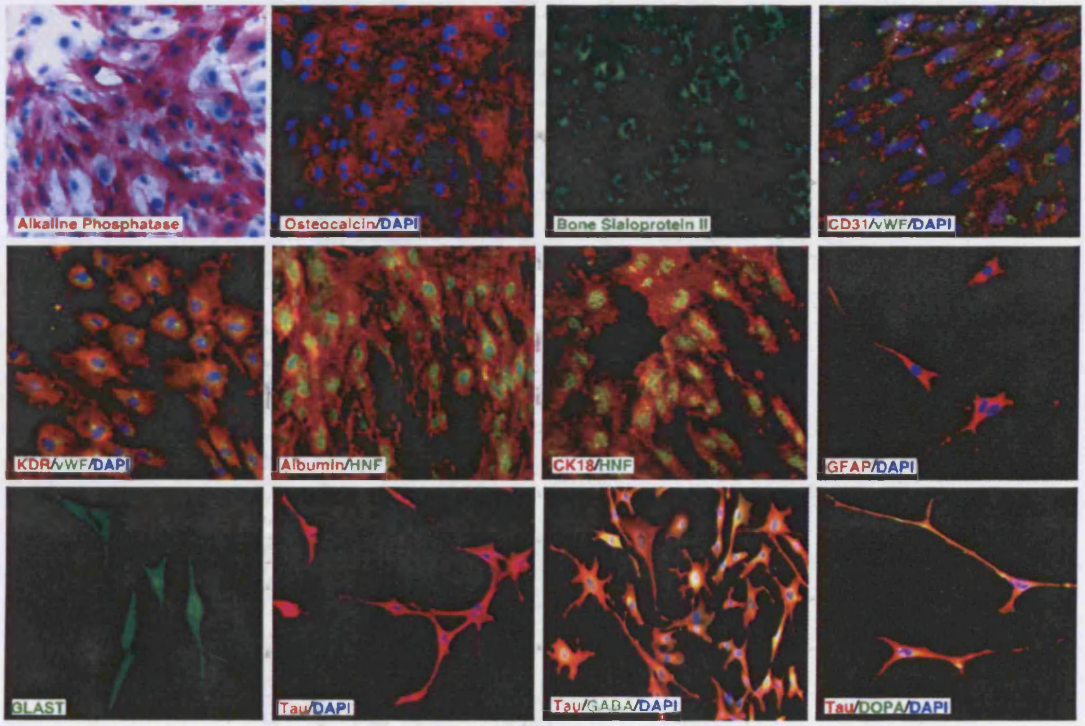


Figure 24. Multipotency of clonally derived SSEA-1^{pos} cells determined by immunocytochemistry analysis

Representative pictures from two independent in vitro differentiation assays performed using clone-3 derived cells. Osteogenic potential was determined using alkaline phosphatase activity (red staining) and staining with osteocalcin-Alexa594 and BSP II-FITC. Endothelial differentiation was confirmed with CD31-Alexa594, KDR-Alexa594 and vWF-FITC staining. Specific staining for hepatocyte-like cells was performed using anti-albumin-Cy3, anti-CK18-Cy3 and anti-HNF-FITC antibodies. Astrocyte/neuronal differentiation was confirmed with staining for GLAST-FITC, GFAP-Cy3. Occasionally some differentiated cells also acquired expression of Tau/GABA and Tau/DOPA respectively. Magnifications: X200.

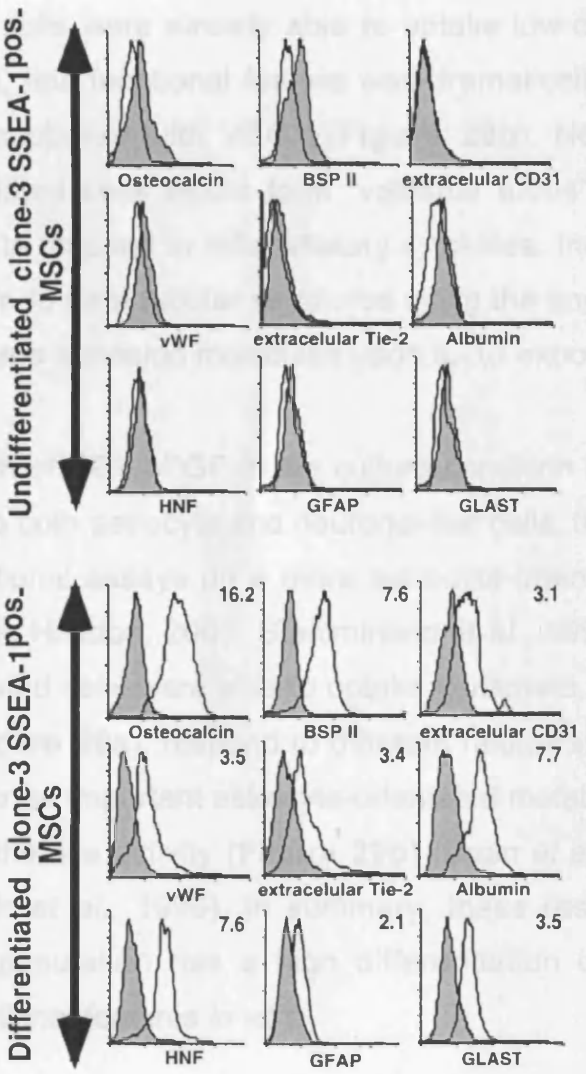


Figure 25. Clonally derived SSEA-1^{pos} cells have high differentiation capacities *in vitro*

FACS histograms showing levels of expression of specific antigens in SSEA-1^{pos} clone-3 derived cells before and after *in vitro* differentiation into osteogenic (osteocalcin and BSP II), endothelial (extracellular CD31, vWF and extracellular Tie-2) hepatocyte (albumin and HNF) and astrocyte (GFAP, GLAST) lineages. The expression of these specific antigens was not detected before *in vitro* stimulation. All FACS plots shown: grey and white areas are the isotype control and specific antibody staining respectively. The numbers shown indicate the relative levels of expression for each antigen, calculated using the M.F.I (mean fluorescent intensity) values obtained from each specific staining over the values from their respective isotype controls.

Although clone-3 cells were already able to uptake low-density-lipoprotein before stimulation, this functional feature was dramatically enhanced after cells had been incubated with VEGF (**Figure 28b**). Next, it was tested whether differentiated cells would form "vascular tubes" when plated on matrigel and also to respond to inflammatory cytokines. Indeed, endothelial-like cells were able to form tubular structures using the angiogenesis assay, and upregulate some adhesion molecules upon IL-1α exposure (**Figure 28a**, **28c**).

The addition of EGF/bFGF to the culture condition induces muMSCs to differentiate into both astrocyte and neuronal-like cells, thus It was chosen to focus the functional assays on a more astrocyte-orientated perspective (Duan *et al.*, 1999; Haydon, 2001; Stanimirovic *et al.*, 1999). For astrocyte lineage, differentiated cells were able to uptake glutamate, specifically using Na^+ channels (**Figure 29a**), respond to different neurotransmitters (**Figure 29c**) and demonstrate important astrocyte-orientated metabolic activity, such as glutamine synthetase activity (**Figure 29b**) (Duan *et al.*, 1999; Haydon, 2001; Stanimirovic *et al.*, 1999). In summary, these results indicate that clonally derived population has a high differentiation capacity with the acquisition of functional features *in vitro*.

5.4 - Clone-3 SSEA-1^{pos}-MSCs can incorporate into different tissues *in vivo*

So far, data from the literature on the contribution of MSCs into different tissue cell-types *in vivo* has been somewhat discouraging. Thus, in order to examine that SSEA-1^{pos} cells have truly multipotential capacities *in vivo,* clone-3 cells were injected into non-irradiated NOD/SCID/β_2^{null} newborn mice and their distribution and contribution to different tissue cell-types was assessed. For this experiment newborn mice were used because their tissues are in a state of rapid growth, thus it might create a better environment for incoming stem cells to be incorporated and proliferate than

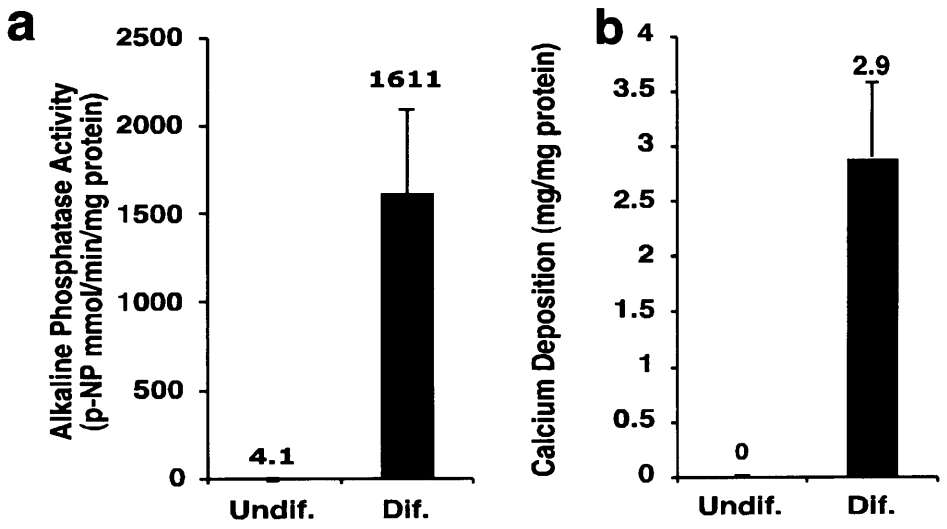


Figure 26. Clone-3 SSEA-1^{pos} cells acquired high levels of AP activity and calcium deposition upon osteogenic differentiation

Representative data from two independent experiments that were carried out with cells from 22 to 35 doublings. After 14 days in the presence of osteogenic induction medium, clonally derived cells acquired high levels of alkaline phophatase activity (**a**) and deposited high levels of calcium (**b**) (Dif. Bars). These functional features were not observed before induction (Undif. Bars). All quantitative results shown are mean ± SD from two independent experiments, each one performed in triplicate.

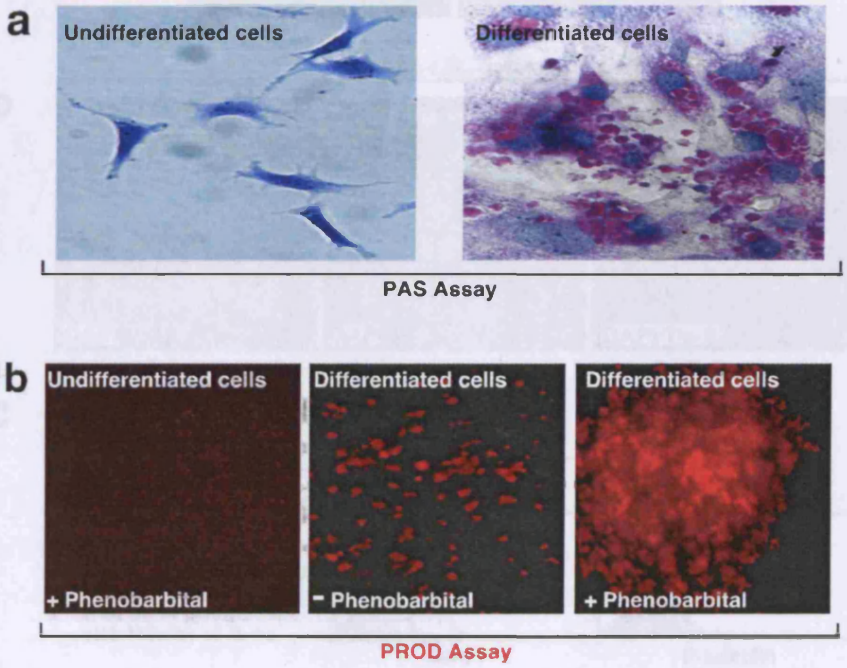


Figure 27. Differentiation of clonally derived SSEA-1^{pos} cells into hepatocyte-like cells with some functional features *in vitro*

Representative data from two independent experiments that were carried out with cells from 22 to 35 doublings. Hepatocyte-like cells derived from clone-3 cells were able to deposit glycogen (**a**; PAS assay; glycogen storage is seen as accumulation of magenta staining) and showed cytochrome P450 activity (**b**; PROD assay; activity is seen as red staining). The cytochrome P450 activity in the differentiated cells can be dramatically induced in the presence of Phenobarbital. None of these assays were positive using undifferentiated cells. Magnification: X200.

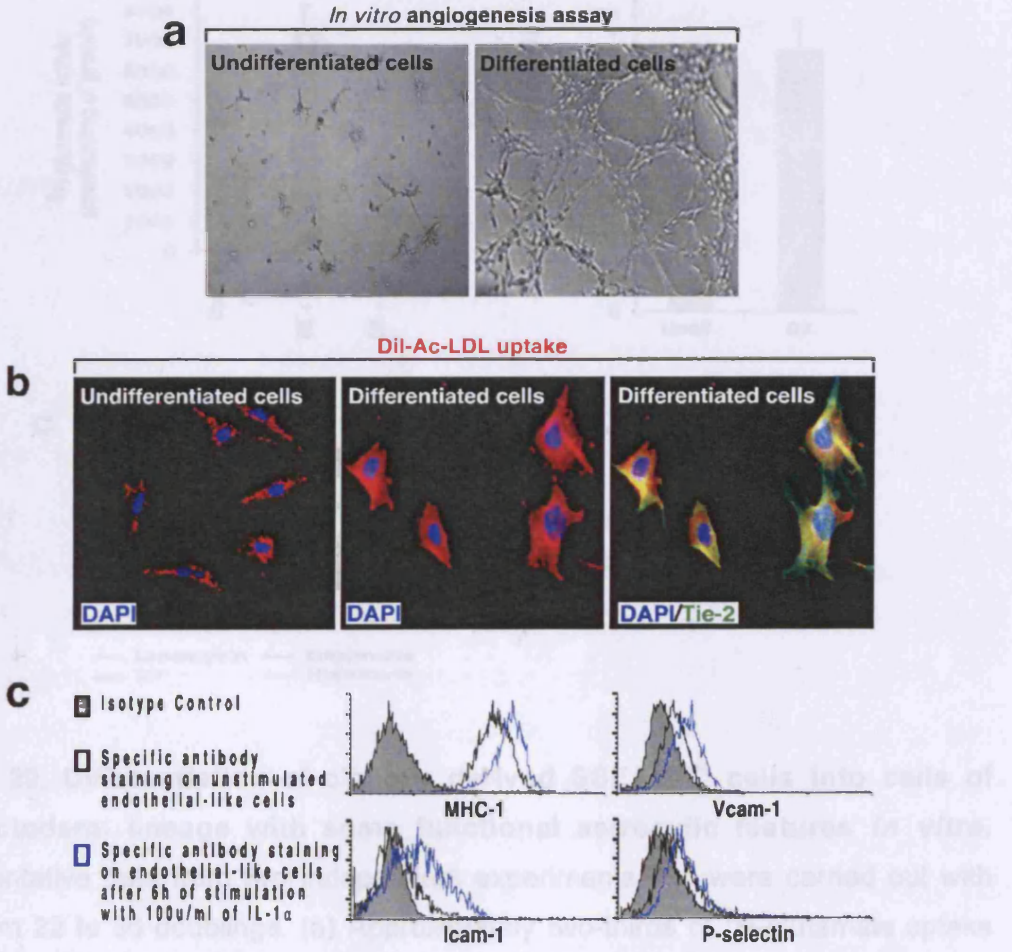


Figure 28. Differentiation of clonally derived SSEA-1^{pos} cells into functional endothelial-like cells *in vitro*

Representative data from two independent experiments that were carried out with cells from 22 to 35 doublings. Although undifferentiated cells were able to occasionally form tubular structures in the angiogenesis assay (**a**) and uptake some LDL (**b**, red staining), these functional features were drastically enhanced after the cells were exposed to VEGF for two weeks. Differentiated cells also acquired expression of Tie-2 (FITC), which co-localized with LDL staining. Moreover, differentiated cells were able to up-regulate MHC-I, VCAM-1, ICAM-1 and P-selectin after 6h of IL-1α exposure (**c**). Magnifications: X100 (**a**; angiogenesis assay and Undif. of Ac-LDL uptake), X200 (**b,** Dif. Ac-LDL uptake).

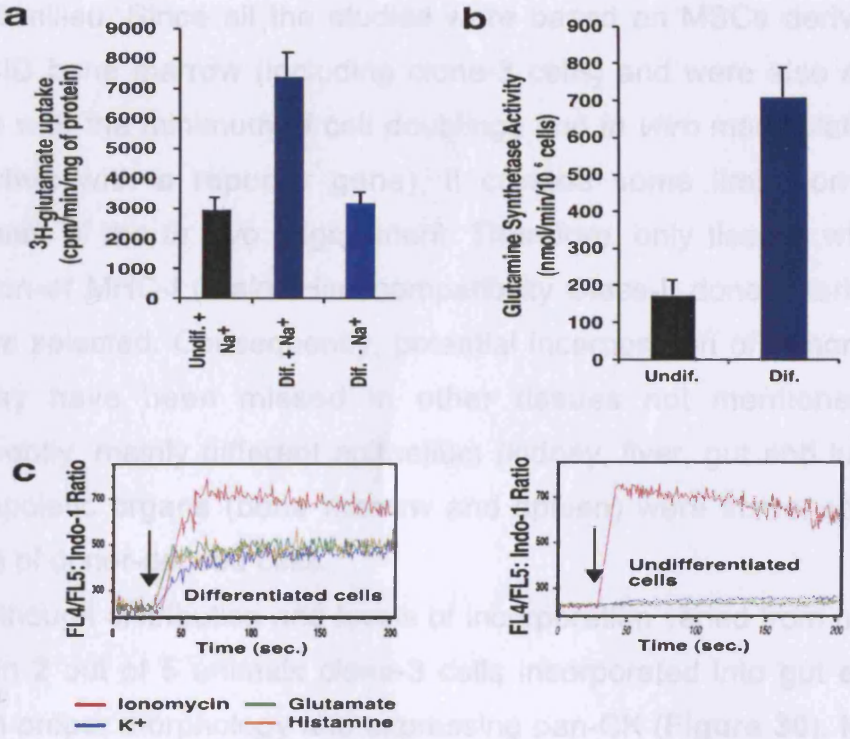


Figure 29. Differentiation of clonally derived SSEA-1^{pos} cells into cells of neuroectoderm lineage with some functional astrocytic features *in vitro*. Representative data from two independent experiments that were carried out with cells from 22 to 35 doublings. (**a**) Approximately two-thirds of ^{3}H-glutamate uptake was Na^+-dependent (dark blue bar); Non-stimulated cells had a basal level of ^{3}H-glutamate uptake (even in the presence of extracellular Na^+; black bar), with values similar to those obtained with differentiated cells when Na^+ was not added to the culture (light blue bar). (**b**) Glutamine synthetase activity in differentiated cells. The activity was determined using the colorimetric assay as described in Materials and Methods. Again, this activity was minimal in undifferentiated cells (black bar) but present in differentiated cells (dark blue bar). (**c**) Astrocyte/neuronal-like cells were also able to respond to different neurotransmitters such as K^+, glutamate and histamine. These responses were measured by intracellular calcium fluxes using flow cytometry. The fluorescence emission spectrum of Indo-1 shifts after binding to calcium; thus, the ratio obtained by dividing the signal collected on FL4 and FL5 channels changed depending on the intracellular levels of free Ca^{2+}. Flow plots show the time course of Ca^{2+} shifts (200 s) with the basal levels obtained before the addition of any substance (time of addition is indicated by the arrows). Ionomycin was used as positive control. All quantitative results shown are mean ± SD from two independent experiments, each one performed in triplicate.

the adult milieu. Since all the studies were based on MSCs derived from NOD/SCID bone marrow (including clone-3 cells) and were also aimed to use cells with the minimum of cell doublings and *in vitro* manipulation (e.g. transduction with a reporter gene), it created some limitation for the assessment of the *in vivo* engraftment. Therefore, only tissues where the expression of MHC-I (Major Histocompatibility Class-I; donor marker) was high were selected. Consequently, potential incorporation of donor-derived cells may have been missed in other tissues not mentioned here. Consequently, mainly different epithelium (kidney, liver, gut and lung) and haematopoietic organs (bone marrow and spleen) were screened for the presence of donor-derived cells.

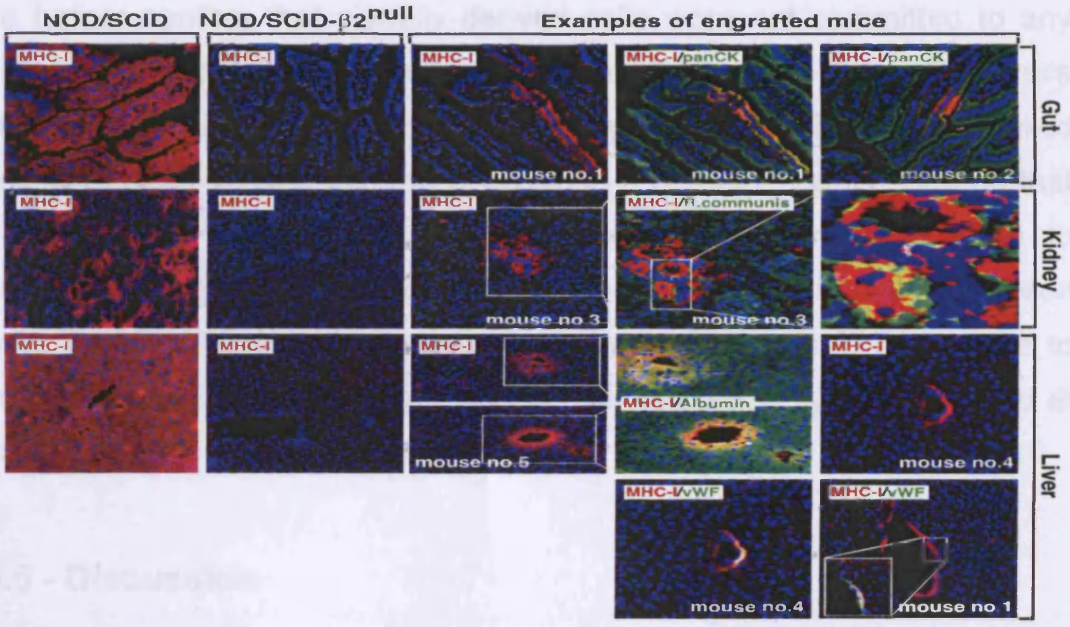
Although distribution and levels of incorporation varied from mouse to mouse, in 2 out of 5 animals clone-3 cells incorporated into gut epithelial cells with proper morphology and expressing pan-CK (**Figure 30**). MHC-I^{pos} cells expressing albumin in the liver of one mouse were also able to be detected. MHC-I^{pos}/vWF^{pos} endothelial-like cells were also detected in the liver (**Figure 32**). In addition, specific MHC-I^{pos} cells were observed in the kidneys of 2 mice. These cells had renal tubular epithelial cell morphology and were seen in clusters. These donor-derived cells stained positively for lectins *Ricinus communis* (**Figure 30**). No lung epithelial cells were detected in any of the animals. At first glimpse of the data presented it appeared that these contribution events were rare but in reality they were actually substantial. Firstly, only 10 non-serial tissue-sections/organ were screened and presence of donor derived cells were found quite easily. Unlike in Chapter-3 using bulk cultures of MSC, where many more animals and tissue-sections/organ were screened, and very little incorporation of donor cells in the recipient's tissues was found.

Due to the nature of the donor cell-type, there was a limitation in addressing whether the events observed were due to the controversial data on fusion with hematopoietic cells (Harris *et al.*, 2004; Jang *et al.*, 2004; Terada *et al.*, 2002; Wang *et al.*, 2003). Despite the limited data reported on the contribution of MSC to different cell-types *in vivo*, so far none seem to be due to fusion (Kogler *et al.*, 2004; Pochampally *et al.*, 2004; Sato *et al.*, 2005). Based on the fact that these same cells have high endodermal and

endothelial capacities *in vitro*, this indicates that under favourable environment they could also differentiate *in vivo* into different tissues. Moreover, the events presented were always seen as clusters of cells and not single cells, therefore it is less likely that fusion had occurred in such a fashion. Although, it cannot exclude the possibility that expansion of fused cells had also occurred. It is possible that both mechanisms could have contributed to such events. Further isolation of SSEA-1^{pos} cells from other strains of mice will be necessary in the future in order to address the mechanisms behind the events observed.

5.5 - Clone-3 SSEA-1^{pos}-MSCs can differentiate into haematopoietic cells *in vivo*

Clonally derived SSEA-1^{pos} cells could also give rise to haematopoietic cells in all the recipient animals. These donor-derived haematopoietic cells were found in the spleen (data not shown) and in the bone marrow (**Figure 31**). Most MHC-I/$CD45^{pos}$ cells had myeloid markers ($CD45^{pos}$/Gr-1^{pos}) (**Figure 31f**), although low levels of $CD45^{pos}$/$CD19^{pos}$ (**Figure 31f**), and $Ter119^{pos}$ (**Figure 31e**) could also be detected. Interestingly, multicolour flow-cytometry analysis revealed high frequency of donor-derived cells with $CD45^{pos}$/KLS phenotype (**Figure 31h, 31g**) (0.6-3.5%). These data suggest that SSEA-1^{pos}-MSCs are capable of differentiating into haematopoietic cells, potentially through an HSC stage. Clonally derived SSEA-1^{pos} were obtained from NOD/SCID and thus these cells have a defect in B and T cell development. Consequently, the only lymphoid cells present could only be pre/pro-B cells. Immunocompetent derived SSEA-1^{pos} cells are required to fully appreciate this conversion, especially to different lymphoid cell-types. Indeed, further detailed characterisation of the cellular/molecular events involved in this mesenchymal-haematopoietic "conversion" is of great the interest to the lab.



Type of Tissue/ Mouse N°.	Liver	Gut	Kidney
1	endothelial cells and dispersed single hepatocyte (~7)	2 groups of epithelial cells (~5 to 15 cells each)	Neg.
2	Neg.	4 groups of epithelial cells (~3 to 5 cells each)	Neg.
3	Neg.	Neg. but with many circulating cells	1 group of tubular epithelial cells (~15 to 20 cells each)
4	endothelial cells	Neg.	Neg.
5	3 groups of hepatocytes (5 to ~15 cells each)	Neg. but with many circulating cells	2 groups of tubular epithelial cells (~15 to 20 cells each)

Figure 30. Engraftment and incorporation of SSEA-1^{pos} clone-3 derived MSCs into different tissues *in vivo*

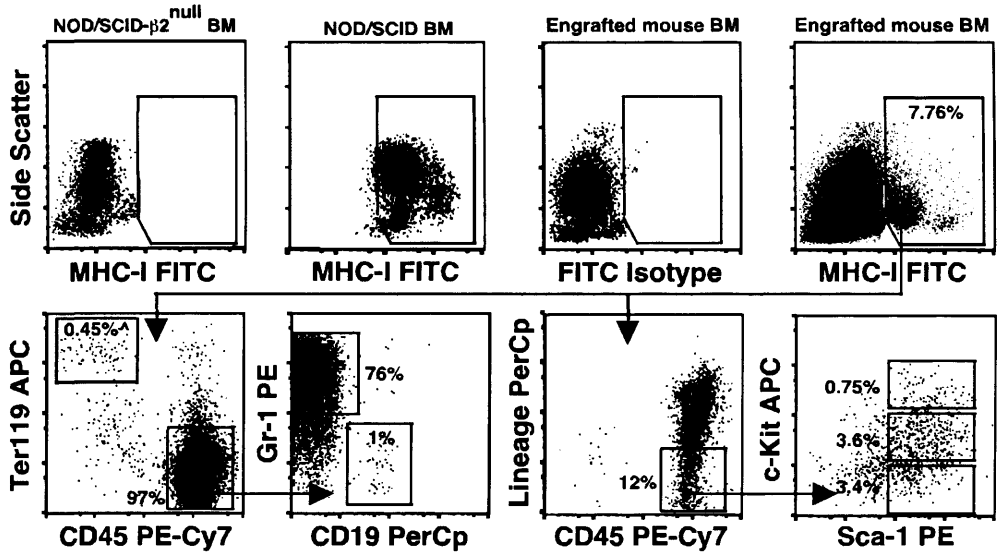
Examples of frozen tissue sections of engrafted mice (NOD/SCID-β2^{null}) 8 weeks after infusion of 2x10^5 clone-3 derived cells (NOD/SCID-derived; at 28 doublings). Similar type of tissues from NOD/SCID and NOD/SCID-β2^{null} were also used as positive and negative controls for MHC-I expression (red staining; Alexa594) respectively. Showings are examples (one or two from different engrafted animals) of areas where incorporation of donor derived cells (MHC-I positive staining; red) were observed. Each example is followed by its respective serial section that was stained with a tissue specific antibody. Sections of gut, kidney and liver were stained with, anti-pan-CK-FITC, lectin *Ricinius communis*-FITC and anti-albumin-FITC/anti-vWF-FITC, respectively. All sections were counterstained with DAPI. Magnifications: X200 (liver and kidney: with the exception of the last highlighted area at X1000) and X400 (gut). Insert table shows the engraftment levels which were determined by immunohistochemistry; estimation of chimerism from the five recipient animals used in this study was accessed by counting the number of MHC-I positive cells seen on 10 non-serial sections screened for the presence of donor-derived cells.

To further confirm that clonally derived cells were not committed to any haematopoietic cells before *in vivo* inoculation, cells were immunophenotyped for several surface antigens (**Figure 32**). Also, detailed cell cycle analysis was performed on clone-3 SSEA-1^{pos} cells showing that the cells had normal DNA content (**Figure 33**). This was done to demonstrate that the contribution events in different tissue cell-types seen were not due to potential participation of polyploid cells in being able to incorporate into different tissues, as recently described by others (Rubio *et al.*, 2005).

5.6 - Discussion

In this chapter it is described that a single SSEA-1^{pos} cell derived population could not only give rise to functional mesenchymal, endothelial, endoderm and neuroectoderm cell lineages *in vitro,* but also could incorporate into different epithelia and endothelium and differentiate into haematopoietic cells *in vivo* when injected into newborn recipients. This is the first demonstration of a single mesenchymal cell derived population, which can be prospectively isolated from early adherent cultures using a defined marker and have multipotent capacities both *in vitro* and *vivo*.

It is also shown that the clonally derived population expresses high levels of Oct-4, Rex-1 and Nanog. Although surprising, these findings were not entirely unexpected. Firstly, they have been expanded for more than 35-45 doublings in MAPC medium; secondly, preliminary immunophenotype results using bone marrow cells from Oct-4-eGFP mice revealed the existence of an Oct-4-eGFP expressing population with considerable eGFP expression; these Oct-4-eGFP^{pos} cells were indeed Lin/CD45^{neg} and SSEA-1^{pos} (personal observations). These results strongly suggest that SSEA-1^{pos} cells in the bone marrow express Oct-4 and therefore support the data that SSEA-1^{pos} cells found in bulk cultures did not emerge from *ex-vivo* conditions. In light of all the data and observations made it suggests that the expression of Oct-4 in SSEA-1^{pos} cells in the bone marrow is present but is somehow downregulated upon exposure to simple culture conditions



Type of engraftment/ Mouse N°.	MHC-I^{pos}	CD45^{pos}	Ter119^{pos}	CD45/GR-1^{pos}	CD45/CD19^{pos}	CD45/KLS^{pos}
1	7.76%	97%	0.45%	76%	1%	3.5%
2	6.8%	97%	0.5%	82.2%	0.8%	1.7%
3	6.1%	95%	1.8%	83.7%	0%	0.6%
4	10.4%	97.8%	0.5%	76.4%	0%	3%
5	2.6%	99.2%	0%	86.5%	0%	0.3%

Figure 31. *In vivo* haematopoietic reconstitution ability of SSEA-1^{pos} clone-3 derived-MSCs

Representative flow cytometry plots of bone marrow engraftment analysis from one of the recipient mice. The assessment of donor-derived cells was determined by the presence of MHC-I expressing cells. (**a**, **b**) NOD/SCID-β2^{null} and NOD/SCID BMMNCs were used as negative and positive controls respectively. BMMNCs from each recipient animal were stained with an appropriate isotype control (**c**) and MHC-I antibodies (**d**) to determine the percentage of donor-derived cells. Multicolour FACS analysis shows that most of donor-derived cells (MHC-I^{pos}; gated area of **d**) were haematopoietic origin, composed mainly by CD45^{pos} cells (**e**). Most of donor-derived CD45^{pos} cells co-expressed the myeloid antigen Gr-1 (**f**) and conversely were also positive for lineage-committed antigens (**h**). However, some cells with HSC/HPC phenotype, KLS (c-Kit$^{low/pos}$/Lineage^{neg}/Sca-1^{pos}) were also found (**g**). (**i**) Level of bone marrow engraftment of the five mice used in this study were determined by FACS; the percentages of MHC-I^{pos} cells represent the number of positive cells found over the total BMMNCs analysed, whereas the other values represent their respective percentage within the MHC-I^{pos} cells.

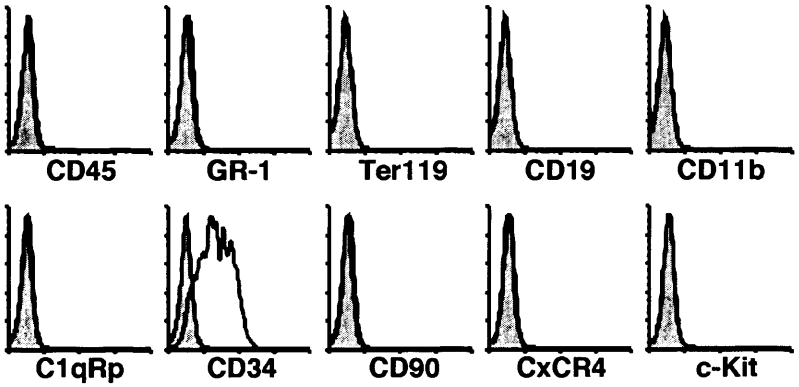


Figure 32 - Clone-3 SSEA-1^{pos} cells were not committed to haematopoietic cells before transplantation

Clone-3 SSEA-1^{pos} cells did not express any specific haematopoietic surface antigens, except for CD34 before *in vivo* transplantation. Grey and white areas are the isotype control and specific antibody staining respectively.

(therefore not appropriate for its maintenance) to the point that this expression was not even detectable when the SSEA-1^{pos} cells were isolated from bulk cultures. However, when SSEA-1^{pos} cells were isolated early enough and placed in a more appropriate culture medium (such as for MAPC), the expression of such embryonic transcription factors could be "rescued" and re-induced in these cells. Even though the exact function these transcription factors might have in the SSEA-1^{pos}-MSCs is not clear, the latest observations indicate they are lost when culture conditions are changed or the cells are forced to differentiate (personal observations). Thus, these transcription factors might have some function in the maintenance of the self-renewal and/or multipotency features in these cells, akin to ES cells (Chambers *et al.*, 2003; Niwa *et al.*, 2000). Evaluation of the gene expression profile of SSEA-1^{pos}-MCSs might uncover important key pathways, which regulate their *in vitro* self-renewal and unique "plastic" properties.

The findings described here will open new questions concerning the hierarchical organisation of MSCs, the role of SSEA-1^{pos}-MCSs in the maintenance of tissue homeostasis and especially in the developmental origin and relationship of these cells with other cell-types, in particular with the haematopoietic cells. In fact, as demonstrated here, a clonally derived SSEA-1^{pos} cell could contribute to haematopoietic reconstitution. Accumulating evidence suggests that a common precursor of mesenchymal and haematopoietic lineages might exist both in mice and humans (Dominici *et al.*, 2004; Jiang *et al.*, 2002; Kogler *et al.*, 2004). This hypothesis could challenge current views on the developmental origin of haematopoietic stem cells. With the use of SSEA-1, and possibly Oct-4, it may be possible to trace the developmental origin of this primitive mesenchymal cell-type.

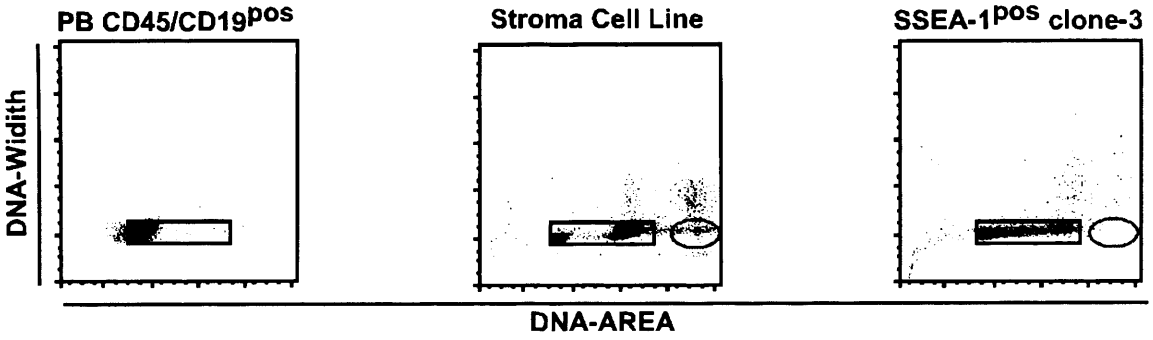


Figure 33. Clonally derived population is not aneuploid

Cell cycle analysis of clone-3 population at 45 doublings showing normal DNA content, without a detectable polyploid peak (highlighted oval area). Peripheral blood (PB) CD45/CD19^{pos} cells (from C57BL/6J) were used as reference of normal DNA content (area highlighted by the black box) and to set the G0/G1 peak and a stroma cell line M210B4 as an example of presence of polyploid peak.

Conclusion

This project was initiated merely by curiosity, no plan or targets were initially drawn up for this particular study. Most of the initial studies on muMSCs aimed to address simple questions, such as characterisation of basic isolation/expansion methods, phenotype and differentiation capacities of these cells, since these points were not properly addressed for muMSCs. During the process of defining culture conditions for isolation and *ex vivo* expansion of adult murine MSCs, a new cell-type was found. Under a conventional isolation/culture system a minor sub-population of muMSCs was identified with MAPCs features (based on the expression of SSEA-1 antigen). MAPCs were characterised by Catherine Verfaillie's group and shown to have the capacity to differentiate into different cell-types of the three-germline layers, both *in vitro* and *in vivo*, thus resembling embryonic stem cells. The presented data strongly suggest that this new subpopulation is the founder of the MSC compartment; as they give rise to the SSEA-1^{neg} cells, with limited differentiation potential, and also they are required to initiate bulk MSC cultures.

These SSEA-1^{pos}-MSCs exhibit some common features in common with MAPCs: they can differentiate into different cell-types of the three-germline layers *in vitro*, incorporate into different epithelia and endothelium and differentiate into haematopoietic cells *in vivo*. Furthermore, they also express specific embryonic transcription factors such as Oct-3/4, Rex-1 and Nanog.

Most importantly, these cells can be found not only after *in vitro* culture but also directly from mouse bone marrow, therefore proving their true existence *in vivo*. Although SSEA-1^{pos}-MSCs described in this study share some features of ES cells but they differ from them by expressing, for example, MHC-I and AP, and having different cell expansion requirements (e.g. confluency). Moreover, it still has to be demonstrated whether these cells can produce chimeras after blastocyst injections. As such, at this point, the results shown are not sufficient to support that these cells are reminiscent

of ES cells in post-natal life. Further experiments are required to address this very important and interesting issue. Hopefully, using the recently acquired Oct-4-GFP mice will give a better understanding on the biology and developmental origin of these cells.

MSCs can indeed incorporate into different tissue-types *in vivo* and therefore the *in vitro* "plastic" features described by many researchers and also reported here seem to be shared *in vivo*. However, it is important to emphasise the weakness of this study concerning the interpretation of the *in vivo* results shown. As mentioned earlier, this study was not designed properly to address and pursue *in vivo* studies. Basically, the lack of appropriate mouse strains (e.g. the cre-lox system) and injury models for targeting specific tissues created a tremendous difficulty to detect and address the mechanisms behind these incorporation events. SSEA-^{pos} cells derived from more suitable mouse strains are required to better understand whether these events were due to differentiation or fusion with resident cells. Also, by focusing (direct inoculation of the cells or use of an injury model or both) on one particular tissue of interest, rather than screening randomly multiple tissues would dramatically ease studies of this kind. The lab is especially enthusiastic on the mesenchymal-haematopoietic conversion, and it will be very interesting to know whether these SSEA-1^{pos} cells could convert into fully functional long-term haematopoietic stem cells. Also, to further support the notion that the newly identified cell-type behaves like ES cells, it will be important to investigate if this conversion follows a similar molecular/cellular pattern as the ES cell.

At last, but not least, the fundamental question, whether these SSEA-1^{pos} cells can fit into the definition of a stem cell. *In vivo* a stem cell is capable of regeneration or maintenance of a tissue compartment at a single cell level, thus it can be transplanted, re-isolated, and serially transplanted. Using this stringent criterion, which is applied for haematopoietic stem cells, SSEA-1^{pos} cells still do not fit into the definition, especially concerning the serial transplantability point of view. Although SSEA-1^{pos} cells have self-renewal properties *in vitro* under the MAPC culture system, it was not tested

here if these cells can "home" and repopulate adult bone marrow and therefore be able to be re-isolated and to engraft into secondary recipients. As shown in Chapter-3, when MSCs were infused intravenously into adult recipient mice, no donor-derived cells were found in the recipient's marrows. Although in these experiments bulk MSCs cultures were used, those cultures contained some SSEA-1^{pos} cells (< 1%). The "homing" properties of MSCs in general might have been lost upon culture and therefore it would be challenging to conduct such studies. Intra-bone administration of SSEA-1^{pos} cells may help to overcome this problem. Secondly, it is not known what role these cells have in the maintenance of the homeostasis and repair of the bone marrow. As shown here, these cells gradually disappear during aging and this finding suggests that they might not play a great homeostatic role during the adult lifetime of the animal. It is tempting to speculate that direct progenitors of these cells (e.g. osteoprogenitors, adipocyte progenitors, etc) might actually participate most (if not to all) the homeostatic and repair functions in the marrow. These experiments are absolutely essential in order to prove that these SSEA-1^{pos} cells are stem cells by definition.

Finally, it is important to emphasise that the nomenclature applied for describing bulk cultures of bone marrow adherent cells (even after the haematopoietic depletion) as MSC is not adequate. As postulated by many researchers and also shown here, what is obtained from the adherent isolation method is a mixture of fibroblastic cells, with most of them not having either stem or progenitor features. Only for the "sake" of a consensual scientific understanding, this commonly used designation was opted for. Perhaps bone marrow mesenchymal cells, BMMCs, might be more appropriate for what has been described as MSC or MPC. However, it is hoped that with further investigation in the near future that the SSEA-1^{pos} cell can be finally named as the true MSC.

Appendix I - Solutions

Hst Buffer

Hank's balanced salt solution (CRUK, central cell services)

20 µmol/ml of HEPES (Gibco)

1 mg/ml of Glucose (Sigma)

10% FBS (Gibco)

AP Buffer, pH7.4

15 mM Tris-HCl (BDH)

1 mM $ZnCl_2$ (BDH)

1 mM $MgCl_2$ (BDH)

1% TX (Sigma)

Krebs Buffer, pH 7.2

4.8 mM KCl (BDH)

1.2 mM KH_2PO4 (BDH)

1.3 mM $CaCl_2$ (BDH)

1.2 mM $MgSO_4$ (BDH)

25 mM HEPES

6 mM Glucose (Sigma)

140 mM NaCl (BDH) or Choline-Cl (Sigma)

GS Buffer, pH 7.2

0.15 M KCl

2 mM DTT (Sigma)

1 mM EDTA (Gibco)

GS Solution, pH 7.2

0.1 M imidazole-HCl (Sigma)

50 mM, L-glutamine (Gibco)

0.4 mM $MnCl_2$ (BDH)

62.5 mM hydroxylamine (Sigma)

10 mM Na_2HAsO_4 (Sigma)

Ferric Chloride Reagent

0.37 M $FeCl_3$ (Sigma)

0.67 M HCl

0.2 M TCA (Sigma)

Resolving gel (10% gel), 25 ml

8.3 ml of 30% acrylamide/bisacrylamide (29:1 mix) (National Diagnostics, Atlanta, US)

3.15 ml of 8x Resolving gel buffer (National Diagnostics)

13.3 ml of water

10 µl of TEMED (Sigma)

250 µl of 10% ammonium persulfate (Sigma)

Stacking gel, 8 ml

1.3 ml of 30% acrylamide/bisacrylamide (29:1 mix)

2 ml of 4x Stacking gel buffer (National Diagnostics)

4.7 ml of water

8 µl of TEMED

80 µl of 10% ammonium persulfate

Appendix II - Primers

Genomic PCR Primers

-eGFP
F: 5'-CAACAGCCACAACGTCTATATCATG-3'
R: 5'-GAAGTTCTAGGCGGTGTTGTA-3'

-muGAPDH
F: 5'-CGGAGTCAACGGATTTGGTCGTAT-3'
R: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

RT-PCR and Real-time RT-PCR Primers
(Unless is stated all the reactions were performed at 60 °C as annealing temperature for 40 cycles)

-Albumin
F: 5'-GCATGAAGTTGCCAGAAGACATCC-3'
R: 5'-TCTGCAGTTTGCTGGAGATAGTCG-3'

-ß-actin (55 °C; 30 cycles)
R: 5'-TTCCTTCTTGGGTATGGAAT-3'
F: 5'-GAGCAATGATCTTGATCTTC-3'

-ß-actin (Real-Time)
F: 5'-ACCAACTGGGACGATATGGAGAAGA-3'
R: 5'-TACGACCAGAGGCATACAGGGACAA-3'

-BSP II
F: 5'-TTTCCACACTCTCGGGTGTAA-3'
R: 5'-CCGCCAGCTCGTTTTCATC-3'

-CD31

F: 5'-CTGCCAGTCCGAAAATGGAAC-3'

R: 5'-CTTCATCCACCGGGGCTATC-3'

-Desmin

F: 5'-TCGGTACCAAGGGCTCCTC -3'

R: 5'-GCGGTCATTGAGCTCTTGC -3'

-Dystrophin

F: 5'-GAAAAGAAAAAGCCAACCATGG -3'

R: 5'-TGCAAAGTTTTCCATCCAGATTT -3'

-GFAP

F: 5'-TCCTGGAACAGCAAAACAAG-3'

R: 5'- GAGGTCCTGTGCAAAGTTGTCC -3'

-GLAST

F: 5'- ACCAAAAGCAACGGAGAAGAG-3'

R: 5'-GGCATTCCGAAACAGGTAACTC-3'

-HNF-1 (56 °C)

F: 5'-AAGCTGGTCTCAGCCACGG-3'

R: 5'-CTGAGGTGAAGACCTGCTT-3'

-Oct-3/4

F: 5'-GAAGCCGACAACAATGAGAAC-3'

R: 5'-CAGAACCATACTCGAACCACA-3'

-Osteonectin

F: 5'-GAACAGACAAGTCCCACACAGC-3'

R: 5'-AGCCAGGGCCAGCAGAG-3'

-Myogenin

F: 5'-CCCAACCCAGGAGATCATTT -3'

R: 5'-GTCTGGGAAGGCAACAGACA -3'

-Nanog

F: 5'-AGGGTCTGCTACTGAGATGCTCTG-3'

R: 5'-CAACCACTGGTTTTTCTGCCACCG-3'

-Rex-1 (55 °C)

F: 5'-CGTGTAACATACACCATCCG-3'

R: 5'-GAAATCCTCTTCCAGAATGG-3'

-Rex-1 (Real-Time)

F: 5'-AAGCGTTTCTCCCTGGATTTC-3'

R: 5'-TTTGCGTGGGTTAGGATGTG-3'

-Tie-2

F: 5'- TCCCTCCTCAACCAGAAAACA-3'

R: 5'- TCTCAGCAAAAATATCCACATGGT-3'

-vWF

F: 5'-CCGGAAGCGACCCTCAGA-3'

R: 5'-CGGTCAATTTTGCCAAAGATCT-3'

Appendix III -Antibodies

Primary Antibodies

Anti-	Format	Clone/Designation	Application/Dilution	Source
α4β7 integrin	Purified	DAKT32	FC (1:50)	Pharmingen
Albumin	Purified	HAS-11	FC (1:10); IF (1:400); IH (1:200); WB (1:400)	Sigma
α-SMA	Purified	A2547	IH (1:100)	Sigma
β7 integrin	FITC	FIB504	FC (1:50)	Pharmingen
β-Actin	Purified	AC-15	WB (1:400)	Sigma
BSPII	Purified	MAP	FC (1:10); IF (1:200); WB (1:400)	CosmoBio
C1qRp/AA4.1	FITC	AA4.1	FC (1:50)	Pharmingen
CD2	PE	RM2-5	FC (1:50)	Pharmingen
CD3e	Cy-Chrome	145-2C11	FC (1:50)	Pharmingen
CD4	PE	GK1.5	FC (1:50)	Pharmingen
CD5	PE	53-7.3	FC (1:50)	Pharmingen
CD8a	PE	53-6.7	FC (1:50)	Pharmingen
CD9	Biotinylated	KMC8	FC (1:100)	Pharmingen
CD11a	Purified	H68	FC (1:50)	CRUK
CD11b	Purified	M1/70	IH (1:25)	Pharmingen
CD11b	PE	M1/70	FC (1:50)	Pharmingen
CD11b	Biotinylated	M1/70	FC (1:100)	Pharmingen
CD11c	FITC	HL3	FC (1:50)	Pharmingen
CD14	PE	rmC5-3	FC (1:50)	Pharmingen
CD19	Biotinylated	1D3	FC (1:50)	Pharmingen
CD25	PE	3C7	FC (1:50)	Pharmingen
CD29	Purified	9EG7	FC (1:50)	Pharmingen
CD31	Purified	MEC13.3	FC (1:30)	Pharmingen
CD31	Biotinylated	390	FC (1:100)	Pharmingen
CD31	FITC	390	FC (1:50)	Pharmingen
CD34	PE	RAM34	FC (1:50)	Pharmingen
CD34	FITC	RAM34	FC (1:50)	Pharmingen
CD43	PE	S7	FC (1:50)	Pharmingen
CD44	APC	IM7	FC (1:50)	Pharmingen
CD45	Purified	30-F11	FC (1:30)	Pharmingen
CD45	Biotinylated	30-F11	FC (1:150)	Pharmingen
CD45	FITC	30-F11	FC (1:50)	Pharmingen
CD45	PE-Cy7	30-F11	FC (1:75)	Pharmingen
CD45	Cy-Chrome	30-F11	FC (1:75)	Pharmingen
CD45R/B220	PE	RA3-6B2	FC (1:50)	Pharmingen
CD49e	PE	5H10-27	FC (1:50)	Pharmingen
CD49f	FITC	G0H3	FC (1:50)	Pharmingen
CD62E	Purified	10E9.6	FC (1:50)	Pharmingen
CD62L	Purified	Mel-14	FC (1:50)	Pharmingen
CD62P	Purified	RB40.34	FC (1:50)	Pharmingen
CD73	PE	TY/23	FC (1:50)	Pharmingen
CD90	FITC	HIS51	FC (1:50)	Pharmingen
CD103	Purified	M290	FC (1:50)	Pharmingen
CD105	Purified	Mj7/18	FC (1:25); IH (1:10)	Pharmingen
CD106	FITC	M/K-2	FC (1:50)	Chemicon
CD117	APC	2B8	FC (1:50)	Pharmingen
CD127	PE	SB/199	FC (1:50)	Pharmingen
CD135	PE	A2F10.1	FC (1:50)	Pharmingen
CK18	Purified	CY-90	IF (1:400)	Sigma
CXCR4	FITC	2B11/CXCR4	FC (1:50)	Pharmingen
DDC	Purified	DDC 109	IF (1:400)	Sigma
Desmin	Purified	33	FC (1:15); IF (1:50); IH (1:80); WB (1:200)	Dako
Dystrophin	Purified	H-300	FC (1:10); IF (1:400); WB (1:400)	S.Cruz
FTM	Purified	MY-32	IF (1:400)	Sigma
GABA	Purified	A2052	IF (1:400)	Sigma
GFAP	Purified	H-50	FC (1:10); IF (1:400); WB (1:400)	S.Cruz
GFP	Purified	FL	FC (1:10); IH (1:400)	Santa Cruz
GFP	Purified	3E1.	FC (1:10); IH (1:400)	CRUK
GLAST	Purified	ab416	FC (1:10); IF (1:400); WB (1:400)	Abcam
GR-1	PE	RB6-8C5	FC (1:50)	Pharmingen
H-2D^b	Purified	HB27	IH (1:200)	CRUK
H-2K^a	Purified	K9-18	IH (1:200)	CRUK
H-2K^d	FITC	SF1-1.1	FC (1:50)	Pharmingen
HNF-1	Purified	H-205	FC (1:10); IF (1:200); WB (1:400)	S.Cruz
I-A	PE	AMS-32.1	FC (1:50)	Pharmingen
Icam-1	Biotinylated	3E7.	FC (1:50)	Pharmingen
Icam-2	FITC	3C4	FC (1:50)	Pharmingen
KDR/FLK-1	Purified	89B3A5	FC (1:500)	Chemicon
Lectin L. esculentum	Biotinylated	L0651	IH (1:100)	Sigma
Lectin L. tetragonolobous	Biotinylated	B1325	IH (1:100)	Vector Laboratories
Lectin R. communis	Biotinylated	B1085	IH (1:100)	Vector Laboratories
MadCAM-1	Biotinylated	MECA/89	FC (1:100)	Pharmingen
Nanog	Purified	?	IF (1:400)	CosmoBio
NeuN	Purified	MAB377	IF (1:400)	Chemicon
NK1.1	PE	PK136	FC (1:50)	Pharmingen
Oct-4	Purified	H-134	IF (1:100)	S.Cruz
Osteocalcin	Purified	M-15	FC (1:10); IF (1:400); WB (1:400)	Santa Cruz
pan-CK	Purified	H-240	IH (1:200)	S.Cruz
Pro-Sulfactan Protein C	Purified	AB3428	IH (1:100)	Chemicon
Rex-1 (ZFP42)	Purified	AP2051b	IF (1:100)	Abgent
Sca-1	Biotinylated	E13-161.7	FC (1:100)	Pharmingen
Sca-1	FITC	E13-161.7	FC (1:100)	Pharmingen
Sca-1	PE	E13-161.7	FC (1:100)	Pharmingen
SSEA-1	Ascites	480	FC (1 μg/10^6 cells)	DSHB
SSEA-1	Purified	480	FC (1:15); IF (1:100); IH (1:50)	Santa Cruz
Tau	Purified	H-150	IF (1:400)	S.Cruz
TER-119	APC	TER-119	FC (1:100)	eBioscience
TER-119	Purified	TER-119	IH (1:25)	Pharmingen
TER-119	PE	TER-119	FC (1:100)	Pharmingen
Tie-2	Purified	H-176	FC (1:10); IF (1:400)	Santa Cruz
Trk (NGFR)	Purified	C-14	FC (1:25): IH (1:10)	S.Cruz
vWF	Purified	H-300	FC (1:10); IF (1:400); IH (1:200); WB (1:400)	Santa Cruz

Secondary Antibodies/Reagents

Anti-	Format	Application/Dilution	Source
Goat IgGs	FITC	FC (1:50); IF (1:400)	Dako
Goat IgGs	AlexaFluor647	FC (1:50)	Molecular Probes
Goat IgGs	Cy3	IF (1:400)	Jackson Immuno.
Goat IgGs	POD	WB (1:800)	Santa Cruz
Mouse IgG1	APC	FC (1:50)	Pharmingen
Mouse IgG1	FITC	FC (1:50)	Pharmingen
Mouse IgG2a	FITC	FC (1:50)	Pharmingen
Mouse IgG2b	FITC	FC (1:50)	Pharmingen
Mouse IgG2b	PE	FC (1:50)	Biocarta
Mouse IgGs	AlexaFluor594	IF (1:400)	Molecular Probes
Mouse IgGs	ALK	IH (1:800)	Vector
Mouse IgGs	Biotinylated	FC (1:100)	DAKO
Mouse IgGs	FITC	FC (1:50); IF (1:400)	Sigma
Mouse IgGs	FITC	FC (1:50); IF (1:400)	Abcam
Mouse IgGs	POD	IH (1:800); WB (1:1000)	Sigma
Mouse IgGs	AlexaFluor488	IF (1:400)	Molecular Probes
Mouse IgM	APC	FC (1:50)	Pharmingen
Mouse IgM	Biotinylated	FC (1:25); IH (1:100)	Sigma
Mouse IgM	FITC	FC (1:25); IH (1:100)	Biocarta
Mouse IgM	PE	FC (1:50)	Biocarta
Mouse IgM	TexasRed	IF (1:100)	S.Cruz
Rabbit IgGs	AlexaFluor488	IF (1:400); IH (1:400)	Molecular Probes
Rabbit IgGs	AlexaFluor647	IF (1:400); IH (1:400)	Molecular Probes
Rabbit IgGs	Cy3	IF (1:400)	Jackson Immuno.
Rabbit IgGs	PE	FC (1:100)	Southern Biot.
Rabbit IgGs	POD	IH (1:800); WB (1:1000)	Sigma
Rabbit IgGs	TRITC	IF (1:400)	Sigma
Rat IgG2a	FITC	FC (1:100)	Serotec
Rat IgG2b	FITC	FC (1:100)	Serotec
Rat IgGs	AlexaFluor488	IF (1:400); IH (1:400)	Molecular Probes
Rat IgGs	AlexaFluor594	IF (1:400); IH (1:400)	Molecular Probes
Rat IgGs	FITC	FC (1:100)	Serotec
Streptavidin	APC	FC (1:100)	Pharmingen
Streptavidin	Cy-Chrome	FC (1:100)	Pharmingen
Streptavidin	FITC	FC (1:100)	Pharmingen
Streptavidin	PE	FC (1:100)	Pharmingen
Streptavidin	PerCP	FC (1:100)	Pharmingen

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