Investigations into the *In Vitro* Developmental Plasticity of Adult Mesenchymal Stem Cells

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Thesis submitted for the degree of Doctor of Philosophy

University of Durham

School of Biological and Biomedical Sciences

2006

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Bone marrow (BM) derived stem cells contribute to the regeneration of diverse adult tissues including heart, liver and brain following BM transplantation. Trans-differentiation is a mechanism proposed to explain how tissue specific stem cells could generate cells of other organs, thus supporting the emerging concept of enhanced adult stem cell plasticity. New studies have demonstrated that spontaneous cell fusion rather than trans-differentiation is the cause of unexpected cell fate changes *in vivo*. In contrast, several authors have reported that trans-differentiation can occur *in vitro* in the absence of cell fusion, including the generation of neural derivatives from non-neural tissues. These findings have profound implications for stem cell biology and cell replacement therapy, and as a result require extensive validation. Mesenchymal stem cells (MSCs) have been isolated from the postnatal BM and more recently many other sites including adipose tissue, skin and placental cord blood. As such these cells have attracted interest as candidates for cell replacement therapies. This interest follows recent observations both *in vitro* and in transplant studies that these cells are capable of broader differentiation potential beyond those cell lineages associated with the organ in which they reside. The aim of the present thesis was to examine the developmental plasticity of MSCs *in vitro* including the capacity of these cells to cross lineage boundaries by differentiating into neuro-ectodermal cell derivatives.

There are no universally accepted procedures for the prospective isolation of these cells. In the present thesis, procedures for the isolation of MSCs from rat BM and optimal conditions for the propagation of these cells in culture without loss of multipotent differentiation potential and proliferative capacity are first described. Secondly, the response of cultured MSCs with a consistent immunophenotype to defined culture conditions, previously reported to induce neuronal differentiation of MSCs are evaluated. Thirdly, evidence is presented that suggests that previous claims of trans-differentiation and apparent changes in cell phenotype have been incorrectly interpreted. Evidence is provided that MSCs respond to neural cues *in vitro* with a stress response, which is characterized by aberrant changes in the expression of constitutive neural proteins, an event previously interpreted as trans-differentiation. MSCs do not have the attributes of early or mature neural derivatives and therefore such changes in protein expression do not equate to true neural differentiation. Finally, evidence is presented that demonstrates that MSCs cultured under defined culture conditions release soluble factors that instruct a neurogenic cell fate decision on neural stem cells (NSCs). In addition, these soluble factors also increase neurite outgrowth of Tuj-1+ differentiating cell progeny. These effects may in part explain the therapeutic benefit of MSC transplantation in animal models of CNS lesions.

Acknowledgements

I would firstly like to thank my supervisor Dr Stefan Przyborski for providing me with many of the opportunities that have made these investigations a success. I would also like to thank Dr Nicholas Hole for insightful and enthusiastic discussions on much of the data presented in this thesis. I also extend my thanks to the members of Lab 9 for their help and support over the past 4 years and I wish them every success in the future. Most importantly, I would like to thank my wife, Lyndsey for her help, understanding and continued support, without which none of this would have been possible.

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Declaration

I confirm that the material presented in this thesis is entirely my own and has not previously been submitted by me for a degree in this or any other University. All published work cited has been acknowledged where appropriate.

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Abbreviations

α-MEM	:	Alpha-Minimal Essential Media
ANOVA	:	Analysis of Variance
aP2	:	Fatty acid binding protein
BDNF	:	Brain Derived Neurotrophic Factor
bFGF	:	Basic fibroblast growth factor
β-Gal	:	β-galactosidase
BHA	:	Butylated Hydroxyanisole
BM	:	Bone marrow
BMDSC	:	Bone Marrow Derived Stem Cells
BME	:	β-mercaptoethanol
BMP	:	Bone Morphogenetic Protein
BMSC	:	Bone Marrow Stem Cells
BrdU	:	Bromodeoxyuridine
Ca^{2+} ion	:	Calcium ionophore
CaMKII	:	Ca 2+ /Calmodulin-Dependent Protein Kinase II
camp	:	Cyclic adenosine monophosphate
CB	:	Cytochalasin B
CCM	:	Complete Culture Medium
cDNA	:	Complementary deoxyribonucleic acid
CD	:	Cluster of differentiation
CFU	:	Colony Stimulating Factor
CFU-F	:	Colony Stimulating Factor Fibroblast
CHX	:	Cycloheximide
ChCl	:	Cherlerythrine Chloride
CMV	:	Cytomegalovirus
CNS	:	Central Nervous System
CNTF	:	Ciliary Neurotrophic Factor
CO^2	:	Carbon Dioxide
COL	:	Colcemid
Cy-3	:	Cyan-3
Cyclic AMP	:	Cyclic Adenosine 3'5' Monophosphate
DAG	:	Diacylglycerol
Dex	:	Dexemathasone
DMEM	:	Dulbecco's Modified Eagle's Medium
DMSO	:	Dimethyl Sulfoxide
DNA	:	deoxyribonucleic acid
E14	:	Embyronic day 14
ECM	:	Extra-cellular Matrix
EDTA	:	Ethylenediaminetetra-acetic acid
EGF	:	Epidermal Growth Factor
eGFP	:	Enhanced Green Fluorescent Protein
ELISA	:	Enzyme linked immunosorbant assay
ES	:	Embryonic stem cells
FACS	:	Fluorescence activated cell sorting
F-actin	:	Filamentous Actin

FAH	:	Fumarylacetoacetate hydrolase
FCS	:	Fetal Calf Serum
FGF-2	:	Fibroblast growth factor-2
FISH	:	Fluoresence in situ hybridization
FITC	:	Fluorescein isothiocyanate
FS	:	Forward light scatter
Gal-C	:	Galactosylceramidase
GAP-43	:	Growth Associated Protein-43
GDNF	:	Glial derived Neurotrophic factor
GFAP	:	Glial Fibrillary Acidic Protein
GFP	:	Green Fluorescent Protein
GGF	:	Glial Growth Factor
HBSS	:	Hank's balanced salt solution
HCl	:	Hydrogen Chloride
HCN	:	Adult Hippocampal Progenitor Cells
HEK	:	Human Embryonic Kidney Cells
hES	:	Human Embryonic Stem Cells
HGF	:	Hepatocyte growth factor
hMSCs	:	Human Mesenchymal Stem Cells
HRP	:	Horseradish peroxidase
HSC	:	Hematopoietic stem cells
IBMX	:	3-isobutyl-1-methylxanthine
IDMM	:	Iscove's Dulbecco-modified medium
IFN	:	Interferon
Ig	:	Immunoglobulin
IGF	:	Insulin-like Growth Factor
IgG	:	Immunoglobulin G
IHC	:	Immunohistochemistry
IL	:	Interleukin
IV	:	Intravenous
JNK	:	c-jun kinase
K+	:	Potassium
kDa	:	Kilodalton
KCl	:	Potassium Chloride
L	:	Litre
Lac-z	:	Gene that encodes β-galactosidase
LIF	:	Leukemic Inhibitory Factor
LIR	:	Leukocyte immunoglobulin receptors
Low Attach	:	Low Attachment
LPL	:	Lipoprotein lipase
LT-HSC	:	Long-Term Hematopoietic Stem Cells
mAb	:	Monoclonal antibody
MAG	:	Myelin associated glycoprotein
MAP	:	Microtubule associated protein
MAPCs	:	Multipotent Adult Progenitor Cells
MAPK	:	Mitogen Activated Protein Kinase
MCAO	:	Middle Cerebral Artery Occlusion
M-CSF	:	Macrophage-Colony Stimulating Factor
MEM	:	Minimal essential Media

MFI	:	Mean Florescence Intensity
mg	:	Milligram
µg	:	Microgram
MgCl	:	Magnesium Chloride
MHC	:	Major histocompatibility complex
ml	:	Milliliter
mRNAs	:	Messenger Ribonucleic Acid
MSC	:	Mesenchymal Stem Cells
MSCs	:	Mesenchymal Stem Cells
N2a	:	Neuroblastoma 2a
Na+	:	Sodium
NCAM	:	Nuclear Cell Adhesion Molecule
ND1	:	NeuroD1
NeuN	:	Neuronal Nuclei
NF	:	Neurofilament
ng	:	Nanogram
NGF	:	Nerve Growth Factor
NIH	:	National Institute of Health
NPC	:	Neuroprogenitor Cells
NSC	:	Neural Stem Cells
NSE	:	Neuron-specific enolase
NT-3	:	Neurotrophin-3
OMgp	:	Oligodendrocyte-myelin glycoprotein
P	:	Passage
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PDGF	:	Platelet Derived Growth Factor
PDL	:	Poly-D-Lysine
PFA	:	Paraformaldehyde
PI	:	Propidium iodide
PKA	:	Protein Kinase A
PKC	:	Protein kinase C
PMA	:	Phorbol 12-myristate 13-acetate
POL	:	Poly-L-Ornithine
PPARy2	:	Peroxisome proliferator activated receptor y2
PVDF	:	Polyvinylidene Difluoride
RA	:	Retinoic Acid
RBC	:	Red blood cell
RIP	:	Receptor interacting protein
rMSC	:	Rat Mesenchymal Stem Cells
rMSCs	:	Rat Mesenchymal Stem Cells
RNA	:	Ribonucleic Acid
rpm	:	Revolutions per minute
RS	:	Rapid Cycling Stem Cells
RT	:	Room Temperature
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SCID	:	Severe combined immunodeficiency
SDS-PAGE	:	Sodium dodecyl sulphate polyacrylamide gel
SE	:	Standard Error

SEM	:	Standard Error Mean
SF	:	Serum Free
SFb	:	Serum Free with basic fibroblast growth factor pre-induction
Shh	:	Sonic Hedgehog
SMA	:	Smooth Muscle Actin
SNAP-25	:	Synaptosome-associated protein of 25,000 daltons
SP Cells	:	Side Population of cells
SS	:	Side scatter
ST-HSC	:	Short-term Hematopoietic Stem Cells
Str	:	Straurosporine
TBS	:	Tris-buffered saline
TC plastic	:	Tissue culture plastic
TGF	:	Transforming Growth Factor
TH	:	Tyrosine Hydroylase
TNF	:	Tumor necrosis factor
TRITC	:	Tetramethyl Rhodamine Isothiocynate
TUJ-1	:	Neuron specific beta III Tubulin
VCAM	:	Vascular Cell Adhesion Molecule
VEGF	:	Vascular endothelial growth factor
VEGFR	:	Vascular Endothelial Growth Factor Receptor
WB	:	Western Blot

Publications arising from this thesis

Papers in peer reviewed journals:

Croft A.P. and Przyborski S.A. (2006) Mesenchymal stem cells expressing neural antigens instruct a neurogenic cell fate decision on neural stem cells. *Stem cells. Submitted.*

Croft A.P. and Przyborski S.A. (2006) Formation of neurons by non-neural adult stem cells: potential mechanism implicates an artifact of growth in culture. *Stem Cells. 24(8):1841-51.*

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Croft A.P. and Przyborski S.A. (2002). Developmental potential of mesenchymal stem cells isolated from human bone marrow and umbilical cord blood. *The 3rd Cord Blood Immunology Group Meeting, London. Oral Communication.*

Other publications:

Croft A.P. and Przyborski, S.A. (2004) 'To be or not to be a neuron' *Scientific review, British Neuroscience Association Newsletter Issue 49 2004 Pages 12-13.*

CHAPTER 1

Literature Review

1.1 Introduction

This chapter entails a concise review of the literature, which has considerably expanded in recent years, in response to observations of adult stem cell plasticity and the motivation to drive these cells into the clinic for therapeutic use. It is not possible to review all the literature in this field and therefore only a select number of seminal studies are discussed to illustrate key developments in the field of adult stem cell biology. MSCs isolated from the postnatal BM are considered in more detail and their ability to differentiate into neuro-ectodermal cell derivatives is comprehensively reviewed. Finally the therapeutic benefit of transplantation of MSCs into animal models of CNS injury is discussed.

1.1.1 The concept of cell plasticity

Cell plasticity has become a central issue in stem cell biology following the demonstration that fully differentiated somatic cell nuclei have the ability to dedifferentiate, when transferred to enucleated oocytes (Munsie et al., 2000; Wakayama et al., 2001) or when fused experimentally with embryonic stem cells (Rideout et al., 2000; Wakayama et al., 1999). However, this remarkable plasticity was only achieved following considerable experimental manipulation. The traditional model of development states that adult stem cells have a differentiation potential restricted to the cell lineages associated with the organ in which they reside. Recent reports have claimed that adult stem cells may differentiate into developmentally unrelated cell types both *in vivo* and *ex vivo* (For review see: Blau 2002; Krause 2002; Anderson et al., 2001; Tsai et al., 2002). The traditional irreversible model of somatic cell differentiation was first questioned by the findings of early cell fusion and nuclear transfer experiments. Gurdon, 1968 demonstrated that when nuclei from amphibian intestine were introduced into enucleated oocytes, feeding tadpoles were generated. The results demonstrated that during cell specialization genetic material was not lost or permanently inactivated but that differentiation was dependent on the regulation of gene activity in response to cytoplasmic cues. The hypothesis stated that somatic nuclei injected into enucleated oocytes were conditioned by the cytoplasm and that

the reacquisition of multipotentiality, involved the activation of previously silent genes.

It has long been known that cell fusion is an effective means of achieving reprogramming of cells (Blau 1989; Blau et al., 1985; Blau 2002; Miller et al., 1988). To determine if the differentiated cell state was irreversible Blau and colleagues generated stable, non-dividing heterokaryons (a cell having two or more genetically different nuclei sharing a common cytoplasm). Non-dividing multinucleate heterokaryons were formed by the fusion of human cells representative of each embryonic germ layer (Keratinocytes, fibroblasts, hepatocytes) with mouse skeletal muscle cells. These heterokaryons expressed muscle genes when activated in primary human diploid keratinocytes, fibroblasts and hepatocytes (Blau et al., 1985; Miller et al., 1988; Pavlath et al., 1989). This reactivation of previously silent genes occurs in the absence of DNA replication. Gene dosage and/or the balance of proteins derived from the two cell types determine which genes are activated. In order for a mature differentiated cell to dedifferentiate into a more primitive cell phenotype or undergo reprogramming, to differentiate into an alternate cell fate requires the expression of an entirely new set of genes. Collectively these experiments involving heterokaryon formation demonstrated that previously silent genes can be activated and expressed in differentiated cell types. Terminal differentiation implies a terminal cell fate decision, however the results of these studies and nuclear transfer collectively challenge the dogma that differentiation is fixed and irreversible, and show that the phenotype of the differentiated somatic cell is plastic.

The issue of adult stem cell plasticity was placed at the forefront by the findings of nuclear transfer experiments (Munise et al., 2000; Wakayama et al., 2001), which involves the transfer of a diploid cell nucleus from a donor cell into another cell that has had its nucleus removed (enucleated). Cloning by nuclear transfer provides a method of creating genetically identical embryos. This technique involves the transfer of a nucleus from an adult somatic stem cell to an unfertilized oocyte devoid of maternal chromosomes. Embryonic development is then achieved under the control of the transferred nucleus. Stem cell lines from the cloned embryo are genetically identical (Wilmut et al., 1997; Wakayama et al., 1998; Wakayama et al., 1999). This

technique not only has implications for cell based therapies such as the creation of genetically identical organs for transplantation but the outcomes of such experiments may give profound insights into cellular plasticity and the mechanisms underlying the reprogramming of mature, differentiated nuclei to a status of totipotency (Rutenberg et al., 2004). Using these methods Munsie et al., 2000 isolated pluripotent mouse embryonic stem cells (ES) (see section 1.1.2.2 for an explanation of embryonic stem cells) from reprogrammed adult somatic nuclei. These cells were capable of generating all three embryonic germ layers in tumors and chimeric embryos. Wakayama et al., 2001 derived ES cell lines *in vitro* from the inner cell mass of blastocysts clonally produced by nuclear transfer. These experiments demonstrate that the adult stem cell is capable of been reprogrammed, an observation that implies plasticity, however it only occurs in response to extensive experimental manipulation.

Recent excitement in the adult stem cell field was initiated by the observations that adult stem cells previously thought only to generate cell types of the organ in which they reside, may in certain defined conditions adopt cell fates other than cell types associated with their dermal origin, both in culture and following transplantation *in vivo*. These observations are consistent with reports from heterokaryon studies that demonstrate the plasticity of the differentiated state (Blau et al., 1985) but suggest that environmental cues may be sufficient to reprogram a somatic cell fate. This type of plasticity is evident during development in which cells respond to micro-environmental factors by specific phenotypic alterations and these changes and subsequent lineage commitments result in the formation of a variety of cell types (Prindull and Zipori, 2004). Recent observations of adult stem cell plasticity suggest that these processes may not be confined to the embryo.

1.1.2 Terminology

There are many inconsistencies in the use of terminology in stem cell biology and therefore it is useful to begin the review by defining key terms used.

1.1.2.1 Stem cells, precursor cells and progenitor cells

A stem cell is an undifferentiated clonogenic, self-renewing progenitor cell with the capacity to generate one or more specialized cell types through asymmetrical cell

division (Anderson et al., 2001). In this way a stem cell can produce either a daughter cell that can either remain as a stem cell (self-renewal, symmetrical cell division) or commit to a pathway of differentiation. The pathway of differentiation and lineage commitment involves the production of a series of precursor cells, which proliferate before they differentiate. As a result these precursor cells are sometimes refereed to as transient amplifing cells because the proliferation amplifies the number of differentiated cells eventually formed. In addition, precursor cells display increasing commitment towards a particular cell lineage and thus concomitantly, their self-renewal capacity and differentiation potential are reduced with each precursor step. The terms progenitor cell and precursor cell have been used interchangeably but progenitor cell will be used in this thesis to describe a less committed cell with a greater differentiation potential than a precursor cell.

The mechanism, which determines self-renewal or commitment to differentiation, is currently not known for all stem cell populations but is dependent on both intrinsic (inheritance of genetic cell fate determinants) or extrinsic cues (environmental factors) (Spradling et al., 2001; Watt and Hogan 2000). The ability to self renew is seen as one fundamental and defining property a stem cell must process however, there is inconsistency as to how sustained the self re-newal should be. In addition, multilineage differentiation potential is often quoted as another defining characteristic but some stem cells produce only one cell type, for example spermatogonial stem cells in the testis produce only spermatozoa (Meachem et al., 2001).

There are also difficulties in identifing stem cells from precursor cells. Telomeres are the mitotic counting mechanism of cells and consist of a complex of guanine-rich repeat sequences and associated proteins, which cap and protect every eukaryotic chromosome against terminal DNA degradation (Blackburn, 2001). Telomeric shortening occurs with each cell division and complete loss of the telomere length is associated with DNA damage that cumulates in growth senescence of somatic cells in culture, after a defined number of cell divisions. Telomerase is an enzyme, present in certain cells including stem cells, which functions to add telomere repeats onto chromosome ends, and prevents replication dependent loss of telomeres and cellular senescence (Blasco, 2005). In most somatic cells except lymphocytes telomerase

activity is suppressed and therefore telomeric shortening occurs with each cell division. In humans and rodents, stem cells maintain telomerase activity (Forsyth et al., 2002) and human ES cells express high levels of telomerase, and as a result have an infinite lifespan *in vitro* when cultured under optimal conditions (Forsyth et al., 2002). Many human precursor cells turn off telomerase activity and are therefore only able to undergo a limited number of cell divisions before replicative senescence (Cong et al., 2002). Many rodent precursor cells maintain telomerase activity and can divide indefinitely in optimal culture conditions (Mathon et al., 2001). It has also been reported that some precursor cells can revert to a stem cell phenotype in defined conditions. If this process also occurs *in vivo* as demonstrated to occur in intestinal crypts following stem cell ablation (Marshman et al., 2002), then the assumption that at least one daughter cell of a stem cell must remain a stem cell to maintain the stem cell pool may be incorrect.

1.1.2.2 Pluripotency, multipotency and unipotency

Mammalian stem cells can be operationally classified according to their developmental potential. ES cells are produced from the inner cell blastocyst (Thompson et al., 1998) and embryonic germ cells are produced from the primordial germ cells of the early embryo (Shamblott et al., 1998). These two cell populations are defined as pluripotent based on the fact that they can generate cell types of all three germ layers of the embryo proper, including germ cells. They cannot however, form the extra-embryonic tissues required for mammalian development and therefore are not considered totipotent. Tissue specific stem cells or adult stem cells are described as multipotent if they produce more than one cell type and unipotent if they produce just one cell type. It is of note that when cell suspensions prepared from postnatal organs are analyzed by florescence activated cell sorting (FACS), stem cells are found as a small side population of cells and are only weakly fluorescent after staining with a fluorescent dye (Goodell et al., 1996; Wolf et al., 1993); these cells express a high level of a specific ABC transporter that actively pumps dye out of the cells (Zhou et al., 2001). This property is often used for the prospective isolation of adult stem cells but the biological significance of this property is unknown.

1.1.2.3 Commitment, differentiation and trans-differentiation

Cell fate is determined by a variety of factors that control gene transcription during embryonic development and normal adult physiology. Cell commitment is therefore viewed as consisting of a series of irreversible steps to differentiation. In the embryo, this process consists of a descending hierarchy of diminishing capacities of differentiation, which begins with the ES cell, and development of all cell types of the embryo. During subsequent organogenesis, the potency of differentiation is further reduced, coupled with diminishing proliferative capacity. Most of these postnatal organs contain a quiescent self-renewing population of stem cells, the differentiation potential of which is restricted to lineages in which the stem cells reside. Trans-differentiation denotes an alteration in the state of differentiation of cells that have already been specialized or programmed to a given cell lineage (Okada et al., 1999). The original quoted example is that of the conversion of pigmented epithelial cells of the iris into lens cells during lens regeneration in newts (Brockes et al., 2002).

1.1.3 Germ layer model of development and the stem cell concept

As described above, in vertebrates ES cells are capable of generating all differentiated somatic cell types in the body. These cells are isolated from the embryo at a stage in development prior to their commitment to differentiate into particular lineages and therefore show a high degree of plasticity, with the capacity to generate somatic cell lineages of all three embryonic germ layers during growth as embryoid bodies *in vitro* (Itskovitz-Eldor et al., 2000) and formation of teratomas *in vivo* (Reubinoff et al., 2000). ES cells give rise to tissue specific adult stem cells. These multi-potent stem cells generate the cell types comprising a particular organ or tissue during embryonic development and in some cases in the adult. Tissue specific stem cells exist in most postnatal organs where they have a haemostatic function regulating cell turnover and in regenerative organs such as the intestine and skin contribute to repair following injury (Blau 2002). Ongoing cell turnover in regenerative organs provides a rationale for the existence of stem cells, whereas in organs such as the heart and brain, which undergo only limited regeneration the presence of stem cells has been proven by their isolation and their subsequent growth in culture, and differentiation into multiple cell lineages *in vitro* or after transplantation *in vivo*. The role these cells play in repair and regeneration following injury is uncertain. Originally the existence of stem cells was implied retrospectively from genome marking experiments (Wu et al., 1968). More

recently stem cells have been prospectively isolated from a number of postnatal organs using cell surface marker proteins (e.g. Shi and Gronthos 2003; Gronthos et al., 2003; Spangrude et al., 1988; Morrison and Weissman, 1994). A long-standing concept has been that adult tissue specific stem cells have a restricted potential to generate differentiated cell types of the organ in which they reside.

1.1.4 Adult stem cell review

1.1.4.1 Introduction

Consistent with embryonic stem cells, adult stem cells have the capacity for self renewal and the capacity to differentiate into mature effector cells (Watt and Hogan, 2000). It was originally thought that adult stem cells resided only in those organs with high cell turnover rates such as the respiratory tract, blood, skin, gut and testis. However, the recent prospective isolation of somatic tissue stem cells from a wide range of postnatal organs suggests that the homeostasis of tissues capable of regeneration is mediated by stem cells. As part of the review of the current literature in the field of adult stem cell plasticity, the major adult stem cells are first introduced briefly with a more extensive review of MSCs in section 2.2 and CNS stem cells in section 2.4. Considered here are only those stem cells involved in plasticity experiments.

Most postnatal organs are primarily comprised of terminally differentiated and postmitoitic effector cells, however many tissues retain a small population of tissue specific stem cells. The function of these cells is regeneration of damaged tissue and tissue homeostasis for example physiological replacement of skin and blood cells. Examples include epithelial stem cells in epidermis and intestinal crypts (Slack, 2000), NSCs in the CNS (Mckay, 1997) and satellite cells in muscle (Charge and Rudnicki, 2004). The BM contains hematopoietic and MSCs (Prockop, 1997). An increasing number of tissues could be added to this list and some stem cells such as MSCs are been isolated from an increasing number of organs (de Silva Meirelles et al., 2006). Despite the isolation of tissue specific stem cells from certain organs, many of the properties of these cells are unknown as a result of the difficulties in the isolation, heterogeneity, identification *in vivo* and tracing of their progeny. The

primary function of tissue stem cells is regeneration of damaged tissue and maintaining tissue homeostasis.

1.1.4.2 Hematopoietic stem cells

Hematopoietic stem cells (HSCs) were the first stem cells to be isolated and characterized and the first stem cell population to be used clinically. HSCs reside within the postnatal BM and are pluripotent stem cells with the capacity to reconstitute the adult blood system by differentiating into multiple hematopoietic cell lineages (Lagasse et al., 2001; Morrison and Weissman, 1994; Weissman, 2000). In early seminal experiments, infusion of BM cells was shown to rescue lethally irradiated mice by reconstituting their blood system (Ford et al., 1956; Nowell et al., 1956; Till and McCulloch 1961). This functional repopulation of the blood system following ablation of the host hematopoietic system is considered the gold standard for the functional characterization of HSCs. Till and McCulloch found that the spleen of BM infused hosts contained macroscopic cell colonies containing differentiated progeny of multiple blood lineages (Till and McCulloch 1961). They were later able to demonstrate that these colonies arose from a single cell, thereby demonstrating that BM infused cells were indeed multipotential with the capacity to generate progeny of multiple cell lineages (Becker et al., 1963). Serial transplantation experiments indicated that at least some of the infused BM cells were capable of self-renewal and retained their differentiation potential over time (Wu et al., 1967).

On the basis of these findings, a model of hematopoietic differentiation was proposed in which HSCs reside at the top of a cellular hierarchy and give rise to progeny with the capacity to differentiate into multiple blood cell lineages. HSCs form mature, terminally differentiated effector cells through a process of cellular differentiation in which progenitor cells become increasing more committed to a distinct cell lineage with a concomitant reduction in their differentiation potential and self renewal capacity. Therefore a model system is proposed in which HSC derived progenitor cells progressively loose stem cell characteristics including loss of multipotentiality as they differentiate and acquire the phenotypic characteristics of mature blood cells. Whilst there is abundant evidence for this model in the hematopoietic system, it is thought that a similar, if not, identical system operates in other mammalian organs.

Purified populations of HSCs have been prospectively isolated from human (Baum et al., 1992) and mouse BM (Spangrude et al., 1988). Two classes of HSCs have been identified on the basis of expression of cell surface antigens and long-term reconstituting ability. Both capable of reconstituting the blood system of mice, but short term (ST-HSC) have only a limited ability (two months) and long-term (LT-HSC) have been shown to reconstitute the blood system for greater than six months (Morrison and Weissman 1994; Lagasse et al., 2001). ST-HSC are considered precursor cells derived from LT-HSC and which subsequently give rise to common myeloid and lymphoid precursor cells, which give rise to progressively more restricted precursor cells and terminally differentiate into effector cells of the blood and immune systems respectively (Weissman et al., 2001).

The isolation of HSC was made possible by the development of *in vitro* and *in vivo* functional assays in which to evaluate lineage potential and self-renewal ability. Isolation protocols have been developed which allow for the prospective isolation of highly enriched (>80%) populations of HSCs (Lagasse et al., 2001). The success of these isolation and enrichment protocols relies on the use of florescence-activated cell sorting (FACS) which allows positive selection of cells based on the expression of a set of cell surface proteins. Lineage depletion is also used to prospectively isolate cells by using panels of monoclonal antibodies to exclude cells expressing markers of mature hematopoietic cells (Matsuoka et al., 2001; Sato et al., 1999). However, the success of this enrichment, is the result of extensive characterization of HSC and progenitor cell surface marker expression, which has been used to distinguish these cells. An analogous system for other adult stem cell populations does not currently exit. As a result, it is not currently possible to achieve such highly purified populations of tissue specific stem cells, although clonal isolation has been achieved.

Such a system however is not faultless and heterogeneity remains a problem even for the HSC system as cell populations enriched for HSCs, using the most optimal isolation procedures are heterogeneous with some cells failing to demonstrate pluripotency or long-term reconstitution ability (Morrison and Weissman, 1994). Despite these technical difficulties HSCs can be enriched up to a 1000 fold and

delivered to marrow ablated recipients to fully reconstitute the blood system (Bryder et al., 2006; Morrison and Weissman, 1994). This ability to reconstitute the blood system is utilized clinically to treat many blood disorders including leukemia and autoimmune disorders. The HSC system remains a model system and the gold standard characterization yet to be achieved for other adult stem cell populations. In addition, many of the cytokines and growth factors that promote the proliferation of different precursor cells have been identified and some are used clinically to mobilize HSCs from the BM into the systemic circulation where they can be harvested for transplantation (Lapidot and Petit 2002).

The hematopoietic system functions with a high cell turnover, as the life span of effector cells is only short. This places great demands on the stem cell system and requires fine haemostatic controls to regulate differentiation. The system of differentiation has a number of advantages. Enormous amplification in numbers of cells is made possible by coupling certain steps in differentiation with increased proliferative potential. This places very little proliferative pressure on HSCs, which have a low cell turnover and primarily reside in the G0 phase of the cell cycle (Bradford et al., 1997). This is thought to protect these cells from the mutagenic hazards of DNA replication and metabolic demands. Despite the success in the use and isolation of HSCs little is known about the regulation of cell fate decisions in these cells or the molecular basis of self-renewal and as a result expansion of purified populations of HSCs in culture is a problem and a limitation to their use (Bryder et al., 2006).

1.1.4.3 Hepatic stem cells

The adult liver is capable of extensive regeneration, an activity evident in physiological response to liver injury in which replication and regeneration promote growth, to restore the liver's functional mass. The source of tissue specific stem cells responsible for this extensive regeneration is uncertain. Unlike most postnatal organs the generation of new cells in the injured liver involves two cell systems comprised of mature functional differentiated liver cells including hepatcytes, bilary epithelial cells and endothelial cells (Taub, 2004; Fausto and Campbell 2003; Fausto, 2000; Michalopoulos and DeFrances 1997). It is thought that these cells provide the primary

response to injury, a phenomenon unique to the liver. Progenitor cells with stem-like activity are located near the bile ducts and give rise to a proliferation of oval cells that subsequently generate hepatocytes and ductular cells. These progenitor cells are thought to function only as a reserve compartment that is activated when the regenerative capacity of hepatocytes is compromised by certain types of injury. The response to surgical resection of the liver is reconstruction of liver mass by the progeny of residual differentiated liver cells. In contrast, injury that impairs hepatocyte proliferation results in a transient amplification of progenitor cells which form new hepatocytes and bilary cells (Kubota and Reid, 2000; Sell, 2001).

These studies raise the possibility that the liver contains stem cells with multilineage potential, which resides in or near terminal bile ductules (Theise et al., 2000). One group has claimed to have isolated hepatic progenitor cells from the adult murine liver without a preceding injury (Wang et al., 2003). In culture, these cells express markers of oval cells and with prolonged culture this expression profile changes. A decrease in oval cell markers is observed and cells begin to express albumin and cytokeratin suggestive of differentiation towards hepatocytic and bilary lineages. However, as yet there is no consensus on the prospective isolation of a purified population of stem cells from the adult liver.

1.1.4.4 Skeletal muscle stem cells

Skeletal muscle cells are referred to as satellite cells and are one of few adult stem cell populations that can be identified prospectively *in vivo* (Bischoff, 1986). They are responsible for postnatal growth and repair (Charge et al., 2004). The satellite cell has been defined as a quiescent mononucleated cell enshealthed under the basal lamina that surrounds multinucleated muscle fibers (Mauro, 1961). These cells are thought to constitute a stem cell population with the capacity to contribute to intact skeletal muscle fibers even after propagation in culture. In response to muscle injury these cells have been shown to proliferate to form myoblasts, which form either new satellite cells (self renewal) or differentiate into muscle fibers through fusion with pre-existing muscle fibers.

Until recently, the difficulty in isolating pure populations of satellite cells has limited their use in cell based tissue repair assays (Montarras et al., 2005). These assays have instead utilized muscle precursor cells that correspond to the progeny of muscle satellite cells, obtained after activation and proliferation in culture, or following dissociation of whole muscle tissue (Qu-Petersen et al., 2002; Mueller et al., 2002; Skuk 2002). The barrier for direct isolation of these quiescent muscle cells has been the lack of definitive cell surface markers. Satellite cells have been shown to express surface markers such as M-cadherin (Morgan et al., 1993), Syndecan 3 and 4 (Cornelison et al., 2001), and CD34 (Beauchamp et al., 2000), however none of these markers permits definitive pure isolation because of a lack of specificity. Recently satellite cells in a subset of muscles have been shown to express Pax-3, a member of the homeodomain/paired box family of Pax proteins (Buckingham et al., 2003; Seale et al., 2000). Recently, Montarras et al., 2005 directly isolated quiescent satellite cells from skeletal muscle using a GFP-tagged Pax-3 mouse line by flow cytometry. Transplantation of these cells into irradiated tibialis anterior muscles of immunodeficient nude mdx mice (mdx nu/nu) led to restoration of dystrophin expression. Mdx mice lack dystrophin, the structural protein that is mutated in Duchene muscular dystrophy. Therefore evaluation of fiber repair in these mice following transplantation provides a functional repopulation assay. Purified satellite cells were found to be more efficient than crude or cultured cell populations in contributing to muscle repair. In addition, it was found that culture of muscle progenitor cells markedly reduces their regenerative efficiency. Self-renewal capacity of engrafted purified satellite cells was also demonstrated, as in addition to contributing to muscle repair some cells persist as progenitor cells, adopting a satellite cell position and expressing Pax-7. Engrafted cells can be recovered from muscle into which they were transplanted and shown to produce muscle cells in culture providing further evidence of self-renewal capacity without loss of differentiation capacity. Despite recent success with the prospective isolation of a subset of purified populations of satellite cells, there is much heterogeneity in this cell population and their may be many subsets of satellite cells in which the markers for isolation may not be consistent (Beauchamp et al., 2000).

1.1.4.5 Neural stem cells

Neurons of the mammalian CNS arise during embryonic development from neuroproliferative zones surrounding the ventricles of the neural tube (Pakkenberg et al., 2003). All cell types of the post mitotic neuron are formed from precursor neuroblasts that exit the cell cycle and migrate from these regions to the adult grey matter. During embryonic neurogenesis there is a massive proliferation of cells but only 15-50% of these post migratory cells survive suggesting that a selection process is occurring. Until only recently, the mammalian CNS was thought to be incapable of significant self-repair or regeneration (Bjorklund and Lindvall, 2000). Recent evidence has shown that NSCs with the capacity for long term self-renewal and multilineage differentiation have been reported to persist throughout adult life (Temple 2001). The adult CNS is also known to retain a range of progenitor cells with more limited capacities for growth and differentiation (Temple 2001). These cells are known to be abundant in the paraventricular areas and recent studies have shown them to be present in the parenchyma of various CNS regions (Palmer et al., 1999; Yamamoto et al., 2001). NSCs have been prospectively isolated from a number of brain regions following dissection and subsequent culture (Lois and Alvarez-Buylla 1993; Morshead et al., 1994; Palmer et al., 1997; Reynolds and Weiss 1992). Proteolytic dissociation of adult brain tissue allows for the isolation of populations enriched for NSCs. However, there is no definitive cell marker for NSCs and therefore current protocols only allow for about 50-fold enrichment in NSCs (Uchida et al., 2000). In culture (Reynolds and Weiss, 1992; Loisand Alvarez-Buylla, 1993; Palmer et al., 1997; Palmer et al., 1999 and Kondo and Raff, 2000) and following transplantation back into the adult brain (Shihabuddin et al., 2000), these isolated NSCs population can generate cell progeny of all three principal neural lineages; oligodendrocytes, astrocytes and neurons.

Consistent with the observations in culture, continuous generation of new neurons has been detected in the adult (Temple, 2001; Gould and Gross, 2002). In the adult CNS *in vivo* however, neurogenesis appears to be restricted at least under normal physiological conditions to two specific regions: the subventricular zone and hippocampal subgranular zone (Gage 2000). Beyond these two regions progenitors in other brain regions give rise to only new glial cells. Interestingly when these dividing cells are isolated from non-neurogenic regions and explanted in culture they are able

to generate all three neural cell lineages under defined culture conditions (Kondo and Raff, 2000) and when transplanted back into the hippocampus are able to form neurons (Suhonen et al., 1996; Shihabuddin et al., 2000). These results are consistent with micro-environmental signals playing a key role in influencing the fate specification of NSCs.

In addition to postnatal neurogenesis recent evidence has shown that the brain responds to injury, but this response is limited and cannot result in full functional recovery. Various insults including ischemic injury have been shown to stimulate the proliferation of endogenous progenitors either in known neurogenic (Gould and Tanapat, 1997; Liu et al., 1998; Magavi et al., 2000) sites or in non-neurogenic sites (Johansson et al., 1999; Yamamoto et al., 2001). Collectively these results are consistent with the existence of multipotent cells with the ability to divide and differentiate into neural cell lineages within the CNS (Gage, 2000). However, the identity of NSCs *in vivo* is controversial (Barres 1999, Laywell et al., 2000) and the ability to culture neurospheres (proliferating neural cells) from regions of the adult brain not normally able to self-renew (Weiss et al., 1996; Palmer et al., 1995) raises the possibility that neurospehres can in fact arise from differentiated neural cells and precursor cells and not necessarily NSCs (Andersen 2001).

1.1.4.6 Skin stem cells

The germinal layer of the epidermis contains stem cells, which support a high epithelial cell turnover. The germinal layer extends from the epidermis to surround the dermal appendages such as hair follicles. It is currently uncertain whether the two tissues are distinct; with each containing a unique stem cell population or that a common stem cell supports both tissue compartments (Rochat et al., 1994). It has been shown that following severe epidermal injuries, that keratinocytes can migrate from the hair follicles to regenerate the epidermis (Taylor et al., 2000). Recently stem cells have been identified in the bulge zone of the follicular epithelium with the capacity to generate hair follicles and epidermis and replenish the epidermal basal layer (Fuchs and Segre, 2000; Lavker and Sun, 2000; Taylor et al., 2000). Current work is focused on determining the relationship and origin of these stem cell populations.

1.1.4.7 Mesenchymal stem cells

A large body of evidence demonstrates that stromal and connective tissue throughout the body contains clonogenic progenitor cells (CFU-F) a proportion of which has the capacity for differentiation into multiple mesenchymal cell lineages including adipose tissue, bone, cartilage, tendon and ligament (Bruder et al., 1998a; 1998b; Prockop 1997). Despite the considerable interest in these cells there is still no well-defined protocol for the prospective isolation of these cells, a problem compounded by the lack of a definitive cell surface marker for the isolation of these cells. As a result, little is known about the precise phenotypic characteristics or localization MSCs *in vivo*. MSCs have been primarily isolated from the adult BM (Pittenger et al., 1999; Kopen et al., 1999), however more recently they have been isolated from other tissues including adipose tissue (Zuk et al., 2001), periodontal ligament (Seo et al., 2005), bronchi (Sabatini et al., 2005) and the synovial membrane (De Bari et al., 2003). A recent study carried out a systematic isolation of MSCs from different organs and tissues and evaluated their characteristics (da Silva Meirelles et al., 2006). They report the isolation of MSCs from brain, spleen, liver, kidney, lung, BM, muscle, thymus and pancreas. Cells isolated from these regions all had the ability to differentiate into bone and fat. However, variation in the degree of differentiation was evident. These authors were also able to isolate MSCs from large blood vessels including the aorta and vena cava as well as capillaries. This data is consistent with the growing evidence that suggests MSCs are related to pericytes. A more in-depth review of MSCs is provided later in section 1.2.

1.1.5 Claims for adult stem cell plasticity

Although nuclear transfer experiments demonstrated that cells are capable of remarkable plasticity following reprogramming this was only possible following extensive experimental manipulation. Evidence that adult stem cells may be capable of differentiating into diverse fates under physiological conditions came following the detection of non-associated cell types in distinct organs following BM transplantation into sub-lethally irradiated host animals. Sub-lethal irradiation of animals results in complete ablation of the cells of the blood system including all hematopoietic stem cells and their differentiated progeny (Ford et al., 1956). Transplantation of BM purified hematopoietic stem cells and their derivatives or single cell transplantation of

HSC results in reconstitution of the blood system and this repopulating assay provides the functional definition of HSCs (Ford et al., 1956; Nowell et al., 1956; Till and McCulloch 1961). If a traditional view of stem cell biology is taken then HSCs can exclusively reconstitute the blood system of an irradiated host, consistent with its developmental potential having been restricted to the organ in which it resides. Early experiments demonstrated that following transplantation of tissues from a particular organ to an ectopic site in the body, the transplanted tissue retained its original phenotypic characteristics (Andersen et al., 2001).

Cells are also known to retain their characteristics following culture *ex vivo*, although stem and progenitor cells may loose their differentiation potential (Blau et al., 1985). Consistent with this view, the definition of a adult stem cell is one in which a cell is capable of extensive self renewal and the capacity to give rise to progeny that can differentiate into multiple cell lineages associated with the organ in which they reside. However a large number of recent reports have described a phenomena in which cells isolated from one organ give rise to, or acquire the characteristics of cells of another organ simply through exposure to new environmental cues. This process is referred to as metaplasia, cell plasticity, or lineage switching or trans-differentiation. It was previously thought that the developmental potential of adult stem cells is restricted to the differentiated elements of the tissue in which they reside. The process or observation in which stem/progenitor cells differentiate into cells that are not of the same dermal origin is referred to here as trans-differentiation. It suggests that extensive experimental manipulation may not be required to reprogram adult stem cells, but that certain stem cell populations retain an intrinsic ability to differentiate into multiple cell lineages outside their organ of residence. It also suggests that micro-environmental signals regulate the cell fate commitment of adult stem cells *in vivo* and exposure to new micro-environmental signals allows them to express a broader differentiation potential.

The first evidence of unexpected cell fate changes in adult stem cell populations came from BM transplantation studies. BM is particularly advantageous for testing the potency of donor cell populations since BM tissue can be ablated by sublethal irradiation of host animals and functional repopulation of the organ by transplanted

donor cells can be evaluated accurately. Eglitis and Mezey, 1997 were one of the first investigators to report unexpected cell fate changes in BM derived stem cells. Donor cells (genetically labeled or sex chromosome detection) were detected in the brains of adult mice following BM transplantation. Engrafted BM derived cells expressed antigenic markers consistent with an astroglial cell fate.

This study provided the first observation that indicated adult stem cells might have a broader differentiation potential than previously appreciated. A large number of publications subsequently reported similar observations in both BM derived stem cells and other adult stem cell populations. Ferrari et al., 1998 reported that mouse BM cells could give rise to skeletal muscle cells when transplanted into a mouse muscle that had been damaged by injection of a muscle toxin. Bjornson et al. 1999 used a BM repopulation assay to test the hypothesis that adult NSCs have the capacity to generate hematopoietic cell lineages. The authors reported that genetically labelled NSCs were capable of producing a variety of blood cell types of both myeloid and lymphoid cell lineages following transplantation of BM into irradiated hosts. Transplanted BM cells were subsequently shown to produce hepatocytes, endothelial, myocardial and CNS and glial cells. It was unknown however, from these studies which stem cell population in BM was responsible for this apparent plasticity. MSCs isolated from the postnatal BM when transplanted into the lateral ventricle or striatum of mice migrate into the brain and differentiate into astrocytes (Kopen et al., 1999). A single transplanted HSC has been shown to contribute to the epithelia of various organs of all three germ layers including liver gut, lung and skin (Krause et al., 2001). NSCs isolated from the adult mouse brain produce skeletal muscle (Galli et al., 2000), hematopoietic cell lineages (Bjornson et al., 1999) and when injected into a blastocyst contribute to cells of all three primary germ layers in the resulting embryos (Clarke et al 2000).

These reports challenged the previous dogma that the differentiation of adult stem/progenitor cells is restricted to cell lineages associated with the organ in which they reside and that somatic cell specialization may not involve irreversible genetic changes. The findings of these studies are complementary to the seminal demonstration of conserved genomic totipotentiality in adult somatic stem cells

following the successful cloning of an adult ewe (Wilmet et al., 1997; Wilmet et al., 2002). Reports of apparent adult stem cell plasticity suggest that reactivation of previously silent genetic programs may not require experimental manipulation of the genome and that adult stem cells have an intrinsic capacity to form cell phenotypes beyond their dermal origin. This genetic potentiality remains silent until reactivation in response to the appropriate pattern of stimulation probably provided by the microenvironment.

The reason why cells with such apparent plasticity have not been observed before is thought to be the result of methodology. In classical transplantation studies whole organs or tissue fragments have been transplanted and therefore transplanted stem or progenitor cells remain within their normal microenvironment even following engraftment into host tissue (Raff 2003). Recent transplantation studies use single cell suspensions and therefore individual donor cells engraft into distinct host microenvironments. It is possible that these new microenvironmental signals influence the reprogramming of cells into distinct cell fates. Recent experiments have also involved genetically labelled donor cells so that rare cells expressing donor-cell genes could be identified in tissue sections. Genetic markers used include Y chromosome detection and transgenes encoding β-galactosidase (β-gal) or green florescent protein (GFP) (Alison et al., 2000; Korbling et al., 2002).

1.1.6 Criteria for establishing the occurrence of cell fate changes

Several authors have emphasized the need to establish clear criteria to prove functional trans-differentiation potential. Andersen et al., 2001, have suggested criteria required to establish that a cell fate transition has occurred. Firstly, the donor cell population must be prospectively isolated and transplanted without intervening culture manipulations. Secondly the transplanted stem cells should give rise to robust and sustained regeneration of target tissues. Thirdly the phenotype of converted cells must be confirmed not only anatomically and molecularly but also functionally, for example the formation of a neuron by a non-neural stem cell must be shown to result in a functional neuron (conducts action potentials). Finally, what is the frequency of trans-differentiated events compared to the total number cells transplanted.

1.1.7 Reproducibility of results

One of the major challenges to observations of adult stem cell plasticity has been the reproducibility of several seminal observations. As described previously Bjornson et al., 1999 demonstrated that single LacZ+ NSCs with the capacity to form neurosphere structures *in vitro* in which all three neural cell lineages were present were shown to have hematopoietic potential when transplanted into sublethally irradiated mice. Clonogenic assays from recipient BM showed that in these studies >95% of the colonies were positive for β-galactosidase, which suggested that their origin was from the NSCs. Cultured NSCs did not display any hematopoietic potential without prior injection into the irradiated mouse host. This suggested that the microenvironment played a crucial role in the process of trans-differentiation of NSCs. However, Morshead et al., 2002 used a similar protocol to Bjornson et al., 1999 and rigorously tested the hematopoietic potential of mouse neurosphere cells and was unable to find any evidence of hematopoietic differentiation in a large group of sublethally irradiated mice each transplanted with 10^6 neurosphere cells, which suggested that hematopoietic potential was not a general property of NSC.

There have also been conflicting observations with reference to the ability of BM to contribute to neural tissue. As described previously, Mezey et al., 2000 using female homozygous PU.1 mice rescued these mice with BM transplants from male wild type donors. 4.6% of cells found in the CNS expressed the Y chromosome and were therefore identified as donor derived and up to 2.3% of these Y-chromosome positive cells possessed the neuronal markers NeuN and NSE. In contrast, Castro et al., 2002 found no neuronal differentiation in eight lethally irradiated recipients of $2x10^3$ SP cells from ROSA26 donors or twelve recipients of whole BM cells even though in both groups neuronal injury was present. These are just two examples of studies in which there has been discrepancy and it is difficult to explain the reasons for these different observations but subtle differences in experimental conditions have been invoked by some authors to explain these differences.

1.1.8 Mechanisms of stem cell plasticity

Several possible mechanisms must be considered when attempting to explain observations of increased stem cell plasticity: (1) multiple tissue-specific stem cells

are present in different organs, (2) plasticity is the result of fusion of donor cells with host recipient cells in the organ under investigation. (3) cells undergo de- and re-differentiation, or (4) true multi- or pluripotential stem cells, which exist in postnatal organs throughout life. Data exists to support all these theories.

1.1.8.1 Multiple tissue-specific stem cells are present in different postnatal organs

HSC exit the BM and circulate in the systemic vasculature where they engraft in peripheral end organs (Weissman et al., 2001). HSC can be found in a number of organs and tissues other than the BM. As result, preparations of stem cells from tissues or organs may in fact be heterogeneous. Heterogeneity is an issue HSC cell biologists have contended with for years however, the relatively recent increase in the number of prospectively isolated adult stem cells means this issue is of significance. It has been proposed that if a stem cell is a single entry it should be possible to isolate prospectively. However, even among tissue specific stem cells there is inherent heterogeneity. In the CNS two distinct cell types have been reported to give rise to neurons, which may reflect the existence of more than one distinct type of stem cell (Alvarez-Buylla et al., 2001; Doetsch et al., 1999; Johansson et al., 1999). It is reportedly difficult to purify stem cells from the nervous system and this may reflect heterogeneity inherent to the stem cell pool (Morrison et al., 1999; Uchida et al., 2000). Satellite cells from skeletal muscle are heterogeneous in respect to their protein expression profiles (Beauchamp et al., 2000). As a result caution must be taken when interpreting possible trans-differentiation events and a consideration must be given to the possibility of a heterogeneous starting cell population. Two studies have shown for example, that the perceived repopulation of the hematopoietic system following transplantation of muscle cells into sub-lethally irradiated mice is the result of HSC that reside within muscle (Kawada et al., 2001; McKinney-Freeman et al., 2002). In addition, the reported contribution of BM-derived cells to liver regeneration may be the result of the presence of oval cells (progenitor cells for hepatic and biliary epithelial cells), which reside in the BM. Therefore rigorous characterization of the starting stem cell population must be carried out prior to transplantation and this is especially true when observing trans-differentiation events. Some investigators have argued that the stem cell property should be considered a cellular function and not an entity (Blau et al., 2002). Accumulating evidence raises the possibility that many

types of cells from distinct organs and at differing stages of differentiation are capable of being recruited to function as stem cells. Thus the ability to act as a stem cell may be shared by numerous cell types expressing diverse genes. If this holds to be true then stem cells should be defined as a cellular function and not an identity.

1.1.8.2 Pluripotent stem cells persist in postnatal organs throughout life

There is a body of experimental evidence, which now supports the notion that the precursors to defined adult somatic stem cells may persist beyond the early steps of embryogenesis (Jiang et al., 2002a; Reyes and Verfallie, 2001). Multipotent adult progenitor cells (MAPCs) are a novel class of stem cells originally isolated from BM where they co-purify with MSCs. However, MAPCs have now been isolated from a number of adult tissues including BM, muscle and brain (Jiang et al., 2002b). MAPCs are pluripotent, and a single MAPC injected into a blastocyst contributes to all somatic tissues (Jiang et al., 2002a). Clonal MAPCs are able to differentiate *in vitro* into various lineages of mesodermal, ectodermal, and endodermal origin and contribute to terminally differentiated tissues grafted into postnatal organs, a capacity, which is increased during injury. Such pluripotent stem cells were not thought to exist in the postnatal organism. ES cells are characterized by their expression at the molecular level of a number of transcription factors thought to be highly specific for these undifferentiated cells, including Oct-4 and Rex-1 (Nichols et al., 1998; Scholer et al., 1989; Rosfjord et al., 1997; Ben-Shushan et al., 1998). Oct-4 is a transcription factor, which usually has a restricted distribution being expressed in the pregastrulation embryo, cells of the inner mass of the blastocyst and embryonic carcinoma cells (Scholer et al., 1989; Rosner et al., 1990). Oct-4 expression is down regulated, when cells are induced to differentiate *in vitro* (Pikarsky et al., 1994). Oct-4 and Rex-1 are required to maintain the undifferentiated phenotype of ES cells and in adults Oct-4 expression is confined to germ cells (Nichols et al., 1998; Rosfjord et al., 1994; Ben-Shushan et al., 1998; Niwa et al., 2000). However, MAPCs isolated from the adult express Rex-1 and Oct-4 consistent with their pluripotent differentiation capacity (Jiang et al., 2002a). Another group has recently reported the isolation of an adult stem cell population from BM under low oxygen tension and defined culture conditions, which has the capacity to generate cells associated with all three germ layers *in vitro* (D'Ippolito et al., 2004). Pochampally et al., 2004 described the

expression of Oct-4 in MSCs maintained in culture for a prolonged period of time in serum free media. It is not known how these stem cells relate to each other and whether or not they share the same identity but the data does suggest the possibility that a pluripotent stem cell with the capacity to generate cells of all three germinal layers persists in the adult BM throughout postnatal life. However, at present it is not unknown whether MAPCs exist *in vivo* and therefore there is currently no definitive proof that true pluripotent stem cells exist *in vivo* during postnatal life.

1.1.8.3 Cell fusion

Two publications in Nature (2002) suggested that the phenomena of the generation of unexpected phenotypes *in vivo* were the result of fusion of donor cells with the host tissue and not trans-differentiation of transplanted adult stem cells (Ying et al., 2002; Terada et al., 2002). When genetically altered BM from transgenic mice expressing GFP and the puromycin resistance gene were co-cultured with mouse ES cells, GFP positive clones were detected within three weeks of puromycin been removed from the culture (Terada et al., 2002). The expression of GFP and resistance to puromycin indicated that the GFP positive embryonic-like cells were derived from BM cells. The BM derived embryonic-like cells also possessed corresponding pluripotency defined by their capacity to spontaneously differentiate into cell types representative of all three embryonic germ layers and form teratomas following transplantation into NOD/SCID mice. Genomic analysis revealed these cells to be tetraploidy, indicating that cells were formed by fusion of BM cells and embryonic stem cells and not transdifferentiation. Similar results were reported when mouse CNS cells (also genetically altered) were co-cultured with ES cells (Ying et al., 2002). These authors found that chimeras generated from these cells were capable of contributing to somatic tissues, including liver, intestine, kidney and heart. In both these studies the frequency of reported fusion was extremely low, 2-11 hybrid clones per 10^6 marrow cells and one event per 10^5 CNS stem cells. This low frequency of fusion events is too low to account for the extensive contributions of somatic stem cells to embryonic tissues reported in previous studies. In both these studies the fused tetraploid cells inherited the selectable markers for both cell types and the properties of ES cells, therefore in both cases the ES genome was dominant.

It was the studies of Alvarez-Dolado et al., 2003 that provided the evidence that cell fusion could occur *in vivo*. Following BM transplantation BM derived cells (BMDCs) fuse *in vivo* with hepatocytes in the liver, purkinje neurons in the brain and cardiac muscle in the heart resulting in the formation of multinucleated cells. These authors employed a cre/lox recombination technique. In this method, transgenic mice expressing Cre recombinase ubiquitously under the control of a hybrid cytomegalovirus (CMV) enhancer B-actin promoter are used in conjugation with the conditional Cre reporter mouse line R26R. In this line, the lacZ promoter gene is only expressed following the excision of a loxP-flanked stop cassette by cre-mediated recombination. As a result, when cre-expressing cells from transgenic mice fuse with R26R cells, Cre recombinase excises the flanked stop cassette of the reporter gene in the R26R nuclei, resulting in the exclusive expression of LacZ in the fused cells. This lacZ staining can then be subsequently detected by X gal staining. These investigators used this method to study cell fusion *in vivo*. R26R reporter mice were sub-lethally irradiated and grafted with BM from mice constitutively expressing Cre recombinase and GFP. All animals displayed significant hematopoietic reconstitution when analysed 2-4 months following transplantation and B-gal positive cells were detected in heart, liver and brain. Analysis revealed these fusion events to be highly specific as BMDCs fused with hepatocytes in the liver, purkinje neurons in the brain and cardiac muscle in the heart resulting in the formation of multinucleated cells. Most importantly this study found no evidence of trans-differentiation without cell fusion in these tissues but did raise the possibility that cell fusion may contribute to the development and maintenance of key cell types and possibly be a source of cell replacement therapy.

Cell fusion may be the mechanism for the generation of unexpected cell fates observed in various organs and as a result the phenomenon presented a major challenge to transdifferentiation. Lagasse et al., 2000 reported that intravenous injection of BM cells into mice, in which the gene for fumarylacetoacetate hydrolase (FAH) had been deleted, rescued the mouse by restoring the biochemical function of its liver. In this study a highly purified fraction of HSCs were used for transplantation and therefore the observation that HSCs regenerate the liver provided some of the best evidence for trans-differentiation. This study was particularly convincing because the

HSCs were purified and the donor type hepatocytes were functional and constituted up to 50% of the liver. However two recent studies revealed the observation to be the result of cell fusion and not an intrinsic capacity of HSCs to trans-differentiate into hepatocytes (Wang et al., 2003; Vassilopoulos et al., 2003). These studies demonstrate that donor type hepatocytes arise by cell fusion and not by differentiation of HSCs or their progeny into hepatocytes.

In summary, cell fusion may be the mechanism that underlies plasticity in injury models and in tissues that tolerate tetraploidy, such as muscle, hepatocytes and purkinje cells. Cell fusion may underlie plasticity where the frequency of trans-differentiation is extremely low but may be insufficient to explain those studies, which claim high levels of trans-differentiation events. Whilst cell fusion may not be the explanation for apparent plasticity in some cases it must be actively excluded, and some recent studies have been able to exclude this possibility.

1.1.8.4 Cells undergo dedifferentiation and re-differentiation

Cloning experiments including 'Dolly' the sheep made it clear that genetic information of a cell can be reprogrammed and somatic cells can dedifferentiate into pluripotent cells (Wilmut et al., 1997). Dedifferentiation is a well-established process in amphibians that can regenerate whole limbs (Verfaillie 2005). A number of studies have suggested that dedifferentiation may operate in mammalian somatic stem cells (Odelberg et al., 2000).

1.2 Mesenchymal stem cell biology

1.2.1 Definition of MSCs

MSCs are described as a self-renewing multipotent stem cell population, which generates progeny with the capacity to differentiate into multiple cell lineages of mesodermal origin (Caplan et al., 1994; Beresford et al., 1992; Friedenstein, 1992; Johnstone et al., 1998; Owen and Friedenstein, 1988). The principle source of adult mammalian MSCs is the postnatal BM stroma (Wexler et al., 2003; Friedenstein, 1990), where these cells are believed to contribute to the regeneration of non-hematopoietic BM tissues such as bone, cartilage, muscle, ligament, tendon, adipose

and the surrounding marrow stroma which functions to support hematopoiesis *in vivo* (Prockop et al., 1997).

1.2.2 Historical background of MSC biology

Circumstantial evidence for the existence of a stromal precursor cell within the BM tissue was first provided by studies demonstrating the regeneration of BM following the ablation of this tissue by various techniques (Knospe et al., 1972; Patt and Maloney, 1978). Such findings were consistent with studies in rodents demonstrating the development of a BM organ following transplantation to an ectopic site (Amsel and Dell, 1971; Tavassoli and Crosby, 1968). More definitive evidence of marrow stromal cell precursors was provided by the pioneering studies of Friedenstein and colleagues, who described the growth *in vitro* of adherent colonies of cells morphologically resembling fibroblasts from BM derived explants (Castro-Malaspina et al., 1981; Friedenstein et al., 1970; Owen, 1988). Friedenstein reported that these colonies formed from the foci of two-four cells, had high replicative potential *in vitro* and formed colonies, which were heterogeneous in appearance (Friedenstein et al., 1976). The authors demonstrated that the clonogenic stromal progenitor cells responsible for colony growth under these conditions, fibroblast colony forming cells (CFU-F) had the ability to differentiate into colonies that resembled small deposits of bone or cartilage (Ashton et al., 1980). Most importantly Friedenstein demonstrated that stromal cells derived from explanted CFU-F could be maintained in culture for 20-30 population doublings and still retain their capacity to differentiate into fibrous tissue, bone and some cartilage when implanted into the peritoneum of rats in a porous capsule (Friedenstein et al., 1987). Collectively these studies demonstrated that the BM contained a cell population that is capable of protracted self-maintenance and capable of differentiation into multiple cell lineages of mesodermal origin.

BM derived CFU-F are consistently heterogeneous in terms of their morphology, size, proliferation rate, expression levels of alkaline phosphatase and developmental potential (Friedenstein et al., 1982). Several groups have reported that stromal cell cultures grown from explanted CFU-F isolated by the relatively crude method of Friedenstein are capable of multilineage differentiation and readily differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes and myoblasts *in vitro* (Owen 1988;

Caplan 1994; Wakitani et al., 1995; Friedenstein et al., 1987; Friedenstein et al., 1976; Pereira et al., 1995; Pittenger et al., 1999; Sekiya et al., 2002). These observations are consistent with the hypothesis that within the marrow stromal tissue, a hierarchy of differentiation exists supported at its apex by a small compartment of self-renewing, multipotent stromal stem cells referred to principally as MSCs (Owen and Friedenstein, 1988).

1.2.3 Differentiation potential of MSCs

However, studies documenting the differentiation potential of stromal cell cultures derived from BM CFU-F have noted extensive functional heterogeneity in both clonal and non-clonal cell populations (Kuznetsov et al., 1997; Phinney et al., 1999). Mauragraglia et al., 2000 examined the differentiation potential of 185 non-immortalized human marrow stromal cell clonal populations derived from single cell derived colonies. Of these clones only half expressed osteo-chondrogenic differentiation potential and less than one third had trilineage potential that is the ability to differentiate into bone, cartilage and fat. In addition, the authors report the loss of multi-potentiality with increasing passage number although clones retained their osteogenic differentiation capacity suggested as the default pathway of differentiation. This finding was consistent with the previous observation that non-clonal populations of BM stromal cells progressively lost differentiation capacity with culture expansion (DiGirolamo et al., 1999). Collectively these findings highlight two important concepts when culturing MSCs. Firstly, stromal cell cultures obtained from BM derived CFU-F are a heterogeneous mix of cells with varying developmental potentials. Secondly, current protocols for *ex vivo* culture expansion of BM stromal cell cultures is not permissive to the long term self renewal of MSCs.

Most experiments examining the differentiation potential of MSCs have been reported using cells isolated by their adherence to tissue culture plastic. Several investigators have attempted to prepare more homogenous populations of cells based on the negative or positive selection of cells by cell surface markers. Pittenger et al., 1999 reported the isolation of an adherent homogenous population of MSCs (homogeneity defined by the presence of consistent set of surface marker proteins), which could be extensively expanded in culture and consistently differentiated into three principal

mesenchymal lineages (fat, cartilage and bone) under defined culture conditions *in vitro*. This tri-lineage differentiation capacity was also demonstrated in culture expanded cells from single cell derived colonies, providing definitive proof that cells with multilineage potential exist in the BM and not just committed progenitors.

1.2.4 Phenotypic characteristics of MSCs derived from marrow stromal cell cultures

Despite the considerable interest in the potential therapeutic applications of MSCs there is still no well-defined protocol for the prospective isolation of these cells prior to cell culture and therefore little is known about the biological properties of these cells *in vivo*. Current procedures for isolation of human or rodent CFU-F are still based on those described by Friedenstein and colleagues which relied upon the rapid adhesion of stromal progenitor cells to tissue culture plastic and their subsequent rapid proliferation in culture (Friedenstein et al., 1982). Such protocols select for the progeny of CFU-F and not directly for the clonogenic precursors themselves. As a result, little is known about the phenotype of the primary clonogenic precursor cells responsible for colony development and stromal cell growth *in vitro*. The majority of information regarding the phenotypic properties of stromal progenitor cells is based on the analysis of marrow stromal cells (i.e. MSCs) in culture. This adherent cell population is, as described above, a heterogeneous starting population of cells in which only a minor fraction represent multipotent stem cells (Gronthos and Simmons, 1995).

Although the precise characteristics of the various progenitor sub-fractions of MSCs in culture are unknown, stromal cell cultures do processes a number of general properties, which can be tested *in vitro*. Cultured MSCs have the capacity to synthesize an extracellular matrix which includes type I collagen, fibronectin, type IV collagen and laminin of basement membranes (Keating et al., 2006). In addition, MSCs in culture express a broad range of cell surface proteins responsible for hetero- and homotypic interactions among cell types and also serve as receptors for growth factors, cytokines, or extracellular matrices. These cell surface proteins are not specific and serve only to characterize MSCs as no specific antigen has been identified which associates the developmental potential of MSCs with a specific phenotypic trait.

Several laboratories have developed monoclonal antibodies, which react against human BM and stromal cells in culture. Antibody SH2 (Haynesworth et al., 1992); identifies an epitope on endoglin (CD105) (Barry et al., 1999), TGFβ receptor III present on endothelial cells, erythroblasts, monocytes and connective tissue stromal cells. SH3 and SH4 antibodies identify epitopes on CD73 a molecule involved in B cell activation and present on cultured stromal cells (Haynesworth et al., 1992). SB10 identifies activated leukocyte cell adhesion molecule, a ligand for CD6 (Bruder et al., 1997). All four of these antibodies are routinely used as characteristic markers of culture expanded MSCs, however they do not represent the heterogeneity in the developmental potential of these cells. SH2 is reported to facilitate the enrichment of stromal progenitors from BM (Chiefetz et al., 1992). One the first antibodies that was shown to enrich for CFU-F in fresh human BM aspirates is STRO-1 a monoclonal antibody which reacts with an unknown cell surface antigen expression by a minor fraction of adult human BM (Simmons et al., 1991). Studies have demonstrated that STRO-1 was unreactive with hematopoietic progenitors but the STRO-1+ cell fraction contained all detectable CFU-F (Simmons et al., 1991). Purging human BM of STRO-1+ cells resulted in a 10 to 20-fold enrichment of CFU-F relative to the incidence of CFU-F in un-fractionated BM. This STRO-1+ population exhibited multilineage differentiation potential *in vitro* when assayed at the clonal level (Simmons et al., 1991; Gronthos et al., 1994) and differentiated into bone tissue when transplanted into SCID mice (Gronthos et al., 2003). The authors conclude that the data strongly suggests that putative BM stromal stem cells with multilineage potential are restricted to the STRO-1+ fraction, however STRO-1 as an antigen to define MSCs is not universally accepted. As a result, it is impossible to determine the proportion of stem cells, multipotential clones, and determined progenitors in stromal cell cultures or the anatomical distribution of these cells within the BM and perhaps other tissues, a significant obstacle in the understanding of stromal progenitor cell function.

1.2.5 Functional definition of MSCs

In the absence of definitive cell surface marker proteins, the demonstration of stem cell functionality in terms of self renewal and multi-lineage differentiation potential

provides the functional definition of MSCs and the gold standard for conformation of their isolation within a culture (Pittenger et al., 1999). In addition, MSCs also have the capacity to produce cytokines and growth factors that support and regulate hematopoiesis within the BM microenvironment a property which when tested *in vitro* can be used as a defining characteristic (Dexter et al., 1977; Gartner et al., 1980; Quessenberry and Lowry 1992). The lack of definition of MSCs is in part due to a lack of suitable assays whereby the properties of MSCs such as self-renewal can be accurately measured. Evidence for the isolation of multipotent HSCs is provided by an *in vivo* assay in which the hematopoietic system is ablated by irradiation and the demonstration of the reconstitution of the system by single HSC transplantation or BM transplantation provides the evidence of multipotentiality of HSCs (Lagasse et al., 2001; Morrison and Weissman, 1994; Weissman, 2000; Ford et al., 1956; Nowell et al., 1956; Till and McCulloch 1961). Such an assay does not exist for MSCs and is probably not feasible since MSCs contribute to many skeletal and connective tissues not easily disrupted in such a definitive manor. As a result, demonstration of the differentiation potential of MSCs at the single cell level is currently reliant on the demonstration of putative cellular differentiation in a number of *in vitro* assays and differentiation into bone and cartilage *in vivo* (Pittenger et al., 1999; Gronthos et al., 2003; Friedenstein et al., 1976; Pereira et al., 1995; Sekiya et al., 2002). In stromal cell cultures demonstration of multi-lineage differentiation *in vitro* is a testament to the presence of multipotent MSC/progenitor cells but not definitive evidence for the presence of multipotent MSCs (Pittenger et al., 1999; Muraglia et al., 2000). However such assays demonstrate the developmental potential of the cultures tested and provides a functional assay for the presence of multipotent stem cells with mesodermal differentiation capacity.

1.2.6 Differentiation of MSCs into mesenchymal cell lineages in vitro

Conditions for differentiating cells *in vitro* are species dependent and are influenced by incompletely defined variables such as fetal calf serum batches (FCS) (Barnes and Sato, 1980; Lennon et al., 1995). Although the culture conditions for differentiation into every mesenchymal cell lineage has not been established for every species studied, mammalian MSCs have been shown to differentiate into bone, cartilage, fat, mytotubules and a mature stromal phenotype under defined culture conditions, as

demonstration of their multipotency (Prockop, 1997). MSCs do not differentiate spontaneously, a property, which allows them to expanded ex-vivo without lineage progression although they do lose their multipotent potential.

Osteogenic differentiation of mammalian BM stromal cells *in vitro* is reported to occur in response to various bioactive factors including osteogenin, BMP-2, osteogenic growth peptide, and the synthetic glucocorticoid dexamethasone (Dex) (Bruder et al., 1998a; Jaiswal et al., 1997; Friedenstein et al., 1987). The effects of Dex are considered particularly relevant in osteogenic culture systems as endogenous glucocorticoids are involved in bone formation during development. The osteogenic potential of human MSCs is well characterized both *in vivo* and *in vitro* (Friedenstein et al., 1987; Bruder et al., 1998; Jaiswal et al., 1997). When cultured in the presence of the synthetic Dex, ascorbic acid, and B-glycerolphosphate, hMSCs differentiate into the osteogenic lineage with the generation bone-like nodules with a mineralized extracellular matrix containing hydroxyapatite, accompanied by a significant increase in alkaline phosphatase activity and the expression of bone matrix proteins (Jaiswal et al., 1997). These phenotypic changes can be used as specific markers of lineage commitment. These osteogenic culture conditions have also been reported as optimal for rodent *in vitro* differentiation.

Chondrogenesis during development is a complex processes in which mesenchymal cells from the lateral plate mesoderm undergo a condensation step at the site destined for bone development (DeLise et al., 2000). The cartilaginous scaffold which forms defines the morphology of subsequent bone development. Induction of cartilage differentiation of MSCs *in vitro* is reliant on recapitulating many of the aspects of cartilage development *in vivo* (Sekiya et al., 2001). MSCs are maintained in a condensed culture system (pellet micromass) under which maturation is sustained to a hypertrophic phenotype. Chondrogenesis is thought to be regulated by a number of growth factors, hormones and bioactive factors some of which are critical to promote cartilage differentiation of MSCs in culture (Johnstone et al., 1998; Barry et al., 1999; MacKay et al., 1998; Yoo et al., 1998). Transforming growth factor Beta has been shown to have a chrondroinductive effect on rat mesenchymal cells *in vitro* and in other animal and human cell systems (Sekiya et al., 2001; Mackay et al., 1998;

Seyedin et al., 1986; Johnstone et al., 1996). Thyroxine is a hormone believed to be involved in hypertrophy and endochondral ossification (Seyedin et al., 1986). In addition, Dex has also been reported to exert important effects on cartilage differentiation *in vitro* (Johnstone et al., 1996). MSC derived chondrocytes produce an extracellular matrix (ECM) rich in type II collagen and aggrecan both of which are essential structural proteins for the normal function of cartilage (Mundlos et al., 1997. Detection of these proteins and the accumulation of safranin O-stained sulfated glycosaminoglycans act as lineage commitment markers (Kiraly et al., 1998).

Adipogenesis has been extensively studied *in vitro* using murine preadipocyte cell lines such as 3T3. When MSCs are cultured in defined medium with dexamethasone, isobutylmethylxanthine, and insulin most cells differentiate into adipocytes in a similar fashion to 3T3-L1 cells (Ailhaud et al., 1995). This differentiation is characterized by the accumulation of cytoplasmic lipid vesicles and the expression of genes that are expressed during adipogenesis such as peroxisome proliferator activated receptor y2 (PPARy2), lipoprotein lipase (LPL), and fatty acid binding protein (aP2) (Pittenger et al., 2000; Pittenger et al., 1999)

1.2.7 Clonal growth of MSCs

To demonstrate the growth and developmental potential of stem cells it is necessary to analyze properties at the single cell level through developing clonal populations expanded from single cell derived clones. The clonal characteristics of MSCs have been determined by analyzing the intrinsic growth and developmental potential of explanted CFU-F *in vitro* (Muraglia et al., 2000). However, explanted marrow stromal cells establish colonies slowly (several days) following attachment to tissue culture plastic (Friedenstein 1976). Several reasons have been postulated to explain this observation including: the cells been quiescent upon initiation of culture, or needing to adjust to the *in vitro* culture conditions. A negative cell cycle regulator may be present in dormant MSCs *in situ* that must undergo turnover before rounds of mitosis can begin in the explanted cells. In addition, BM stroma may contain small groups of cells which are initially dormant, but which begin rapid proliferation. Clonal isolation studies require cells to be plated at low cell densities. Van den Bos et al., 1997 reported that single hMSCs might undergo apoptosis prior to colony formation. This

observation suggests that cell contact and/or release of paracrine or autocrine factors by hMSCs, or elements of the stromal environment *in situ*, may provide survival signals sufficient to support the growth of MSCs. In addition, the marrow stromal environment is a complex environment with several cell types and a complex stroma or extracellular matrixes. Some authors have reported the isolation of more primitive cells / stem cells from the BM stroma by explanting these cells on substrates such as fibronectin, an extracellular matrix component formed by MSCs themselves (Jiang et al., 2002a; D'Ippolito et al., 2004).

Despite the difficulties in clonal isolation, many investigators have been successful. Colony formation has been extensively studied in the guinea pig and rabbit (Friedenstein et al., 1970; Friedenstein 1976; Ashton et al., 1980; Owen et al., 1987; Owen and Friedenstein 1988). Clonal analysis of rabbit MSCs indicated that epidermal growth factor (EGF) increases colony size and reduces spontaneous differentiation (Owen et al., 1987). In serum free conditions, human MSCs required dexemethasone and L-ascorbate for colony formation and their growth was most responsive to a combination of platelet derived growth factor (PDGF) and EGF (Gronthos and Simmons, 1995). Robey and colleagues isolated 34 individual clones from BM derived cells and evaluated their potential to differentiate into an osteogenic phenotype following their seeding into porous ceramics carriers which were implanted *in vivo* (Kuznetsov et al., 1997). Only 20 of the clones used in the studies produced bone following 8 weeks of implantation. This data suggests that not all CFU-F from bone marrow has an osteogenic potential. Pittenger et al., 1999 tested clonal populations of homogenous MSC preparations (homogeneity defined by consistent expression of over 50 surface markers) from human BM. Of the 6 clones analyzed, 3 differentiated into bone, fat and cartilage under defined culture conditions whereas 2 formed only bone and fat and 1 only bone.

The traditional model for MSC differentiation proposes that lineage progenitors are directly derived from MSCs. If this model were true, a random combination of phenotypes would be expected upon appropriate stimulation of clonal MSCs. However evidence presented by Muraglia et al., 2000 suggests a deterministic model of MSC differentiation. As described previously these authors demonstrated multiple

differentiation at the clonal level of human MSCs and that clones with the potential to generate cartilage, fat and bone occur at high frequency in the BM. However, following prolonged periods in culture clones enter senescence and lose differentiation potential with multi-lineage potential giving rise to bi-potential and uni-potential clones. They also report the existence of fixed combinations of lineages (i.e. osteo-adipo-chondro, osteo-chondro and osteogenic clones). Based on these findings they propose a model of differentiation in which cells proceed through a spontaneous process leading to osteogenic differentiation and progressively lose their multipotentiality. A hierarchy is evident in their differentiation in which the adipogenic lineage branches earlier than the osteogenic and chondrogenic lineages, which proceed together until possibly branching later. These features are consistent with a deterministic model of MSC differentiation.

1.2.8 Global gene analysis of MSCs

Efforts at defining the phenotypic characteristics of MSCs have been compounded by the fact that these cells display a variety of morphologies and express various cell lineage antigens that can vary between different preparations and as a function of time in culture, leading to different conclusions about the biological nature of these cells. More recent efforts to define MSCs and characterize their phenotypic properties have relied on analyzing the expression of different gene families or classes of biological molecules. Human MSCs cultured under conditions that inhibit differentiation were shown to express numerous cytokines including interleukin 6 (IL-6), IL-11, G-CSF, macrophage colony-stimulating factor (M-CSF), stem cell factor, and leukemia inhibitory factor (LIF) (Haynesworth et al., 1996). Other investigators have reported the expression of a complement of expressed receptor tyrosine kinases and insulin-like growth factor binding proteins in both clonal and non-clonal populations of MSCs (Grellier et al., 1995; Satomura et al., 1998). Pittenger et al., 1999 analyzed the expression of 50 genes including cytokine and mitogen receptors, matrix molecules and integrins in a homogenous population of human MSCs.

The completion of the human genome, together with technologies to simultaneously measure the expression of thousands of genes has facilitated a global approach to complex biological questions. This systems biology approach has been utilized by

many stem cell biologists to begin to characterize the molecular signatures of stem cell populations. Such investigations will allow us to elucidate the genes involved in essential properties such as self-renewal and various programs of differentiation. In addition, such studies may offer unique signatures in which to identity and define stem cells. In this context a small number of studies have aimed to determine the microanatomical identity of MSCs by global gene expression arrays. Three papers have principally addressed the question of defining the molecular signature of MSCs by global gene expression analysis, each by alternate methods. Tremain et al., 2001 carried out a serial analysis of gene expression (SAGE), which provides the means to analyze the entire complement of expressed transcripts in a cell. A catalog of 2,353 expressed transcripts was analyzed in a single cell derived colony of human stromal cells elaborated from low-density cultures. Analysis of the transcript profile of a single cell-derived colony of human MSCs cultured under conditions that prevent differentiation, revealed that a single MSC colony expresses mRNAs of multiple cell lineages including mesenchymal cell lineages, chondrocytes, myoblasts, osteoblasts, and hematopoietic supporting stroma as well as various transcripts characteristic of endothelial, epithelial and neural cells. The authors note that it is surprising to find that these cells simultaneously expressed mRNAs characteristic of various committed mesenchymal cell lineages. They suggest that one explanation may be that cells within the colony individually enter into distinct differentiation programs leading ultimately to the generation of a molecularly heterogeneous cell population. Interestingly if the expression does indeed constitute differentiation then such events have occurred in the absence of external cues suggesting that fate determination in MSCs may be governed by intrinsic mechanisms as described for other stem cell populations. In addition, to mesenchymal lineage specific transcripts, MSCs also expressed mRNAs common to non-mesodermal tissues including neural cells. This expression is consistent with reports of enhanced MSC plasticity (as discussed in later sections) and suggests MSCs at least have the genetic potential for a broader differentiation potential however it is unclear from these studies whether expression of these transcripts reflects entry of MSCs into a developmental program that specifies neurogenic cell fates. In contrast to previous reports many cytokine receptors, integrins, and matrix molecules reportedly expressed in stromal cell cultures passed at high density were not expressed in the single cell derived colony.

The authors suggest that the explanation for these differences is the method of culture and that a single cell derived colony from low-density cultures is comprised of distinct subsets of cells.

DNA micro-arrays allow large-scale gene expression analyses. Wieczorek et al., 2003 analyzed 3 independent samples of MSCs by cDNA micro-arrays. Their analysis revealed a high concordance with previous expression studies and analysis revealed the expression profile to be closely related to vascular pericytes indicating that MSCs resemble pericytes. Seshi et al., 2003; consistent with previous studies also found using single cell micro-array analysis that MSCs simultaneously express transcripts associated with osteoblast, fibroblast, muscle, and adipocyte differentiation. These cells also expressed transcripts associated with all three germinal layers. Collectively these expression studies have been interpreted by some as indicating that MSCs represent a multi-differentiated cell type and this property has been evoked to explain the observations of plasticity in these stem cells.

1.2.9 Other BM derived stem cell populations

The developmental potential of CFU-F in culture is consistent with the hypothesized existence within the BM of a hierarchy of differentiation supported at its apex by a small compartment of self-renewing, multipotent MSCs (Owen and Freiedenstein, 1988). The lack of defining characteristics of MSCs and the different methodologies used to cultivate and characterize MSC-related cell types means there is a lack of consensus on the hierarchy intrinsic to the MSC compartment. This is reflected by the reported findings of similar cells including multipotent adult progenitor cells (MAPC) (Reyes and Verfaillie, 2001) and marrow isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al., 2004). These cell populations have been described under highly defined culture conditions. Jiang et al., 2002 demonstrated that MAPCs, copurified with MSCs from BM could, at the single cell level, differentiate *in vitro* into cells with characteristics of mesodermal, ectodermal and endodermal cell lineages. MAPCs also contribute to most if not all cells when injected into the blastocyst. MAPCs have been isolated from muscle, brain and BM and all express pluripotent stem cell markers Rex-1 and Oct-4, markers that are usually confined to germ cells in the adult (Jiang et al., 2002). This expression and differentiation

potential is more consistent with an ES cell phenotype. It is not known what relationship theses cells have to MSCs. They may be a more primitive cell type than MSCs and in fact MSCs may derive from these cells. In addition, in culture these cells may represent an MSC-like de-differentiated phenotype. D'lppolito et al., 2004 also isolated a cell population from adult BM under low oxygen tension and defined culture conditions. This cell population displayed pluripotent differentiation potential *in vitro* and expressed Oct-4 and Rex-1 however, the developmental potential of these cells following transplantation or *in vitro* is unknown. In summary, there may be numerous adult stem cell populations in BM and their relationship to MSCs is currently unknown.

1.2.10 Ontogeny and anatomical location of MSCs

As discussed previously, due to the lack of specific defining antigen for MSCs, which associates their developmental potential with a specific phenotypic trait, it is impossible as yet to determine the exact anatomical location of these cells *in vivo*. The lack of precise knowledge regarding the anatomical distribution of these cells in the BM is a major obstacle to their study. [3H]-thymidine labeling studies in rodents have demonstrated that CFU-F *in vivo* is essentially in a non-cycling state (Friedenstein et al., 1974). Despite the fact that the exact nature and localization of MSCs *in vivo* remains poorly understood, a large body of experimental evidence indicates a relationship with pericytes (vascular smooth muscle cells) (Doherty et al., 1998; Farrington-Rock et al., 2004). One approach to the study of MSCs *in vivo* has been the use of specific markers known to be expressed by MSCs *in vitro* in order to locate positive cells *in vivo*. Gronthos et al., 2003 reported that STRO-1bright VCAM-1+ cells isolated from fresh human BM were a morphologically homogenous population of cells which expressed collagen type-1, but which lacked phenotypic characteristics of leukocytes or vascular endothelial cells. Interestingly, over 70% of the cells expressed α-SMA and 50% of these cells were capable of clonogenic growth *in vitro*. This suggests that a significant proportion of CFU-F must express α-SMA. In the adult, under steady state conditions the expression of α-SMA in human BM has a restricted distribution and is limited to vascular smooth muscle cells in the media of arteries, pericytes lining capillaries, and occasional flattened cells on the endosteal surface of bone (Bianco et al., 2001). No expression is found in other marrow stromal

elements such as reticular cells within hematopoietic cords, adipocytes or vascular endothelial cells. It is important to note however that α-SMA+ cells have been reported that are not associated with vasculature for example, in the fetal BM (Schmitt-Graff et al., 1989). These observations have been interpreted as suggesting two possible identities and locations for MSCs *in vivo*, endosteal cells or vascular smooth muscle cells (pericytes). The expression of bone sialoprotein and osteonectin that was reported in freshly isolated STRO-1 bright VCAM-1+ cells is consistent with a bone cell phenotype but inconsistent with a pericyte origin (Gronthos et al., 2003). As yet, there has been no direct demonstration of the histological expression of STRO-1 expression on pericytes and/or endosteal cells in BM and therefore this cannot be confirmed. Recent evidence has reported that the STRO-1 antibody does bind to blood vessel walls in frozen sections of human BM (Shi et al., 2003). The precise nature of the cells binding STRO-1 is unknown.

Several observations support the interpretation that MSCs are indeed vascular pericytes. Previous studies have demonstrated co-expression of STRO-1 and α-SMA in a proportion of human BM cells *in vitro* (Simmons et al., 1991). Studies using the STRO-1 antibody have provided useful information, however, this approach is sensitive but may be non-specific, as the cell marker is only specific in a given context and STRO-1 is not universally accepted as a marker of MSCs. A number of other observations are consistent with a pericyte origin. Cultured MSCs also express h-caldesmon, metavinculin, calponin, and smooth muscle myosin heavy chains in addition to the expression of α-SMA (Galmiche et al., 1993). This expression profile is consistent with a vascular pericyte phenotype. There are also a number of similarities in the properties of the two cells (vascular pericytes and MSCs). Cultured pericytes and MSCs synthesize a number of extra cellular matrix proteins comprising a mixture of basal laminins and interstitial collagens (Zuckerman et al., 1983). In addition, MSCs undergo clonal expansion when grown at low density in the presence of PDGF-BB under serum free conditions (Gronthos and Simmons, 1995). Data from mouse knockouts indicates PDGF-BB acts to recruit pericytes and maintain their viability *in vivo* (Hellstrom et al., 1999). Therefore MSCs and vascular pericytes have similar responses to PDGF. Pericytes participate in the maintenance of blood vessel homeostasis and recent evidence suggests that these pericytes may also be capable of

mesodermal proliferation and clonal growth in culture similar to CFU-F (Sims, 1991; Diaz-Flores et al., 1991; Doherty et al., 1998). Pericytes isolated from bovine retinal capillaries are STRO-1+ and exhibit the potential for differentiation into a variety of cell types including osteoblasts, adipocytes, chondrocytes and fibroblasts (Doherty et al., 1998; Brighton et al., 1992).

An alternative approach to these investigations has been to infuse marked cultured cells *in vivo* and to subsequently analyze their tissue distribution. Anjos-Afonso et al., 2004 purified MSCs from mouse BM and transduced these cells using a lentivirus vector expressing the eGFP reporter gene. These transduced cells retained their ability to differentiate *in vitro* and were injected systemically into minimally injured syngeneic mice. The highly enriched mouse MSC population incorporated into several tissues after systemic infusion into recipient animals that only received sub lethal irradiation. Donor derived cells engrafted in numerous organs and acquired the morphological and antigen profiles of hepatocytes, lung epithelial cells, myofibroblasts, myofibres and renal tubular cells in the recipient mice. Whilst this study provided an important observation it is not an accurate analysis of the natural distribution of MSCs *in vivo* because the cells might engraft non-specifically in different locations.

A more accurate approach is the prospective isolation of cells with MSC characteristics from different organs and tissues and subsequent analysis of their phenotypic characteristics. MSCs have been isolated from a number of postnatal organs and tissues including adipose tissue (Zuk et al., 2001), tendon (Salingcarnboriboon et al., 2003), periodontal ligament (Seo et al., 2004), synovial membrane (De Bari et al., 2003) and lung (Sabatini et al., 2005). A recent report has been published in which the authors systematically isolated MSCs from a number of organs and tissues in the adult mouse and evaluated their characteristics *in vitro* including immunophenotype and ability to differentiate towards an adipogenic and osteogenic cell fate (da Silva Meirelles et al., 2006). The investigators established long-term cultures from brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus and pancreas. Variations in immunophenotype and differentiation potential were reported and were related to the site of origin suggesting that this may be a

functional role and organ specific. This study demonstrated that MSC cultures could be established from organs and tissues regardless of embryonic origin. The cells were operationally defined as MSCs on the basis that they could differentiate into mesenchymal cell lineages *in vitro* and were capable of prolonged self-renewal. However, despite a relatively consistent surface marker profile and morphological appearance, variation was reported in the frequency of cells, which actually differentiated into fat or bone and the degree of differentiation. To test the hypothesis that MSCs could be derived from the vasculature long-term cultures were derived from large blood vessels such as the aorta and vena cava as well as capillaries such as the kidney glomeruli. MSCs were not detected in peripheral blood ruling out the possibility that they were derived from circulating blood. These observations collectively are consistent with the distribution of MSCs throughout the postnatal organism been related to their existence in the perivascular niche and provide evidence that the MSC compartment is more widely distributed than previously appreciated.

Da Silva Meirelles et al., 2006 suggest a working hypothesis on the basis of their findings in the context of the literature, which is important to consider. In this model MSCs act as a functional reservoir of undifferentiated cells which supply the cellular demands of the tissue in which they reside. In doing so they acquire the phenotypic characteristics of the local tissue and give rise to committed progenitors that gradually integrate into the tissue. The model suggests that a proportion of postnatal stem cell diversity may be attributed to local perivascular MSCs behaving as tissue specific stem cells. However, the contribution these cells make to regeneration following injury and normal cell turnover in these organs is currently unknown.

1.2.11 Therapeutic applications

Within the past decade, MSCs have been recognized as potential candidates for cell and gene therapy. Unlike many other adult stem cells, MSCs are contained within a clinically accessible site and allow relatively straightforward isolation. Furthermore, MSCs can be readily expanded *ex vivo* to generate clinically relevant numbers *in vitro*, whereas this is currently technically challenging for other stem cell types,

including human ES cells. These features make human MSCs attractive for use in the clinic, especially for autologous therapies.

The practical significance of MSC differentiation was demonstrated in animal models and a smaller number of human clinical studies. Bruder and colleagues (1998) demonstrated that MSCs grown on an appropriate scaffold had the capacity to repair a 8mm defect in the femur of a rat. After 8 weeks the bone defect had completely healed compared to bone implanted with control cells. Other studies on the repair of segmental bone defects have been demonstrated in canines where implantation of allogenic MSCs resulted in enhanced bone formation (Bruder et al., 1998; Rombouts and Ploemacher 2003). In addition, similar reports of the repair of focal defects have been reported in articular cartilage and tendon (Krebsbach et al., 1998; Young et al., 1995). In an animal model of osteoarthritis involving damage to the meniscus tissue, administration of MSCs by intra-articular injection resulted in engraftment and repair of the meniscus tissue (Murphy et al., 2003). Studies by Horwitz et al. (1999) demonstrated that genetically labeled MSCs showed long-term engraftment in patients with osteogenesis imperfecta with beneficial effects such as increased skeletal strength.

For cell replacement to be effective in the allogenic setting it is also important to consider the host immune response to implanted cells. MSCs and their differentiated derivatives do not express HLA-Class II antigens *in vitro* and possess only low-level expression of co-stimulatory molecules (Majumdar et al., 2003). In addition, these cells were found not to elicit an allo-reactive lymphocyte proliferative response in culture (Tse et al., 2001). This finding suggested that human MSCs may be non-immunogenic which has profound implications for their use in allogenic cell therapy and infusion of cells derived from a mismatched donor into a recipient. Engraftment of allogenic MSCs has been reported in patients with osteogenesis imperfecta, in patients with Hurlers syndrome or metachromatic leukodystrophy and idiopathic aplastic anemia without any evidence of graft verses host disease or immune rejection but with significant improvement in the clinical outcome (Horwitz et al., 1999; Quarto et al., 2001; Koc et al., 2002; Fouillard et al., 2003). In addition to the absence of class II HLA antigens, MSCs can also suppress primary mixed lymphocyte reactions

involving autologous or allogenic T cells or dendritic cells (Di Nicola et al., 2002). This suggests that MSCs may also be useful in suppressing ongoing immune cell induced reactions, including treatment of graft verses host disease.

Administration of cells may be direct to the site of damage or injury or in other cases systemic administration may both be necessary and appropriate. MSCs have the capacity to engraft into various tissues and organs when infused systemically and this engraftment has been shown to be stable in the long-term (Liechty et al., 2000). Even more impressively, MSCs administered to the peripheral circulation have the capacity to migrate to a specific site of injury. This has been reported in animal models of bone fracture, cerebral ischemia and myocardial infarction (Shake et al., 2002; Wang et al., 2002). It has also been demonstrated that this homing of cells to a site of injury can also result in actual MSC mediated functional repair. MSCs administered by intra-articular injection into the knee joint following injury are capable of specific migration, engraftment and repair of the damaged meniscus and cartilage (Agung et al., 2006). Rombouts and Ploemacher (2003) reported the expansion and migration of MSCs to a wound environment by demonstrating that in an irradiated mouse host there was migration and expansion of donor GFP expressing MSCs within the spleen and bone marrow in contrast to non-irradiated hosts. However, the homing of the donor cells decreased with long-term ex-vivo expansion that obviously has relevance when considering the preparation of clinical grade MSCs.

There is also evidence that MSCs contribute to the repair of tissues other than those that they are generally accepted to form. For example, Orlic et al. (2001a; 2001b) reported that the local administration of MSCs to the heart could generate *de novo* myocardium suggesting that these cells may be useful in treating myocardial heart disease. Furthermore, infusion of BM cells into the infarct zone of patients following myocardial infarction appeared to have a beneficial effect on global heart function (Stamm et al. 2003). As mentioned above, MSCs have also been reported to differentiate into neural tissues. Subsequent to transplantation, MSCs have been shown to enter the brain and generate neurons (Mezey et al. 2003) whereas Chen et al., 2001 demonstrated that systemic infusion of MSCs aids recovery in animal

models of stroke. These findings collectively suggest that MSCs may be useful in the repair of the CNS following trauma or neurodegenerative disease.

1.2.12 Concluding remarks

The data presented in this literature review provides an overview of MSC biology. As described many questions remain, there are still uncertainties as to the origin and nature of these cells, a problem compounded the lack of a defining antigen. This lack of consensus on the nature of MSCs and their anatomical location *in vivo* is one major obstacle to the study of these cells, since experimental comparison is difficult with different laboratories using different methodologies to isolate MSCs. However, MSCs do represent a source of stem cells that are readily available from the adult. Evidence shows that these cells can be expanded in culture and that they retain their ability to differentiate both *in vitro* and when transplanted into the appropriate site *in vivo* (Pittenger et al., 1999; Gronthos et al., 20003). MSCs may be used routinely in the clinic but standardized procedures for their prospective isolation from tissues must be developed to further understanding of their biology.

1.3 Proposed neural differentiation of MSCs

1.3.1 How to define a neuronal cell in vitro

Many different criteria have been used to define and characterize neuronal cell populations *in vitro* including cell morphology, expression of neuronal specific markers and functional assays. Neurons display considerable heterogeneity in morphology and this asymmetry is a characteristic hallmark of these cells, a neuron possesses a cell body from which extends a single axon (Sevendsen et al., 2001). Also extending from the cell body are numerous dendrites that form synapses and communicate with other cells. Cell type specific markers can be used to assign phenotype to neural cells however some markers are expressed in other tissues. Nestin an intermediate filament protein is highly expressed in NSCs (Lendahl, 1990) and is used to identify these cells in culture but its expression has been found in other tissues (Kornblum et al., 2001). NSCs also express vimentin and the RNA binding protein Musashi that is highly expressed in precursor cells capable of generating both neurons and glia during embryonic and postnatal development (Kaneko et al., 2000). Tuj-1 is

one of the earliest markers expressed on newly formed neurons (Song et al., 2002). In more mature neurons the axonal filament proteins (neurofilaments), NF-L, NF-M and NF-H are expressed along with microtubule-associated proteins in the axons (TAU) and dendrites (MAP-2) (Sevdsen et al., 2001). MAP-2c is expressed neurons and neuro-progenitors that can generate neurons and glia. MAP2ab is specific for more mature neurons (Rosser et al., 1997). Functional criteria include the ability to fire action potentials and communicate with other cells through synapses. Collectively Reh et al 2002 suggested that that neuronal cells should be post mitotic, polarized with a single axon and multiple dendrites and able to fire action potentials and communicate with other neurons through synapses, requiring both neurotransmitter release and neurotransmitter receptors.

1.3.2 The possibility of adult stem cell plasticity

Investigations using animal and human BM transplants suggest that BM can differentiate into neurons and glia in the adult brain (Krause 2002). Using either genetic (Y chromosome) or reporter genes (GFP or LacZ) to track donor cells investigators claimed that BM derived stem cells contributed to the regeneration of several non-hematopoietic tissues. Three criteria are required to demonstrate somatic stem cell plasticity (Andersen et al., 2001), it is first necessary to identify the differentiated cells as being donor derived; secondly, to show that the cell is phenotypically similar to resident cells and thirdly, to prove functionality. Donor cells can be identified and distinguished from those of the recipient by using male cells transplanted into female recipients or transgenic donor cells into wild-type recipients. Retroviral marking can also be used to mark cells. Phenotypic markers of differentiation are usually examined by immuno-cytochemistry for organ specific proteins or *in situ* hybridization for cell type specific RNA expression. Functionality of donor-derived cells has proved a more problematic criterion to fulfill. Studies that have most successfully demonstrated functionality of donor-derived cells have used animals that are mutant for normal cell function in the target organs so that transplanted cells restore function to the recipients (For review see Corti et al., 2002).

However, many of the claims of BM derived stem cell plasticity have failed to be reproduced and the possibility that these trans-differentiation events could be

attributed to cell fusion has been raised (Ying et al., 2002; Terada et al., 2002). Cell fusion was raised as an issue by two papers, which demonstrated that adult stem cells could fuse spontaneously *in vitro* with ES cells and take on their characteristics (Ying et al., 2002; Terada et al., 2002). Furthermore, more recent studies have demonstrated that cell fusion can occur *in vivo* and in particular BM derived stem cells can fuse with purkinje neurons assuming their characteristics (Alvarez-Dolado et al., 2003). These heterokaryons are stable and the purkinje cell, which becomes dominant, activates the purkinje cell specific transgene from the donor cell nucleus. As previously described, the use of the Cre/lox recombination system demonstrated that BM derived cells fuse spontaneously with hepatocytes in the liver, purkinje neurons in the brain and cardiac muscle in the heart, resulting in the formation of multinucleated cells. They found no evidence of trans-differentiation without cell fusion.

MSCs isolated from the postnatal BM have been reported to adopt neural phenotypes *in vitro* and *in vivo*. Kopen et al., 1999 first reported that mouse MSC were able to adopt an astrocytic cell fate following injection into the lateral ventricle of neonatal mice. The authors reported the presence of BrdU labelled donor MSCs expressing the astrocytic marker GFAP within the striatum and hippocampus of the host CNS. The donor cells were therefore shown to migrate throughout the forebrain and cerebellum of the host CNS. Donor derived cells expressing neuronal and glial cell markers where detected in the adult mouse brain following intravascular administration of whole adult mouse BM into lethally irradiated hosts (Brazelton et al., 2000).

Studies in Drosophila and mammalian skin, intestine, BM and brain reveal that these inherent stem cell features of self renewal and differentiation are tightly regulated by the cells and proteins which constitute the extra cellular environment in which the stem cells reside (the stem cell niche) (Fuchs et al., 2004). The premise of trans-differentiation is based on the idea that the developmental potential of stem cells is dictated by the environment in which these cells reside. Removal of stem cells from their normal environment and either *ex vivo* culture or transplantation into an ectopic site can allow stem cells with a broader differentiation potential to adopt cell fates outside their normal organ of reside. Following the finding that adult BM derived

cells, when transplanted systemically migrate to the CNS and acquire the characteristics of astrocyte grafts (Eglitis and Mezey, 1997), a large number of experiments have been published which describe the ability of MSCs isolated from the adult BM to differentiate into neuroectodermal derivatives including neuronal (e.g. Woodbury et al., 2000), glial (e.g. Wislet-Gendebein et al., 2003) and schwann cells (e.g. Caddick et al., 2006).

1.3.3 Neuro-ectodermal differentiation of MSCs in vivo

The first evidence that adult stem cells from the BM may generate neuroectodermal CNS cell types came from studies of whole BM transplantation. Eglitis and Mezey, 1997 reported the generation of glial cells (cells expressing GFAP) from BM in the brains of immunodeficient adult mice that received intravenous (IV) transplantation of genetically marked whole BM. Eglitis and Mezey set out to investigate the relationship between microglia and BM. Microglia are believed to derive from a hematopoietic cell line, whereas astrocytes and oligodendrocytes are believed to be derived from embryonic neuroectoderm and developmentally distinct from microglia. Some microglia are thought to have a neuroectodermal origin and Mezey and Eglitis sought to determine the extent to which cells outside the CNS could contribute to the maintenance of microglia in adult mice. BM cells were labelled with a retroviral vector carrying the gene for neomycin resistance or male donor marrow cells were used followed by *in situ* hybridization with a probe specific for the Y chromosome, for detection of male cells within the female recipient. Retroviral labelled male cells were infused into the systemic vasculature (tail vein) of sublethally irradiated female mice. Adult BM cells migrated to the CNS and engrafted within the brain parenchyma. Cells were detected throughout all regions of the brain and the level of engraftment paralleled the vascularity of a given region. Some donor-derived cells expressed the microglial antigenic marker F4/80 antigen whilst others expressed the astroglial antigenic marker glial fibrillary acidic protein (GFAP). Approximately 10% of engrafted cells acquired microglia antigenic markers (GFAP or F4/80 antigen). The identity of the remaining cells is unknown. The authors concluded that some astroglia and microglia could arise from a precursor in the BM. They also considered this effect to be a normal process because the number of donor derived cells detected in the brain

increased over time and did not appear to be a consequence of the transplantation procedure.

In addition, to astrocytic differentiation under normal physiological conditions Eglitis et al., 1999 demonstrated that BM derived cells could generate GFAP-positive cells in an ischemic lesion model. Using the male BM to donate to female recipients followed by detection of the Y-chromosome by *in situ* hybridization to track donor cells, they demonstrated a preferential homing of donor BM cells to the site of hypoxic/ischemic lesion within the CNS. Following acute middle cerebral artery occlusion to model stroke, donor derived cells were detected around the focal lesion. 2.8% of the total cells within the ischemic hemisphere were donor derived in contrast to 1.8% of cells in the intact, unlesioned side. Therefore the ischemic lesioned side of the brain attracted 55% more donor derived cells. In addition, the percentage of total GFAP+ cells that were double labelled (Y-chromosome+/GFAP+) was 161% greater on the lesioned side of the brain. This data suggests not only that BM derived stem cells may have an intrinsic capacity for trans-differentiation into astrocyte cell fates but that this process may contribute to repair following injury.

Two papers subsequently published in 2000 presented the first evidence of neuronal conversion *in vivo*. These studies relied on the replacement of BM with donor BM cells expressing a GFP transgene or sex mismatched. These studies collectively demonstrated that adult BM derived stem cells could migrate from the systemic vasculature, engraft and trans-differentiate into neurons as well as glial cells. Brazelton et al., 2000 demonstrated that BM derived cells migrated into the brain and differentiated into cells expressing neuronal antigens NeuN, Tuj-1 and NF-200, following intravascular transplantation into irradiated normal adult mouse hosts. Adult BM was harvested from transgenic mice expressing enhanced green fluorescent protein (GFP) and administered to recipients by tail vein infusion. Donor cells were subsequently detected months after transplantation. Donor cells were detected within the hippocampus, cortical areas, olfactory bulb and cerebellum. 20% of GFP+ cells that engrafted within the CNS were negative for the hematopoietic markers CD45 and CD11b suggesting that these cells responded to the brain microenvironment by differentiating into novel cell phenotypes. Only 0.2-0.3% of the total neurons within

the olfactory bulb were of donor origin by 8-12 weeks after transplantation. It is argued that the low conversion may be the result of experimentation on control animals and the number may be higher in lesioned animals. Neurons of donor origin displayed only an immature neuronal phenotype with minimal axonal branching. A proportion of the cells expressed multiple neuron specific genes including NF-200 and Tuj-1. No GFAP positive donor derived cells were detected suggesting only neuronal differentiation and not astrocytic as previously described.

Mezey et al., 2000 transplanted mice, homozygous for a mutation in the PU.1 gene with whole BM from normal adult hosts. PU.1 is a member of the ETS (DNA binding domain) family of transcription factors expressed exclusively by the hematopoietic system. Mice homozygous for this mutation are incapable of generating cells of the myeloid and lymphoid lineages and therefore these animals can be transplanted without irradiation. Within 24 hours after birth, PU.1 homozygous recipients were given systemic infusions of BM from wild type male mice. 1-4 months after transplantation brains were harvested and analysed for the presence of donor-derived cells. 2.3-4.6% of donor derived cells were present in the host brain and 0.2-0.3% co-expressed NeuN and Y-chromosome. Donor derived cells were detected throughout the brain in both cortical and sub-cortical brain areas and in the cerebellum. Unlike previous transplantation studies no GFAP positive cells were detected.

Nakano et al., 2001 transplanted GFP-marked BM into lethally irradiated mice by either systemic infusion or direct injection into the striatum. Twenty-four weeks after intravenous transplantation only donor-derived microglia were detected. However, 12 weeks following direct injection into the corpus striatum of irradiated mice, cells co-expressing GFP and astrocytic and oligodendrocytic markers were detected. Therefore BM derived cells were shown to differentiate into three distinct glial cell phenotypes (oligodendrocytes, astrocytes and microglia). Priller et al., 2001a reported that only a minor proportion of BM derived cells expressing the neuronal marker NeuN were detected 4 months after BM transplantation using GFP-marked cells. However, fully differentiated BM-derived Purkinje neurons positive for calbindin and glutamic acid decarboxylase were found in the cerebellum 12-15 months after transplantation. BM-

derived microglia were detected in three animal models of CNS lesions (Priller et al., 2001b).

Whole BM contains several cell types including: HSCs, MSCs and their differentiated cell derivatives such as hematopoietic cells, osteoblasts, endothelial cells and fibroblasts. Studies using whole BM were unable to demonstrate which stem cell population was responsible for observations of neuroectodermal trans-differentiation. Infusion of MSCs isolated from adult BM directly into the rodent brain has been shown to result in migration of cells from the site of administration and engraftment into the host brain (Azizi et al., 1998 and Kopen et al., 1999). Systemic infusion of MSCs into irradiated 3-week old mice was followed by the appearance of progeny of the donor cells in a variety of non-hematopoietic tissues including the brain (Pereria et al., 1998). MSCs isolated in culture by selective adhesion to tissue culture plastic and then labelled with bisbenzamide were directly injected into the corpus striatum of the rat. 5-72 days later approximately 20% of infused cells had engrafted in the host brain (Azizi et al., 1998). The cells had migrated from the injection site to the corpus collosum, contralateral cerebral cortex and ipsilateral temporal lobe. After engraftment cells lost markers characteristic of a mesodermal derivatives such as immunoreactivity to fibronectin and collagen-1 by 30 days and acquired characteristics of astrocytes. In contrast, implanted fibroblasts continue to synthesize fibronectin and collagen-1. Whilst this study demonstrated that MSCs isolated from the adult BM could migrate in a manor similar to paraventricular astrocytes, an effect not replicated by fibroblast implantation, the ability of the cells to adopt neural cell fates was not determined.

Kopen et al., 1999 grafted purified MSCs directly into the lateral ventricle of neonatal mice. In these experiments the stromal cell fraction was depleted of CD11b+ cells (a marker of myelopoietic cells) and cells were labeled with bisnenzamide or bromodeoxyuridine (BrdU) prior to grafting in order to track cells. Transplanted cells were found to migrate throughout the forebrain and cerebellum without disrupting the host brain. MSCs displayed a characteristic migration pattern consistent with that of neural progenitor cells during development in early postnatal life. MSCs were found to line white matter tracts and the ependyma throughout the ventricles. BrdU labelled

cells expressing GFAP were detected in the corpus striatum, the molecular layer of the hippocampus and the cerebellum, suggesting that engrafted donor cells in these regions had differentiated into astrocytes. Donor MSCs were also detected in postnatal neurogenic regions including the olfactory bulb and ventral forebrain. In the cerebellum cells were found in the internal and external granular layers but not in the purkinje cells. Occasional sections of the brain stem contained BrdU labelled cells expressing neurofilament consistent with neuronal differentiation. The authors discuss the importance of the neonatal brain microenvironment in directing the migration and differentiation of MSCs in a manor consistent with normal developmental processes.

1.3.4 Differentiation in vitro

Studies that attempted to differentiate MSCs into neuro-ectodermal derivatives *in vitro* can be broadly placed in two categories (See Table 1.1; Figure 1.1), those that use chemical agents to induce a presumptive neuronal phenotype and those, which use growth factors and defined culture conditions consistent with those used to differentiate neural progenitor cells *in vitro*. Those using chemical induction describe the rapid and direct differentiation of cells into neural cell derivatives. In contrast, several more recent investigations have developed multiple stage protocols, which describe the differentiation of MSCs towards neural cell lineages through a series of progressive steps including progenitor cells.

Woodbury et al., 2000, was the first to describe an *in vitro* protocol to differentiate MSCs into neural cell lineages using chemical agents, which resulted in a rapid change in cell phenotype. This presumptive neuronal phenotype was defined by the acquisition of a neuronal morphology and expression of pan-neural markers. The characteristic feature of this chemical induction was the rapidity of the effect, which occurred within hours. Woodbury et al., 2000 described a protocol in which rat MSCs acquired a neuronal phenotype following exposure to antioxidant compounds in serum free media. The neuronal phenotype was confirmed by the expression of NSE, NeuN, NF-M and TAU. MSCs were expanded in culture for more than 20 passages and subsequently exposed to serum free inductive media containing 1-10 mM β-mercaptoethanol (BME). Within 60 minutes of exposure to antioxidant compounds morphological changes were observed in exposed MSCs. Cells displayed a retraction

in cytoplasm with the concomitant formation of process-like extensions. Over the first 3 hours cells progressively assumed morphological traits characteristic of neuronal cells. The cell bodies became increasingly spherical and refractile and exhibited a typical neuronal perikaryal appearance. A variety of neuronal morphologies were evident including simple bipolar neurons through to large extensive multipolar neurons. A small number of cells exhibited pyramidal cell morphologies whereas a large number of cells elaborated long processes with varicosities clearly evident. The investigators claimed that the differentiation was exclusively neuronal since no GFAP immunoreactivity was detected. The investigators suggested that one of the possible mechanisms by which this transition in cell phenotype may occur may be related to the antioxidant properties of BME. Consistent with this conclusion, other substances including dimethyl sulfoxide (DMSO) and butylated hydroxyanisole (BHA) either alone or in combination were also found to be effective inductive agents. The most effective treatment was found to be 2% DMSO and 200μM BHA which resulted in 78% of cells expressing NSE and assuming a neuronal morphology. Differentiation under these conditions was characterized by short cell survival presumably as a result of either the toxicity of antioxidant compounds and/or the removal of serum from the culture.

For long term differentiation and promotion of cell survival cells were pre-induced in DMEM/20% FCS/10ng/ml bFGF for 24 hours followed by exposure to serum free inductive medium. This pre-induction protocol was shown to increase the number of cells responding to neural induction. During long-term studies of differentiation (6 days) cells were cultured in a cocktail of inductive agents including: 2% DMSO/ 200μM BHA / 25mM KCl / 2mM valproic acid / 10μM Forskolin / 1μM hydrocortisone / 5μg/ml insulin. Rapid change in cell morphology was still observed and the expression of nestin in MSC presumptive neurons progressively decreased. Induced cells displayed increased expression of Trk A, the high affinity nerve growth factor receptor, which was detectable at 5 hours and continued to be expressed at 6 days. This data was consistent with responsive cells acquiring a more mature neuronal phenotype when maintained under differentiation conditions. The characteristic features of this protocol in particular were the rapid change in cell morphology and exclusive neuronal differentiation and the high number of cells, which responded to

the inductive medium (78-79%). This early study indicated that MSCs might have an intrinsic neurogenic potential.

Consistent with these observations a number of other investigators have used chemical agents to induce presumptive neural differentiation of adult MSCs. Deng et al., 2001, reported neural differentiation in 25% of human MSCs exposed to isobutylmethylxanthine (IBMX) and 1 mM dibutyl cyclic AMP (dbcAMP) in serum free media. IBMX and dbcAMP increase intracellular cyclic AMP. This treatment increased NSE and vimentin levels in induced MSCs. However no change in MAP1B or Tuj-1 was observed and other neuronal proteins such as NF-M, MAP-2, TAU, GFAP and MBP were not detected. Deng et al., 2001 claimed that an increase in cyclic AMP produces phenotypic changes in MSCs consistent with early neurons and glia. The morphological changes observed were consistent with those reported by Woodbury et al., 2000. Neural-like cells were detected following two days differentiation and by 6 days accounted for approximately 25%. Neural-like cells displayed highly refractile cell bodies, had long branching processes with growth cone like structures that made contact with undifferentiated MSCs. A reduction in cell proliferation was reported but there was no significant increase in cell death. Although Deng et al., 2001 demonstrated only limited neuronal differentiation based on morphological and marker expression, a potential mechanism was indicated. The finding that cyclic AMP induces a neuronal phenotype in MSCs is consistent with reports that cyclic AMP elevation of exogenous administration is associated with the terminal differentiation of neural cell lines including neuroblastoma and progenitor cells (Moore et al., 1996; Bang et al., 1994). Consistent with findings by Woodbury et al., 2001 no GFAP expression was detected.

Hung et al., 2002 used a 3 μm filter to size select a homogenous population of human MSCs. The purified MSCs have been referred to as size sieved stem cells and they have a greater self-renewal capacity than heterogeneous MSCs. These cells are multipotential and lack expression of hematopoietic markers but express Thy 1 and CD44, CD105 and CD29 consistent with a stromal cell identity (Hung et al., 2002) These cells were fibroblastic in appearance and when subjected to a pre-induction medium containing BME (with or without retinoic acid) followed by serum

deprivation, underwent morphological differentiation. Cells acquired a neural-like morphology within 2-3 hours, greater than 95% of cells acquired a neural-like morphology but this morphological change spontaneously reverted back to normal by 5 hours. These cells expressed Tuj-1, NeuN, Nestin and NSE prior to differentiation. Following exposure to the inductive agents there were elevations in the expression levels of nestin, NeuN, Tau and NSE proteins after 5 hours. Cells serum deprived for 5 days expressed neurofilaments, but not MAP-2 and the levels of Tuj-1 and NeuN expression decreased. Theses investigators provided some of the first evidence of neuronal functionality following differentiation. The induced cells exhibited voltage sensitive ionic currents, and intracellular calcium levels could be elevated by a high K+ buffer and glutamate in the medium. Despite the demonstration of some electrical excitability the lack of expression of common neuronal markers makes it difficult to assign a final identity to these induced cells.

Kohyama et al., 2001 described the differentiation of MSCs into neurons using the demethylating agent 5-aracytidine (5-Aza-C) or transfecting MSCs with the neural inducer, noggin. These investigators isolated clonal lines from murine MSCs, which were fibroblastic in appearance. 20% of these cells when exposed to 5-Aza-C and neurotrophic growth factors; neuronal growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) acquired a neural-like morphology progressively over 9 days. The differentiated cells expressed neural markers GFAP, Tuj-1, NeuN and Gal-C. This data indicates that MSCs can also differentiate into cells with the phenotypic characteristics of oligodendrocytes, and astrocytes, in addition to neurons. This was the first study to demonstrate that clonal lines isolated from MSCs have a trilineage neurogenic potential. Each line had a different neuronal versus glial marker expression. In addition, after 28 days of induction, the cells exhibited resting potential, rectifying potassium current and could respond to neurotransmitters. These cells therefore appeared to have differentiated at least to some extent towards the neural lineage. MSCs have also been shown to assume morphology consistent with a schwann cell phenotype and express schwann cell markers such p75, GFAP, S-100, O4 and P0 in response to culture with BME/retinoic acid and subsequently forskolin, bFGF, platelet-derived growth factor (PDGF) and heregulin.

It has long been known that the stem cell niche provides important regulation of stem cell proliferation and differentiation, a concept that continues to be supported by recent evidence (For review see: Wurmser et al., 2004). The studies in which chemical induction was used to induce phenotypic changes in MSCs such as those used by Woodbury and others suggested that MSCs isolated from postnatal BM may have an intrinsic neurogenic potential and therefore their differentiation potential exceeds the traditional potency attributed to these cells. Although the mechanism, which this change in cell phenotype occurred, was not determined by these studies, the data suggests that removal of cells from their normal micro-environment may release cells from the inhibitory niche signals which regulate their developmental potential and dictate their cellular plasticity.

Sanchez-Ramos et al., 2000 demonstrated that a subset of both human and murine BM has the capacity to differentiate into early neural cells and express markers consistent with early neuronal development under defined culture conditions. The conditions used to differentiate MSCs in this study were consistent with conditions used to differentiate primary neural progenitor cells and embryonic stem cells towards a neuronal cell fate. Proliferation of cells was induced in response to epidermal growth factor (EGF). Before induction of differentiation, cultures were enriched in fibronectin immunoreactive cells and depleted of hematopoietic contaminants. Treatment of cultures with RA and BDNF resulted in a decreased number of fibronectin positive cells. This loss of fibronectin expression correlated with the progressive loss of large flat fibronectin positive cells with the appearance of small oval cells, which were process bearing and fibronectin negative. The authors claimed that these cells represented a population of early neuronal cells. Consistent with this conclusion western blot analysis of protein expression from cultures treated with proliferation or differentiation medium revealed the expression of nestin, NeuN and GFAP expression. Subsequent treatment with RA or RA + BDNF decreased the expression of the nestin protein and analysis of GFAP and NeuN by immunocytochemistry revealed that 0.5% of cells were NeuN positive and 1% were GFAP positive. NeuN and GFAP expression was also found at low levels in control

conditions (proliferative medium). This study provided the first evidence of early neural differentiation under physiological conditions.

Long et al., 2005 demonstrated increased RNA expression of neuronal specific markers nestin, MAP-2 and TH when cells were cultured with EGF and FGF in serum free media for 14 days. Using mono-cellular culture conditions Jin et al., 2003 investigated the induction of neuronal properties in whole BM stem cells *in vitro*. They evaluated the effects of different growth factors on the presumptive differentiation of MSCs and reported that certain growth factors induced both a neural-like morphology and expression of neural markers, however the localization of these neural markers including neuronal proteins was not consistent with the pattern of expression observed in primary neurons.

1.3.5 Co-culturing of MSCs with primary neural cell populations may enhance neural cell trans-differentiation in vitro

A number of studies have indicated that co-culture with neurons or astrocytes may be necessary for the full expression of a neural phenotype in MSCs induced to differentiate under defined culture conditions (Sanchez-Ramos et al., 2000; Rivera et al., 2006a, 2006b; Wislet-Gendebien et al., 2005). Early studies such as that of Sanchez-Ramos reported that the fraction of cells expressing NeuN when cultured in the presence of EGF or retinoic acid/BDNF increased when the cells were co-cultured with rat fetal midbrain cells (Sanchez-Ramos et al., 2000). MSCs have been shown to express components of the Sonic Hedgehog pathway (Shh) and retinoic acid nuclear receptors (Kondo et al., 2005). Consistent with this observation, a combination of RA and Shh synergistically induced the expression of a set of glutaminergic sensory neuron markers in MSCs primed with FGF-2 and forskolin (Kondo et al., 2005). More recently, Hermann et al., 2004 observed the formation of neurosphere-like structures expressing nestin after 5-7 days in MSC cultures maintained in serum-free media supplemented with bFGF and EGF. Kabos et al., 2002 also observed sphere formation under similar conditions but with whole BM. In both studies neurosphere-like structures could be differentiated *in vitro* into neurons and glia. In addition, Hermann et al., 2004 were able to demonstrate that differentiated neurons were capable of dopamine production and release, and of inward and outward currents. Wislet-

Gendebein et al., 2003 observed that serum withdrawal resulted in nestin expression and this expression was a pre-requisite for the formation of neurosphere-like structures and progression towards further neural differentiation. Co-culturing of these cells with nestin positive NSCs resulted in the formation of heterogeneous cellular spheres which when plated out and differentiated, the MSCs expressed high levels of GFAP indicating increased astroglial differentiation. In fact, nestin positive MSCs were shown to increase astrocytic differentiation of NSCs in co-culture by releasing BMP-4 (Wislet-Gendebein et al., 2004). When nestin positive MSCs were co-cultured with cerebellar granule cells, the nestin positive MSCs expressed neuronal markers and exhibited both inward and outward currents and were responsive to neurotransmitters (Wislet-Gendebein et al., 2005).

Alexanian, 2005 recently emphasized the importance of juxtacrine and paracrine interactions in the neural induction of MSCs. They demonstrate that MSCs co-cultured with mouse proliferating NSCs or NSC conditioned media adopt a neural-like morphology and express high levels of sox-2 and nestin and eventually differentiate into Tuj-1 and GFAP expressing cells. Rivera et al., 2006 reported that soluble factors derived from different brain regions are sufficient to induce a presumptive neuronal phenotype in MSCs during co-culture *in vitro*. The effect of conditioned media derived from the hippocampus, cortex or cerebellum on the phenotype of MSCs was tested. Hippocampal conditioned media had the strongest inductive effect and induced MSCs to adopt a neuronal fate defined by morphology and immunolabelling of neural markers such as growth associated protein 43 (GAP-43) and neurofilaments. Although the mechanism is unknown, BDNF and NGF could not induce the same effect when added to the culture. Abouelfetouh et al., 2004 reported the generation of neuron-like cells in MSCs co-cultured with a hippocampal brain slice. Suzuki et al., 2004 reported the formation of MSC derived cellular spheres with the capacity to differentiate into neurons, astrocytes and oligodendrocytes using chemical induction. However, proof of concept in this study was confined to pan-neural marker expression and morphology. These studies collectively suggest that signaling from the microenvironment is vital for the induction of neural cell fates by MSCs. It also indicates that more mature differentiation requires signaling provided by other cell types *in vitro* or neighboring

neural cells *in vivo*. The environment in which the cells are initially grafted may affect the neural cell fate specification of transplanted MSCs.

1.3.6 Experimental manipulation prior to transplantation

A number of recent studies have used a two-stage approach to investigate the differentiation potential of MSCs. These studies first removed MSCs from their normal microenvironment and placed them in culture. *In vitro* these cells were provided with new environmental cues and stimulation with growth factors and then cells were subsequently transplanted *in vivo* in an attempt to stimulate differentiation of these cells. Munoz-Elias et al., 2004 reported that MSCs treated with 5ng/ml bFGF in culture and then transplanted into the lateral ventricles of E15.5 rats in *utero* formed spheres expressing vimentin and nestin but not mature neuronal markers within two days. Within two months, MSCs had migrated extensively throughout the embryonic brain and expressed more mature neural markers including NeuN. Deng et al., 2006 recently reported that MSCs spontaneously express neural proteins in culture and retain the expression of these proteins following transplantation into the neonatal mouse brain where these cells extensively migrate throughout the tissue. Suon et al., 2006 recently reported the formation of MSC neurosphere-like structures in defined culture conditions. These cells were capable of expression of dopaminergic and GABAergic traits *in vitro*. After transplantation of MSC neurospheres into a parkinsonism animal model, MSCs retain GABAergic traits but lose dopaminergic traits.

1.3.7 Mechanisms of proposed neuro-ectodermal differentiation of MSCs

1.3.7.1 Trans-differentiation of MSCs into neuro-ectodermal derivatives

Cells may differentiate (trans-differentiate) into distinct phenotypes not normally found within a particular tissue without first de-differentiating to a more primitive phenotype. Trans-differentiation involves the removal of inhibitory factors or exposure of cells in new environmental signals that allow cells to express an intrinsic differentiation potential. Embryonic development involves a progressive restriction in developmental potential, however evidence has shown that these restrictions are not absolute and can be modified by altering single or multiple genes. For example, expression of NeuroD1 in the ectoderm can turn cells fated for epidermis into neurons

(Lee et al., 1995). As described previously a wide range of chemicals have been used to induce neural differentiation including antioxidants and chemicals that increase intracellular cyclic AMP (Deng et al., 2001). Recent findings case doubt on whether these chemicals truly induce neural differentiation of MSCs. Time lapse microscopy revealed the neural-like morphology to be the result of a collapse in the cytoskeleton and not differentiation (Bertani et al., 2005; Neuhuber et al., 2004). In addition, this effect was demonstrated in fibroblasts and in response to other agents such as detergents (Lu et al., 2004). Neural protein expression such as NSE was also expressed under these conditions suggesting that the MSC phenotype as defined by morphology and neural protein expression can be mimicked in culture.

1.3.7.2 Dedifferentiation

As discussed previously, cells may revert to an earlier phenotype and become less restricted in terms of their developmental potential. Under these conditions it is thought that MSCs may re-differentiate into other cell fates in response to new environmental cues such as humeral or cellular signals. For example, oligodendrocytes progenitors from the optic nerve can be induced to acquire a wider range of stem cell characteristics when maintained in serum free media in low-density culture conditions (Tang et al., 2001). These more primitive cells can be re-differentiated into neurons and glia. It is possible that a similar mechanism operates that accounts for the neural differentiation of MSCs in culture.

1.3.7.3 Rare pluripotent stem cells reside in adult tissue

As described in the previous section there is evidence for the presence of pluripotent stem cells in BM (Jiang et al., 2002a). Isolation and enrichment of these cells involved the selection of a very small fraction of marrow mononuclear cells by immunomagnetic sorting to eliminate cells of blood lineages and proliferation for more than 20 population doublings. This resulted in a homogenous population of cells with the capacity to generate cells of all three primary germ layers in culture under defined conditions and when injected into the early blastocyst contributed to somatic cells of all three germinal layers. These cells are referred to as MAPCs but their relation ship to MSCs is not known and whether such cells exist *in vivo* is also currently unknown. However, it is possible that it is these cells and not MSCs in

culture that generate these neural differentiated cells. This is unlikely however, in cultures in which the proposed trans-differentiation frequency is high. In addition, maintenance of MAPCs in culture required highly defined culture conditions not consistent with normal MSC growth.

1.3.7.4 Concluding remarks

The ability of MSCs to generate neural cell derivatives both *in vivo* and *in vitro* is an unexpected observation of which there is some doubt as to its validity in light of recent findings. As a result careful interpretation of results obtained both *in vitro* and vivo is required. Recent results however have been encouraging and some investigators have attempted to demonstrate functionality. The mechanism by which MSCs could adopt neural cell fates in culture is currently unknown but is likely to involve one of the following. (1) Residual populations of pluripotent stem cells reside in adult tissues, which retain the ability to differentiate into multiple cell types either when grafted into ectopic sites *in vivo* or differentiated under defined experimental conditions. (2) Tissue specific stem cells normally with a restricted differentiation potential can generate other cell lineages when exposed to new environmental conditions, a process referred to as trans-differentiation. (3) Presumptive isolation of adult stem cells from a particular tissue is contaminated with progenitor cells of a different embryonic germ layer. (4) Transformation of grafted cells occurs by cell fusion with host cells, mimicking differentiation. (5) Adult stem cells can undergo dedifferentiation and redifferentiate in response to new environmental cues. These issues will not be resolved until the molecular mechanisms of proposed trans-differentiation events are determined.

1.4 Effects of MSCs following neural injury

1.4.1 Introduction

MSCs have previously been shown to improve neurological outcome and functional recovery in animal models of stroke (Chen et al., 2003; Li et al., 2001; Li et al., 2002), spinal cord injury (Chopp et al., 2000; Hofstetter et al., 2002; Neuhuber et al., 2005; Lu et al., 2005) and brain trauma following whole BM transplantation or administration of *ex vivo* expanded purified populations of MSCs isolated from the

postnatal BM (Mahmood et al., 2001; Lu et al., 2001). Higher cell engraftment was achieved when MSCs were infused into the rodent brain compared to whole BM infusion (Chen et al., 2001). The protective effects conferred by marrow-derived stem cells were both obvious and rapid (Chen et al., 2001). The mechanism of the therapeutic benefit of MSCs in these animal models is unknown.

1.4.2 Effect of MSCs transplantation in animal models of CNS injury

In rats subjected to stroke and trauma, intravenously injected MSCs pass through the blood brain barrier, migrate selectively to the damaged tissue and improve functional recovery (Li et al., 2001; Lu et al., 2001). Whole BM cells and *ex vivo* expanded MSCs intra-cerebrally transplanted into the ischemic boundary zone of adult rats and mice 24 hours following middle cerebral artery occlusion (MACO) express a neural phenotype (expression of key neural markers) and promote functional recovery (Chen et al., 2000; Chen et al., 2001). Li et al., (2000) transplanted adult BM MSCs pre-labeled with BrdU directly into mouse striatum following MACO. BrdU reactive cells were detected 28 days following the insult. The cells had successfully survived and migrated from the graft site to the ischemic region. Transplanted animals displayed a significantly increased neurological outcome as accessed by the neurological severity score (motor, sensory functions and reflexes). 1% of BrdU reactive cells expressed NeuN whereas 8% expressed GFAP. The effect was not attributable to a reduction in the infarct size since no significant difference in infarct volume was found in transplanted animals. These results were also found to be consistent in the rat MACO model in which intra-cerebral implantation of MSCs was associated with improved functional recovery (Chen et al., 2000). After 14 days a minimal proportion of MSCs expressed NeuN, MAP-2 and GFAP. In addition, a composite graft of BDNF and adult BM resulted in superior grafting and improved functional recovery. NeuN and GFAP BrdU positive cells were detected at 7 days post MACO (Chen et al., 2000).

MSCs are also able to migrate selectively to the site of the ischemic lesion following administration into systemic vasculature (Li et al., 2001; Lu et al., 2001). BM cells injected intravenously into rats subjected to MACO migrate across the blood-brain barrier into damaged ischemic tissue. Intravenously injected cells can be found in many organs including kidney, spleen, liver and BM (Lu et al., 2001). Few are

however found in the parenchyma with the majority encircling the microvessels in these organs. As few as 1.5-3% of 3 million implanted MSCs are found in the brain parenchyma 14-35 days following treatment (Eglitis et al., 1999). In the injured brain there is however selective migration to the injured hemisphere with 80% of cells within the area around the ischemic lesion (Mahmood et al., 2001). Li et al., 2001 reported that intra-carotid infusion of MSCs 24 hours post ischemia improved the neurological outcome of rats subjected to transient MACO at 14 days compared to controls. Outcome measures were behavioral testing and neurological severity score. Therefore MSCs delivered to the carotid artery enter the ischemic adult rat brain. These cells survive and a small number of MSCs express proteins phenotypic or astrocytes or neurons. Intra-arterial administration appears to be a more superior method of transplantation, with intra-arterially administered cells migrating extensively throughout the ischemic core and penumbral regions compared with intra-cerebrally grafted cells (Lu et al., 2001).

During stroke the permeability of the blood brain barrier may be increased and chemokines released from neural tissue may attract MSCs to damaged tissue (Webb et al., 2000). It is thought that MSCs may use the same transport systems as inflammatory cells (a process of diapedesis) as there is an up regulation of adhesion molecules in local vasculature. MSCs are also capable of passing through the intact blood-brain barrier and migrate throughout the neonatal forebrain and cerebellum (Kopen et al., 1999). Adhesion molecules and their receptors are expressed on inflammatory cells and guide cells to injured tissue and transport them across the vascular endothelium including the blood-brain barrier (Webb and Muir 2000). Targeting adhesion molecules work in concert with chemokines. Using a two chamber cell migration assay (microchemotaxis chamber) MSCs have been found to respond in a dose dependent manor to chemotactic agents such as intercellular adhesion molecule 1, monocyte chemoattractant protein 1 and macrophage inflammatory protein 1 which are known to be expressed by ischemic brain tissue (Kim et al., 1995). These agents significantly increased MSC chemotaxis, as did ischemic brain tissue extracts. This ischemic brain induced chemotaxis can be effectively blocked using antibodies to the chemoattractant agents (Wang et al., 2002). These results suggest that MSCs utilize the same mechanisms as inflammatory cells to migrate into the CNS and provide

insights into how MSCs selectively target their migration to injured tissue. Therefore any injury or disease that results in an inflammatory response may be characterized by MSC migration, the extent of which is dependent on the degree of the inflammatory response which is in turn related to the severity of the tissue injury.

1.4.3 Potential mechanisms of functional recovery

1.4.3.1 Trans-differentiation

MSCs and whole BM cells grafted either directly or indirectly into the rodent brain following injury contain a small number of cells that express neuronal and glial cell specific markers (Chen et al., 2001; Chopp and Li, 2002; Li et al., 2001; Chen et al., 2003). The expression of these markers appears to be dependent on specific microenvironment signals. As it appears that neuronal replacement may be one of the principal mechanisms by which functional recovery is achieved, the possibility that MSCs can differentiate into neuronal and glial cell phenotypes must be considered. MSCs have been reported that express neural markers in the intact CNS following systemic infusion (Kopen et al., 1999; Azizi et al., 1998; Deng et al., 2006) and following injury (Chen et al., 2001 and Li et al., 2001) however expression of these limited number of markers is not sufficient evidence in itself to represent true differentiation. The beneficial effects of MSCs are seen within a few days and thus even differentiated cells are highly unlikely to integrate fully into the host tissue and form the complex connections required to improve function. Even if the transplanted MSCs can trans-differentiate into neural cell types the number of cells transplanted and the number of cells expressing neural proteins are too few to replace lost cells. As described previously many of these previously reported trans-differentiation events may be attributed to cell fusion with host cells rather than differentiation. However several independent reports have also demonstrated engraftment of MSCs with the expression of neural proteins and a neural-like morphology independent of cell fusion (Deng et al., 2006; Munoz-Elias et al., 2004). However, the question of whether this represents true differentiation remains to be established. Thus, tissue replacement as the mechanism by which MSC promote their beneficial effects is unlikely, although it is still possible that transdifferentiation contributes a small proportion of cells to neuron replacement.

MSCs are described as 'small molecular factories', which describes their secretary function in which MSCs release cytokines and trophic factors (for review see: Chopp and Li, 2002). It is believed that the release of these factors from MSCs within cerebral tissue or the microvasculature of the injured brain is the mechanism by which MSCs effectively promote restoration of function (Chopp and Li, 2002). MSCs have been shown to produce hepatocyte growth factor, VEGF, NGF and BDNF among other trophic and growth factors (Chen et al., 2001b; 2002). It appears that it is the combination of trophic factors that has the beneficial effect rather than any single growth factor. More importantly, the combination of trophic factors released by MSCs is dependent on the specific micro-environmental signals (Chen et al 2001c). MSCs when cultured in different ionic microenvironments respond to the cues by adjusting growth factor expression (Chen et al., 2001c). As a result of this finding it is proposed that MSCs regulate their secretion of growth factors in response to tissue specific cues and thus secretion is dependent on the extent of tissue injury and the degree of disruption of the ionic microenvironment. This hypothesis is supported by several lines of experimental evidence in which culture of MSCs with tissue extracts from brains affected by stroke or injury significantly increased the release of MSC derived trophic factors and the pattern of secretary response differs according to the time the tissue is extracted from the affected brain (Chen et al., 2002; Chen et al., 2002b). Consistent with these findings the overall expression of trophic factors in injured brains is significantly higher in MSC treated animals compared to non-MSC treated animals (Chen et al., 2005; Munoz et al., 2005).

Thus current experimental evidence supports a hypothesis in which MSCs home to sites of CNS tissue injury from the periphery in a chemoattractant mechanism similar to that described for inflammatory cells in response to chemokines released at the site of injury. Following these chemoattractant gradients MSCs enter the CNS passing through the blood-brain barrier and congregate at the ischemic boundary where they release growth factors in response to external tissue specific cues including changes in the ionic microenvironment. MSC secretary products encourage endogenous regeneration and repair including synaptogenesis, angiogenesis, neurogenesis, dendritic arborisation and a reduction in apoptosis.

1.4.3.2 Neurotrophic factor production by MSCs

Previous studies have observed dense innervation by nerve fibers in BM, which lie in intimate association with marrow stromal cells (Afan et al., 1997). It has been hypothesized that MSCs produce neurotrophic factors to promote and maintain nervous innervation of BM and guide fibre development during growth or following injury (Tabarowski et al., 1996).

Tremain et al., 2001 using SAGE analysis to analyze the transcript profile of a single cell derived colony of human MSCs detected the expression of several neurotrophic factors, including pigment epithelial derived factor, glial derived nexin 1α, and macrophage migration inhibitory factor. These products have been shown to promote neuronal cell survival (Houenou et al., 1995; Fujimoto et al., 1997) as well as induce neurite outgrowth in cultured neurons (Monard et al., 1983; Houenou et al., 1999). The expression of these products may in part contribute to the therapeutic benefit of MSCs when transplanted into the damaged CNS.

In response to media conditioned with traumatic brain extracts MSCs increase their release of BDNF, NGF, VEGF and HGF in culture (Chen et al., 2002; Chen et al., 2002b). The levels of these growth factors were dependent on the time of exposure. These observations demonstrate that human MSCs are sensitive to both the normal brain and the traumatic brain and respond by significantly increasing the production of many growth factors. MSCs are known to survive for prolonged periods of time in the injured brain and therefore this provides injured tissue with a continuous and microenvironment responsive secretion of neuroprotective and angiogenic factors at the local level, which may be the key to their functional benefit (Chopp and Li, 2002). More recently, Chen et al., 2005 using *in vitro* studies detected the synthesis of various growth factors, including NGF, BDNF, GDNF and NT-3 by MSCs in culture. Following intra-ventricular injection of MSCs, NGF levels were increased significantly in cerebrospinal fluid as detected by ELISA. Therefore in these studies the therapeutic benefit of MSC transplantation appeared to be mediated at least in part by their ability to increase brain NGF concentration. The effects of neurotrophic factors on brain plasticity are shown in Table 1.2.

1.4.3.3 Effects of MSCs on angiogenesis in the injured brain

Stroke induces angiogenesis, and angiogenesis is associated with improved neurological outcome (Zhang et al., 2000; Zhang et al., 2002). Under normal circumstances, injury-induced angiogenesis is insufficient to support the brain plasticity required for functional recovery. However, MSCs induce angiogenesis of brain endothelial cells *in vitro* and stimulate angiogenesis of an avascular cornea following transplantation (Hamano et al., 2000; Villars et al., 2000; Leung et al., 1989). Angiogenesis is significantly increased in the ischemic brain compared to the intact brain when MSCs are infused intravenously 24 hours post MACO in the rat (Chen et al., 2003). The mechanism of MSC-induced angiogenesis is an increase in endogenous rat VEGF and VEGFR2 expression. Newly formed vessels improve tissue perfusion around the ischemic boundary zone and enhanced angiogenesis following stroke improves neurological recovery. Therefore MSC induced angiogenesis probably contributes to the improved outcome following MSC transplantation. MSCs are reported to significantly increase the vascular perimeter and numbers of capillaries in animal models of stroke. VEGF is a potent angiogenic factor and its expression is increased by bFGF (Tamada et al., 2000; Hamano et al., 2000), which is one of the trophic factors released by MSCs in response to injury and a possible mechanism by which MSCs up regulate endogenous VEGF and induce angiogenesis. VEGF has been shown to improve functional outcome when administered 1 day or more after stroke in animal models (Zhang et al., 2000; Zhang et al., 2001). These findings demonstrate that some of the factors released by MSCs may act directly and others indirectly, by evoking the expression of trophic factors by endogenous cells, particularly astrocytes and endothelial cells.

1.4.3.4 Effect of MSCs on neurogenesis in the injured brain

In highly regenerative tissues such as the skin the progeny of proliferating resident stem cells continually replaces dead or injured cells. The adult CNS however, was considered to be incapable of significant regeneration and self-repair. However, within the last four decades evidence has accumulated which demonstrates that neurogenesis persists within the postnatal brain (Altman and Das, 1965; Altman and Das, 1967) and more recently definitive evidence that new neurons are generated from stem cells within discrete regions of the adult brain (Temple, 2001). NSCs

capable of long-term self-renewal and multipotentiality have been found to persist in the adult mammalian CNS, including the subventricular zone and hippocampus (Gage, 2000; Rao et al., 1999; Gould et al., 1998; Mckay, 1997; Momma et al., 2000). The adult CNS also contains a range of progenitors with more limited capacities for growth and differentiation (Temple 2001). These adult neural progenitors are known to be abundant within the periventricular areas (as described above) and recent studies have revealed widespread occurrence within the parenchyma of various CNS regions (Palmer et al., 1999; Yamamoto et al., 2001). Consistent with these findings neuronal cell turnover within the CNS has been demonstrated (Gould and Gross, 2002). Although adult neurogenesis had originally been thought to occur in only a small number of discrete regions, more recent studies have demonstrated production of new neurons in many other CNS regions (Rietze et al., 2000; Pencea et al., 2001; Gould and Gross, 2002). More importantly, CNS injury stimulates the proliferation of endogenous progenitor cells in both known neurogenic sites (Liu et al., 1998; Magavi et al., 2000; Arvidsson et al., 2002; Nakatomi et al., 2002) and in regions where neurogenesis does not occur under normal physiological conditions (Johansson et al., 1999; Yamamoto et al., 2001). These observations have been met with much excitement because of the potential to induce endogenous regeneration following injury. However, initial studies have shown regeneration of new neurons to occur at low frequency or simply the survival of existing neurons (Magavi et al., 2000). It is thought that one reason for this limited endogenous response to injury is the lack of trophic support and the presence of inhibitory signals within the brain microenvironment. Consistent with this concept, intra-ventricular infusion of growth factors can recruit endogenous neural progenitor cells and induce massive regeneration in the hippocampus following a transient ischemic event (Jin et al., 2002; Benraiss et al., 2001).

Long-term recovery of function will require neuronal replacement. Induction of neurogenesis by MSCs may contribute to functional improvement following stroke or neural injury. Transplantation of MSCs amplifies the endogenous response to injury possibly through the production of soluble growth factors that promote neurogenesis (Chen et al., 2003; Chen et al., 2005, Chen et al., 2001). Following transplantation of MSCs a significant increase in cell number was measured in the subventricular zone

after stroke (Chen et al., 2003). Many of these cells had markers of newly formed neuronal cells such as Tuj-1. Neurogenesis within the adult brain is mediated by trophic influences (Hsieh 2004; Takahashi et al., 1998). Neurotrophic factors produced by MSCs appear to induce neurogenesis in the adult brain following injury, amplifying the endogenous response; this may be a direct effect on resident NSC/progenitor or as a result of providing a more favorable microenvironment for the normal endogenous response to be more effective.

1.4.4 Concluding remarks

A large body of evidence demonstrates that MSC transplantation in various animal models of CNS injury has a therapeutic benefit, promoting functional recovery. The mechanism of this therapeutic benefit remains elusive and may result from a number of effects on endogenous brain plasticity. The contribution made by trans-differentiation of MSCs remains to be determined but current evidence suggests that this process is insufficient to fully explain functional recovery following injury.

Treatment	Starting Material	Verification of neuronal/glial phenotype	References
Chemicals			
Isobutylmethylxantine (IBMX) + dibutyryl c AMP	Human BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB	Deng et al. (2001) Rismanchi et al (2003)
β-Mercaptoethanol (BME) followed by butylated hydroxyanisole (BHA) and dimethylsulfoxide (DMSO)	Rat BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC	Woodbury et al. (2000,2002) Munoz-Elias et al (2003) Rismanchi et al (2003) Jori et al. (2005)
5-Azacytidine	Clonal lines derived from murine BMSCs	Neuron-like morphology, expression of neuronal markers determined by RT-PCR and IHC, electrophysiological characterisation and calcium uptake imaging	Kohyama et al (2001)
β-Mercaptoethanol (BME) + retinoic acid (RA)	Size-sieved Human BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC, electrophysiological characterisation	Hung et al (2002a)
Butylated hydroxyanisole (BHA) + dibutyryl c AMP + Isobutylmethylxantine (IBMX) + retinoic acid (RA) + ascorbic acid	Mouse BMSCs	Expression of NSE and NeuN	Levy et al. (2003) Hellmann et al. (2006)
Neurotrophic (or glial) factors/gene transfection			
Combination of GDNF, PACAP and dibutyryl c AMP	Size-sieved BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC	Tzeng et al (2004)
Generation of neurospheres by culturing with b-FGF/EGF and induction with RA and sonic hedgehog	Mouse BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC	Locatelli et al (2003)
Transfection with the Notch intracellular domain (NICD) followed by treatment with neurotrophic factors (such as BDNF)	Human, mouse and rat BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC, electrophysical characterisation	Dezawa et al (2004) Kamada et al (2005)
Transfection with noggin	Clonal line-derived from murine BMSCs	Neuron-like morphology, expression of neuronal markers determined by IHC, calcium uptake imaging	Kohyama et al (2001)
Glial growth factor (GGF)	Rat BMSCs	Cells exposed to GGF-expressed GFAP and S100	Tohill et al (2004)
Human neural stem cell (NSC) culture conditions	Human BMSCs	Formation of neurosphere-like aggregates with expression of proneural genes	Hermann et al (2004)
Co-culture with neural cells			
Co-culture with rat or mouse fetal midbrain cultures	Human and mouse BMSCs	Morphology and IHC labelling of neuronal markers	Sanchez-Ramos et al (2000)
Co-culture with astrocytes	Murine bone marrow multipotent adult progenitor cells (MAPCs)	Neuronal morphology, expression of neuronal markers determined by IHC, electrophysiological characterisations	Jiang et al (2002a, 2003)
Co-culture with mouse NSCs	Rat BMSCs	Nestin-positive cells selected in classical NSC medium could differentiate into GFAP-positive expressing astroglial-like cells when co-cultured with mouse NSCs	Wislet-Gendebien et al (2003)
Co-culture with cerebellar granule neurons	Rat BMSCs	Nestin-positive cells selected in classical NSC medium could differentiate into GFAP-positive astroglial-like cells or NeuN-positive and electrically excitable neuron-like cells when co-cultured with mouse NSCs	Wislet-Gendebien et al (2005)

Table 1.1 Methods used for the induction of a neuronal phenotype in MSCs. Adapted from Chen et al., 2006

Treatment	Starting Material	Verification of neuronal/glial phenotype	References
Chemicals			
Isobutylmethylxantine (IBMX) + dibutyryl c AMP	Human BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB	Deng et al. (2001) Rismanchi et al (2003)
β-Mercaptoethanol (BME) followed by butylated hydroxyanisole (BHA) and dimethylsulfoxide (DMSO)	Rat BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC	Woodbury et al. (2000,2002) Munoz-Elias et al (2003) Rismanchi et al (2003) Jori et al. (2005)
5-Azacytidine	Clonal lines derived from murine BMSCs	Neuron-like morphology, expression of neuronal markers determined by RT-PCR and IHC, electrophysiological characterisation and calcium uptake imaging	Kohyama et al (2001)
β-Mercaptoethanol (BME) + retinoic acid (RA)	Size-sieved Human BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC, electrophysiological characterisation	Hung et al (2002a)
Butylated hydroxyanisole (BHA) + dibutyryl c AMP + Isobutylmethylxantine (IBMX) + retinoic acid (RA) + ascorbic acid	Mouse BMSCs	Expression of NSE and NeuN	Levy et al. (2003) Hellmann et al. (2006)
Neurotrophic (or glial) factors/gene transfection			
Combination of GDNF, PACAP and dibutyryl c AMP	Size-sieved BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC	Tzeng et al (2004)
Generation of neurospheres by culturing with b-FGF/EGF and induction with RA and sonic hedgehog	Mouse BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC	Locatelli et al (2003)
Transfection with the Notch intracellular domain (NICD) followed by treatment with neurotrophic factors (such as BDNF)	Human, mouse and rat BMSCs	Neuron-like morphology, expression of neuronal markers determined by WB and IHC, electrophysical characterisation	Dezawa et al (2004) Kamada et al (2005)
Transfection with noggin	Clonal line-derived from murine BMSCs	Neuron-like morphology, expression of neuronal markers determined by IHC, calcium uptake imaging	Kohyama et al (2001)
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Co-culture with neural cells			
Co-culture with rat or mouse fetal midbrain cultures	Human and mouse BMSCs	Morphology and IHC labelling of neuronal markers	Sanchez-Ramos et al (2000)
Co-culture with astrocytes	Murine bone marrow multipotent adult progenitor cells (MAPCs)	Neuronal morphology, expression of neuronal markers determined by IHC, electrophysiological characterisations	Jiang et al (2002a, 2003)
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Co-culture with cerebellar granule neurons	Rat BMSCs	Nestin-positive cells selected in classical NSC medium could differentiate into GFAP-positive astroglial-like cells or NeuN-positive and electrically excitable neuron-like cells when co-cultured with mouse NSCs	Wislet-Gendebien et al (2005)

Table 1.1 Methods used for the induction of a neuronal phenotype in MSCs. Adapted from Chen et al., 2006

(Trophic) Factors	Action on	Recent reviews
EGF	Stem cell proliferation, migration, differentiation and survival. Neural/glial precursor cell proliferation, migration, differentiation and survival.	Xian and Zhou, 2004, *Front Biosci* 9:85-92 Wong, 2003, *Cell Mol Life Sci* 60:113-8
bFGF	Neural induction Stem cell proliferation, migration Neural/glial precursor proliferation	Akai and Storey, 2003, *Cell* 115:510-2 Reuss and von Bohlen and Halbach, 2003, *Cell Tissue Res* 313:139-157
NGF	Neuronal plasticity Regulation of neuron number Neuronal maturation and survival	Jaaro et al., 2001, *Trends Neurosci* 24:79-85 Kruttgen et al.,2003, *Neuropathol Exp Neurol* A62:340-50 Heerssen and Segal, 2002, *Trends Neurosci* 25: 160-5
BDNF	Neuronal plasticity, maturation and survival	Kruttgen et al., 2003, *Neuropathol Exp Neurol* A62:340-50 Heerssen and Segal, 2002, *Trends Neurosci* 25: 160-5
CTNF	Neural development and repair Astrocytes differentiation Neuronal survival	Murphy et al.,1997, *Prog Neurobiol* 52:355-78 Sleeman et al., 2000, *Pharm Acta Helv* 74:265-72
RA	Neurogenesis onset Ventral neural patterning Amplification of response to neurotrophic factors Neural differentiation (motor neuron subtype)	Appel and Eisen, 2003, *Neuron* 40:461-4 McCaffrey et al., 2003, *Europ J Neurosci* 18:457-472
IGF-1	Neuronal development, Growth, elongation, and branching Neuronal cell death (prevention)	Carro et al., 2003, *Mol Neurobiol* 27:153-62 Rabinovsky, 2004, *Neurol Res* 26:204-10
LIF	Neural precursor cell differentiation Neural development and repair Neurotransmitter phenotype	Metcalf, 2003, *Stem cells* 21:5-14 Murphy et al., 1997, *Prog Neurobiol* 52:355-78
GDNF	Neuronal morphology, survival, development, and regeneration	Markus et al., 2002, *Curr Opin Neurobiol* 12:523-531 Kirik et al., 2004 *Nature Neurosci* 7:105-110

Table 1.2: The effect of trophic factors on stem cell populations. Adapted from Bossolasco et al., 2005

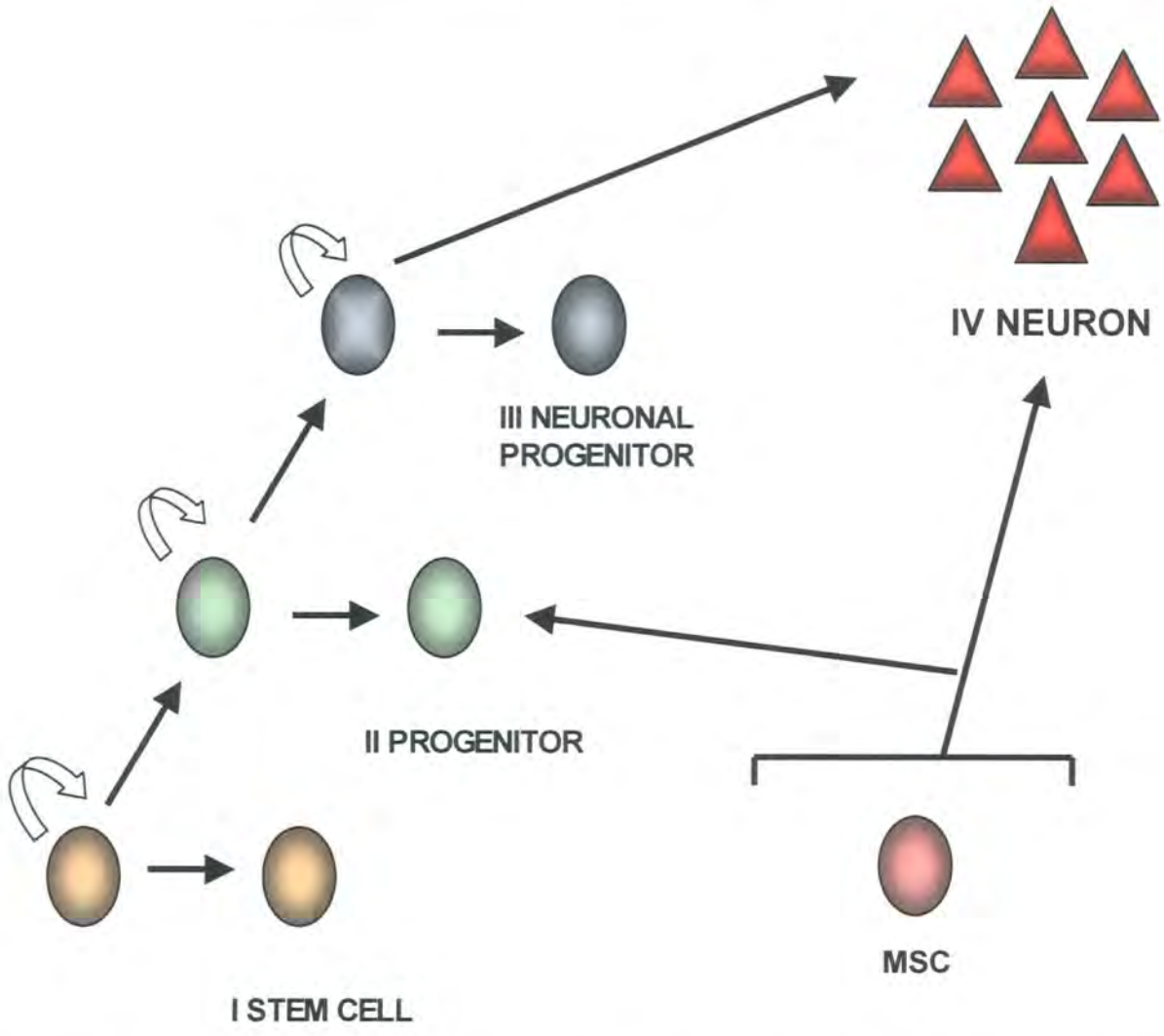


Figure 1.1: Potential mechanism of MSC neural differentiation. It has been proposed that an MSC may acquire a neuronal cell fate through either a series of progressive differentiation steps involving a number of progenitor cell stages to form a neuronal cell or an MSC may directly acquire a neuronal cell fate through a single step process.

CHAPTER 2

Isolation and Characterisation of Postnatal BM Derived Mesenchymal Stem Cells *In vitro*

2.1 Introduction

In order to define a cell as a multipotent stem cell, it must have the capacity to self renew and the ability to generate progeny of multiple cell lineages (Wagers and Weissman, 2004). The BM contains two prototypical stem cell populations: the well characterized HSCs which give rise to cells of all blood lineages and are able to reconstitute the hematopoietic system of irradiated mice (Till et al., 1964). The non-hematopoietic stem cells of the BM stroma provide the structural and functional support for hemapoesis (Tavassoli and Friedenstein 1983). These latter populations of cells are derived from the complex stroma, and are referred to as MSCs because they can differentiate into multiple cell lineages of mesodermal origin (Prockop 1997).

MSCs have been isolated from a number of adult (da Silva Meirelles et al., 2006; Zuk et al., 2001; Seo et al., 2005; Sabatini et al., 2005; De Bari et al., 2001) and fetal tissues (Miao et al., 2006; Fan et al., 2005); however the principal source of MSCs in the postnatal organism remains the stromal tissue of the bone meduallary cavity (Wexler et al., 2003). The BM contains the highest numbers of colony forming progenitors compared to placental cord and peripheral blood when accessed by a CFU-F assay (Wexler et al., 2003). Despite considerable interest in the potential therapeutic applications of MSCs, there is still no well-defined method for the isolation and expansion of these cells in culture. Friedenstein et al., 1970, 1982 initially isolated MSCs by their adherence to tissue culture plastic and these same procedures have been employed by many subsequent investigators (Piersma et al., 1983; 1985; Howlett et al., 1986; Mardon et al., 1987; Beresford et al., 1992). The original reports by Friedenstein have been extensively replicated and extended by a number of other studies in which it was established that MSCs isolated by their adherence to tissue culture plastic were multi-potential with the capacity to differentiate into multiple cell lineages of mesodermal origin. This was observed both in culture (Pittenger et al., 1999; Bruder 1998; Muraglia et al., 2000) and following implantation *in vivo* (Haynesworth et al., 1992; Friedenstein et al., 1974b). The relative ease of isolation and high proliferative potential in culture (Smith et al.,

2004), led many investigators to evaluate the potential use of these cells as effective vehicles for cell and gene therapies (Nandoe et al., 2006; Vilquin and Rosset, 2006).

The definitive evidence that BM contains cells with the capacity to differentiate into fibroblasts and other cells of mesodermal cell origin was first demonstrated by Friedenstein et al. (1974). Friedenstein reported the presence of adherent cells with a fibroblast-like morphology that grew as colonies and had the ability to differentiate into cells which resembled bone (Friedenstein et al., 1974; 1974b; Friedenstein et al., 1987). It was further noted that these cells could be maintained in culture for 20-30 population doublings and still retain their capacity for differentiation, which is a testimony to the stem cell-like characteristics of these cells (Friedenstein et al., 1987). As described previously, colonies of adherent cells were first described as CFU-F (Friedenstein et al., 1974). The CFU-F assay has since been used by many as a functional method to study mesenchymal progenitors (Clarke et al., 1989). These cells were later termed MSCs or marrow stromal cells, based on their ability to self renew and differentiate into mesenchymal cell lineages (Prockop et al., 1997).

The identity of an MSC still remains controversial as there is no single universally accepted definition and little standardization in the procedures used to isolate and culture these cells (For review see: Short et al., 2003). BM stroma contains a mixture of mature cell types and progenitor cells at various stages of differentiation including fibroblasts, adipocytes, and osteogenic cells. MSCs can be isolated from a BM aspirate by preferential adherence to tissue culture plastic or by differential centrifugation through a percoll or ficoll density gradient to obtain a nucleated cell fraction (Pittenger et al., 1999). Cultures established by preferential adhesion are often heterogeneous in the first instance and difficult to clone (Friedenstein et al., 1976). Cultures may only contain a limited number of cells with true CFU-F that would define them as a true stem cell (Wexler et al., 2003). Most researchers consider this population too crude to be referred to as a pure population of MSCs (Phinney 2002). One problem is that the adherent stromal cells possess many of the same characteristics of MSCs in terms of morphology, their ability to differentiate and expression of certain cell surface markers. As a result, the terminology is confusing and terms used may be inaccurate. This is further complicated by the fact that many independent investigators work with different starting populations of cells which

makes comparison of findings between studies and laboratories more difficult, although efforts have been made to rectify this. Notably, Pittenger et al. (1999) provided what is considered the 'gold standard' for the functional definition of human MSCs. It was reported that a phenotypically homogenous population of MSCs could be isolated from a human BM aspirate using density centrifugation and that this population of cells had the capacity to differentiate into three principal cell lineages, oesteoblasts, chondrocytes and adipocytes, whereas fibroblasts (mature mesenchymal cells) did not process this ability (Pittenger et al. 1999). Furthermore, clonal analysis demonstrated that single cell colonies had a multipotent capacity confirming the identity of MSCs (Pittenger et al. 1999). In this study, we will refer to the adherent cell population obtained following percoll density centrifugation and differential adhesion as BM stromal cells, whereas MSCs should be considered a sub-population of this adherent culture with the ability to form colonies and exhibit the potential to form the three principle mesenchymal cell lineages (bone, fat and cartilage).

In humans, MSCs are primarily isolated from aspirates of BM harvested from the superior iliac crest of the pelvis (Digirolamo et al., 1999; Pittenger et al., 1999). In addition, MSCs can be isolated from the tibial and femoral marrow compartments (Murphy et al., 2002) as well as the thoracic and lumbar spine (D'Ippolito et al., 1999). Pittenger et al. (1999) showed that MSCs represent only 0.001-0.01% of the total number of nucleated cells in the BM (Pittenger et al., 1999). However, this population can be expanded ex vivo and enriched by standard culture conditions. Whole BM samples obtained from aspirates are usually subjected to fractionation on a density gradient solution such as percoll (Lange et al., 2005). The mononuclear cell fraction obtained at the interface is then explanted ex vivo by plating at densities from $1x10^4$ cells/cm^2 to $0.4x10^6$ cells/cm^2 (Lodie et al., 2002; McBride et al., 2003). Cells initially adhere to the tissue culture plastic, possess a fibroblastic appearance and develop into symmetrical colonies between 5-7 days after plating. At this stage the cell population also contains a relatively high fraction of hematopoietic cells, most of these are lost in the first 2 weeks of culture.

Contamination of cultures with hematopoietic cells can be monitored by staining for cell surface markers, including CD34, CD45, CD11b and CD14, which are present on

hematopoietic cells but are not expressed by MSCs (Ortiz et al., 2003). Several earlier investigations have determined the ideal culture conditions for selection of MSCs (Friedenstein et al., 1976) and include the culture of cells in basal medium in the presence of 10% FCS (Pittenger et al., 1999). Under these conditions, MSCs develop as an adherent phenotypically stable monolayer of cells. It has been found that human MSCs proliferate most rapidly, and maximally retain their multipotentiality if cultured at relatively low cell densities (Sekiya et al., 2002). Under these conditions, MSCs can be propagated for 20-30 population doublings in an undifferentiated state and retain their capacity for multi-lineage differentiation (Coulter et al., 2000; Coulter et al., 2001).

There remain a number of obstacles in the isolation and culture of MSCs, which limit both our understanding of their biology and the potential therapeutic application of these cells. This is reflected in the lack of universally accepted definition on their phenotype, a problem which itself arises from the extensive heterogeneity of MSC cultures. A consistent feature of explanted CFU-F colonies is their considerable heterogeneity in terms of their morphology, phenotype and developmental potential. A large number of studies have characterized the multi-lineage differentiation of MSCs *in vitro* derived from these adherent colonies, and these and other studies have observed functional heterogeneity in both clonal and non-clonal cell preparations (Phinney et al., 1999; Muraglia et al., 2000). A recent study reported the derivation of 185 immortalized MSC cell clones derived from human BM (Muraglia et al., 2000). Less than one third were capable of differentiating into bone, cartilage and fat *in vitro*. In addition, the authors reported that these multipotential clones lost their capacity for chondrogenic and adipogenic differentiation with increasing passage number but retained their osteogenic differentiation as a default pathway of lineage commitment. This finding is consistent both with previous studies demonstrating a loss in multipotentiality in MSC cultures with expansion under standard conditions (DiGirolamo et al., 1999) and the initial reports of osteogenic differentiation of MSCs implanted *in vivo* following extensive ex vivo expansion (Friedenstein et al., 1987; Kuznetsov et al., 1997). Current methodologies for the putative isolation of MSCs select for the progeny of CFU-F and not the clonogenic precursors themselves and such starting populations are heterogeneous and there is no consensus of the

phenotype and therefore definition of MSCs. This problem is compounded in part by the lack of definitive cell surface markers.

As described above, the property of plastic adherence itself is not sufficient to allow for purification of MSCs. Despite the fact that adherent BM stromal cells appear to possess the ability to differentiate, only a small proportion of such cells are CFU-F positive, indicating the BM stroma consists of a heterogeneous mixture of cells at different stages of development and maturation. As a consequence, many investigators have developed procedures to test for MSC enrichment and identification using selective cell surface markers. Stem cells and their differentiated progeny are often identified on the basis of the presence or absence of cell surface markers. However, there is currently no single antigenic marker that is specific to the MSC population. A number of antibodies have been developed in an effort to isolate and characterize human MSCs. For example, Stro-1 was developed by Simmons et al., 1991) and was found to react against non-hemapoetic progenitor BM stromal cells. Antibody SB-10 recognizes the CD166 antigen (activated leuckocyte cell adhesion molecule) that is present on undifferentiated MSCs but is absent from cells that embark upon the formation of bone (Bruder et al., 1998c). Caplan and co-workers developed the SH-2 antibody that reacts against an epitope of the transforming growth factor beta receptor, endoglin (CD105), (Haynesworth et al., 1992) and the SH-3 and SH-4 antibodies, which recognize distinct epitopes of CD73 (ecto-5'-nucleotidase). Neither of SH2, SB-10, SH3 or SH4 reacted with hematopoietic cells or mature osteocytes. Pittenger et al. (1999) reported that CD44, CD90 and CD29 are also important antigenic determinants, but that none of these antigens are exclusive to MSCs and can be found on a number of cell types including mature mesenchymal derivatives in addition to endothelial, epithelial and muscle cells. MSCs do express a large number of adhesion molecules, extra cellular matrix proteins, cytokines and growth factor receptors all associated with their function and cell interactions within the BM stroma. As a result antibodies that recognize cell surface antigens as described above cannot be used independently to examine the phenotype of MSCs or for direct cell isolation but must be used in combination to achieve enrichment.

A number of laboratories have developed protocols for enrichment of the MSCs based on the expression or absence of cell surface markers, including immunodepletion of CD34/CD45/CD11b (hematopoietic cell markers) positive cells and selection of CD105 positive cells using immunomagnetic sorting (Cheifetz et al., 1992). However, the exact phenotype of the cells isolated by these procedures has yet to be confirmed and standardized across different laboratories. Indeed, a lack of standardization between laboratories, in terms of cell isolation, enrichment and immunophenotype, makes it difficult to compare results between different studies and draw firm conclusions. The lack of definitive markers that associate the developmental potential of MSCs with a specific phenotypic trait means that functional characterization of MSCs is the gold standard for proof of cell identity. This involves testing the developmental potential of MSCs in culture.

2.2 Materials and Methods

2.2.1 Isolation of MSCs from postnatal BM

Rat MSCs were isolated using established procedures with modification (Javazon et al., 2001). Cells were obtained from 6-8 month old Wistar Rats, which were individually euthanised by CO_2 asphyxiation. The femurs and tibiae were removed, cleaned of all connective tissue and placed on ice in 20 ml of collection media. Collection media consisted of RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 12 μM L-glutamine. The ends of each femur and tibia were clipped to expose the BM. BM aspirates were obtained by inserting a 21-gauge needle into the shaft of the bone and flushing it with 10 ml of collection media, the subsequent aspirate from one rat was plated into one T75 cm^2 flask. The cells remained in the collection aspirate for 2 days, allowing stromal cells to adhere to the tissue culture plastic. The cells were then washed using complete culture medium (CCM) consisting of Dulbecco's Modified Egales Medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 12 μM L-glutamine and 1x non-essential amino acids to remove non-adherent hematopoietic contaminants.

The cells were subsequently grown in CCM at 37°C and 5% CO_2 for 3 days, the medium was replaced with fresh medium, the adherent cells were grown to 90% confluence were they were defined as passage 0 (P0). For passing, MSCs were washed with phosphate-buffered saline (PBS) and detached by incubation with 0.25% trypsin and 0.1% ethylene diamine tetraacetic acid (EDTA) for 5 to 10 minutes at 37°C. CCM was added to inactivate the trypsin. The cells were centrifuged at 250 x *g* for 5 minutes, the medium was removed and cells were resuspended in 1 to 10 ml of CCM. The cells were subsequently seeded at 10,000 cells/cm^2 between P0 and P1 and then at 10 cells/cm^2 during subsequent passing to initiate expansion, unless otherwise stated.

2.2.2 Effect of plating density on population growth dynamics

Passage 2 MSCs were plated in triplicate at initial seeding densities of 5, 10, 50, 100, 500 and 1000 cells/cm^2 in CCM. The media was changed every 3 days for 15 days. To assay cell number, cells were lifted with trypsin/EDTA, and cells from each plate were counted in duplicate with a hemocytometer. Phase contrast images were also obtained of cultures plated at different initial plating densities at each time point using a Nikon 330 inverted microscope.

2.2.3 Selection of culture media

Passage 2 MSCs were plated at 10 cells/cm^2 on 60cm^2 tissue culture dishes in 1 of 4 basal media compositions: α-modified minimal essential medium (α-MEM) (Invitrogen), DMEM (Sigma), IDMM (Sigma) or RPMI-1640 (invitrogen). Each medium was supplemented with 10% FCS (unless otherwise stated), 100 U/ml penicillin, 100 μg/mL streptomycin and 12 mM L-glutamine. The media was changed every 3 days for 15 days. Cell number was determined every 3 days using a hemacytometer.

2.2.4 Selection of serum concentration

To assay the effects of serum concentrations, cells were cultured in DMEM supplemented with either 1,2,5,10,20 or 30% FCS and 100 U/ml penicillin, 100 μg/mL streptomycin and 12 mM L-glutamine. Cells were maintained under these

conditions for 15 days and media was changed every 3 days, with cell numbers assayed every 3 days using a hemacytometer.

2.2.5 Immunocytochemical staining

PBS washed cells were fixed in 4% paraformaldehyde (PFA, in PBS) for 1 hour at room temperature followed by immunocytochemical staining as previously described (Palmer et al., 1999). After fixation, cells were incubated with primary antibodies overnight at 4°C in blocking buffer (5% goat serum, 0.2% Triton X-100 in PBS). Cells were then incubated for 1 hour with secondary antibodies conjugated to fluorescein isothiocyanate (FITC). Primary antibody concentrations used are as follows: Mouse anti-smooth muscle actin (SMA, Sigma, 1:200), mouse anti-fibronectin (Sigma, 1:500) and mouse anti-collagen-1 (Sigma, 1:100). Secondary antibodies were purchased from Jackson Immuno-Research laboratories and used at a concentration of 1:250 dilution.

2.2.6 Flow cytometry analysis of cell surface markers

Cells required for flow cytometry analysis were washed and resuspended in FACS medium (6.6g NaCl, 1.5g Na_2HPO_4, 0.2g KH_2PO_4, 1g BSA, 1g Sodium azide, 1000ml ddH_20, filtered through a 0.2μ filter) at a concentration of 1 x 10^6 cells/ml. 200μl of cell suspension (2 x 10^5 cells) was pipetted into each well of a 96-well plate (Greiner), centrifuged at 300g at 4°C for 10 minutes and the supernatant discarded. 50μl of the required primary mouse monoclonal antibody was added to the cells at the appropriate concentration and the plate incubated on ice for 20 minutes. Antibodies included (all anti-mouse monoclonal): CD90 (Chemicon, 1:100), CD45 (Chemicon 1:100), CD44 (Chemicon 1:50), CD29 (Sigma, 1:100). TRA 2-54 (1:200), CD56 (1:50), CD73 (1:100), CD11b (1:50) and HLA Class I (1:200) All antibodies unless stated otherwise were obtained from the developmental hybridoma bank. Following 2 washes with 150-200μl FACS medium, 50μl secondary FITC antibody (anti-mouse Ig Fab_2 fraction, FITC conjugated - DAKO) was then added to the cells (1:20) and incubated on ice in the dark for 20 minutes. The cells were then washed twice with FACS medium and transferred in 500 μl aliquots to 5ml centrifuge tubes (Greiner) for flow cytometry analysis. 10μl of 0.25mg/ml propidium iodide (Sigma) was added to

each sample to assess viability of cell populations. Analysis was performed using a coulter EPICS XL flow cytometer. FITC and Cy-3 were identified by using a 530 and 585 band pass filter respectively, and quantification was completed using CellQuest Software (Becton Dickinson). Ten thousand events were acquired per sample with fluoresence measured on logarithmic scales. Forward and side light scatter gates were set to exclude dead debris and clumps of cells. To calculate the percentage of positive cells, linear gates were set at 0.1%, on samples stained only with secondary antibodies, and expression corresponding to a fluoresence signal exceeding this percentage was measured.

2.2.7 Propidium iodide for quantification of cell viability

To assess cell death cells were trypsinized, washed and processed for flow cytometry by forming a single cell suspension. The suspension was maintained on ice prior to analysis and stained with 1 ug/ml propidium iodide (PI) (Sigma) just prior to analysis by flow cytometry.

2.2.8 Effect of growth factors on population growth dynamics

To assess the short-term effects of growth factors on the expansion of MSCs, cells were grown in DMEM medium supplemented with 10% FCS and 100 U/ml penicillin, 100 μg/mL streptomycin and 12 mM L-glutamine. In addition, the media was supplemented with either basic fibroblast growth factor (bFGF, Invitrogen), epidermal growth factor (EGF, Invitrogen), platelet derived growth factor (PDGF, Invitrogen) or leukemia inhibitory factor (LIF, Sigma) at concentrations ranging from 0.2-1ng/ml. Cell numbers were assayed every 3 days for 15 days using a hemacytometer. In experiments examining the long-term effects of growth factor supplementation, MSCs were maintained continually from the time of isolation in a growth factor supplemented media.

2.2.9 Determination of population doublings and average population doubling time

The mean population doubling time was calculated according to the equation: $TD = t\ {}_p\log 2/(\log Nt - \log No)$, where No is the number of seeded cells, Nt the number of harvested cells, and t is the time of the culture (in h). The finite population doubling was determined by cumulative addition of total numbers generated from

each passage. To calculate the number of seeded cells for primary culture the cells, MSC cells were cultured in a 25-cm^2 plastic flask in complete medium. Three days after culture initiation, i.e., when confluency had been achieved, the cells were trypsinized and counted and the remainder split between two 25-cm^2 flasks. The number of the cells in the time of confluency was taken as the number of harvested cells. Data were presented as mean±standard deviation.

2.2.10 Colony forming unit (CFU) assays

CFU-F assays were carried out as previously described according to Javazon et al., 2001. Rat MSCs were washed with phosphate buffered saline (PBS) and detached by incubation with 0.25% trypsin and 0.1% EDTA (Sigma) for 5-10 minutes at 37°C. Complete serum containing medium was added to inactivate the trypsin. The cells were centrifuged at 250 x *g* for 10 minutes, the medium was removed, and the cells were re-suspended in 10 ml complete medium. The cells were counted in duplicate using a hemacytometer and then 100 cells were plated in a 60cm^2 tissue culture dish. Cells were cultured for 12 days with medium replaced every 3 days. After 12 days, the media was removed, and cells were fixed in 100% methanol for 10 minutes at -20°C. Culture dishes were subsequently stained with 0.5% crystal violet solution in 100% methanol for 10 minutes. The cells were washed twice with distilled water, and the number of colonies were counted. Colonies less than 2mm in diameter and faintly stained colonies were ignored. To access the incidence of CFU-F in cultures established in the presence of various growth factors, cells were isolated and immediately cultured in media supplemented with either FGF-2, EGF, PDGF or LIF at a concentration of 1ng/ml. 100 P2 cells isolated and cultured under these conditions were then transferred to 60cm^2 tissue culture dishes and maintained for 14 days in growth media devoid of growth factors.

2.2.11 Osteogenic assay

Cells were seeded at a density of 3 x 10^3 cells/cm^2 in tissue culture dishes and cultured in DMEM media supplemented with 10% FCS, 100 nM dexamethasone (Sigma), 50 μM ascorbic (Sigma) acid 2-phosphate (Sigma), 10nM β-glycerophosphate (Sigma). The cultures were maintained for upto 3 weeks and the culture medium was replaced every three days. After either 7, 14 or 21 days of

culture, cells were rinsed twice with PBS, and fixed with 10% buffered formalin for 10 minutes at room temperature. Cells were stained with 5% silver nitrate solution for von kossa staining. Cells were incubated in 5% silver nitrate solution for 10 minutes in the dark, washed thoroughly and then exposed to bright light for 15 minutes. Examining 10 non-overlapping visual fields and determining the % area in each field that was mineralized, as identified by Von Kossa staining, quantified osteogenic differentiation.

2.2.12 Adipogenic assay

Cells were seeded at a density of 2 x 10^4 cells/cm^2 in tissue culture dishes. When the cell culture was confluent, the adipogenic differentiation was initiated by three cycles of induction/maintenance culture. Each cycle consisted of 3 days of culture in the induction media (DMEM 10% FCS, 1μM dexamethasone, 0.2 mM indomethacin, 10 ug/ml insulin and 0.5mM 3-isobutyl-1-methylxanthine) followed by 2 days culture in maintenance media (DMEM with 10% FCS and 10 μg/ml of insulin). After either 7,14 or 21 days culture, cells were rinsed twice with PBS, and fixed with 10% buffered formalin for 10 minutes at room temperature. Fixed cells were then stained with 0.3% Oil-red-O (RA Lamb) to identify lipid accumulation within cells. The number of adipocytes generated following differentiation was quantified by examining 10 non-overlapping visual fields (approximately 25 cells/field of view) at x 100 magnifications. The number of cells, which contained oil-red-O positive lipid droplets, was recorded.

2.2.13 Image analysis

Labeled cells were visualized using an inverted fluorescent microscope (model E660 Nikon) and a CCD camera (Spot RT; diagnostic instruments) with individual filter sets for each channel. Color images were generated using Adobe Photoshop (Adobe systems, Mountain View, CA). Phase contrast images were obtained either on the same microscope or an inverted bright field microscope Nikon 330. Phase contrast images were also generated using Adobe Photoshop.

2.2.14 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism Software version 4. Results were analysed for statistical significance using ANOVA and all error bars are expressed as standard error +/- mean (Mean±SEM). All data unless stated otherwise is expressed as Mean±SEM. Post hoc analysis was done using Bonferroni or Dunnetts post hoc analysis for corrected planed comparison.

2.3 Results

2.3.1 Isolation of MSCs from rat postnatal BM

MSC isolation from postnatal BM is classically performed by adhesion to tissue culture plastic as originally described by Friedenstein and colleagues (Friedenstein et al., 1982). As described previously MSCs are isolated from the stromal fraction of BM and constitute a distinct population of cells from cells of the hematopoietic lineage. As a result, the principal contaminants when using BM explants to isolate MSCs are hematopoietic cells mainly erythrocytes. Hematopoietic cells do not adhere to tissue culture plastic therefore this property of selective adhesion to tissue culture plastic can be used as a means of isolating MSCs.

In the current studies initial cell explants were obtained by flushing the cut ends of the long bones using sterile culture medium. The BM is a sterile site, and sterility is only compromised once the bone end is cut. Therefore to maintain optimal sterility the bones were dissected from the animal and then transferred to a class II biological hood were the bones were cut and aspirated. The aspirate was then centrifuged and a single cell suspension was generated by mechanical dissociation. There is little extra-cellular matrix present in marrow and gentle mechanical disruption (by pipetting and passage through a 21*g* syringe needle) can readily dissociate stroma and Hematopoietic cells into a single cell suspension.

The single cell suspensions were subsequently plated at a density of 10^4-10^5 cells/cm^2 in T75 cm^2 tissue culture flasks. Under these conditions stroma cells rapidly adhere and can be easily separated from non-adherent hematopoietic cells by repeated washing. Preliminary isolation studies showed that this initial seeding density was

optimal for obtaining the maximal number of non-overlapping colonies. At higher seeding densities colonies overlapped and hematopoietic contamination was higher. In addition, a lower cell density was associated with low cell viability and therefore a low cell yield. Following centrifugation, and re-suspension, the total number of cells counted was between approximately 800,000 and 1000,000 cells.

Within 24 hours of plating a number of adherent cells, mostly spindle shaped, although cell morphology was heterogeneous were apparent at the bottom of the tissue culture flask (Figure 2.1a). Other cells present in the culture (approx 30%) were highly refractile (phase bright) and remained in suspension (Figure 2.1a,b, c and d). these cells did not adhere to the bottom of the tissue culture dish. These cells are contaminating cells of the Hematopoietic lineage most of which are erythrocytes. By 48 hours (Figure 2.1b) adherent cells spread and acquired a more stromal-like morphology with foci of 2-4 cells. The cells in these foci remained relatively dormant for 2 to 4 days and then began to multiply rapidly. At 6 days small cell colonies (10-20 cells) morphologically resembling fibroblasts were evident at low magnification (x10) (Figure 2.1c). These colonies continued to grow in size (50-100 cells in size) and eventually overlapped and merged forming a monolayer culture by 10-12 days following initial plating. At this stage isolated cells were described as P0 and still contained a high fraction of contaminating cells (approx >10%). However, all of the readily identifiable hematopoietic cells were lost as cells were maintained as primary cultures for 2-3 weeks (Figure 2.1e,f).

When P0 cultures reached confluence, cells were passaged by enzymatic dissociation (Trypsin). Single cell suspensions were then replated at the appropriate seeding density. For the first passage (P0-P1), cells were replated at high cell density (1,000 cells/cm^2) to maintain high cell viability. The culture reached >70% confluence within 3 days and was subsequently replated for expansion at low cell densities (10-50 cells/cm^2). P1 cells were heterogeneous in morphology and consistent with previous reports (Javazon et al., 2001; Sekiya et al., 2002), three different phenotypes were observed: (1) fibroblastic elongated cells, (2) large flattened cells and (3) thin star shaped cells (Figure 2.1e). The predominant cell phenotype observed in early growth phases of the culture was spindle shaped cells (Figure 2.1f).

2.3.2 Verification of MSC phenotype

In the current study MSC identity was confirmed by flow cytometry for MSC associated, cell surface markers (Figure 2.2), immunofluorescent staining of extra-cellular matrix proteins (Figure 2.3 a-c), CFU-F assays (Figure 2.3 d-f) and the ability of cells to differentiate into mesodermal cell lineages (Figure 2.4). Although there are no definitive markers for the identification of the MSC phenotype an analysis of markers expressed by MSCs can be used monitor consistency between cell preparations and an analysis of hematopoietic surface markers was used to monitor hematopoietic contamination within the cell preparations. Examples of surface molecule profiles for selected markers are shown in Figure 2.2. CD45 and CD11b were used to monitor contamination of cultures with cells of the hematopoietic cell lineage. CD45 (leukocyte common antigen) is known to be highly expressed on all hematopoietic cells (Trowbridge et al., 1993). CD11b (MAC-1) is expressed on granulocytes, monocytes, NK cells and a subset of T and B cells (Corbi et al., 1988). P2 MSC cultures were negative for the hematopoietic markers CD45 and CD11b. These markers were only detected in early cultures (data not shown). TRA 2-54 is a mouse monoclonal antibody, which is a non-isotype specific antibody against alkaline phosphatase (Andrews et al., 1984). All cells analyzed expressed TRA 2-54 (alkaline phosphatase), a marker commonly expressed by undifferentiated stem cells that decreases on differentiation (Andrews et al., 1984). TRA 2-54 was expressed by all cells (>98%) in the culture but at a relatively low level of expression. CD90 (Thy 1.1) is highly expressed in connective tissue, fibroblast and stromal cell lines, and is also expressed by hematopoietic cells (Reif 1989; Criag et al., 1993). Its use therefore as a marker of MSCs is not in distinguishing these cells from hematopoietic contaminating cells but in determining the undifferentiated status of cells. MHC-class I and CD90 (Thy 1.1) were expressed at high levels on P2 cells (>98%). The mean florescence intensity (MFI) of this staining has high as indicated as indicated by the rightward shift of the positive curve (solid peak). This expression was consistent with the undifferentiated state of the cells. CD29, CD73, CD56 and CD44 are all expressed to varying degrees on MSCs isolated from a number of different sources and species (da Silva Meirelles et al., 2006; Peister et al., 2004). Their expression does not necessarily relate to function but their expression coupled with the absence of hematopoietic markers is consistent with an MSC phenotype, but not definitive. The expression of

CD29 (β-1 integrin), CD56 (N-CAM), CD73 (Ecto-5'-nucleotidase) and CD44 (surface glycoprotein) when present displayed moderate intensity staining, however not all cells in the culture expressed these markers. CD29 was expressed by 81% of cells; CD56 was expressed by 92%, CD44 79% and CD73 81%. The reason why a proportion of cells do not express these markers is currently unknown but the variable expression levels of these markers within the culture and the expression profile as a whole is consistent with previous reports for the isolation of rat MSCs (Javazon et al., 2001).

Cultured MSCs synthesize an extra cellular matrix and therefore detection of these proteins can be used to confirm the identity of the cell population (Owen 1988). Using monoclonal antibodies directed at fibronectin and type-1 collagen we found that MSCs isolated by differential adhesion, all expressed these extra cellular proteins as detected by immunofluorescence staining (Figure 2.3a,c). MSCs isolated by this procedure also expressed αSMA (a vascular smooth muscle cell marker, Figure 2.3b) at high levels consistent with their relationship to perivascular cells (Gronthos et al., 2003).

A large body of evidence demonstrates that stromal tissue isolated from postnatal BM contains clonogenic progenitor cells, some of which are multi-potent with the capacity to differentiate into a range of mesenchymal cell lineages (Bruder et al., 1994; Prockop, 1997). The colonies of cells morphologically resembling fibroblasts which develop from single cells in early cultures (Figure 2.1) are described as colony forming cells (CFU-F) and are the colongenic stromal progenitor cells responsible for colony growth. These progenitor cells therefore have the capacity for self-renewal and mesodermal differentiation.

The colony forming ability of MSCs isolated using differential adhesion was evaluated by plating cells at low density (100 cells/60 cm^2 dish) and accessing colony growth 12 days later by staining cells with crystal violet and counting the number of colonies formed. The incidence of CFU-F was calculated per 100 cells and expressed as a percentage (%) (Figure 2.12b). The morphology of cells within early colonies was heterogeneous containing both spindle shaped cells and larger flattened cells

(Figure 2.3d). However, continued colony growth was the result of the proliferation of spindle shaped cells (Figure 2.3e) evident because cells with this morphology dominated late stage colony growth. These cell phenotypes dominated the centre of proliferating colonies whereas at the periphery of colonies where growth had slowed or ceased, cells with a large flattened morphology were evident (Figure 2.3f). The CFU-F frequency of P2 cells was highly variable but was within the range of 12-28%.

Functional characterization of MSCs is the gold standard of which to test the stem cell characteristics of isolated presumptive MSCs (Pittenger et al., 1999). Cells were differentiated into either the osetogenic lineage (Figure 2.5) or adipogenic lineage (Figure 2.4). To induce adipogenic differentiation, cells were transferred to a serum, dexamethosone and insulin-containing medium for 3 weeks. Lipid droplet accumulation within cells was then evaluated. The presence of lipid droplets following differentiation was confirmed by oil red O positive staining. Lipid droplets were evident after only 7 days (Figure 2.4b) but 3 weeks was necessary to achieve maximal lipid accumulation (Figure 2.4d). Control cells maintained in non-inductive growth medium were negative for oil-red-O staining (Figure 2.4a). Lipid droplet accumulation progressively increased over the 3 weeks (Figure 2.4b,c, d).

To induce osteogenic differentiation, confluent P2 cells were cultured in osteoinductive medium consisting of growth medium supplemented with β-glycerophosphate, dexamethasone and ascorbic acid (Jaiswal et al., 1997). Osteogenic differentiation was confirmed by the detection of a mineralized extra cellular matrix (Figure 2.5). Von Kossa staining was used to visualize the deposition of a calcified matrix on the surface of the culture dish as previously described (Jaiswal et al., 1997). The degree of extra cellular mineralization progressively increased from week 1 to week 3 of induction. These investigations collectively demonstrated that MSCs isolated by our procedures were capable of forming colonies (CFU-F, self renewal) and differentiating into bone and fat (multipoteniality).

2.3.3 Effect of plating density

Passage 2 rMSCs were plated at varying densities in standard culture medium (DMEM/10% FCS/L-Glutamine) on 75cm^2 plates, and cell numbers were assayed

every 3 days for 15 days. As previously reported for human (Sekiya et al., 2002), rat (Javazon et al., 2001) and mouse MSCs (Peister et al., 2004), cultures showed a lag period in which little cell growth was evident for the first 3 days of culture (Figure 2.6a). Thereafter a rapid expansion in cell number was evident (Figure 2.6a,b). The rates of expansion of the cultures were extremely sensitive to the initial plating density with significant differences in cell growth between cultures plated at different densities (Figure 2.6a,d). Rat MSCs showed decreased growth rates as the initial plating density of the culture increased (Figure 2.6a) with cultures seeded at $5cm^2$ and 10 cm^2 showing the greatest fold increase in cell number over the 15 day period (Figure 2.6b). Cell expansion was significantly less in cultures with an initial seeding density of 500 cm^2 or 1000 cm^2 however cell growth was still apparent. Cells plated at an initial density of 10 cells/cm^2 expanded 1828.3$\pm$114.4 fold over 15 days growth, in contrast to cells plated at a density of 1000 cells/cm^2 which expanded 32.5$\pm$0.9 fold over 15 days growth. Therefore low initial plating densities are associated with higher growth rates and greater fold expansion over a given time period. Consistent with these observations the total numbers of cells generated over 15 days showed the same pattern as observed for fold expansion (Figure 2.6b). The yield per cell following 15 days growth was also greater for cells plated at a density of 5 cells/cm^2 and decreased as initial plating density was increased (Figure 2.6e). We next tested the ability of single cells to reform colonies following growth at different initial plating densities. A CFU assay was used as previously described. Cells were seeded at densities ranging from 5-1000 cells/cm^2 and cultured for 15 days. On day 15, 100 cells from each culture was transferred to a $60cm^2$ dish and incubated for 14 days. After 14 days, dishes were stained with crystal violet to evaluate colony growth. There was considerable variation in the colony forming efficiency of cells grown from cultures plated at varying initial plating densities. The CFU-F efficiency ranged from 34.7$\pm$2.4 (in cells grown from seeding densities of 5 cells/cm^2) to 5.33$\pm$1.5 (in cells grown from an initial plating density of 1000 cells/cm^2). Therefore consistent with the results of expansion studies CFU-F efficiency was higher in cells grown from low initial plating densities. 1 way analysis of variance followed by Bonferroni post hoc analysis revealed that the CFU-F of cells seeded at 5 cells and 10 cells/cm^2 was significantly ($P<0.01$) higher than cultures initially seeded at 50, 100 and 500 cells/cm^2. Cultures seeded at 1000 cells/cm^2 had a CFU-F significantly ($P<0.01$) lower than all other

cultures. No significant difference in CFU-F efficiency was observed between cultures seeded at 50, 100 and 500 cells/cm^2. In summary, the CFU-F efficiency of cultures grown from high seeding densities is significantly lower than those grown from low seeding densities.

2.3.4 Correlation of growth rate with morphology

Visual evaluation of cultures of MSCs by phase contrast microscopy was used to monitor changes in the morphology of cells with changes in plating density and subsequent propagation (Figure 2.7). Evaluation of cultures as a function of both plating density and incubation time showed a transition among the three morphologically distinct cell types. Cells in cultures at low confluence are predominately spindle shaped and cultures at this stage are associated with high growth rates. As cultures reach confluence and the cell growth rate decreases, larger flattened cell types dominate the cultures. Consistent with these observations, analysis of the morphology of cultures seeded at low cell density (5 cells/cm^2) revealed cells to have a predominately spindle shaped appearance (Figure 2.6a). These cultures were associated with the greatest fold increase in cell number (Figure 2.6d). In contrast, cultures seeded at an initial plating density of 50 cells/cm^2 contained cells with a predominately star shaped morphology (Figure 2.7b). Cultures plated at a higher seeding density (1000 cells/cm^2) were dominated by cells with a flattened wide spread morphology (Figure 2.7c). These cultures were associated with a low growth rate. Morphological data therefore correlated with the overall growth rate of cultures. Cultures containing cells with a predominately spindle shaped morphology have a high proliferate capacity, whereas those cultures containing mainly flattened wide spread cells have a low proliferate capacity. Cultures established from low initial plating densities contained a higher fraction of spindle shaped cells compared to those initiated from high seeding densities, which contain a higher fraction of flattened well spread cells and have a low proliferate capacity. The presence of a high fraction of spindle shaped cells is an indicator of the proliferate capacity of the culture.

2.3.5 Effect of media composition on MSC growth

Basal media composition has been shown to have a significant effect on the expansion of MSCs isolated from adult BM (Peister et al., 2004; Javazon et al., 2001 and Sekiya

et al., 2002). To define the optimal media for expansion of cells, passage 2 rat MSCs were plated at a density of 10 cells/cm^2 in T75cm^2 tissue culture flasks and incubated in different media compositions including α-MEM, DMEM, IDDM or RPMI-1640 for 15 days. Cell counts were carried out every 3 days to assess culture growth. Significant cell growth was observed under all conditions over 15 days, however significant differences in growth rate and total fold expansion was observed. Consistent with our previous findings a lag period of growth was evident for the first 3 days under all conditions. During this period no significant difference in growth rate was observed between different basal media compositions. The relative growth rates of MSCs in different medias were DMEM > α-MEM > IDMM > RPMI (Figure 2.8a).

Cells grown in RPMI media had the lowest growth rate compared to other basal media compositions. Cells grown in RPMI media expanded 500 fold over 15 days and significant proliferation was observed at 6, 9, 12 and 15 days compared to the 3 day time point ($P<0.01$ ((for each tie point versus 3 days) 1 way-ANOVA with Dunnetts post hoc). However, the increase in proliferation with time in culture was significantly less than that achieved in other basal media conditions for the 6, 9, 12 and 15 day time points ($P<0.001$ for comparison of fold increase in cell number at each time point for each media condition; 2 –way ANOVA followed by Bonferroni post hoc test).

Cells maintained in IDMM, α-MEM or DMEM all showed significant expansion in cell number with time in culture ($P<0.01$ for comparison of fold increase in cell number at 6, 9, 12 and 15 compared to the 3 day time point; 1-way ANOVA, Dunnetts post hoc test). However, analysis of the fold increase in cell number achieved at each time point in each culture condition revealed no significant difference between cells grown in IDMM, DMEM or αMEM at 3, 6, 9, or 12 days culture (2-way ANOVA; Bonferroni post hoc comparison). Therefore growth rates were comparable between cells maintained in IDMM, αMEM and DMEM at early time points (3,6,9 and 12 days).

Analysis of the total fold increase in cell number by 15 days revealed significant differences in the proliferation under different culture conditions (Figure 2.8b). There

was no significant difference in the fold increase achieved in αMEM compared DMEM following 15 days culture although the greatest fold expansion was observed when cells were maintained in DMEM (370-fold increase in cell number compared to 330 fold increase when cells were maintained in αMEM). However the fold increase in cell number observed in cells maintained in DMEM was significantly greater than RPMI ($P<0.01$; 1-way ANOVA Bonferroni post hoc comparison) and IDMM ($P<0.01$ 1-way ANOVA Bonferroni post hoc comparison) at the 15-day time point. Therefore the greatest proliferation was observed when cells were maintained in DMEM basal media. The ability of cells to form single cell colonies (CFU-F) was analyzed following expansion in different media compositions and was assessed by plating 100 cells in a $60cm^2$ tissue culture dish following 15 days expansion. There were no significant differences in colony forming ability of cells grown in different media compositions (Figure 2.8c).

2.3.6 Effect of serum concentration on MSC growth

Serum (FCS) concentration and quality are known to have effects on the proliferation and differentiation potential of MSCs in culture (Barnes and Sato, 1980; Lennon et al., 1995). Serum used in the current study was lot selected for optimal MSC growth and differentiation potential. To determine the optimal serum concentration for growth, cells were grown in DMEM media supplemented with either 1, 2, 5, 10, 20 or 30 % FCS and cell numbers were assayed every 3 days for 15 days. Surprisingly, there was no significant difference in the growth rates between cultures for the first 12 days (Figure 2.9a). However by day 15, there were significant differences between the total fold expansion observed in each of the cultures (Figure 2.9b). MSCs grown in the presence of 10, 20 or 30% FCS displayed significant growth compared to those cultures maintained in either 1, 2, and 5% FCS ($P<0.01$ 1-way ANOVA followed by Bonferroni post hoc analysis). There was no significant difference in cell growth between cultures maintained in 10, 20 and 30% FCS. There was also no significant difference in cell growth between cultures maintained in 1-5% FCS. 10% FCS was the critical level at which significant cell growth was observed, increasing the serum concentration above 10% FCS did not result in any further increase in cell growth (figure 2.1).

CFU-F efficiency in cultures maintained in different concentrations of serum was variable (Figure 2.9c). Cultures grown in 5% FCS had a significantly greater CFU-F when compared cells grown in 1 and 2% FCS. However, cells grown in 10, 20 and 30% FCS had a significantly higher CFU-F compared to cells grown in 1, 2 or 5% FCS. There was no significant difference in CFU-F between cultures maintained in 10, 20 or 30% FCS. Therefore 5% FCS, was critical for CFU-F growth below which CFU-F growth was significantly reduced.

2.3.7 Effect of passage on MSC growth

Passage of MSCs in culture has been associated with loss of differentiation potential and proliferative capacity (DiGirolamo et al., 1999). These effects have also been reportedly associated with distinct morphological changes, which can be used to predict the expansion potential of cultures. P2 cultures (approximately 4 population doublings) contained cells with heterogeneous morphology with both spindle shaped cells, star-shaped cells and larger flattened cells were evident. The predominant phenotype in P2 cultures was spindle shaped cells (Figure 2.10a,e) and P2 cell cultures displayed a high proliferation potential. In contrast, P12 cultures displayed reduced proliferation (Table 2.1) and analysis of cell morphology revealed the presence of only large flat cells (Figure 2.10b,f). Table 2.1 shows the average population doubling time with serial passage. The average population doubling time increased with increasing passage. The average doubling time increased from 1.8 days at P2 to 13.1 days at P14. To examine the colony forming ability of cultures at different passages, P2 or P12 cells were re-plated at low density and colony growth examined 12 days later. P2 expanded cells retained the capacity to form colonies when re-plated at low cell density (Figure 2.10c). These colonies contained mainly spindle shaped cells with a high proliferative potential. In contrast, P12 cultures did not reform clearly defined colonies upon re-plating and the morphology of cells was large and flat (Figure 2.3d).

Therefore increasing passage was associated with a reduction in colony forming ability and proliferation. These effects correlated with clear changes in morphology of cells within the culture. High passage and cultures with a low proliferative potential contained large flat cells and low passage cells with a high proliferative potential

contained a high proportion of spindle shaped cells. Immuno-phenotyping for surface markers was accessed using flow cytometry to characterize cultured 2^{nd}, 8^{th} and 12^{th} passage MSCs (Table 2.2). Cells at all passages were negative for CD45 and CD11b. Expression of CD44, MHC Class I, CD56, CD29 and TRA 2-54 was high in almost all cells and remained at a constant level of expression with increasing passage. CD73 and CD90 labeling slightly decreased with increasing passage.

Examining the capacity of MSCs to generate bone and fat assessed the potency of MSCs at different passages. Cells from P2, P8 and P12 cultures were subjected to osteogenic and adipogenic differentiation. P2 MSC cultures retained the capacity to generate both bone and fat as did P8 cells as verified by oil red O (adipogenic differentiation) and von kossa staining (osteogenic differentiation). P12 cells retained only the capacity to generate bone and lost Adipogenic potential as confirmed by negative oil-red-O staining. Control P2, P8 and P12 cells not exposed to either adipogenic or osteogenic differentiation did not stain positive for either oil-red-O or von kossa staining demonstrating that MSCs maintained in culture retain their undifferentiated status and spontaneous differentiation was not evident.

PI staining and quantification by flow cytometry was used to determine the level of cell death in cultures at different passages. The number of cells, which incorporated the fluorescent exclusion dye PI, was calculated. PI is excluded from healthy cells but enters dead cells following their loss of membrane integrity. It binds to exposed DNA and becomes highly fluorescent. This fluorescence was examined by flow cytometry, which determined the number of cells, which had incorporated PI and was therefore a measure of cell death. Analysis revealed a significantly ($P<0.05$, 1 way ANOVA, bonferroni post hoc analysis) higher level of cell death in P12 cultures when compared to P2 and P8 cultures (Figure 2.12, a). Cell death increased from $7.8\pm2.3\%$ at P2 to $15.9\pm2.1\%$ at P12. To examine the effect of passage on colony forming efficiency, cells were plated at low density (100 cells/60cm^2 dish) and colony growth evaluated 14 days later (Figure 2.12b). CFU-F efficiency was significantly reduced in P8 cultures compared to P2 cells ($P>0.05$, 1 way ANOVA followed by Dunnetts post hoc analysis). CFU-F efficiency was reduced from 20.8 ± 2.2 (P2) to 10 ± 2.8 (P8). Therefore between passage 2 (4 population doublings) and passage 8 (25 population

doublings) the CFU-F efficiency was reduced by half. No colonies were detected on replating P12 cells at low density.

The differentiation potential was quantified for both Adipogenic and osteogenic differentiation of cells at different passages. Adipogenic differentiation was quantified by counting the number of oil red-O positive cells in 25 non-overlapping visual fields in three independent experiments. The number of adipocytes significantly ($P<0.05$, 1 way ANOVA followed by Dunnetts post hoc analysis) decreased from 26.8±4.5% in P2 cells to 13.3±3.8% in P8 cells (Figure 2.12c). No Adipogenic differentiation was detected in P12 cultures. Osteogenic differentiation was quantified by determining the % area of the visual field that was mineralized. 25 non-overlapping visual fields were analysed in three independent experiments. The % mineralization was approximately 35% at all passages (Figure 2.12d). There was no significant difference in the % mineralization between cultures at different passages. The effects of passage are summarized in Table 2.3.

2.3.8 Long-term growth potential of MSCs

To access the long-term growth potential of MSCs cultured under optimal conditions as defined in previous experiments, cultures derived from 5 independent donor rats were maintained in culture until growth ceased (senescence). As indicated in figure 2.13 there was a moderate variation in population doublings obtained with samples from different donors. Cultures expanded through 31-41 population doublings. The replicative potential of cultures is not reflected in their initial growth rates, which are comparable between cultures until 30-35 days in culture (after approx 25 population doublings). Cells were isolated from donors of the same age and sex as described in materials and methods. Primary cultures (P0) reached confluence in about 1 week and 2 population doublings. The average population doubling time at P0 was 1.2 days. Following growth to confluence and subsequent re-plating MSCs slowed their proliferation rate and by passage 5 (16 population doublings) the average population-doubling rate was 3 days. At passage 10 (approx 30 population doublings) was average population doubling time had increased to 8.9 days. Therefore with time in culture and increased passage (re-plating) the proliferation rate progressively decreased as indicated by a progressive increase in the average population doubling

time. The average population doubling time increased from 1.2 days (P0, 2 population doublings) to 13.1 days by passage 14 (39 population doublings).

2.3.9 Effect of growth factors on the expansion of MSCs in culture

To overcome the loss in differentiation potential and reduction in proliferation with serial passage in culture, MSCs were cultured in the presence of a number of different growth factors including either bFGF, EGF, PDGF and LIF at concentrations of 0.2-1 ng/ml. With the exception of growth factor supplementation the cells were cultured under identical conditions to those used for previous studies. P2 MSCs were plated at 10 cells/cm^2 in DMEM supplemented with 10% FCS (control). In parallel cultures, the media was supplemented with either bFGF (FGF-2), EGF, LIF or PDGF at a concentration of 0.2, 0.5 or 1 ng/ml. Cell number was assayed every 3 days for 15 days (Figure 2.14). Following plating initial cultures (0-9 days) supplemented with growth factors displayed increased growth rate when compared to control cells for all conditions tested (Figure 2.14). The total cell number at each time point for each culture condition was greater than the cell number achieved when cultures were maintained under standard conditions (control). This increased growth rate in response to growth factor supplementation was a dose dependent response. As the concentration of growth factor increased from 0.2-1 ng/ml the growth rate of the cells increased as indicated by an increased slope on the graph with increasing growth factor concentration. The pattern of this effect and concentration response was consistent for all growth factors examined. Despite the pattern of changes in cell number been consistent between conditions the absolute number of cells attained under each condition was significantly different. At a maximal concentration of 1 ng/ml the cell number following 9 days culture under each culture condition was greatest in FGF-2 > PDGF > EGF > LIF. FGF-2 treated cultures contained 6519$\pm$766 cells at 9 days compared to 1246$\pm$116 in LIF treated cultures. During expansion in growth factor supplemented media cells maintained a constant immuno-phenotype comparable with cells maintained under standard growth conditions (Table 2.4).

Analysis of the total fold increase in cell number at 15 days showed significant variation in the expansion potential of MSCs cultured in the presence of different growth factors. Treatment of cultures with either bFGF, PDGF or EGF all

significantly increased cellular proliferation compared to cultures maintained under standard growth conditions (Figure 2.15). FGF treatment was only effective at 0.5 and 1 ng/ml. Fold increase in cell number was significantly increased in these cultures ($P>0.01$; 1 way ANOVA, Dunnetts post hoc test) but not in cultures maintained in media supplemented in 0.2 ng/ml bFGF when compared to control cultures. Cell expansion in response to FGF-2 treatment was therefore dose dependent. Cell number increased approximately 4000 fold in the presence of 0.5 ng/ml FGF-2, 6000 fold in the presence of 1 ng/ml bFGF compared to the 2000 fold increase in cell number observed under standard culture conditions following 15 days growth in culture. A similar pattern was observed in cultures treated with PDGF but the fold increase in cell number was less overall. At 0.5 ng/ml PDGF resulted in approximately 4000-fold increase in cell number and 1 ng/ml a 4500-fold increase in cell number. Therefore treatment with 0.5 or 1 ng/ml PDGF significantly ($P>0.01$, 1-way ANOVA, Dunnetts post hoc test) increased fold expansion in cell number compared to control conditions. Treatment with 0.2 ng/ml PDGF did not significantly increase cell number compared to control. EGF resulted in a significant increase in cell number when used at a concentration of 1 ng/ml. Although treatment of cultures with LIF appeared to increase initial growth rates following plating analysis of cell number at 15 days did not reveal any significant difference in fold increase in cell number for any concentration of LIF. Therefore addition of LIF (0.2-1 ng/ml) did not result in any significant additional increase in cell number compared to control cultures. In summary, addition of either bFGF (at 0.5 or 1 ng/ml), PDGF (at 0.5 or 1 ng/ml) or EGF (1 ng/ml) resulted in a significantly higher fold increase in cell number over 15 days growth in culture compared to cultures maintained under standard growth conditions.

Following the demonstration that MSCs cultured in the presence of FGF, EGF and PDGF show increased proliferation, we next attempted to analyze whether this increased proliferation was the result of the proliferative activity of stromal progenitor cells (CFU-F). It is possible that the addition of growth factors provides the appropriate trophic support to retain the long-term progenitors that support long-term proliferation in cultures. We therefore examined the incidence of CFU-F in cultures which were isolated and immediately cultured in media supplemented with either

FGF-2, EGF, PDGF or LIF all at 1 ng/ml. 100 P2 cells isolated and cultured under these conditions was transferred to 60cm^2 tissue culture dishes and maintained for 14 days, following which the number of colonies formed was determined (Figure2.17a). The CFU efficiency of control treated cultures was 22.6±0.67 %. The incidence of CFU-F in FGF-2 treated cultures was significantly higher (30.33±1.33 %; P>0.05 1-way ANOVA Dunnetts post hoc test) compared to control cultures. In addition, the incidence of CFU-F in cultures maintained in the presence of LIF was also significantly greater (32.67±2.91; P>0.05 1-way ANOVA, Dunnetts post hoc test) than cells maintained under control conditions. The incidence of CFU-F in cultures maintained either in EGF or PDGF was not significantly different from the control cultures. Therefore the presence of either FGF-2 or LIF significantly increased the incidence of CFU-F when examined at passage 2.

The size of colonies was then determined in order to examine the proliferative potential of cells isolated and maintained under these conditions (Figure 2.17b). The diameter of CFU-F colonies was measured and colonies were placed in 3 size categories: 2-3, 4-5 or 6-7 cm. There was a large degree of variation in the size of colonies obtained under each culture condition examined. Of the 60 colonies analysed from cells cultured in the presence of 1ng/ml LIF, 43 colonies (71.7%) were between 2-3cm in diameter. Only 14 colonies were between 4-5cm (23.3%) and 3 colonies (5%) between 6-7cm. In contrast, the majority of colonies derived from FGF-2 treated cultures were large colonies. 6.7% (4 colonies) of colonies were between 2-3cm in diameter, 31.7% (19 colonies) were between 4-5cm in diameter and 61.7% (37 colonies) were between 6-7cm. In PDGF treated cultures the predominant colony size was between 6-7cm (51.7%) whereas in EGF treated cultures the predominant colony size was between 4-5cm (52%). Very few colonies from EGF (18.3%) treated cultures or PDGF (16.7%) treated cultures were between 2-3%. 31.7% of colonies derived from PDGF treated cultures were between 4-5% and 51.7% of colonies derived from EGF cultures.

This data indicates that LIF and/or FGF-2 supplementation of media in early passage cultures maintains a higher incidence of CFU-F when compared to cultures maintained under standard culture conditions. However, consistent with our previous

results CFU derived from cultures maintained in the presence of 1ng/ml LIF has only a low cell turn over with low replicative potential. It therefore appears that LIF may provide trophic support that maintains the MSC stem cell pool *in vitro* but does not provide signals that promote proliferation. The proliferative potential of colonies derived cells grown in the presence of LIF was less than that observed for control cells with 25% of control colonies between 6-7cm compared to 5% of colonies derived from LIF cells. This suggests that whilst addition of LIF may provide survival signals that maintain a higher number of CFU-F with propagation in culture it may also inhibit the proliferation of these cells promoting only a low CFU cell turnover.

2.3.10 Effect of passage on CFU-F potential when cells are cultured in growth factor supplemented media

As described previously serial passage is associated with a reduction in CFU-F potential. To examine whether addition of particular growth factors to the basal media retains CFU-F potential, cells maintained in either 1-ng/ml bFGF, EGF, PDGF and LIF were re-plated at low cell density and colony growth accessed after 14 days (Figure 2.17). Analysis revealed that there was no significant difference in CFU-F efficiency between P2 and P8 cells in all growth factor supplemented conditions. In contrast colony formation was virtually undetectable in P12 cells cultured under either standard culture conditions in or in the presence of 1 ng/ml EGF, PDGF or LIF. However cells continually cultured in the presence of 1 ng/ml FGF retained CFU-F potential at levels comparable with P8 cell cultures.

2.3.11 Differentiation potential of MSCs maintained in growth factor supplemented media following serial passage in culture.

Cells were propagated under defined and optimal growth conditions in growth factor (either 1ng/ml EGF, FGF-2, PDGF or LIF) supplemented media. At P2 a sample of cells were cultured either in osteogenic medium or adipogenic medium for 3 weeks (Figure 2.18). Osteogenic differentiation was confirmed by von kossa staining and adipogenic differentiation was verified by oil-red-O staining. Positive von kossa and oil red-O staining was detected in all culture conditions. Consistent with previous findings early passage cultures of MSCs differentiated into fat and bone regardless of the addition of growth factors to the growth medium. However, P12 cells grown

continually in 1ng/ml EGF or PDGF or LIF supplemented media only differentiated into bone and lost the ability to differentiate into fat. The exception however, was P12 cells grown continually in the presence of 1ng/ml FGF-2. These results show that only FGF treated cells retained their capacity to differentiate into multiple cell derivatives following serial passage in culture.

2.3.12 Lifespan of cultures maintained in growth factor supplemented media

The lifespan of cultures maintained in growth factor supplemented media was analyzed by recording the number of population doublings before senescence under defined culture conditions. Cells were first isolated from 5 animal donors and cells from each donor were grown in the presence of either 1ng/ml bFGF, EGF, PDGF or LIF under standard tissue culture conditions. Control cultures were grown under standard culture conditions. The average lifespan of cells maintained under control conditions was 32.6 population doublings; in contrast, the average lifespan of bFGF treated cultures was 41.8, EGF, 34.1, PDGF, 36.3 and LIF 39.3 population doublings (Table 2.5). Therefore, growth factor supplementation resulted greater expansion potential prior to senescence. Consistent with the results of CFU-F assays, LIF and FGF treated cultures displayed the greatest expansion potential. Donor variation in the expansion potential of isolated cells can be excluded as the cause since cells from each individual donor were all plated under the conditions to be tested. Some degree of donor variation was evident in the response to growth factors. For example analysis of donor 1 cultures revealed that the lifespan for LIF treated and PDGF treated cells was not any greater than cultures maintained under control conditions. Despite these small variations, in general the expansion potential was greater in growth factor supplemented media.

2.4 Discussion

Presumptive isolation of MSCs has been described for a number of different species including: human, mouse, rat, dog, pig, sheep, goat, cat and rabbit. In these studies MSCs were isolated by selective adherence to tissue culture plastic as described by Friedenstein, 1974 and shown to differentiate into cell progeny of multiple mesenchymal cell lineages including bone, fat and cartilage. Despite the great interest

in MSCs there is still no well-defined protocol for the prospective isolation and expansion of these cells in culture. This difficulty is compounded by the lack of definitive cell surface markers. Most experiments have been carried out with cultures of MSCs that are isolated primarily by their tight adherence to tissue culture dishes. Several groups of investigators have developed protocols to prepare more homogenous populations, but none of these protocols has gained wide acceptance (Pittenger et al., 1999; Gronthos et al., 2003; Colter et al., 2000; Smith et al., 2004).

In the present study, we first present data concerning the isolation of a fibroblastoid cell population from adult rat BM. This cell population displayed characteristics in culture, which allowed them to be classified as MSCs operationally. The cells sustain prolonged self-renewal in culture maintaining their ability to differentiate into an osteogenic phenotype after >35 population doublings. A proportion of these cells (ranging from 22-35%) were capable of forming colonies plated at low cell density. The incidence of cells which give rise to these colonies (CFU-F), which represent the clonogenic progenitor cells, some of which are considered multipotent, was lower than that previously reported in some studies for rat stromal cell cultures (Javazon et al., 2001), however the incidence was consistent with that reported by both mouse and human MSCs cultured under similar conditions (Sekiya et al., 2002; Peister et al., 2004). This cell population also expressed a number of MSC associated cell surface markers including: CD44, CD56, CD29, TRA 2-54 and consistent with their undifferentiated status expressed high levels of CD90 and CD73. In addition, hematopoietic contamination was eliminated from the culture by 2-3 weeks as demonstrated by the absence of hematopoietic markers CD45 and CD11b. The cells were also positive for the extracellular markers; collagen-1, fibronectin and smooth muscle actin. More importantly these cells could differentiate into osteogenic and Adipogenic cell lineages when induced under defined culture conditions. Osteogenic differentiation was confirmed by the deposition of a mineralized extra cellular matrix on the culture dish by Von Kossa staining. Adipogenic differentiation was confirmed by the accumulation of oil-red-O positive lipid droplets within differentiated cells. This multipotency and self-renewal allowed for an operational definition MSCs.

Whilst MSCs have been isolated successfully from a number of different species and a number of postnatal organs, there are a number of species and strain differences in the properties of MSCs and relatively subtle differences in the protocols for isolation and expansion, which have made results from experiments conducted in different laboratories difficult to compare (Peister et al., 2004). Optimal expansion of MSCs during culture is still difficult to achieve since they tend to loose both their differentiation potential and proliferative capacity when cultured under standard conditions (10% DMEM on tissue culture plastic) (DiGirolamo et al., 1999).

In the current study we describe optimal conditions for the expansion of MSCs isolated from adult rat BM. Whilst the conditions used were optimized solely for MSCs isolated using our protocols, they highlight the importance of optimizing growth conditions and are consistent with many of the properties reported for expansion and culture of mouse and human MSCs (Sekiya et al., 2002; Javazon et al., 2001; Peister et al., 2004). Consistent with previous reports, rat MSCs were prepared by plating all marrow cells at high density and incubating them until cultures reached about 90% confluence before expanding cells at low density. In contrast, mouse MSCs have proved more challenging to isolate and expand in culture, a technical difficulty that until recently, limited their use in transgenic mouse studies (Phinney et al., 1999). Human MSCs are reportedly isolated under the same conditions except a density gradient is used to isolate the mononuclear cell fraction, which is subsequently plated at high density (Sekiya et al., 2002).

In order to establish the optimal conditions for the expansion of MSCs in the present study, cells were first plated at varying initial seeding densities, and the fold expansion in cell number was assayed 15 days later. The rat MSCs isolated in this study were similar to mouse and human MSCs in that they expanded more readily at low plating density, an observation also consistent with previous reports for rat MSCs (Javazon et al., 2001). The cells expanded more rapidly if plated at very low initial plating densities, they readily formed single cell derived colonies and differentiated into osteogenic and Adipogenic cell lineages. The CFU-F potential (incidence of CFU-F) was greater in cells plated at low cell density. This indicates that low initial plating densities not only promote cell growth but also promote the growth of

clonogenic progenitor cells responsible for the differentiation potential of MSC cultures and their long-term propagation. The incidence of CFU-F has previously been shown to be dramatically affected by small changes in plating density for rat MSCs (Javazon et al., 2001). In contrast, human MSCs also expand more rapidly when plated at low cell density, but plating density had little effect on the incidence of CFU-F (Sekiya et al., 2002). We therefore determined that the optimal initial plating density for expansion was very low plating densities between 5-10 cells/cm^2. Under these optimal growth conditions, rat MSCs could expand >1800 fold following 15 days culture. Human MSCs have been reported to have a much lower expansion potential even when cultured at low plating densities (Prockop et al., 2003). The expansion potential of rat MSCs in this study was comparable with that reported for mouse MSCs plated at low cell densities (Peister et al., 2004).

There have been several studies in human and rat MSCs which have described conditions for the optimal growth of these cells and have described evidence for a link between expansion potential and the morphology of certain subpopulations of MSCs within the cultures (Sekiya et al., 2002; Javazon et al., 2001). MSC cultures are known to be morphologically heterogeneous in culture and Mets and Verdonk 1981 reported the presence of two morphologically distinct cell types. Type II cells were identified as large flat cells that propagated only very slowly in culture, in contrast to Type I cells, which are smaller spindle shaped cells which proliferated rapidly in culture. Colter et al., 2000 identified a subpopulation of cells in cultures of human MSCs that are small and proliferate rapidly, undergo cyclical renewal and when plated at low density are the precursors of more mature cells in the same cultures. These cells have been refereed to as recycling stem cells (RS-1 cells). Flow cytometry analysis revealed that stationary cultures of MSCs contained a large proportion of large flat cells and only a minor population of small agranular cells (RS-1 cells). As cultures approach senescence, large flat granular cells become the dominant phenotype. In rapidly proliferating cell cultures, the dominant cell phenotype is RS-1, which are small and agranular cells. In the present study, no flow cytometry analysis was carried out specifically to look at the proportions of these cell types. Visual evaluation of cultures initiated from different plating cell densities demonstrated

changes in the morphology of cells with time in culture, and between cultures initiated from different seeding densities.

Evaluation of cultures as a function of both time in culture and initial plating density revealed the transition among three morphologically distinct cell types: thin spindle shaped cells, star-shaped cells and large flattened cells. High initial plating densities were associated with low expansion potential and cultures contained predominately large flat granular cells with low proliferative potential. In contrast, cultures plated at low initial plating densities were associated with high expansion potential and contained a very high proportion of spindle shaped cells, which were the predominant cell phenotype. Therefore, we conclude that low initial plating densities allow for the growth of spindle shaped cells, which have a high proliferative potential. The effect of plating density on the growth of cells may be the result of cell to cell contact signaling or due the release of factors into the media, but this is still under investigation. In summary, there is a correlation between expansion potential and the proportion of spindle shaped cells in the culture. Conditions that maximize the growth of these small spindle shaped cells maximizes the expansion potential of MSC cultures.

Basal growth media composition and serum (FCS) concentration also had a significant effect on the expansion potential of MSCs even when plated at low initial plating densities, consistent with previous findings (Peister et al., 2004; Jaiswal et al., 1997). Optimal MSC growth was achieved when cells were cultured in DMEM media, however, comparable expansion in cell number was achieved when cells were grown in the presence of IDMM and αMEM although growth rates were greater in DMEM media. Growth was however, significantly reduced when cells were grown in RPMI media. This media has been used as a growth medium in early cultures of mouse MSCs to retard the growth of hematopoietic contaminants. Basal media composition had no effect on CFU-F potential. Serum concentration and quality have been reported to have significant effects on the growth potential of MSCs (Barnes and Sato, 1980; Lennon et al., 1995). We found that a minimum concentration of 10% FCS was required for the growth of MSCs. This concentration was also required for growth of CFU-F in colony assays and the incidence of CFU-F was significantly reduced if the serum concentration was less than 10%. Increasing the serum

concentration above 10% had little additional effect on growth, or CFU-F potential and 10% FCS has previously been reported to be effective in promoting MSC growth but retarding the growth of hematopoietic contaminating cells, which require higher serum concentrations for growth (Prockop, 1997).

Analysis of the growth kinetics of MSC cultures maintained under optimal growth conditions revealed that cultures can be grown to approximately 30-40 population doublings before senescence (cessation of growth). At senescence, cells still remain viable but growth ceases. The initial growth rate was comparable between cultures and therefore initial growth did not reflect long-term growth potential. The expansion potential of rat MSCs in this study was greater than the replicative potential previously reported for human MSCs (DiGirolamo et al., 1999; Banfi et al., 2000). Human MSCs have been expanded for approximately 20-30 population doublings (Banfi et al., 2000) and mouse MSCs can be expanded for approximately 20-30 population doublings (Tropel et al., 2004). The reduction in growth rate when cells reach confluence and are subsequently re-plated is consistent with previous findings for both mouse and rat MSCs (Javazon et al., 2001; Sekiya et al., 2002). Concurrent with the slowing of growth rate, MSCs showed a change in appearance, from the initial spindle shape to the more flattened morphology which dominates slowly replicating cultures. Expansion of adult BM derived stem cells beyond the hayflick limit for primary cultures of 50 population doublings (Hayflick and Moorhead, 1961) has been reported in MAPC and MIAMI cells. However, there is no evidence that these cells are present in our MSC cultures since isolation of these cells requires highly defined conditions not consistent with standard MSC culture (Jiang et al., 2002a; D'Ippolito et al., 2004).

Serial passage of MSCs is associated with a loss in multipotentiality and expansion potential (DiGirolamo et al., 1999; Banfi et al., 2000). In the present study, morphological analysis of the effects of passage revealed that early passage (P2) cultures that displayed a high proliferation potential with an average population doubling time of 2.3 days contained a high proportion of spindle shaped cells and a comparatively lower proportion of large flat cells. In contrast late passage cultures (P12) contained mainly large flat, granular cells with low proliferation potential.

Therefore as MSCs are propagated in culture over multiple passages, growth potential is lost even under conditions that promote optimal expansion in early cultures. The loss of growth potential correlates with the appearance of large flat cells, which become the dominant phenotype. Therefore the conditions used in this study and in other studies are not optimal for the long-term propagation of CFU-F.

Consistent with this observation we also found that colony development was not detected in colony assays of P12 cells and was significantly reduced in P8 cultures. The average population doubling time increased from 1.2 days at P0 to 13.2 days at P14. MSCs also lost their ability to differentiate to adipogenic cell phenotypes by P12 but retained their ability to generate osteoblasts consistent with previous reports that the osteogenic differentiation pathway is the default pathway and adipogenic differentiation potential is lost first (Maurglia et al., 2000). The reason for a slight decrease in the expression of CD73 and CD90 with prolonged time in culture is unknown. However, CD90 is known to be expressed on undifferentiated MSCs and lost with commitment to differentiation (Chen et al., 1999) and therefore it may indicate spontaneous differentiation with increasing passage in culture. However, no differentiation into bone or fat was detected in these cultures. Changes in cell surface marker expression have reported in rat MSCs with increased passage in culture (Zohar et al., 1997; Vogel et al., 2003).

To overcome the problems of reduced multipotentiality and loss of expansion potential in culture, investigators have used a number of approaches. One approach is the supplementation of the growth medium with mitogenic agents that increase self-renewal of MSCs. An increasing number of growth factors and hormones have been shown to regulate CFU-F proliferation *in vitro*. PDGF has been shown to be a potent mitogen for CFU-F by numerous investigators (Castro-Malaspina et al., 1981; Gronthos and Simmons, 1995). EGF has been shown to increase colony size and reduce the spontaneous expression of alkaline phosphatase (a marker of osteogenic differentiation) and therefore maintaining the undifferentiated cell state (Owen et al., 1987) in CFU-F colonies isolated from rabbit BM. EGF was shown to be a mitogen for an enriched (STRO-1+) population of human CFU-F under serum free culture conditions (Gronthos and Simmons, 1995). Similarly, bFGF (FGF-2) is a potent

mitogen for CFU-F isolated from multiple species including mouse, rat and human resulting in extended growth of cells in culture (>50 population doublings) (Tsutsumi et al., 2001). More importantly, cells maintained their differentiation potential during this extended growth in culture. PDGF and EGF have also been shown to act synergistically when added to growth medium (Gronthos and Simmons, 1995). Both stimulate equivalent numbers of colonies but when added together significantly increased average colony size compared to that achieved when either mitogen was added alone. LIF has also been shown to regulate the self re-newal of MSCs in culture (Majumdar et al., 1998).

In the current study, the ability of growth factor supplementation to increase the expansion potential of MSCs without loss of potency was evaluated. FGF-2, EGF, PDGF and LIF were all examined. MSCs grown in the presence of FGF-2, EGF or PDGF all showed increased growth rates with significantly greater fold expansion in cell number evident at 15 days, when compared to cultures maintained under standard growth conditions. For all three growth factors the effect on expansion potential and growth rate was dose dependent. Growth rate of early cultures (1-9 days) increased with increases in growth factor concentration. Examination of fold increase in cell numbers at 15 days revealed that bFGF and PDGF were only effective at concentrations of 0.5-1 ng/ml and EGF was only effective at 1 ng/ml. At these concentrations there was a significantly greater expansion in cell number at 15 days compared to cultures maintained under standard growth conditions for this period. LIF supplementation did not result in a significant increase in fold expansion in cell number at any of the concentrations tested compared to control cultures, although there was a slight dose dependent increase in growth rate following initial plating.

Cells cultured in the presence of FGF-2 showed the greatest fold increase in cell number and long term FGF-2 treatment increased the lifespan of cultures. Consistent with this observation a number of studies have reported that supplementation of the culture media with FGF-2 at concentrations ranging from 0.1-1 ng/ml increases the proliferation of MSCs, which retain their differentiation potential (Tsutsumi et al., 2001). In this present study, we found that the loss multipotentiality observed with serial passage in culture was not evident in cells maintained in FGF supplemented

media. Under these conditions cells differentiated into both osteogenic and adipogenic cell lineages even when propagated to P12. In contrast, under all other culture conditions tested, cells lost their adipogenic differentiation potential. The degree of osteogenic differentiation observed in P12 cells was also higher when these cells were maintained long term in FGF-2 supplemented media.

The incidence of CFU-F in cultures is the primary determinant of long-term replicative potential (DiGirolamo et al., 1999). However CFU-F incidence did not predict the loss of multipotentiality with serial passage in culture. Samples with high CFU-F efficiency have the greatest replicative potential and consistent with this observation we found colony growth was the result of the proliferation of spindle shaped cells. We found that cells cultured in the presence of either FGF-2 or LIF from the time of isolation displayed a higher incidence of CFU-F when tested at P2. In addition, the loss of CFU-F potential with serial passage was not observed in cultures maintained in FGF-2 supplemented media in which the incidence of CFU-F at P12 was comparable with that observed at P2. This observation is consistent with the finding that FGF-2 treated cells retain their multilineage differentiation potential and expansion potential with serial passage in contrast to cells maintained under standard culture conditions. In summary, CFU-F potential is a good predictor of the growth potential of MSCs in culture and their ability to differentiate into mesenchymal cell lineages. FGF-2 was shown to increase colony size compared to control cells consistent with its ability to promote the growth of CFU-F in culture.

Ex vivo expansion is necessary to obtain a sufficient number of cells for cell replacement therapies. However, we are only recently starting to understand the effects of culture on stem cell potentiality and self-renewal. Developing protocols that overcome the loss in potentiality and expansion potential with prolonged culture will be vital in the future to provide adequate cell numbers for therapy.

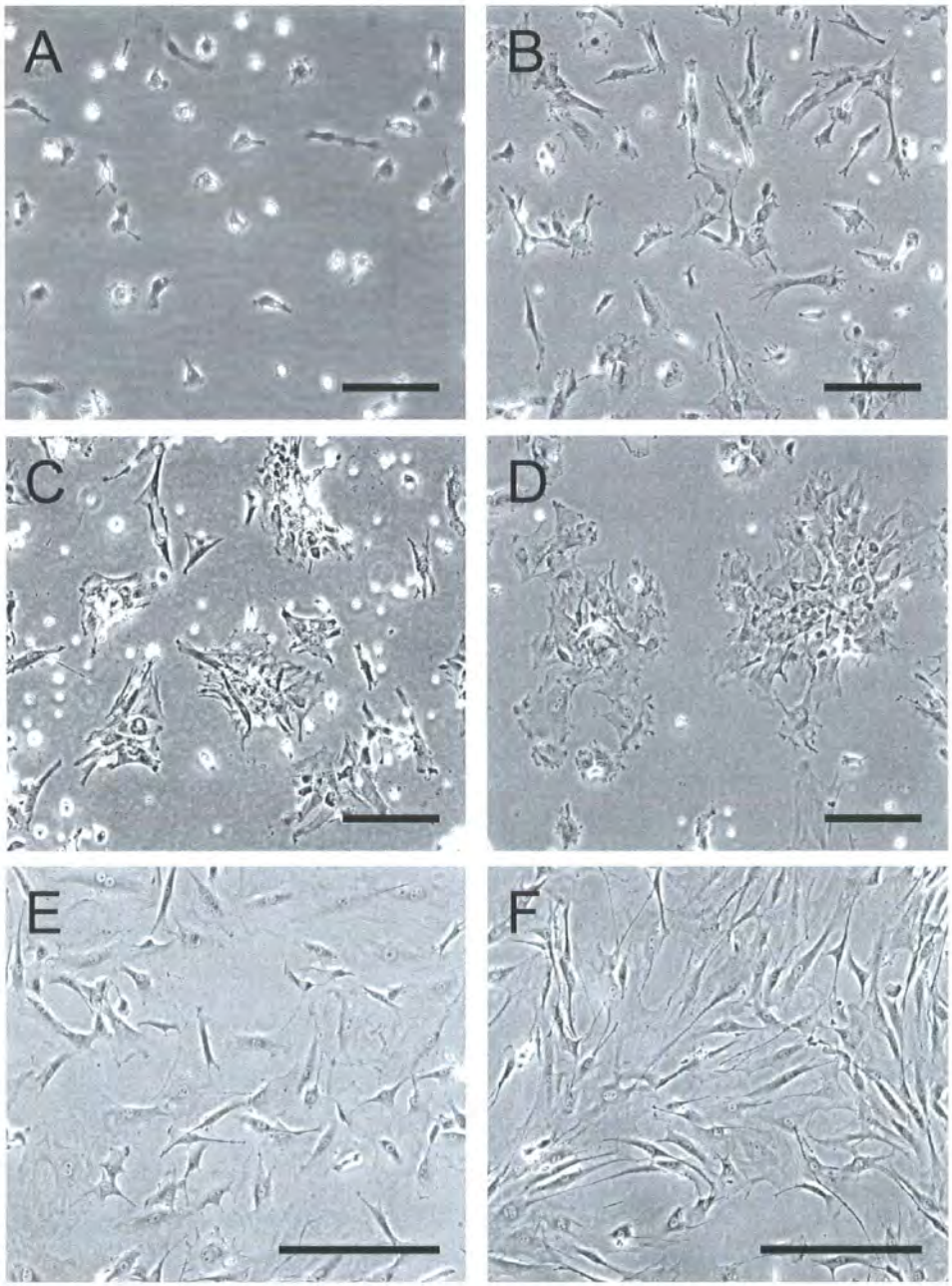


Figure 2.1: MSCs harvested from postnatal rat BM and isolated by differential adhesion to tissue culture plastic. Whole BM aspirate was re-suspended in culture media and plated at high density (100,000 cells/cm^2) in a T75 cm^2 culture flask. (**A**) P0 MSCs adhered to the tissue culture plastic as seen at 12 hours. (**B**) At 48 hours adherent cells displayed a fibroblastic morphology and could be readily distinguished from non-adherent cells, which were highly reflective and remained in suspension in the culture media. (**C**) Single cell derived colonies of 10-20 cells were evident at 5 days of culture. These colonies increased progressively increase in size. (**D**) By 12 days colonies were 150-200 cells in size. Cultures were grown to >80% confluence (5-6 days). (**E**) P0 cells were passaged and re-plated at 1,000 cells/cm^2 (P1). (**F**) P1 rMSCs at 70% confluence 3 days following first passage. Spindle shaped cells dominated the culture at P1. *Scale bars are 50 μm.*

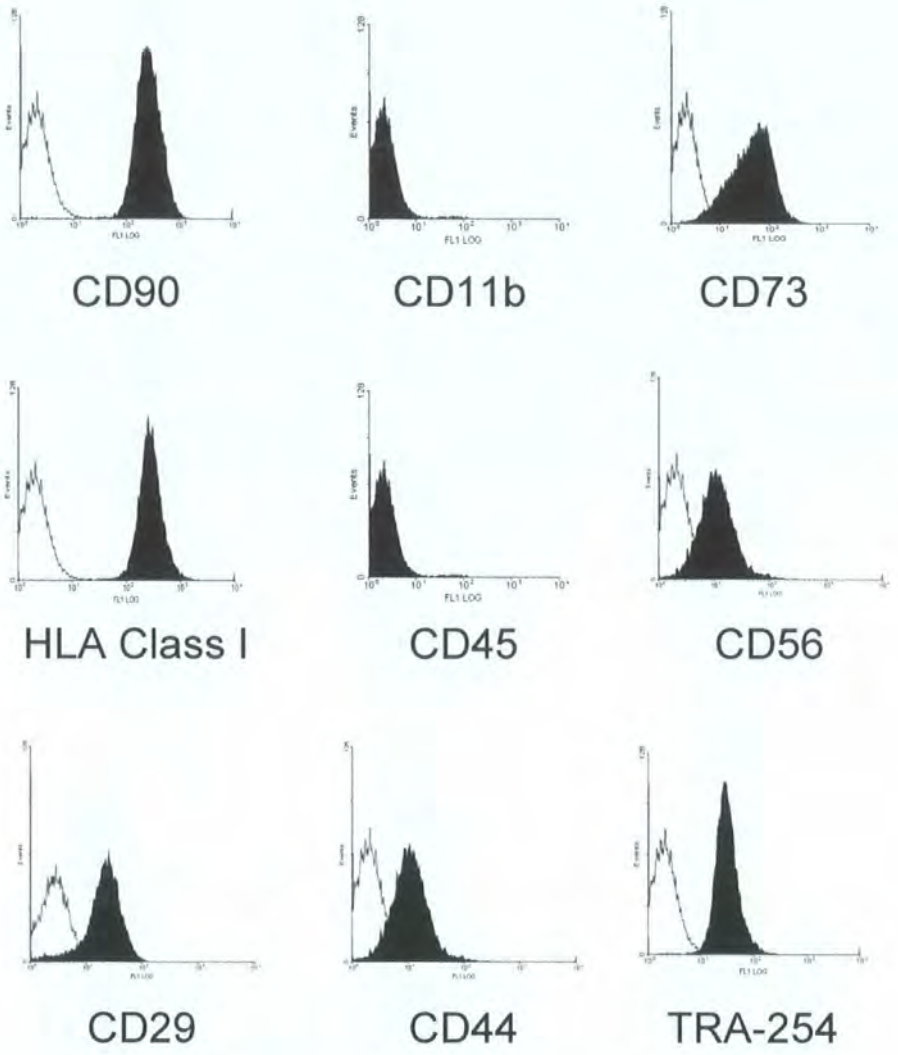


Figure 2.2: Epitope analysis of passage 2 postnatal rat MSCs. Cell surface antigen expression of passage 2 rat MSCs was accessed by immuno-fluorescence detected by flow cytometry. Cytometric traces show the fluorescence intensity (x-axis) and the number of cells (y-axis). Characterisation of P2 rat MSCs was examined by employing monoclonal antibodies directed against CD44 (Hyaluronan receptor), CD90 (Thy 1.1), CD45 (leukocyte common antigen) and alkaline phosphatase (non-specific isotype) (TRA-2-54), CD73 (Ecto-5-nucleotidase), CD11b (MAC-1), HLA-Class I, CD29 (VLA beta chain) and CD56 (N-CAM) to access the expression of these surface markers (solid peaks). A primary isotype matched antibody control was included in each analysis to exclude non-specific binding and background fluorescence (open peaks). MSCs were positive for CD90, HLA class I, TRA-2-54 and negative for the haematopoietic lineage markers CD45, CD11b. In addition, cultures were positive for CD73, CD56, CD29 and CD44 although heterogeneous expression was found with these markers.

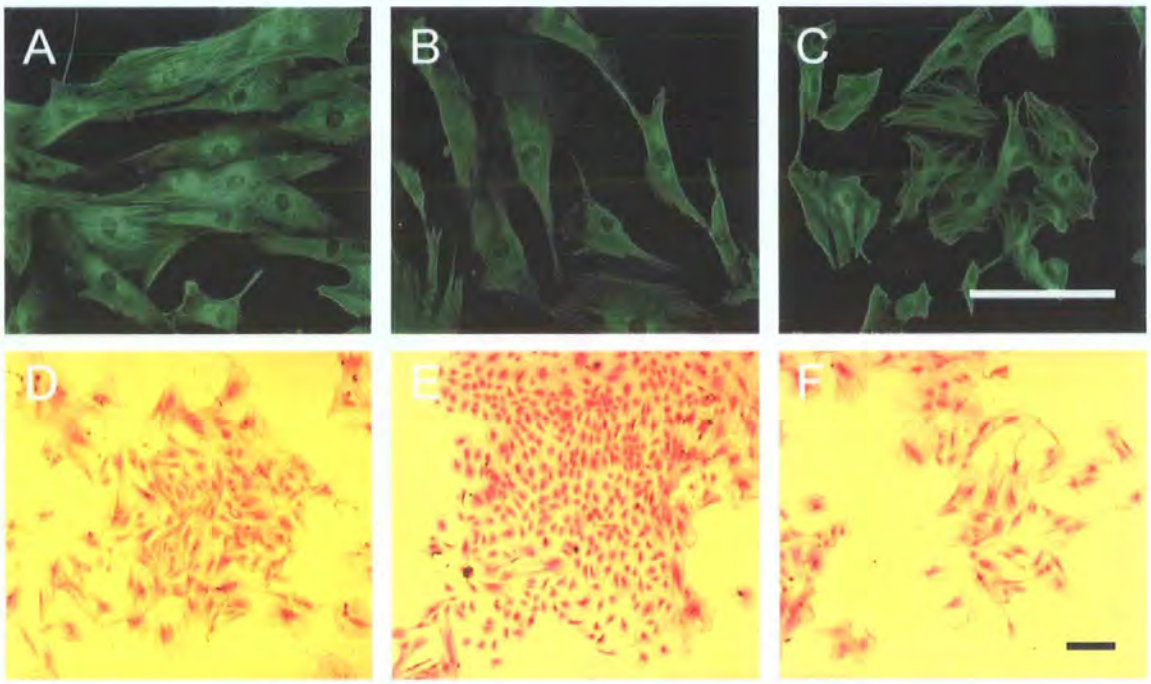


Figure 2.3: Morphological and immuno-cytochemical analysis of P2 rat MSC. P2 MSCs grown on tissue culture plastic were fixed in 4% PFA and processed for immuno-cytochemical analysis of mesodermal proteins using standard procedures. Following immuno-detection using monoclonal antibodies, P2 rat MSCs were found to express fibronectin (**A**), smooth muscle actin (**B**) and collagen-1 (**C**). The expression of these proteins was homogenous within the culture and consistent between cultures derived from animal donors (n = 8). The expression of these proteins is consistent with a mesodermal phenotype. For morphological analysis colony forming assays (CFU-F) were performed. 100 cells were transferred into 60cm^2 dishes and cultured for 12 days, methanol-fixed and stained with 0.5% crystal violet. MSCs following isolation and propagation retained the capacity to form single cell derived colonies (**D**). These early colonies and early passage cultures were dominated by spindle shaped cells (**E**). Cells with a flattened, more wide spread morphology are found at the periphery of colonies and are more evident as cultures reach confluence (**F**). *Scale bars 50 μm.*

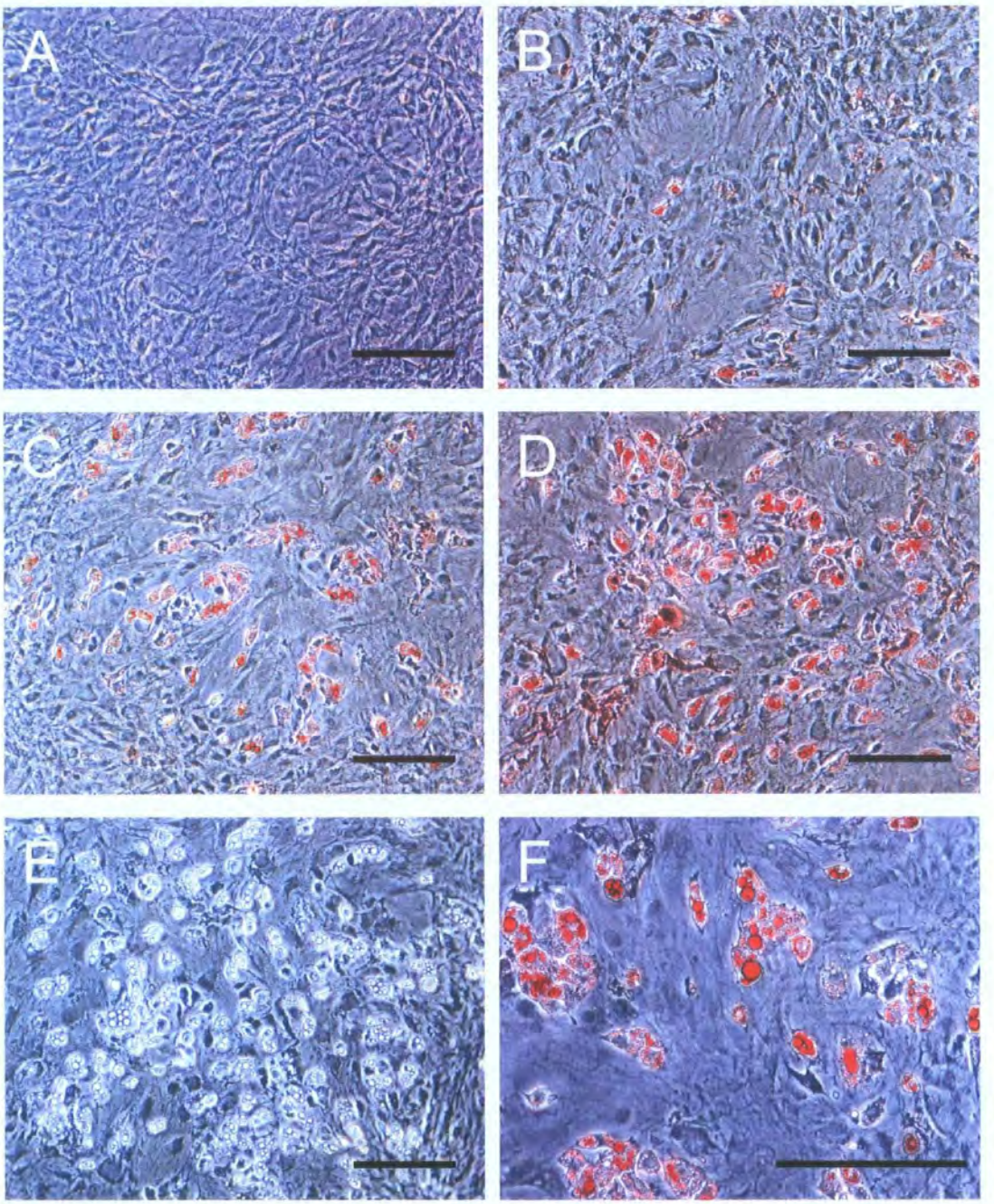


Figure 2.4: Adipogenic differentiation of P2 MSCs isolated from rat postnatal BM. Rat MSCs were cultured as mono-layers in standard culture medium (DMEM 10% FCS) and allowed to become confluent (**A**). Adipogenic differentiation was initiated by three cycles of induction/ maintenance culture. Each cycle consists of 3 days of culture in induction media followed by 2 days of culture in the maintenance media. Adipogenic differentiation was demonstrated by the accumulation of lipid vesicles (**B-F**) confirmed by oil red O histochemical staining (**B,C,D,F**). Multiple treatments resulted in increasing numbers of adipocytes, as shown by oil red O staining at 1 week (**B**), 2 weeks (**C**) and 3 weeks (**D**) differentiation. Phase images were also obtained prior to staining to confirm presence of vacuoles within cells (**E**). Higher magnification (x20) reveals single MSCs with extensive accumulation of oil red O positive vacuoles (**F**). *Scale bars: 50μm.*

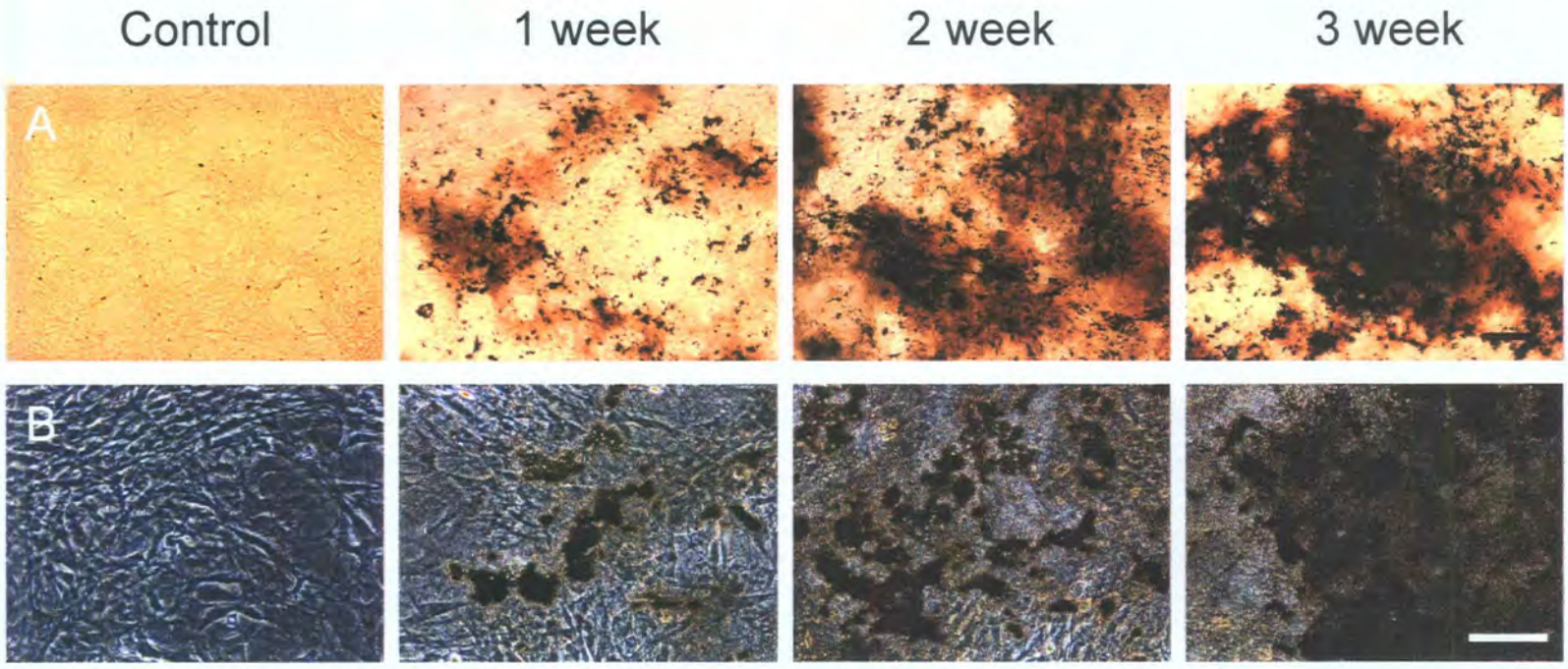


Figure 2.5: Osteogenic differentiation of P2 MSCs isolated from rat postnatal BM. Rat MSCs were cultured as mono-layers in standard culture medium (DMEM 10% FCS) and allowed to become confluent. Seeding cells at a density of 3 x 10^3 cells/cm^2 and maintaining cells in an osteogenic induction medium initiated differentiation. Osteogenic differentiation was demonstrated by the clacification of the extracellular matrix (**A x5 magnification, B, x10 magnification**) confirmed by Von Kossa histochemical staining. Continuous treatment resulted in an increasing area of calcification, as shown by Von Kossa staining at 1 week, 2 weeks, and 3 weeks differentiation. *Scale bars: 50μm.*

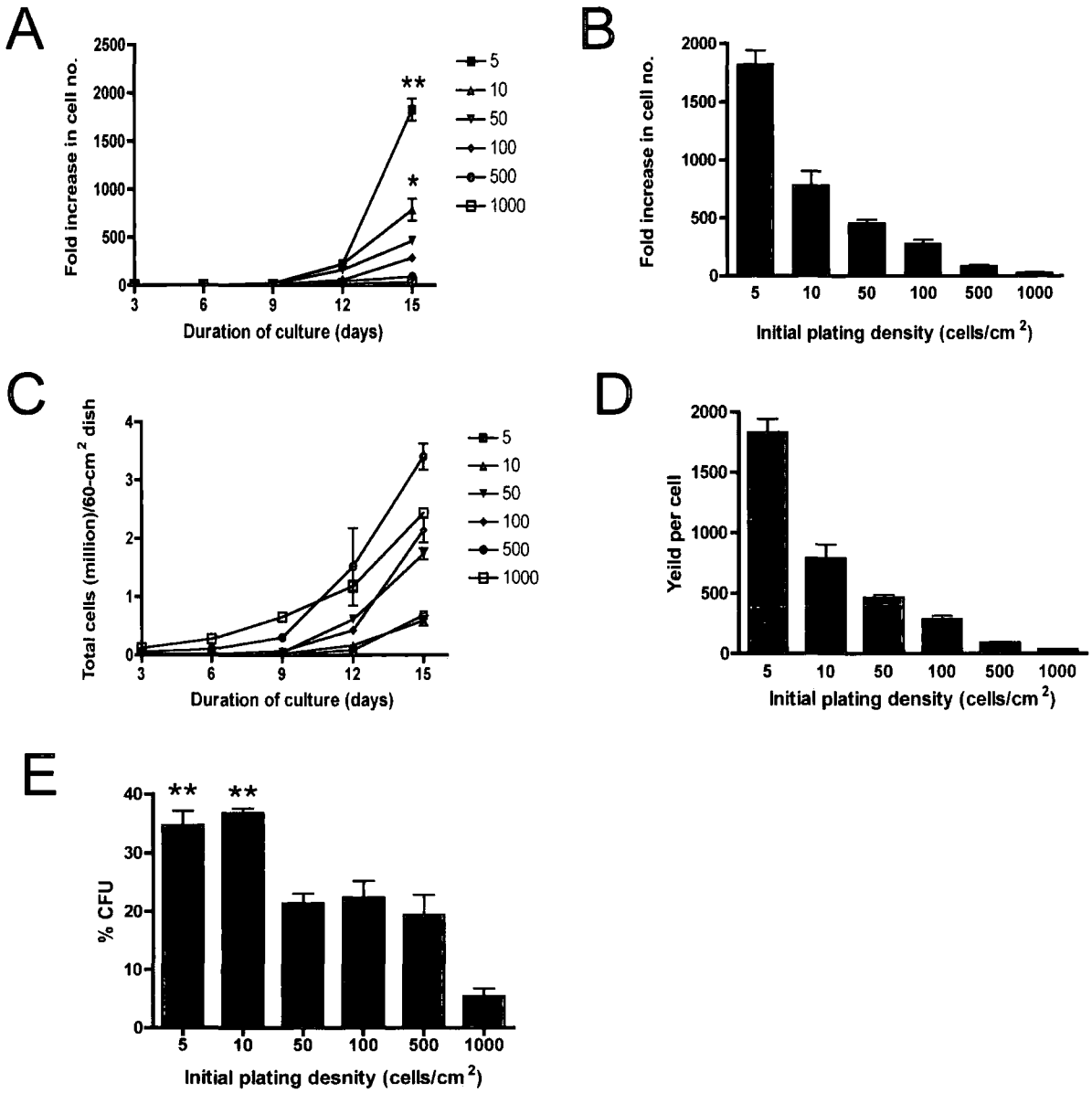


Figure 2.6: Effects of initial plating density on MSC expansion. P2 cells were seeded at varying initial plating densities (5, 10, 50, 100, 500 and 1000) in DMEM media supplemented with 10% FCS and maintained in culture for 15 days. Cell numbers were assayed every 3 days for 15 days. The fold increase in cell number from the initial plating density is shown for each time point and each culture condition (**A**). The total fold increase in cell number for 15 days for each culture condition is shown in (**B**). The total cell number increase from the initial plating density for each time point over 15 days is shown (**C**). In addition the total yield per cell is also indicated (**D**). Following expansion for 15 days colony forming ability was assayed. 100 cells were transferred into 60 cm^2 dishes, cultured for 14 days and stained with crystal violet. Colony forming efficiency is shown (**E**). All data are mean values + SEM from 3 independent experiments in parallel cultures. Significant differences from the control group (1000 cells/cm^2) are indicated with an asterisk (*$P<0.05$ **$P<0.01$).

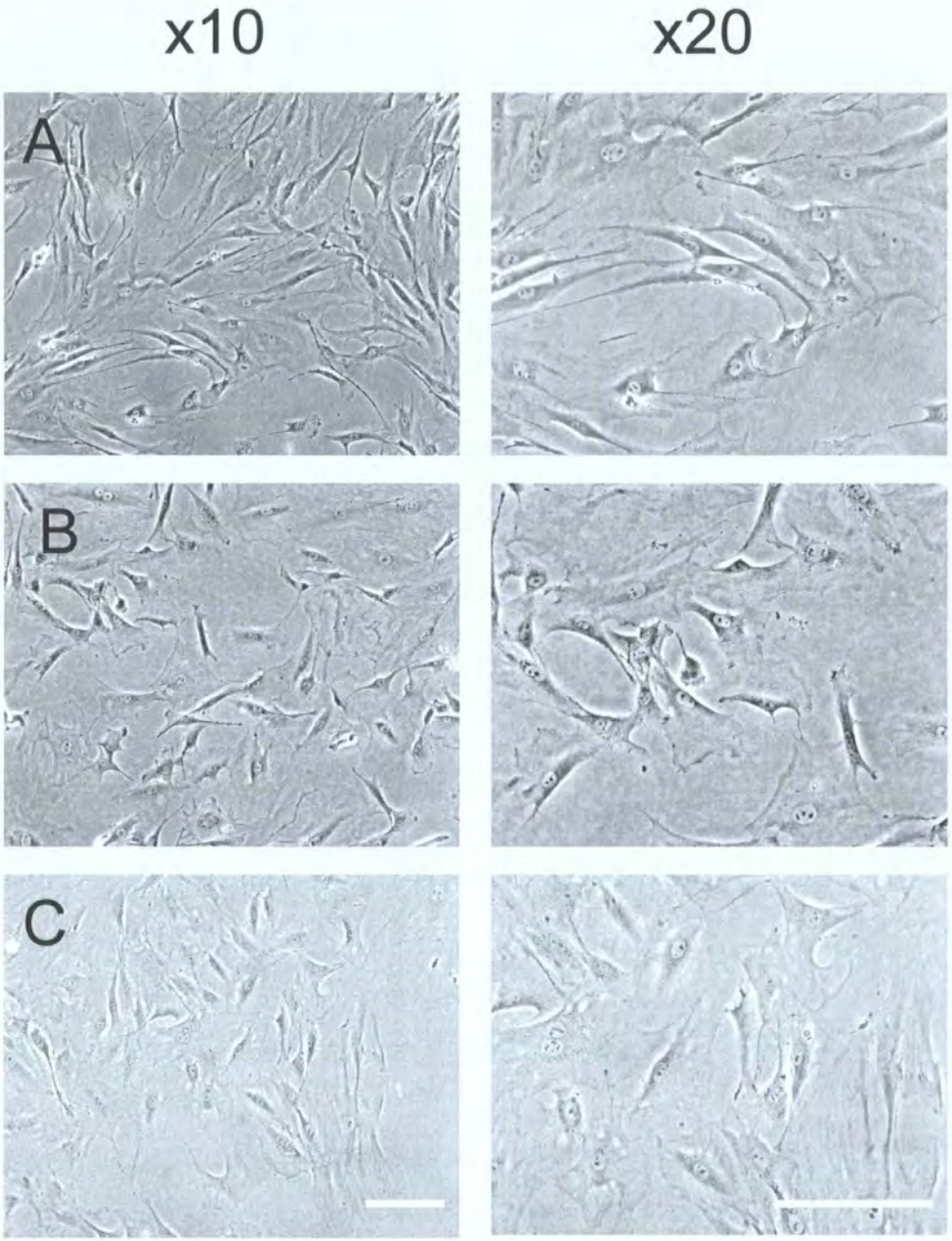


Figure 2.7: Effect of initial plating density on MSC morphology. P2 cells were plated at 10, 50 and 1000 cells/cm^2. Representative phase contrast micrographs are shown of (**A**) 10 cells/cm^2, (**B**) 50 cells/cm^2 and (**C**) 1000 cells/cm^2 at x10 and x20 magnification. All images were acquired 3 days following initial plating. *Scale bars: 50μm.*

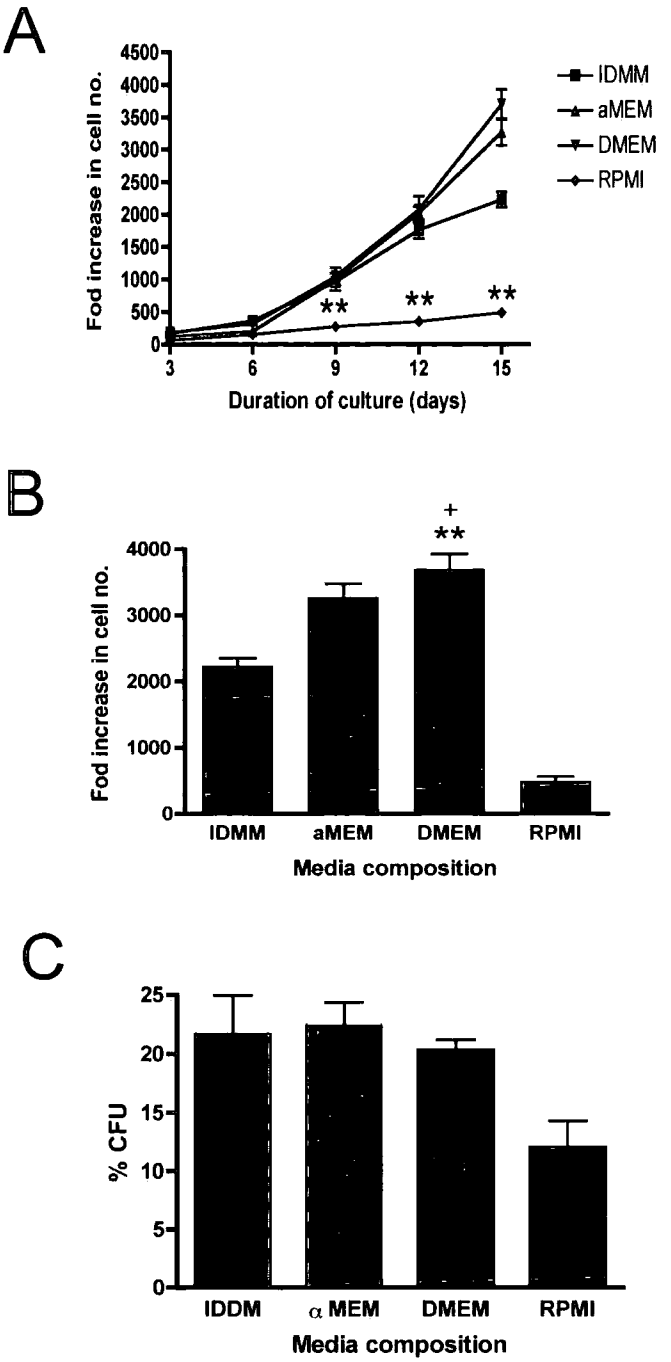


Figure 2.8: Effects of media composition on MSC expansion. P2 cells were seeded at a density of 10 cells/cm^2 in different basal media compositions (IDMM, α-MEM, DMEM or RPMI) supplemented with 10% FCS. Cell numbers were assayed every 3 days for 15 days. The fold increase in cell number from the initial plating density is shown for each time point and each culture condition (**A**) (**P<0.001 RPMI cultures compared to IDMM, alpha-MEM and DMEM). The total fold increase in cell number for 15 days for each culture condition is shown in (**B**) (**P<0.001 DMEM compared to RPMI cultures, +P<0.05 DMEM compared to IDMM). Following expansion for 15 days colony forming ability was assayed. 100 cells were transferred into 60 cm^2 dishes, cultured for 14 days and stained with crystal violet. Colony forming efficiency is shown (**C**) (no significant differences were identified between cultures). All data are mean values + SEM from 3 independent experiments in parallel cultures.

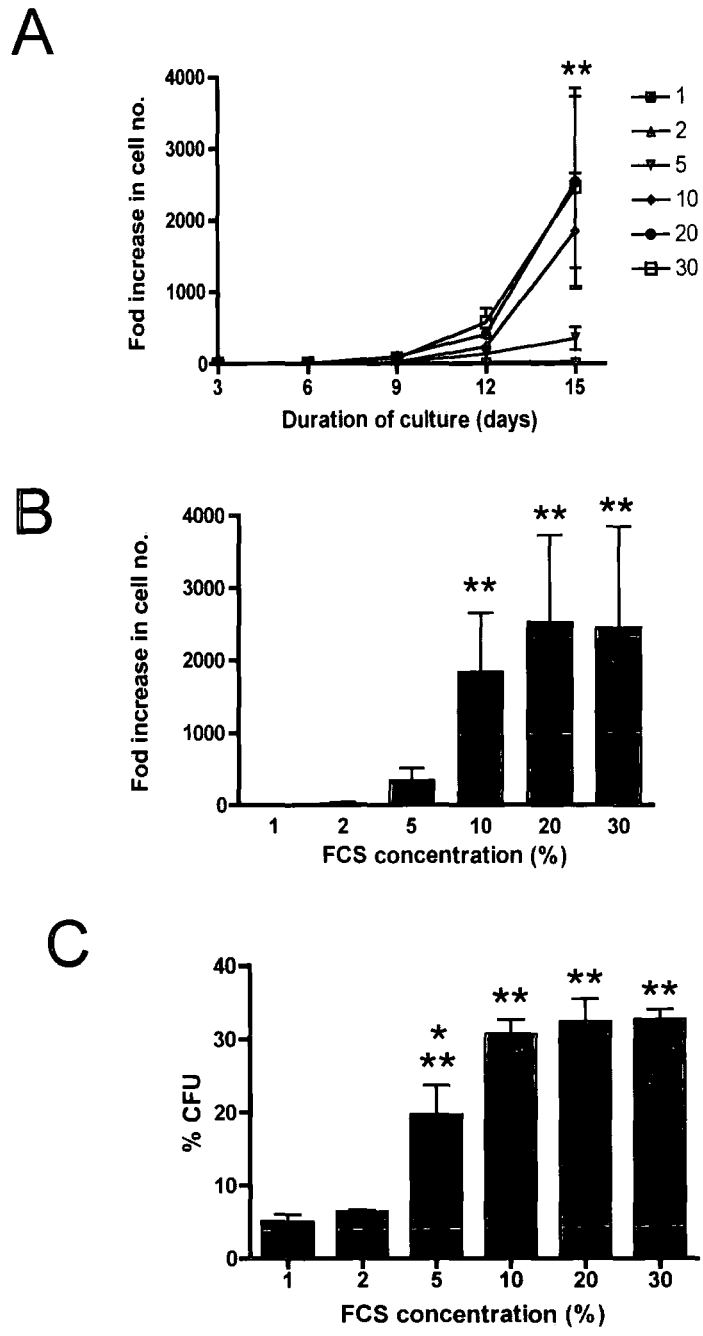


Figure 2.9: Effects of serum (FCS) concentration on MSC expansion. P2 cells were seeded at a density of 10 cells/cm^2 in DMEM supplemented with 1, 2, 5, 10, 20, or 30% FCS. Cell numbers were assayed every 3 days for 15 days. The fold increase in cell number from the initial plating density is shown for each time point and each culture condition (**A**). The total fold increase in cell number for 15 days for each culture condition is shown in (**B**). (**P<0.001 at day 15 5,10,20 and 30% cultures had significantly greater cell growth compared to 1 and 2% cultures. No other significant differences were found using 2-way ANOVA). Following expansion for 15 days colony forming ability was assayed. 100 cells were transferred into 60 cm^2 dishes, cultured for 14 days and stained with crystal violet. Colony forming efficiency is shown (**C**). (*P<0.05 compared to 10,20 and 30% cultures, ** compared to 1 and 2% cultures) All data are mean values±SEM from 3 independent experiments in parallel cultures.

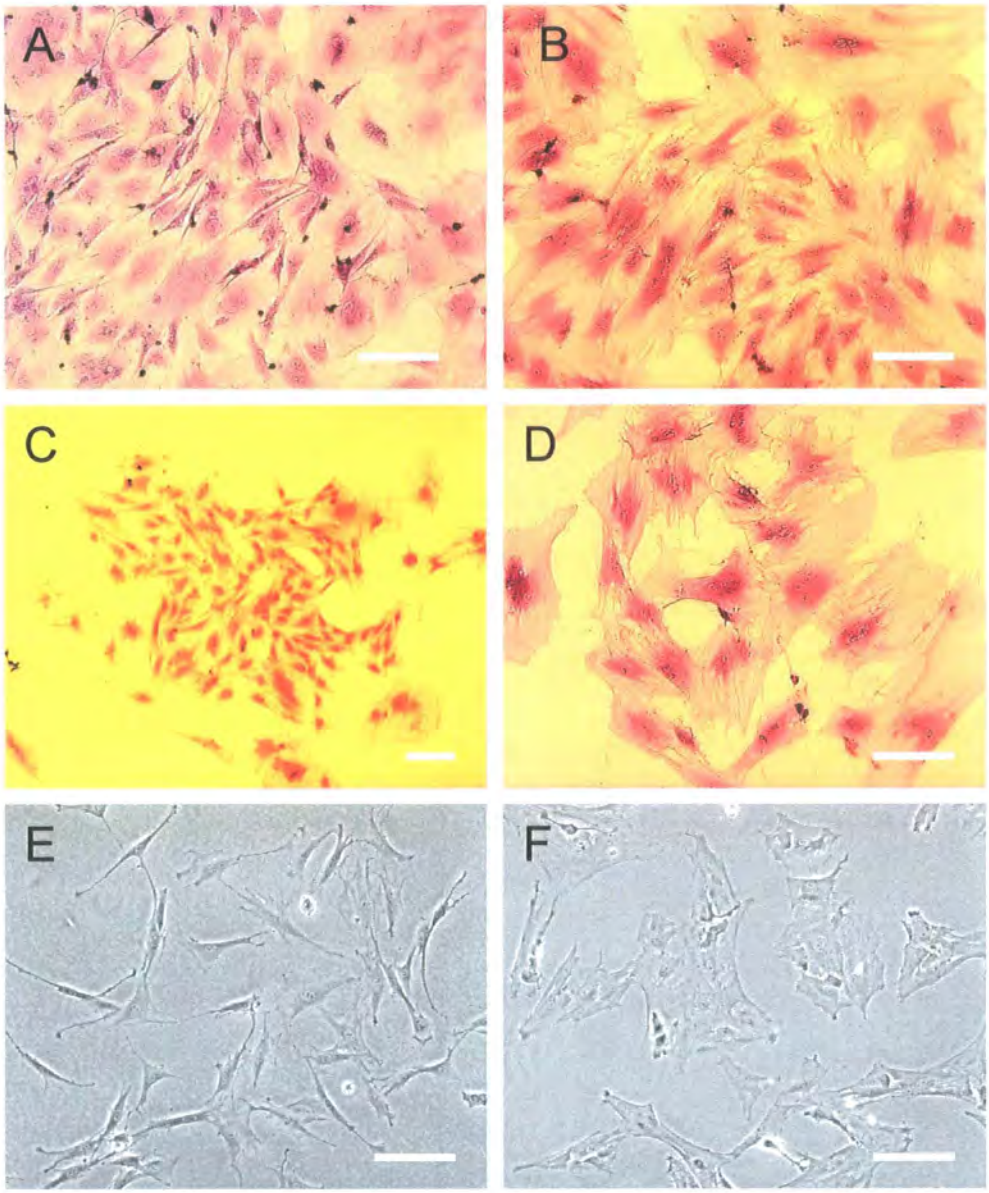


Figure 2.10: Effects of passage on morphology of MSCs in culture. P2 were cells grown to confluence as a monolayer followed by methanol fixation and staining with 0.5% crystal violet. Staining revealed a heterogeneous morphology (**A**). P2 cultures were dominated by spindle shaped cells, but also contained larger flattened cells. P2 cells seeded at low-density formed single cell derived colonies, which were dominated by rapidly proliferating spindle shaped cells (**C**). Morphological analysis by phase contrast analysis of P2 cultures revealed three types of cells based on morphology: spindle shaped cells, star-like cells and large flattened cells (**E**). Large flat slowly proliferating cells dominated P12 cultures (**B**). Re-plating of P12 cells at low density did not result in the generation of well-defined colonies. The colonies that did form remained small and dominated by large flat slowly proliferating cells (**D**). Phase contrast analysis also confirmed that P12 cultures contained mainly cells with a large flat morphology (**F**). *Scale bars 50μm.*

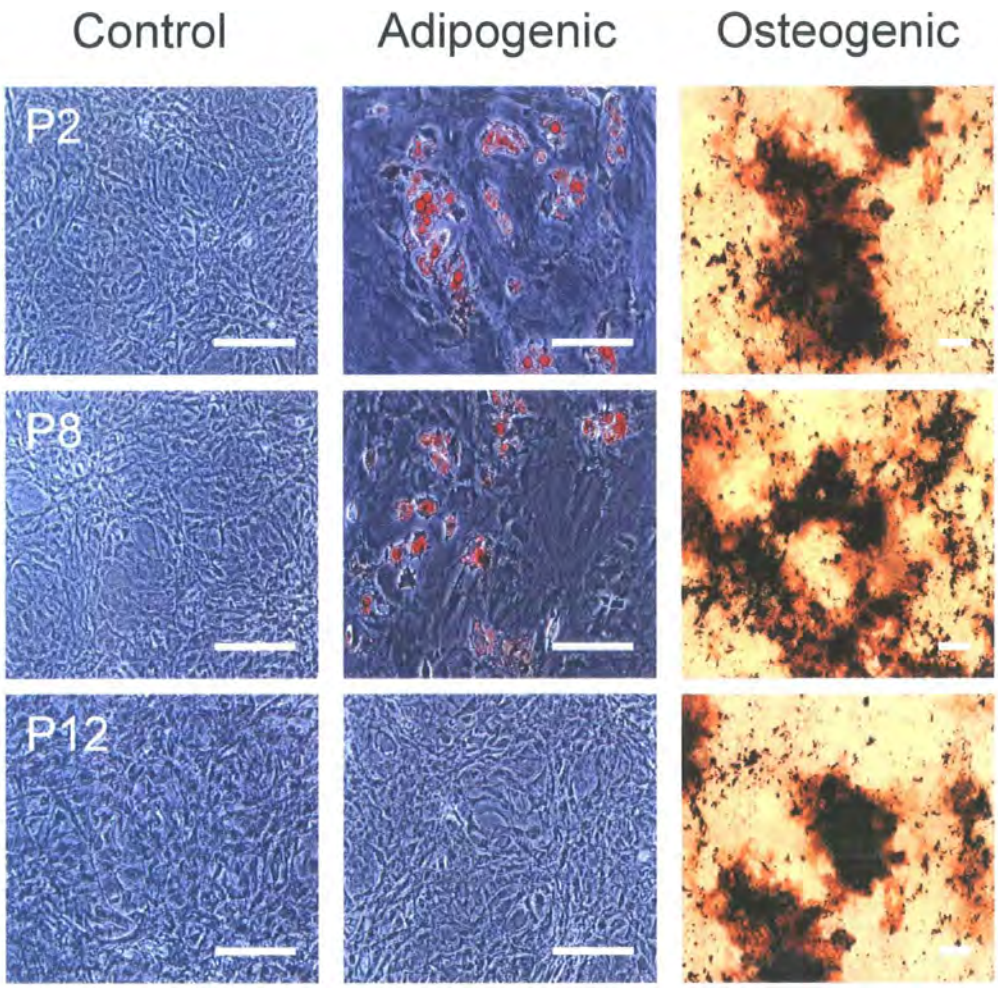


Figure 2.11: Differentiation potential of rat MSCs at different passage numbers. To evaluate the differentiation potential of culture expanded MSCs, cells were isolated from the same donor and expanded in parallel cultures up to passage 2 (P2), 8 (P8) and 12 (P12) according to optimal culture conditions. At the desired passage number cells were differentiated into either adipocytes or bone. Differentiation was confirmed by using the histological satin oil red O for adipogenic differentiation or Von Kossa staining for osteogenic differentiation. Control cultures were maintained under standard culture conditions and were negative for both oil red O staining and Von Kossa. The same pattern of differentiation was observed in 3 independent experiments carried out under identical conditions. *Scale bars: 50μm.*

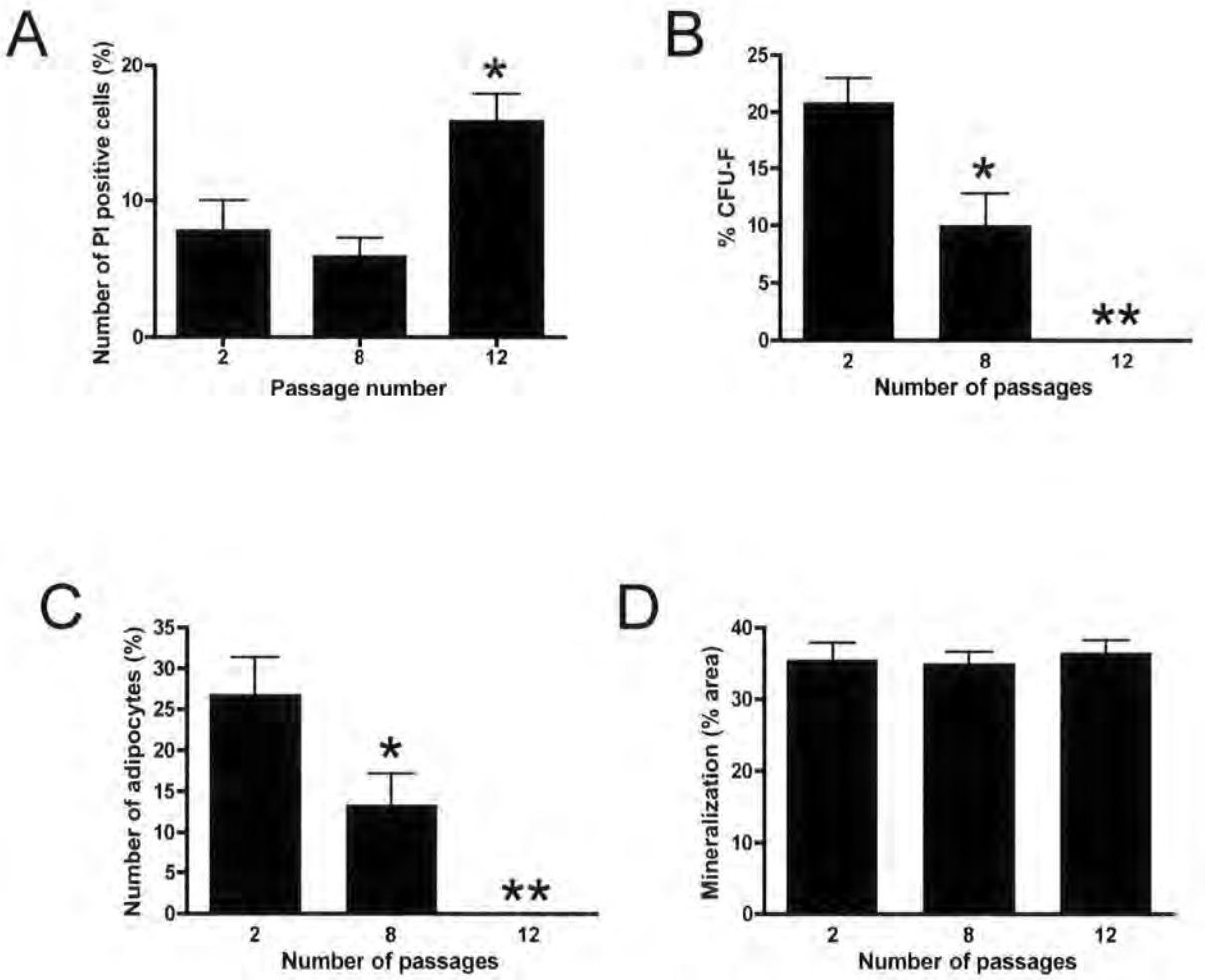


Figure 2.12: Quantification of the effects of culture expansion on the differentiation and CFU-F potential of rat MSCs. (**A**) Effect of passage on viability of cell cultures. Samples of cells from P2, P8 and P12 cultures were stained with PI and the number of cells positive for PI was quantified using flow cytometry. (**B**) 100 cells from P2, P8 and P12 were transferred to 60 cm^2 dishes and cultured for 14 days (n=3, carried out in triplicate). The number of colonies formed was accessed following fixation in PFA and staining with crystal violet. (**C**) Quantification of the number of adipocytes following adipogenic differentiation of P2, P8 and P12 cells. Adipocytes were verified by oil-red-O staining and the numbers of adipocytes (%) was quantified by the visualisation of 10 random fields of view (approx 25 cells/field of view). (**D**) Quantification of osteogenic differentiation. Calcified extra-cellular matrix was stained using von Kossa staining. The % area of each visual field (10 non over lapping visual fields) staining positive was determined. All data are mean values$\pm$SEM from 3 independent experiments in parallel cultures. Significant differences from the control group (P2 cells) are indicated with an asterisk (*$P<0.05$ **$P<0.01$).

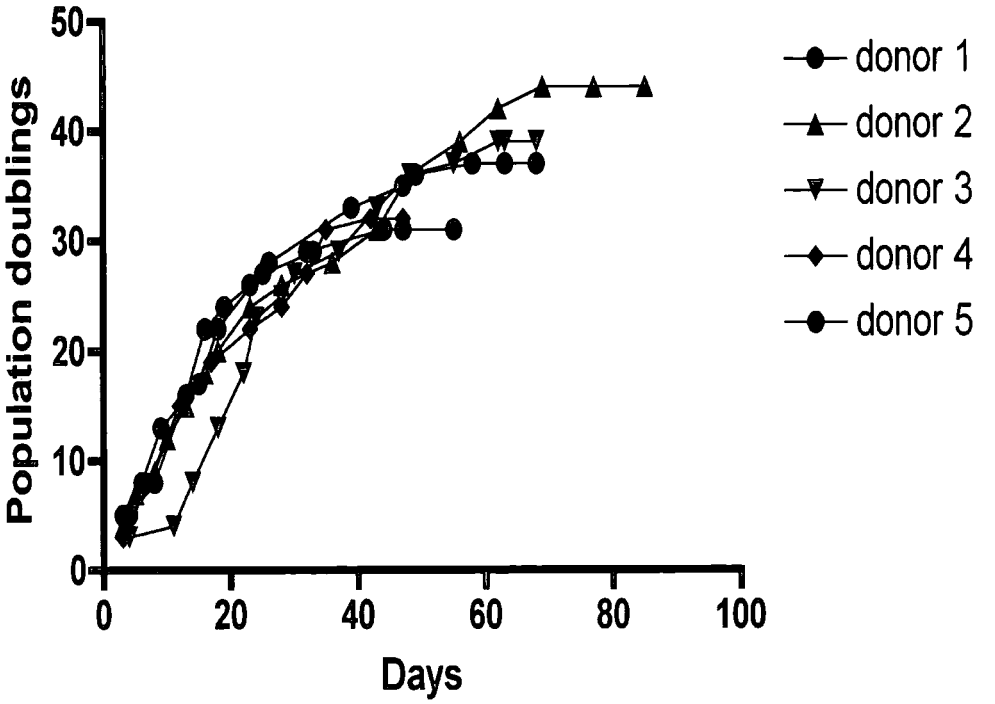


Figure 2.13: Population doublings of rat MSCs. Population doublings of rat MSCs in vitro. Values indicate population doublings after MSCs were grown to confluence and subsequently replated.

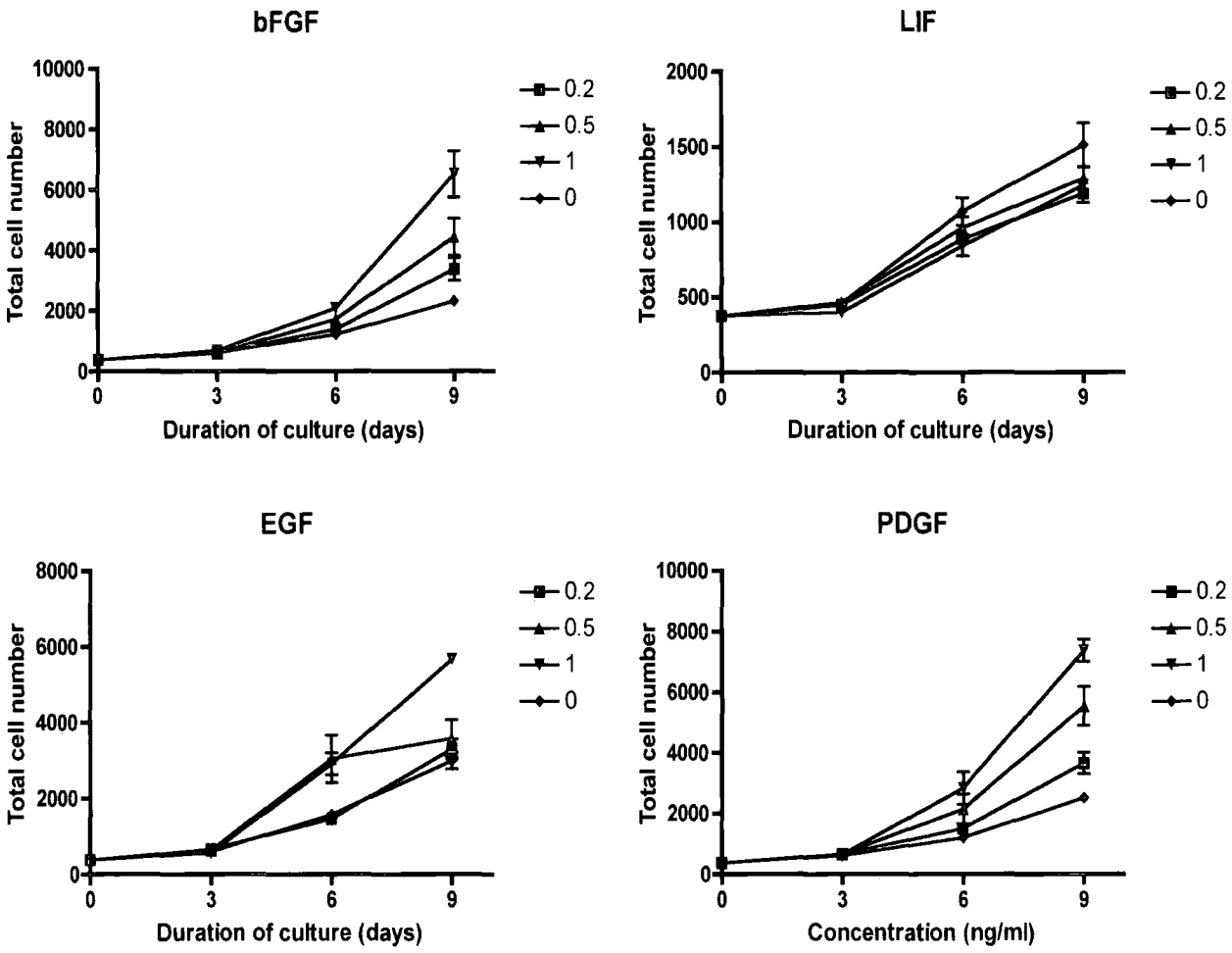


Figure 2.14: Effects of various growth factors on expansion of MSCs in culture. P2 MSCs were cultured in basal DMEM media supplemented with either FGF, EGF, PDGF or LIF. Cell numbers were assayed every 3 days for 15 days. The fold increase in cell number from the initial plating density is shown for each time point and each culture condition. All data are mean values + SEM from 3 independent experiments in parallel cultures.

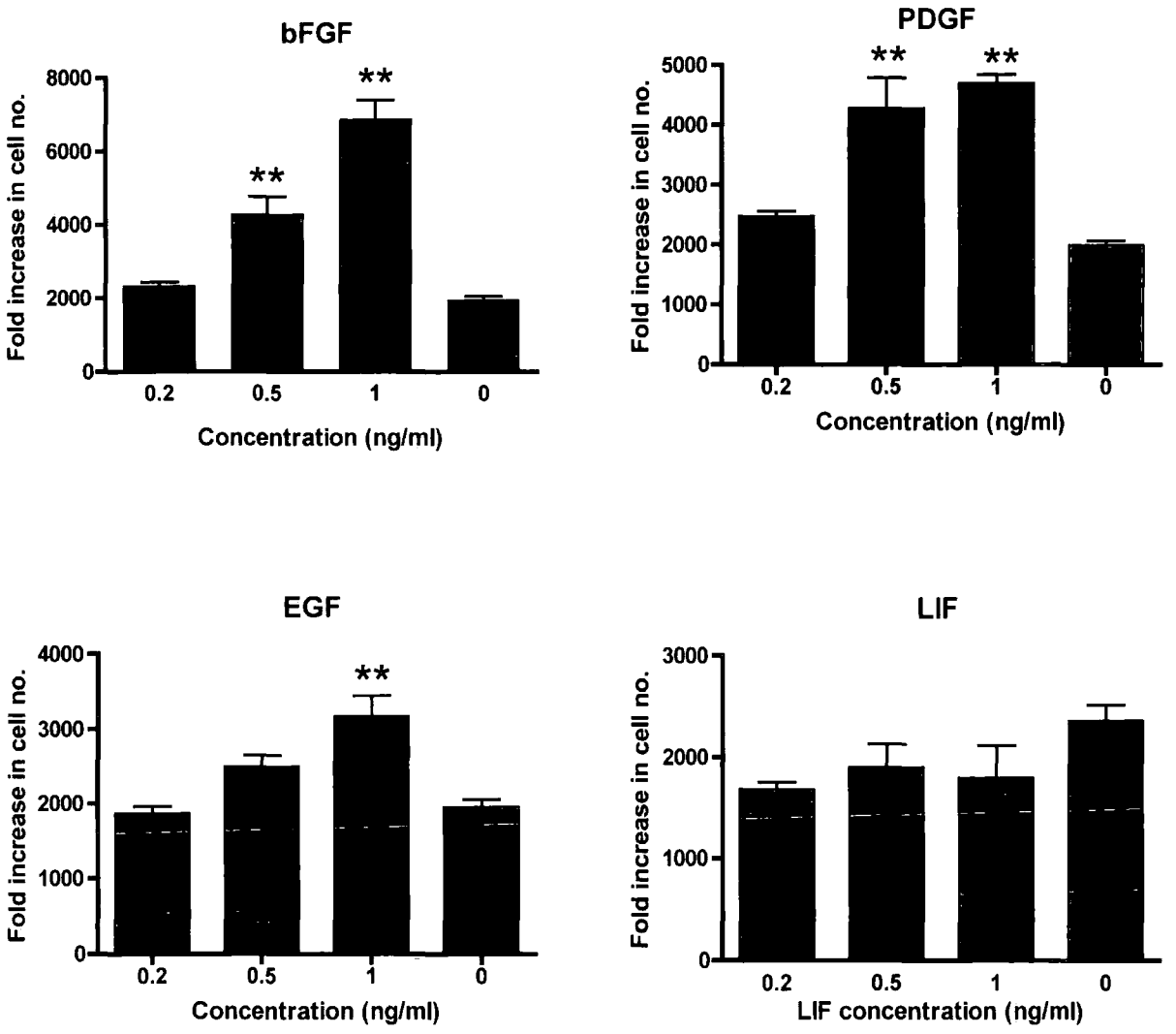


Figure 2.15: Effects of growth factors on MSC expansion. P2 cells were seeded at a density of 10 cells/cm^2 in the presence of different growth factors, either FGF-2, EGF, PDGF or LIF supplemented media. Cell numbers were assayed every 3 days for 15 days. The total fold increase in cell number from the initial plating density is shown for each culture condition. All data are mean values±SEM from 3 independent experiments in parallel cultures. Significant differences from the control group (0) are indicated with an asterisk (**P<0.01).

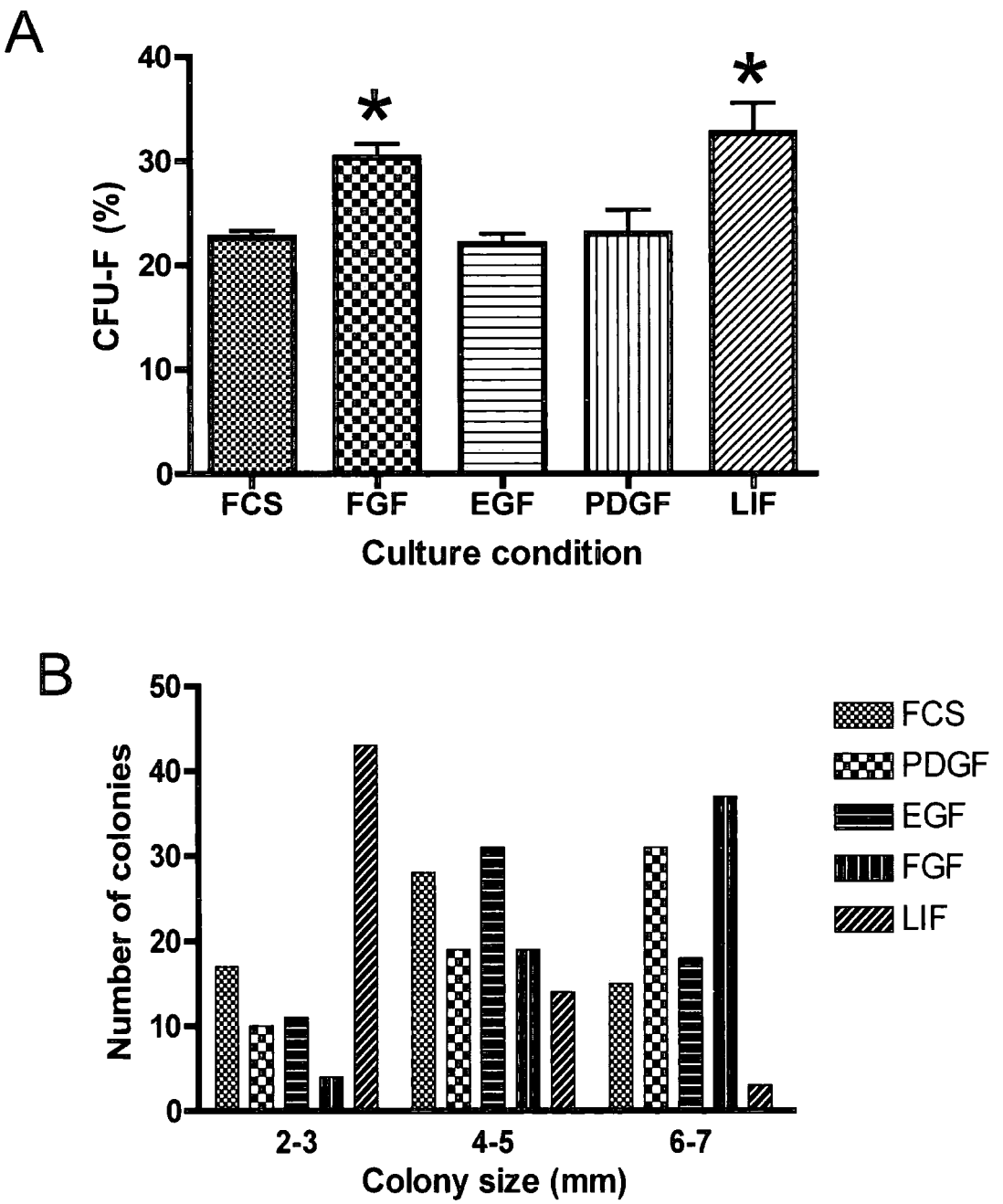


Figure 2.16: Single cell colony forming ability of MSCs maintained in growth factor supplemented media. Cells were maintained under either normal culture conditions (FCS) or in growth factor supplemented media (EGF, FGF, PDGF or LIF all at 1ng/ml) immediately following isolation and culture and remained under these conditions until P2. Then 100 cells were transferred into 60cm^2 dishes, cultured for 14 days under standard culture conditions (no growth factors) and stained with crystal violet. Colony forming efficiency (%, colony numbers/100) for cells maintained under each culture condition is shown (**A**). All data are mean values±SEM from 3 independent experiments in parallel cultures. Significant differences from the control group (FCS) are indicated with an asterisk (*$P<0.05$). Size distribution of colonies obtained in a CFU assay (**B**). Values are from 35 colonies per plate (n=3). Values are largest diameters of the colonies.

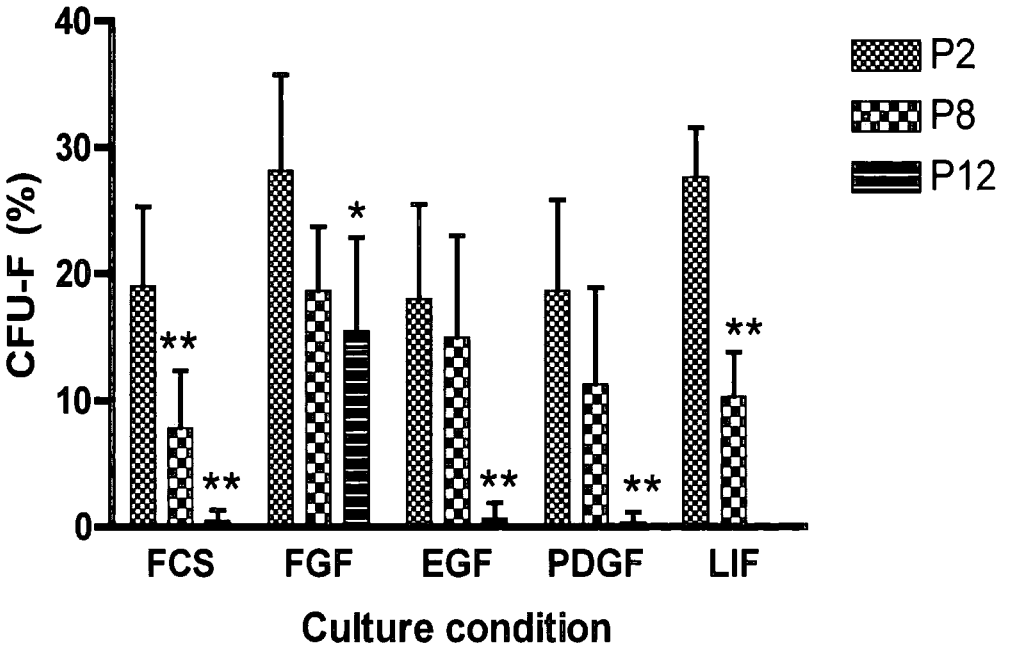


Figure 2.17: Colony forming ability of cells maintained in growth factor supplemented media. Cells previously isolated and expanded in growth factor supplemented media (either EGF, FGF, LIF and PDGF) were grown to confluence. Control cells (FCS) were maintained under standard culture conditions (10% FCS supplemented DMEM). 100 cells from cultures at different passage numbers were then transferred to 60cm^2 dishes, cultured for 12 days and stained with crystal violet. Colony forming efficiency (CFU-F %) is shown. All data are mean values$\pm$SEM from 3 independent experiments in parallel cultures. *P<0.05, **P<0.01 compared to P2 within each growth factor treatment using 1-way ANOVA and post hoc analysis.

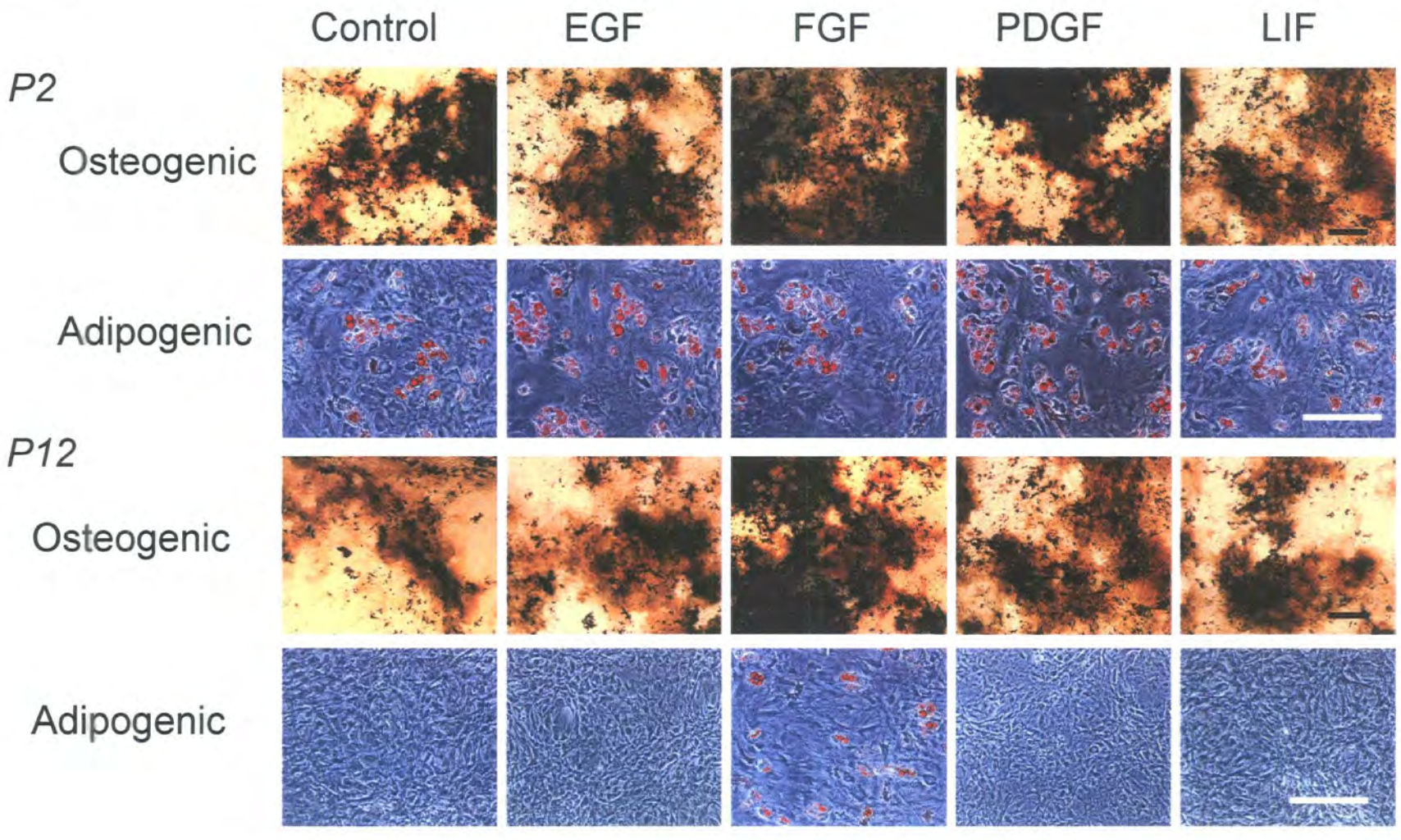


Figure 2.18: Mesodermal differentiation potential of MSCs maintained in growth factor supplemented media. Cells were maintained in growth factor supplemented media (bFGF, EGF, PDGF or LIF, all 1ng/ml) from initial isolation. P2 or P12 cells grown under these conditions were subsequently differentiated into either bone (osteogenic) or fat (adipogenic) for 3 weeks. Differentiation was verified by Von kossa staining (bone) or oil red O (fat). *Scale bars 50μm.*

Chapter 2 tabulated data

Passage	Total doublings	Average doubling time (days)
0	2	1.2
1	4	1.8
2	7	2.3
3	9	2.2
4	14	2.8
5	16	3.1
6	19	3.2
7	24	3.7
8	27	3.8
9	29	5.2
10	32	8.9
11	34	9.3
12	37	10.1
13	38	12.3
14	39	13.1

Table 2.1: Growth kinetics of one MSC primary culture

Passage	2	8	12
CD44	78.9 ± 5.6	76.8 ± 11.2	79.8 ± 8.6
CD11b	0	0	0
CD73	73.2 ± 5.6	63.7 ± 9.5	56.7 ± 8.9
HLA Class I	98.9 ± 8.3	88.4 ± 7.9	79.8 ± 8.8
CD45	0	0	0
CD56	68.8 ± 7.7	66.5 ± 2.3	72.3 ± 9.3
CD29	90.8 ± 7.1	93.2 ± 3.9	88.7 ± 9.7
CD90	99.2 ± 3.4	97.6 ± 2.3	77.6 ± 8.4
TRA 2-54	96.7 ± 2.3	92.4 ± 9.3	94.5 ± 9.7

Table 2.2: Effect of passage on the expression of cell surface markers. The number of cells positive for each cell marker as determined by single colour flow cytometry is shown. All data is expressed as mean + SEM from three independent experiments.

Passage	Cell death (%)	CFUs (%)	Adipocytes (%)	Mineralisation (% area)
2	8±2	21±3	27±5	35±2
8	6±1	10±3	13±4	35±3
12	16±2	0	0	37±2

Table 2.3: Differentiation of early and late passage MSCs

Condition	Control	FGF	EGF	PDGF	LIF
CD44	76.5 + 6.7	79.7 + 2.3	81.2 + 7.8	83.2 + 9.3	78.6 + 4.5
CD11b	0	0	0	0	0
CD73	49.8 + 9.5	74.3 + 11.2	55.4 + 8.7	78.9 + 4.7	88.7 + 10.2
HLA Class I	97.6 + 5.5	94.5 + 7.8	98.7 + 3.4	92.3 + 7.6	99.7 + 5.7
CD45	0	0	0	0	0
CD56	74.8 + 9.1	77.8 + 5.7	71.2 + 6.9	87.3 + 11.2	76.5 + 9.3
CD29	91.2 + 6.7	87.6 + 8.8	98.7 + 8.9	92.1 + 8.7	89.9 + 6.8
CD90	71.2 + 4.4	95.6 + 12.3	82.3 + 9.5	77.6 + 3.4	90.8 + 7.4
TRA 2-54	98.7 + 7.5	93.4 + 7.8	92.3 + 5.5	98.7 + 7.9	92.3 + 4.2

Table 2.4: Effect of growth factors on the expression of cell surface markers. The number of cells positive for each cell marker as determined by single colour flow cytometry is shown. All data is expressed as mean + SEM from three independent experiments.

Donor	Control	bFGF	EGF	PDGF	LIF
1	33.4	37.3	34.7	32.5	33.6
2	29.3	48.4	28.3	36.2	37.4
3	36.3	42.3	38.4	38.6	39.7
4	32.8	44.9	34.3	41.2	44.2
5	31.5	36.3	33.9	32.9	42.5

Table 2.5: Number of population doublings before senescence of MSCs under defined culture conditions.

CHAPTER 3

Generation of Neuro-Progenitor-Like Cells from Mammalian Bone Marrow

3.1 Introduction

Embryonic stem cells (ES) are pluripotent cells derived from the inner cell mass of blastocyst stage embryos that can be propagated as a continuous cell line in an undifferentiated state (Thomson et al., 1998). ES cells isolated from human embryos have been shown to differentiate into somatic cell lineages of all three embryonic germ layers during growth as embryoid bodies *in vitro* (Itskovitz-Eldor et al., 2000) and the formation of teratomas *in vivo* (Reubinoff et al., 2000). Whilst there is enormous potential for the use of human ES cells both in research and in the clinic, their use has encountered numerous ethical concerns (Frankel, 2000). In addition, the requirement for autologlous stem cell therapy to overcome immunological considerations, has led to the search for alternative, easily accessible stem cell populations within post-natal adult tissues.

In the adult, stem cells exist in many tissues and organs where they remain as a self renewing population producing a narrow range of differentiated cell types associated with the tissue in which they reside (Gage 2000; Weissman, 2000, Potten, 1998; Watt, 1998; Alison, 1998; Pittenger et al., 1999). However, the concept that adult stem cells have a limited repertoire for differentiation is being challenged by the growing body of evidence which suggests that tissue specific stem cells have a broader capacity for differentiation and can give rise to cell lineages not associated with their germ line origin (review: Weissman et al., 2001). Accordingly, some stem cells in adult tissues appear to possess a greater plasticity than previously envisioned. For example, hematopoietic stem cells (HSCs) appear to have the capacity to form a wide variety of alternative cell types, including tissues of all three germ layers, notably endothelium (Orlic et al., 2001a; Jackson et al., 2001; Lin et al., 2000); skin epithelia (Krause et al., 2001), cardiac and skeletal myoblasts (Ferrari et al., 1998; Gussoni et al., 1999), hepatic epithelium (Petersen et al., 1999; Lagasse et al., 2000) and neuroectodermal cells (Kopen et al., 1999; Mezey et al., 2000; Sanchez-Ramos et al., 2000). In addition, neural stem cells have been shown to differentiate into hematopoietic cell lineages *in vitro* (Bjornson et al., 1999; Morshead et al., 2002) and transplanted muscle cells can reconstitute the hematopoietic system of irradiated mice (Jackson et al., 1999; Kawada et al., 2001). It has also been demonstrated that neural stem cells

injected into the early blastocyst contribute to a number of embryonic tissues in the resulting chimera (Clarke et al., 2000).

MSCs are a prototypical stem cell population that reside in the bone marrow and are distinct from HSCs. Besides co-habiting with one another, MSCs provide the structural and functional support for HSCs and hemopoiesis (Tavassoli and Friedenstein, 1983). MSCs were first described in 1974 by Friedenstein (Friedenstein et al., 1974), who characterized them as fibroblastic stem cells capable of forming colonies. This designation also includes stromal bone marrow fibroblasts or mesenchymal stem cells (MSCs) (Luria et al., 1987; Caplan, 1994). Although MSCs have a low frequency in the adult bone marrow tissue, they can be readily explanted and cell numbers expanded *ex vivo* by virtue of their high proliferative capacity. Cultured MSCs possess a distinct immunophenotype and can be characterized by their expression of various cell surface markers. For example, a typical expression profile for a population of MSCs would include CD34-, CD45-, CD44+, CD90+, CD44+ and HLA class I+ (Wexler et al., 2003). Cultured bone marrow-derived MSCs are classed as being typical multipotent stem cells in that they retain their ability to differentiate into a range of mesenchymal tissue types, including osteocytes, chrondrocytes, adipocytes, tenocytes and stromal cells able to support hematopoesis (Pittenger et al., 1999).

Like HSCs, several groups have shown that MSCs also have a greater plasticity than had previously been contemplated. MSCs have been reported to differentiate into hepatocytes (Petersen et al., 1999), cardiac and skeletal muscle (Orlic et al., 2001a; Ferrari et al., 1998; Makino et al., 1999; Wakitani et al., 1995), as well as, neural- and glial-like cells (Sanchez-Ramos, 2000; Woodbury et al., 2000). The generation of neuroectodermal tissue from MSCs is of particular interest, especially as a candidate for autologous stem cell therapy to treat neurological disorders. Even though the mammalian central nervous system (CNS) is capable of regeneration following injury, this is of limited capacity and only occurs in distinct regions of the brain (Eriksson et al., 1998). Whilst it is now possible to establish cultures of neural stem cells from adult brain tissues (Westerlund et al., 2003; Carpenter et al., 1999; Uchida et al., 2000), it is difficult to isolate such cells. Accordingly, the ability to produce neural

tissues from a readily accessible tissue such as bone marrow opens up an exciting possibility to produce neural cells for autologous transplantation.

During the past few years, several studies have presented evidence for the generation of neurons from MSCs both *in vitro* and *in vivo*. However, more recent experiments have questioned the validity as to whether transplanted MSCs do differentiate to form somatic cells or whether grafted cells simply fuse with host tissues thus giving the appearance of taking on the identity of a differentiated cell type (Jiang et al., 2002a; Reyes and Verfaillie 2001; Labat et al., 2000; Ying et al., 2002; Terada et al., 2002). Whilst this important debate continues it should not deter us from the fact that the production of neural and glial cells has also been reported in mono-cellular cultures *in vitro*. These cell culture systems are free from the possibility of cell fusion with host tissues and have the advantage of control in terms of the homogeneity of the starting population of cells.

Sanchez-Ramos et al., 2000 first produced neural precursor cells from MSCs by culturing them under growth conditions known to induce the differentiation of neural derivatives from ES cells and neural stem cells *in vitro*. In response to retinoic acid and brain derived neurotrophic factor (BDNF), a small proportion of human and murine MSCs form neural-like cells expressing pan-neural markers. This percentage was further increased by co-culture with fetal mesencephalic cells. Alternatively, Woodbury et al. (2000) described a method in which neural cells are produced from MSCs following treatment with antioxidants in serum-free conditions, including 2% DMSO which has been shown to induce differentiation in several neuroblastoma cell lines (Oh et al., 2006; Bolduc et al., 2001). Exposure of MSCs to antioxidants resulted in over >70% of the cell population assuming a neural-like morphology within 5 hours of treatment. Several groups have independently shown that cultures of MSCs treated in this way, markedly up-regulate their expression of neuronal proteins, including markers such as nestin, β-III-tubulin and NeuN (Woodbury et al., 2000). In addition, no evidence of glial fibrillary acidic protein (GFAP) expression has been reported, suggesting that only neuronal differentiation occurs under these growth conditions. Deng et al., 2001 documented a similar neural induction procedure in which exposure to isobutylmethylxanthine (IBMX) (which has the effect of increasing intracellular cAMP) resulted in 25% of the BMSC population adopting a

neuronal morphology with expression of neural specific enolase but in the absence of GFAP expression. Using an alternative approach, Kohyama et al. (2001) described the differentiation of neural cell types following transfection with the neural inducer noggin. Noggin transfection produced neural-like cells that expressed neural proteins and possessed electrical characteristics normally associated with functional neurons.

It is apparent that cultures of mammalian MSCs respond to a variety of stimuli and have the capacity to form cells that morphologically resemble neurons. However, it is currently not known whether such a transformation in cell identity is an artifact of the growth conditions experienced by the cells or is in fact real cell differentiation. Confounding this issue is the variability between existing studies. For example, there is little rationale for the evolution and the development of the strategies used to induce the formation of neural-like cells from MSCs and the technical merits of these approaches have not been clearly justified. Moreover, aspirates of bone marrow stroma are known to be heterogeneous and contain a broad range of cell types. Several investigators have gone to varying lengths to ensure that HSCs are reduced to a minimum and some effort has been made to immunophenotype the starting population (Woodbury et al., 2000; Deng et al., 2001). Some workers have produced enriched populations of MSCs using cell type markers known to be expressed by MSCs *in vivo*, whilst others have isolated MSCs on the basis of their preferential adherence to tissue culture plastic, or have isolated the mononuclear fraction of cells by ficoll density centrifugation (Kohyama et al., 2001). Accordingly, there are likely to be significant differences in the starting population of MSCs between independent studies assessing their ability to form neural derivatives. In our laboratory, we have attempted to standardize the starting population of MSCs before examining their ability to form alternative cell types. Here we report our preliminary findings comparing the ability of mammalian MSCs to form neural cell types using a range of different growth conditions.

3.2 Materials and Methods

3.2.1 Materials

Tissue culture reagents and other materials were acquired from Sigma (Sigma-Aldrich, Poole, UK) unless otherwise stated. All substances were of the appropriate

chemical, molecular biological or tissue culture grade. Colcemid (COL) was dissolved in ethanol and used at a final concentration of 1 μg/ml unless otherwise stated. Laminin, fibronectin and Polyornithine were purchased from sigma and were used to coat tissue culture plastic. Laminin was diluted in media and used at a final concentration of 5μg/ml to coat flasks overnight at 37°C. Fibronectin and polyornithine were used at final concentrations of 25μg/ml and 10μg/ml respectively. PMA (Sigma) was prepared in ethanol and used at a final concentration of 10μM.

3.2.2. Cell Culture

Rat MSCs (rMSCs) were isolated from the femurs and tibiae of 6-8 month old Wistar rats. The bone marrow (BM) was aspirated with 20 ml collection media (RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 12 μM L-glutamine) into a T75cm^2 flask to allow stromal cells to adhere to the culture surface. Adherent cells were then washed and maintained in complete culture medium (CCM: Dulbecco's Modified Eagles Medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 12 μM L-glutamine and 1x non-essential amino acids) at 37°C in 5% CO_2. Isolation of rMSCs was verified by their capacity to differentiate into mesodermal derivatives (bone, fat) Passage 8 (approximately 25 population doublings) cells were used in the experiments described herein.

The mouse neuroblastoma N2a cell line and human neuroblastoma cell line was originally obtained from the American Tissue Culture Collection. Cells were seeded in T-25 tissue culture flasks (Gibco) plates at a density of 3 x 10^4 cells/cm^2 and grown in DMEM (Gibco) supplemented with 2 mM L-glutamine, penicillin (20 units/mL), streptomycin (20 mg/mL) and 10% FCS (Invitrogen). Cells were maintained in a humidified atmosphere containing 5% CO^2 at 37°C and grown to >70% confluence.

NSC/progenitor cells were isolated from the mesencephalon of day 14 rat embryos. Pregnant female Wistar rats at the specified gestational age of 14 days (E14) (the day of conception was confirmed by the presence of a vaginal plug, embryonic day 0) were killed by cervical dislocation and the uteri were aseptically removed and

transferred to Petri dishes containing sterile Dulbecco's phosphate buffered saline (PBS) with 30% glucose and penicillin (20 units/mL), streptomycin (20 mg/mL). E14 striata were isolated and triturated in DMEM/F12 with a sterile Pasteur pipette. The cell suspension was filtered with a 70 μm-mesh and viable cells were estimated by typan blue exclusion. The cells were plated (1 x 106 cells/75-cm^2 uncoated tissue culture flask (Nunc)) in a chemically defined serum-free medium DMEM/F12 including 0.6% glucose, 2mM L-glutamine, 3mM sodium bicarbonate and 5mM HEPES buffer, supplemented with N-2 (a multi-component cell culture supplement), EGF (10ng/ml, Sigma) and FGF (10ng/ml, Sigma) and 2 μg/ml heparin. When the size of neurospheres reached approximately 50 cells, they were dissociated into a single cell suspension by titration and replated in fresh culture medium. Cultures were maintained in 37°C in a humidified incubator with 5% CO_2. Neurospheres with a maximum of 3 passages were used in this study. Differentiation of E14 derived neurospheres was carried out as described in the results section, where appropriate.

3.2.3 Analysis of cell surface marker expression

Cells required for flow cytometry analysis were washed and resuspended in FACS medium at a concentration of 1 x 10^6 cells/ml. 200μl of cell suspension (2 x 10^5 cells) was pipetted into each well of a 96-well plate (Greiner), centrifuged at 300g, 4°C for 10 minutes and the supernatant discarded. 50μl of the required primary mouse monoclonal antibody was added to the cells at the appropriate concentration and the plate incubated on ice for 20 minutes. Antibodies include: (all anti-mouse monoclonal): A2B5 (1:50), B159 (N-CAM) (1:100), VIN-US-53 (N-CAM, 1:200) and VIN-US-56 (glycoporetin, 1:25) and were all obtained from the developmental hybridoma bank. Following 2 washes with 150-200μl FACS medium, 50μl secondary FITC antibody (anti-mouse Ig Fab_2 fraction, FITC conjugated - DAKO) was then added to the cells (1:20) for a 20 minute incubation on ice in the dark. The cells were then washed twice with FACS medium and transferred in 500μl aliquots to 5ml centrifuge tubes (Greiner) for flow cytometry analysis. 10μl of 0.25mg/ml propidium iodide (Sigma) was added to each sample to assess viability of cell populations. Analysis was performed using a coulter EPICS XL flow cytometer. Using a 530 and 585 band pass filter respectively identified FITC and Cy-3, and quantification was completed using CellQuest Software (Becton Dickinson). Ten thousand events were

acquired per sample with fluoresence measured on logarithmic scales. Forward and side light scatter gates were set to exclude dead debris and clumps of cells. To calculate the percentage of positive cells, linear gates were set at 0.1%, on samples stained only with secondary antibodies, and expression corresponding to a fluoresence signal exceeding this percentage was measured.

3.2.4 Western Blot Analysis

Protein extracts (30μg per lane) were separated by electrophoresis and transferred onto PDVF membrane (Amersham). For immunoblotting, membranes first incubated in blocking solution (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) containing 5% milk powder, 0.2% Tween 20) for 1 hour followed by primary (TuJ1 (Convance), 1:5000; Nestin (Chemicon), 1:1000; β-Actin (Sigma), 1:5000), MAP-2 (Sigma, 1:1000), Synaptophysin (Sigma, 1:1000), PKC (Cell signaling 1:1000), NF-160 (Chemicon 1:500), GAP-43 (Sigma, 1:1000) S100 (Chemicon 1:1000) and secondary (mouse or rabbit IgG-HRP (Amersham, 1:1000) antibody. Protein-antibody binding was detected on film (Hyperfilm ECL, Amersham) using chemiluminescence (Amersham). Densitometry following western blot analysis was carried out on blots obtained from three independent experiments and where analysed by ImageJ.

3.2.5 Differentiation of MSCs into the osteogenic cell lineage

Cells were seeded at a density of 3 x 10^3 cells/cm^2 in tissue culture dishes and cultured with DMEM media supplemented with 10% FCS, 100 nM dexamethasone, 50 μM ascorbic acid 2-phosphate, 10nM β-glycerophosphate. The cultures were maintained for upto 3 weeks and the culture medium was replaced every three days. After 7, 14 or 21 days of culture, cells were rinsed twice with PBS, and fixed with 10% buffered formalin for 10 minutes at room temperature. Cells were stained with 5% silver nitrate solution for von kossa staining. Cells were incubated in 5% silver nitrate solution for 10 minutes in the dark, washed thoroughly and then exposed to bright light for 15 minutes.

3.2.6 Immuno-fluorescence microscopy

For F-actin staining or α-tubulin staining it was necessary to preserve the structure of the microtubule cytoskeleton, cells were washed in PBS, extracted with 1% Triton X-

100 in microtubule stabilizing buffer (PEM: 1 mM $MgCl_2$, 5 mM EGTA, 80 mM K-pipes, pH 6.8). After permeabilisation cells were fixed with 0.5% gluteraldehyde in PBS. Free aldehyde groups were blocked by sodium borohydride (10 minutes) and lysine (2% solution) for 1 hour. For other staining, cells were washed with PBS and fixed in 4% PFA solution for 30 minutes followed by post fixation and permeabilisation in 0.5% Triton X-100 in PBS for 15 minutes. All samples were subsequently rinsed three times in blocking/wash buffer (2% PFA in PBS), incubated with monoclonal mouse antibody directed against either α-Tubulin DMIA (Sigma, 1:100), Nestin (Chemicon, 1:200), Synaptophysin (Sigma, 1:100), NeuN (Chemicon, 1:100), NF-160 (Chemicon, 1:200), Vimentin (Sigma, 1:200), Fibronectin (Sigma, 1:200), GAP-43 (Sigma 1:100), Tuj-1 (Convance, 1:8000), GFAP (Sigma 1:500), MAP-2 (Chemicon, 1:100) and TAU (DHB, 1:50). FITC-conjugated goat antibodies against mouse IgG (Sigma, 1:100) were used as secondary antibodies. To visualize the F-Actin cytoskeleton, cells were stained with TRITC-labeled phalloidin. Labeled cells were cells visualized using an inverted fluorescent microscope (model E660 Nikon) and a CCD camera (Spot RT; diagnostic instruments) with individual filter sets for each channel. All images were captured using the same collection parameters for quantitative comparisons. The fraction of positive cells was determined for each culture condition by counting 10 non-overlapping microscopic fields (>20 cells/field) for each condition in at least three independent experiments. Colour images were generated using Adobe photoshop (Adobe systems, mountain view, CA)

3.2.7 Intracellular staining for flow cytometry

Suspensions of rMSCs (2 x 10^6 cells PBS washed cells) were pelleted in a 12 x 75 mm culture tube. The pellet was re-suspended in 0.875 ml of cold PBS. Then 0.125ml of cold 2% PFA was added and the suspension incubated at 4°C for 1 hour, centrifuged for 5 minutes at 250g, then the supernatant was removed. For permeabilisation cells were incubated with Triton X-100 (0.2% in PBS) at 37°C for 15 minutes. To terminate permeabilisation 1ml of buffer (1xPBS + 2% goat serum + sodium azide) and the suspension was centrifuged for 5 minutes at 250 x g. The supernatant was removed and internal staining then proceeded as described for immunocytochemistry. Non-specific binding was blocked by incubation with 5% goat serum in PBS for 1 hour at room temperature. A primary isotype matched control was

included in each experiment to exclude any background fluoresence. The cells used for this control analysis were fixed, permeabilised under identical conditions to experimental samples. For quantification of cells expressing a given marker, flow cytometry analysis was performed. The specificity of the assay was confirmed by the use of negative controls.

3.2.8 Flow cytometry analysis

All samples were maintained on ice prior to analysis. Analysis was performed using a coulter EPICS XL flow cytometer. FITC and Cy-3 were identified by using a 530 and 585 band pass filter respectively, and quantification was completed using CellQuest Software (Becton Dickinson). Ten thousand events were acquired per sample with fluoresence measured on logarithmic scales. Forward and side light scatter gates were set to exclude dead debris and clumps of cells. To calculate the percentage of positive cells, linear gates were set at 0.1%, on samples stained only with secondary antibodies, and expression corresponding to a fluorescence signal exceeding this percentage was measured.

3.2.9 Measurement of neurite outgrowth

N2a cells were differentiated either with DMSO or in the presence of DMSO and myelin associated glycoprotein (MAG, 0.3µg/ml, R&D). After 6 days cells were fixed in 4% PFA and processed for immunocytochemical staining with Tuj-1. Cells were viewed using an inverted fluorescent microscope and images acquired using digital camera. Images were taken of 10 non-overlapping visual fields (x10 magnification) for each culture condition and in 3 independent experiments in cells were cultured in parallel. The neurite lengths of every Tuj-1+ cell (10-15 cells/field approx) within each field of view was determined. Neurites exhibited by differentiating neuronal progeny from cultures of DMSO induced serum deprived N2a cells, that were immunopositive for TUJ1 were analysed using ImageJ 1.33 software, a public domain JAVA image processing program (NIH, USA). Pixel scale was set to microns according to image magnification. JPEG files obtained from light or fluorescent microscopy were opened in ImageJ and neurite lengths were measured by tracing along neurites with the freehand line tool then measuring length using the measurement tool.

3.2.10 Analysis of cell death

Cells were trypsinized, washed and processed for flow cytometry by forming a single cell suspension and maintained on ice. Cells were stained with 1 ug/ml propidium iodide (PI) (Sigma) just prior to analysis by flow cytometry. Annexin V staining was carried out according to the manufactures instructions. Briefly cells suspensions were washed in ice cold buffered PBS and centrifuged at 500 g at 4°^{c} for 5 minutes. The supernatant as discarded and the cell pellet was finally resuspended in ice cold binding buffer to a cell concentration of 5 x 10^5 cells/ml. Cells were then incubated with annexin V solution and PI for 10 minutes on ice in the dark. Cell samples were then diluted in ice-cold binding buffer and analysed by flow cytometry.

3.2.11 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism Software version 4. Results were analysed for statistical significance using ANOVA and all error bars are expressed as standard error +/- mean. All data unless stated otherwise is expressed as Mean±SEM. Post hoc analysis was done using Bonferroni corrected planed comparison.

3.3 Results

3.3.1 Transition in cell morphology in response to chemical induction

It has been demonstrated both by ourselves and others that exposure of cultured mammalian MSCs to antioxidant compounds or compounds that increase intracellular cyclic AMP (Deng et al., 2001) results in generation of cells which display a morphology and protein expression profile consistent with cells of a neural phenotype (Woodbury et al., 2000, Rismanchi et al., 2003, Qian et al., 2004; Croft and Przyborski, 2004). We describe these cells as neuroprogenitor-like cells. To induce this phenotype, MSCs were maintained as sub-confluent cultures in DMEM, 10% FCS media supplemented with 5ng/ml basic fibroblast growth factor (bFGF) for 24 hours prior to neural induction (in some studies 1 mM BME has been used as the pre-inductive agent and is reported to have the same effect). Formation of the neural-like morphology was induced by the removal of serum and bFGF and the introduction of an antioxidant compound B-mercaptoethanol (BME, 0.1-10 mM) (Figure 3.2b, 3.3a), dimethyl sulphoxide (DMSO, 0.1-2.0% v/v) (Figure 3.1, 3.2a, 3.3b) or butylated

hydroxyanisole (BHA, 10-200 μM) (Figure 3.2c and Figure 3.3c) at varying concentrations. Following this procedure, a transition in cell phenotype occurs rapidly and stromal cells adopt a neural-like morphology over the following 5 hours (Figure 3.1, 3.2, 3.3). For example, in response to 2% DMSO in serum free media, responsive cells drastically changed their morphology (Figure 3.1). During the first 1-2 hours, responsive cells adopt a multipolar phenotype radiating peripheral process-like extensions. Over the next 3 hours the perikaya of responsive cells becomes more spherical and refractile in nature. This process continues and cells display secondary (figure 3.1c, arrows) and tertiary branching, resulting in the formation of extensive network of neurite-like processes (Figure 3.1d). Within the first 5 hours the majority of cells alter their morphology, although there is variability between cells in terms of the onset of induction but it is unclear why this occurs. In our experiments, we observe that type I MSCs, that are characterized by their more spindle shaped morphology (Figure 3.1b, arrows), are the first cells to respond to this treatment whilst larger flat cells show a delayed or inhibited response. It is unclear at this stage why this occurs. Staining for the pan neural marker Neuron-specific enolase (NSE, enolase enzyme associated with neuronal cells) was used to confirm these cells had a neural identity and consistent with the detection of increasing numbers of neural-like cells in the culture in response to DMSO the number of cells positive for NSE increased with time in culture.

Figures, 3.1, 3.2 and 3.3 which collectively provide an analysis of the effects of DMSO, BME, BHA and forskolin on the morphology of MSCs in culture using phase contrast microscopy, Within 30 minutes of exposure of cells to chemical inductive agents in serum-free media, changes in morphology of some rat MSCs were observed and as described above spindle shaped cells tended to be the first to respond. As cells assumed a neuronal morphology the cytoplasm retracted towards the nucleus, forming a contracted multipolar, cell body, leaving membranous, process-like extensions, which radiated from the cell body. These radial processes resembled neurities. By 3 hours the cell bodies of MSCs became progressively refractile, exhibiting what has been described as a typical perikaryal appearance. With increasing time of exposure (3-5 hours) the apparent processes became more elaborate, displaying primary and secondary branching and in some cases growth cone like structures appeared to be present on terminal processes, which is consistent with axonal growth. In summary, in

preliminary findings we were able to recapitulate the earlier observation of Woodbury et al., 2000 in which antioxidant compounds induce a presumptive neuronal phenotype as defined by morphology and the expression of pan neural markers.

3.3.2 Efficiency in inducing changes in cell morphology:

Treatment of cells with either antioxidant compounds or forskolin resulted in comparable morphological changes regardless of the agent used and these changes occurred over a consistent time span (5 hours). (Figure 3.2, 3.3) However, the absolute number of responding cells and the total number of neural-like cells identified after 6 hours induction was different depending on the agent used. Evaluation of cultures of MSCs treated with antioxidant compounds as function of time and concentration revealed that the number of neural-like cells increased as a function of these two parameters. As of induction increased from 0-6 hours the number of neural-like cells progressively increased but reached a plateau in all cases between 5-6 hours. In the presence of 10μM BME the number of neural-like cells increased from 9.8±1.8 to 72.8±4.9% between 30 minutes and 6 hours. Similar results were obtained for 4% DMSO (42.6±6.9 to 86.7±3.6% between 30 minutes and 6 hours) and 200μM BHA (14.8±1.8 to 67.8±8.3%, between 30 minutes and 6 hours). In addition, a dose response curve was demonstrated for all 3 antioxidant compounds (BME, DMSO and BHA). As the concentration increased the total number of neural-like cells increased for each time point analyzed. This suggested that the limiting factor to this acquisition of a neural-like morphology was not time but the concentration of the inducting agent.

The same pattern of response was demonstrated for cells induced with forskolin in serum free media. A dose response curve was demonstrated for forskolin (figure 3.3d, 3.2d) with the number of neural-like cells increasing in proportion to the final concentration of forskolin, however a maximal response was observed between 50-100μM. The absolute number of cells acquiring a neural-like morphology in response to forskolin treatment were significantly lower compared to the efficiency of induction using antioxidant compounds. The number of neural-like cells increased from 7.4±1.9 to 32.7±5.1 between 30 minutes and 6 hours post induction with 200μM forskolin. This observation is consistent with the report of Deng et 2001. These

investigators used IBMX, a compound that increases intracellular cyclic AMP through activation of Protein kinase A found that about 25% of cells assumed a neural-like morphology in response to this compound. The reason for the differences in the level of induction observed in antioxidant treated cultures compared to forskolin treated cultures is known but is likely to be linked to differences in the mechanism of action of the compounds.

To examine whether the removal of serum contributed to the induction of a neural morphology in MSCs, controls were included in analysis in which cells were cultured under identical conditions as chemically induced cells and received the same pre-conditioning treatment as induced cells but where subsequently cultured only in serum free media. 19.8±3.1% of neural-like cells wee found in cultures maintained only in serum free media. The morphology of these neural-like cells was comparable with those in induced cells, however, a large number of cells in serum free media showed a degree of cytoplasmic retraction but not a full neural-like morphology. Although only 20% of cells (approx) responded simply to the removal of serum the responding cells tended to be grouped in the culture and their small groups of 5-6 responding cells were found with the culture. In addition, a small proportion of neural-like cells (ranging from 0.3-06% of cells) were reported in cultures maintained under control conditions, suggesting that MSCs may have an intrinsic neurogenic potential and can differentiate into neural derivatives.

The most effective inducing agent was DMSO. 86.2% of cells treated with 4% DMSO acquired a neural-like morphology (figure 3.2a). Therefore only approximately 15% of cells did not respond and there was no apparent reason from image analysis, which examined why these cells did not respond. Non-responding cells were evident under all inductive conditions tested and ranged from 15-50% in number depending on the agent used. Image analysis at 24 and 48 hours revealed that neural-like cells were still present in cultures and were viable. However, it was evident that some spontaneous reversion had occurred and some had reverted to a stromal cell phenotype with a concomitant reduction in the number of neural-like cells present in the culture. This effect occurred in all inductive conditions, but was least evident in DMSO treated cells. Some of the cells partially lost contact with the substratum and others completely detached. For further investigation DMSO was chosen as the inductive

agent because of the efficiency of induction achieved and because DMSO is known to induce neuronal differentiation of neuroblastoma cell lines. The concentration chosen was 2% because 4% DMSO resulted in high levels of substrate detachment.

Cell counts revealed that between 70-90% of cells treated with either BME, DMSO or BHA adopted a neural-like phenotype, which is consistent with the findings of other investigators (Woodbury et al., 2000; Rismanchi et al., 2003; Qian et al., 2004). However, there is considerable variability between experiments in the proportion of cells that respond to the treatment. We have identified a number of variables that may account for these changes in induction efficiency. One of the primary factors that appeared to influence the percentage of responding cells was the time that the cells had spent in culture (ie. number of population doublings). Earlier work has shown that MSCs gradually lose their multipotent potential for differentiation during ex-vivo cell expansion (Digirolamo et al., 1999; Sekiya et al., 2001). In agreement, the efficiency of inducing neural-like phenotypes in response to antioxidants was highest in cultures with minimal ex-vivo expansion. In general, the majority of experiments we report here are within 3-4 passages of the primary explant culture (unless otherwise stated, in later experiments P8 cells are used following a report that prolonged culture is required for efficient nestin expression in response to defined culture conditions (Wislet-Gendebein et al., 2003). Cell density and confluence of the culture also have a considerable affect on induction efficiency, as well as the formation of neurite-like processes and establishment of an intricate network. We found consistent results and a high percentage induction of neural-like morphologies when MSCs were seeded at a density of 10,000 cells/cm^2 and grown to 80% confluence on tissue culture plastic prior to treatment with antioxidants. Under these growth conditions >85% of cells respond and form secondary and tertiary branches as described earlier.

3.3.3 Characteristics of MSC presumptive neurite

To further examine the structural alterations in response to DMSO treatment and the validity of this response, cells were stained for cytoskeleton proteins characteristic of neuronal development. Cells were first pre-treated with 5ng/ml bFGF for 24 hours in serum containing media. Cells were then transferred to serum free media supplemented with 2% DMSO for 5 hours following which cells were fixed and

processed for immuno-cytochemistry (Figure 3.4). The microtubule system was visualized using a α-tubulin antibody. Microtubules were collapsed around the cell body and although staining was detected in the processes radiating from the cell body, this staining was less dense compared to the cell body staining (Figure 3.4a). This is in contrast, to neuronal cells, in which developing neurites are microtubule rich. MSC neural-like cells, expressed a neuronal specific microtubule Tuj-1, the intensity of this staining was again concentrated in the cell body as opposed to the radiating cytoplasmic processes (Figure 3.4b). To visualize the actin cytoskeleton, cells were labeled with a TRITC conjugated Phalloidin which binds to F-actin (Figure 3.4c). Consistent with neurite development, presumptive MSC neurites contained a high concentration of F-actin. Cells also expressed GAP-43 however, the localization of this protein was not consistent with neuronal development and as the expression was not confined to the terminal portion of developing processes and in fact these regions were weakly stained in MSC presumptive neuronal cells (Figure 3.4d).

To examine if the developmental potential of MSCs is affected by chemical induction, induced MSCs (5 hour treatment with 2% DMSO) were differentiated in osteogenic medium (Figure 3.4e,f). Neural-like cells were capable of forming osteoblasts in culture as verified by a calcified extra-cellular matrix. Chemically induced cells therefore do not loose their developmental potential and retain their capacity to differentiate into mesenchymal cell lineages. The presumptive neural-like morphology of induced MSCs was also found to be fully reversible following the re-introduction of serum and removal of the inductive agent. This reversibility occurred within the same time frame as chemical induction i.e. 5 hours (Figure 3.4g,h).

To investigate the role of pre-induction and serum on the acquisition of a neural-like morphology MSCs were induced by 2% DMSO in either the presence or absence of serum (10% FCS) (Figure 3.5). The number of neural-like cells was determined by image analysis of cultures every hour for 6 hours following exposure to DMSO. The number of neural-like cells was greater in cultures induced in the absence of serum at each time point compared to induction in the presence of serum. By 6 hours, 83.3±2.9% of cells had acquired a neural-like morphology in the absence of serum in contrast, to 44.9±12.5% of cells that acquired a neural-like morphology when induction was carried out in the presence of serum. To examine the effect of bFGF

treatment on induction, cells were cultured in the presence of 5ng/ml bFGF for 24 hours prior to induction (pre-induction). The number of neural-like cells formed under these conditions was compared to non-pre-induced cells. The number of neural-like cells formed in cultures pre-induced (87.2±5.6%) with 5ng/ml bFGF was significantly ($P<0.05$ Students T-test) higher than non pre-induced cell cultures (77.1±3.1%). Therefore consistent with previous reports, FGF-2 pre-induction significantly increased the number of neural-like cells formed in response to chemical induction (Woodbury et al., 2000).

3.3.4 Cell viability following 5 hours DMSO treatment

Antioxidant compounds are cytotoxic at high concentrations. We therefore examined the effect of DMSO induction and serum removal on cell viability (Figure 3.6). Cells were cultured in either: DMEM + 10% FCS + 2% DMSO or DMEM + 2% DMSO. Cell viability was assessed using propidium iodide (PI). PI is excluded from healthy cells but enters dead cells following their loss of membrane integrity. It binds to exposed DNA and becomes highly fluorescent. This fluorescence was examined by flow cytometry, which determined the number of cells, which had incorporated PI and was therefore a measure of cell death. Cells were harvested every hour for 6 hours following induction under these conditions. The cells were then processed for flow cytometry. The number of PI+ cells in induced cultures was compared to non-induced MSCs. The number of PI+ cells ranged from 4-12% in induction carried out in serum containing media and there was no significant difference in the number of PI positive cells in induced versus non-induced MSCs at any time point. Cell death was higher when induction was carried out in serum free media and ranged from 5-19%. However, there was no significant difference between cultures induced in serum free media and control cells, although cell death was higher in cultures induced for 1 hour. Cell death was lower at subsequent time points.

3.3.5 Expression of neural proteins in chemically induced MSCs

To further investigate the phenotype of MSC derived presumptive neurons, the expression of neural and mesodermal proteins in chemically induced MSCs was examined by western blot analysis (Figure 3.7), intracellular staining quantified by flow cytometry (Figure 3.8) and immuno-fluoesecnce (Figure 3.9). Several independent investigations have demonstrated that changes in morphology subsequent

to treatment with antioxidants are also accompanied with the expression of a range of proteins commonly expressed by neural cell types (Woodbury et al., 2000; Lu et al., 2004; Neuhber et al., 2004; Bertani et al., 2005). For western blot analysis cells were treated under a range of conditions including: control MSCs (FCS), serum free media for 5 hours (SF), pre-induced and then cultured in serum free media for 5 hours, preinduced for by exposure to antioxidants (BME, DMSO, BHA) for 5 hours in serum free media (Figure 3.7). Western blot analysis revealed that undifferentiated (non-induced) MSCs expressed of GFAP, Tuj-1 and NF-160 at low levels when cultured under standard culture conditions. This expression was confirmed by flow cytometry following intracellular staining (Figure 3.8). Which demonstrated that small subpopulations of cells (12-45%) expressed low levels of synaptophysin, NeuN, NF-M, GAP-43, Tuj-1 and a minor population of cells (4-19%) express TAU and/or MAP-2 at very low levels. These results are consistent with a number of studies that have reported that MSCs spontaneously express neural proteins in culture (Ratajczak et al., 2004; Tondreau et al., 2004; Deng et al., 2006).

Western blot analysis revealed no change in the expression of GFAP in response to any of the culture conditions and inductive agents tested, however a basal expression level was detected (Figure 3.7). Nestin expression was not detected in control conditions (serum containing media), however its expression was detected when cells were cultured in serum free media and was highly expressed in cultures treated for 5 hours with antioxidant compounds in serum free media. Neurofilament 160 (NF-M) expression was detected at very low levels in cells maintained in serum containing media (control, FCS). However removal of serum and/or treatment with antioxidants was associated with an up-regulation in the expression of NF-M. Tuj-1 expression showed a similar pattern, low levels were detected in control cells and higher levels of expression were identified following serum withdrawal and treatment with antioxidants.

The effect of serum removal and neural induction in the presence of 2% DMSO in serum free media on the induction of neural protein expression by MSCs was confirmed by flow cytometry (Figure 3.8). 5-hour serum withdrawal without antioxidant treatment was associated with an up regulation in the expression of neural proteins by MSCs above the basal level of expression (Figure 3.8). An increased

expression as defined by an increase in the number of cells positive and an increase in the intensity of the fluorescence staining (Right shifted solid curve). Increased expression was observed for all neural proteins tested including synaptophysin, NeuN, NF-M, GAP-43, Tuj-1, GFAP, Nestin, MAP-2 and TAU. This increase in neural protein expression in response to serum withdrawal was greater in the presence of 2% DMSO. The number of positive cells and the mean fluoresce intensity of neural protein expression by DMSO induced MSCs was greater than in non-induced cells. These observations correlate with the effects of neural induction and serum removal on morphology. Cells with a neural-like morphology were observed in cultures in which serum was removed for 5 hours, and consistent with this finding serum removal was associated with an increased expression of pan-neural markers. However, the number of neural-like cells (level of induction) was greater in serum free cultures exposed to 2% DMSO for 5 hours (Figure 3.8). The number of neural-like cells was higher and the associated induction of neural protein expression was greater. Following both serum withdrawal and DMSO induction there was a concomitant down regulation in the expression of the mesodermal marker vimentin. This is consistent with the commitment of MSCs towards a neural lineage with a down regulation in mesodermal properties.

Localization of the expression of these proteins in response to serum removal and neural induction was confirmed by immuno-staining and florescence microscopy (Figure 3.9). The level of immuno-florescence staining was quantified by flow cytometry and the temporal expression of markers analysed over 5 hours (Figure 3.10). The number of positive cells for each marker was assayed every hour for 5 hours in response to DMSO induction in serum free media compared to cells maintained under standard culture conditions. No significant difference in the number of positive cells was observed for GAP-43, Synaptophysin, TAU and Tuj-1. Although the level of expression in those cells positive was increased in response to induction quantification of these effects revealed the number of positive cells for these markers is not significantly different to the number of cell positive under control conditions. However, consistent with the increase in the number of neural-like cells between 1-5 hours following DMSO induction the number of cells positive for MAP-2, NeuN, GFAP, NF-M and Nestin significantly increased over 5 hours compared to the number of cells positive for these markers under control conditions. In addition, we

observed a concomitant down regulation in the number of cells positive for vimentin. The absolute number of cells expressing these markers showed considerable variability depending on the marker analysed. The results are consistent however with the number of neural-like cells increasing in response to DMSO induction and thus the number of cells expressing neural markers.

3.3.6 Effect of the growth substrate

To examine whether the formation of neural-like morphologies was influenced by the substrate on which cells were grown, we plated MSCs on tissue culture plastic coated with either poly-D-lysine (10μg/ml), Laminin (5μg/ml) or fibronectin (25μg/ml) prior to exposing the cells to treatment with antioxidants. None of these substrates had any significant effect on the percentage of cells that responded and form neural-like cells by 12 hours (Figure 3.11b). However, coating of tissue culture plastic with a substrate did slow the acquisition of a neural-like morphology by MSCs as the number of neural-like cells at 6 hours was lower in cultures induced on a substrate compared to cells induced on tissue culture plastic (control).

3.3.7 Comparison of the effects of DMSO induction on N2a cells versus MSCs

N2a mouse neuroblastoma cells differentiate into neuronal cells when exposed to serum free culture or DMSO (Evangelopoulos et al., 2005). N2a and MSCs were induced under identical conditions. Both cells were exposed to 2% DMSO in serum free media for 5 hours. After 5 hours cells were fixed and processes for immuno-cytochemistry (Figure 3.12). N2a cells underwent significant structural alteration including the extension of processes. After 5 hours most N2a cells had assumed a neuronal morphology, MSCs under the same conditions also assumed a neural-like morphology in this time period and both populations of cells expressed GAP-43, NeuN, Tuj-1, NF-M, MAP-2 and NF-L. There were however, differences in the intensity of this staining in different regions of the cells i.e. there were differences in the localization of these proteins. In MSCs most staining was confined to the cell body and staining that was evident in processes was less intense compared to that at the cell body. In contrast, NF-L, NF-M, MAP-2 and GAP-43 was highly expressed in the developing neurites of N2a cells consistent with the role of these proteins in neurite development.

Because of these differences in the localization of key neural proteins involved in neuronal development especially the development of neurites we next chose to examined the validity of presumptive neurite in MSCs compared to N2a cells (Figure 3.13a). Neurite development in N2 cells is microtubule dependent (Diaz-Nido et al., 19991) and as shown in Figure 3.13a treatment of induced N2a cells with a colcemid, a microtubule disruptive agent causes retraction of developing neurites. In contrast the neurite development in MSCs induced by DMSO is not microtubule dependent as treatment of induced neural-like cells with colcemid did not disrupt the MSC presumptive neurite. Following DMSO induction immuno-staining for microtubules revealed intense staining the neurite of N2a cells and not in MSCs (Figure 3.13b). Tuj-1 was also highly expressed in the neurites of N2a cells in contrast to MSCs were the cell body was more intensely stained. Neuroblastoma derived neurite continually undergo reorganization by a process of actinoplast-tubuloplast segregation (Tint et al., 1992). These segregations appear as bulb-like structures along the axon. Similar structures were observed in MSC processes. In N2a cells neurite outgrowth involved the clear extension of growth cone structures on the terminal portions of the developing processes, which highly expressed the growth cone protein GAP-43. MSCs did not however processes GAP-43 positive growth cones on the terminal portions of their neurites (Figure 3.13b). This is not consistent with neurite development and extension.

Three myelin-associated inhibitors of regeneration have been identified. These are myelin-associated protein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo-A (Chen et al., 2000b; GrandPre et al., 2000; Prinjha et al., 2000), and oligodendrocyte myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002). In culture these inhibitors can be used to inhibit neurite development and outgrowth. To test the validity of MSC presumptive neurite we tested the ability of MAG to inhibit their outgrowth. MSCs and N2a cells were induced to differentiate in the presence of DMSO either alone in serum free media or in the presence of MAG (0.3μg/ml) for 6 days. MAG significantly inhibited neurite outgrowth in differentiating N2a cells in contrast MAG had no effect on neurite outgrowth of MSCs (Figure 3.14). Therefore MSC presumptive neurite were not MAG responsive.

3.3.8 Effects of serum on the expression of the neural marker, nestin:

The formation of neural-like cells from MSCs proceeds maximally in the absence of serum. In agreement with other studies (Wislet-Gendebien et al., 2003), we have noted that withdrawal of serum from the media up-regulates the expression of nestin, an intermediate filament protein that is known to be expressed in neuroprogenitor cells (Figure 3.15). We suggest that the expression of nestin is a pre-requisite for the induction of a cell to adopt a neural-like morphology. Interestingly, we have also observed that cells that do not respond to neural induction agents do not up-regulate nestin subsequent to serum removal. Although it remains unclear what mechanism is responsible for the activation of nestin expression, it appears that induction of nestin expression is a key event that precedes further differentiation and dictates the percentage of cells that adopt the neural-like fate. Additional experiments in our laboratory have shown that administration of bFGF (5ng/ml) to cultures 24 hours prior to exposure to antioxidants also increases the expression of nestin even in the presence of serum (Figure 3.15).

3.3.9 Expression of neural proteins following 6 days DMSO neural induction

6 days differentiation was carried out in serum free media supplemented with N2 and 2% DMSO. Under these conditions MSCs expressed neuronal proteins and assumed a neuronal-like morphology consistent with the results for 5 hours induction. The number of neural-like cells does decrease with time indicating that MSC neural-like cells spontaneously revert back to an MSC phenotype and that the conditions may be insufficient for long-term induction however the protocol used is identical to that used by several investigators. Nevertheless, neural-like cells expressing neuronal proteins were detected in culture following 6-day induction (Figure 3.16). Temporal analysis of this expression as quantified by flow cytometry to determine the number of cells positive for each marker showed the at the number of synpatophysin and MAP-2 positive cells was not significantly increased by 6 day induction compared to the number of cells positive for these markers in control cells (Figure 3.17). The number of nestin positive cells decreased with time consistent with the induction of a more mature neuronal cell fate. In addition there was a concomitant decrease in vimentin expression at 1 and 3 days post induction. However, vimentin levels returned to control levels by 6 days consistent with spontaneous reversion of neural-like MSCs to an MSC phenotype. NF-M was also highly expressed at day 1 but returned to control

levels by 6 days. In contrast the number of cells expressing GAP-43 was only significantly increased above control levels at the day 6-time point.

These results were confirmed by western blot analysis (Figure 3.18). Down-regulation in the protein kinase C a signal transduction enzyme which activates pathways leading to gene transcription and in some cases induction of growth and differentiation has been shown, to be associated with the neuronal differentiation of neuroblastoma cell lines in response to serum withdrawal (Charkravarthy et al., 1995). We analysed the expression of PKC using a pan-PKC antibody, which is no specific for PKC isotypes. In MSCs induced for 6 days in the presence of DMSO the expression of GAP-43 is increased but begins to return to control levels by 6 days. Tuj-1 and NF-M shows a similar pattern whereas MAP-2 and synpatophysin show little increase from control levels. PKC was however down regulated following 6-day induction, although early induction was associated with increased expression of PKC this decreased by the day 6-time point. SHSY-5Y cells, a neuroblastoma cell line which undergoes neuronal differentiation in response to DMSO and serum deprivation were cultured and induced to differentiate under identical conditions to the MSCs for 6 days. SHSY-5Y cells differentiated into neuronal cells during the 6 days DMSO induction and this was associated with a progressive increase in the expression of GAP-43, Tuj-1, NF-M, MAP-2 and synaptophysin. This protein expression was associated as it was in MSCs with containment down regulation in the expression of PKC. The down-regulation of PKC with differentiation is consistent with the reports of previous investigators. Therefore despite the similarity in the acquisition of neuronal-like morphologies the temporal expression of neural proteins in MSCs was not consistent with neuronal induction with the expression of Tuj-1, GAP-43 and NF-M decreasing over 6 days. Also the molecular mechanism of neural protein expression may be different in these cell populations since in MSCs neural differentiation was associated with a down regulation in PKC whereas in MSCs early differentiation involved induction and as the number of neural-like cells decreased over 6 days the 6-day time point was associated with lower levels of PKC expression.

We have also used flow cytometry to examine the expression of cell surface antigens previously described to be associated with the neuronal phenotype on MSCs treated with antioxidants over a 6 day period (Figure 3.19). Expression of N-CAM (neural

cell adhesion molecule) was monitored using the monoclonal antibodies B159 and VINIS-53. VINIS-53 staining increased progressively over the 6-day period and correlated with the increased expression of NSE. The neuronal gangliosides, GD2 and GD3, are recognized by the antibody VINIS-56 and showed marked increases in expression over the 6-day period. Similarly, A2B5, a marker of early neurons and oligodendrocytes, was also increased following exposure to antioxidants. These patterns of immunoreactivity are consistent with the generation of neuronal cells.

3.3.10 Effect of 6-day chemical induction on cell viability

Cell death in induced cultures was determined both by PI staining and Annexin V (an apoptosis marker) (Figure 3.20). DMSO induction carried out in the absence of N2 was associated with higher levels of cell death than neural induction carried out in the presence of N2 supplementation. This indicates that it is the removal of serum that compromises long term viability and not the inductive agent. Replacement of serum with a defined culture supplement reduced the cell death associated with 6-day induction.

3.3.11 Comparison of the localization of neural proteins in induced MSCs and differentiated SHSY-5Y cells.

Analysis revealed significant differences in the intracellular distribution of key neuronal proteins (Figure 3.21). Tuj-1 was intensely expressed in SHSY-5Y neurites in contrast to MSCs in which staining was predominantly found in the cell body. In SHSY-5Y cells, synaptophysin, GAP-43 and SNAP-25 where expressed both in the cell body but not only found on the terminal portions of developing neurites consistent with a synaptic location. However, in MSC presumptive neurons the staining for equally distributed between the cell body and process-like extensions. Both SHSY-5Y cells and MSC presumptive neurons expressed vimentin, NF-M, MAP-2 and NF-L. However, in SHSY-5Y cells, NF-M, NF-L and MAP-2 were highly expressed in developing neurites in contrast to MSCs in which processes were only densely stained. Therefore the intracellular distribution of neural proteins in MSCs is not consistent with primary neuronal cells and does not appear to correlate with potential function.

3.3.12 Generation of neurosphere-like structures from MSC cultures

Experiments by others and us have shown that mammalian MSCs up-regulate the expression of nestin in response to serum withdrawal or treatment with basic fibroblast growth factor (Woodbury et al., 2000), suggesting that MSCs may have the capacity to generate a population of neuroprogenitor cells. We hypothesized that MSCs may also produce neuroprogenitor cells when cultured under conditions used to maintain neural stem cells. Neuroprogenitor cells can be routinely grown in serum free, non-adherent conditions where they proliferate in response to epidermal growth factor (EGF) and (bFGF) (Carpenter et al., 1999). Under these growth conditions neuroprogenitor cells express high levels of nestin (Figure 3.22). Kabos et al., 2002 identified that unfractionated bone marrow contains cells with the capacity to form free-floating cellular spheres morphologically and phenotypically similar to neurospheres generated from fetal neural stem cells. Whole bone marrow plated on poly-D-lysine coated surfaces in a defined serum-free growth medium (DMEM/F12 supplemented with B27 (Invitrogen)) containing bFGF and EGF gave rise to aggregations of cells that detached from the culture flask and became free floating clusters (Kobas et al., 2002). These aggregates of cells resembled neurospheres in morphology and expressed nestin. In addition, dissociation of the primary aggregates gave rise to secondary spheres demonstrating that a single cell had the proliferative capacity to form a new cell aggregate. Floating spheres of cells were also capable of differentiation into neurons and glia when plated on to laminin and maintained in medium containing retinoic acid and cAMP or bFGF (Kobas et al., 2002). Furthermore, these cells were also capable of engrafting into the hippocampus following transplantation into the right hemisphere of syngenic animals (Kobas et al., 2002). To address the issue as to which stem cells in bone marrow had given rise to the sphere, Kabos and co-workers examined the expression of stromal and hematopoietic cell markers. From the resulting expression data, it was concluded that because cells in the spheres expressed CD90 and only a low levels of fibronectin that such cells were not stromal in origin but representative of the hematopoietic lineage. However, others and we have found CD90 is expressed at high levels on MSCs cells and that generation of neural precursor cells down-regulates fibronectin expression (Sanhez-Ramos et al., 2000). Therefore it is unclear whether a contribution from the BMSC cell population can be discounted.

3.3.13 Method to produce free floating cellular spheres:

In our laboratory, we have investigated the potential of MSCs to form free floating cellular spheres representative of neuroprogenitor cells (Figure 3.22). Pure populations of MSCs were isolated and plated at 8,000 cells per cm^2 and grown to >80% confluency. At this stage the cells were dissociated by 0.05% trypsin and 0.04% EDTA in phosphate buffered saline, neutralised with DMEM plus 10% FCS, collected by centrifugation and replated on to poly-D-lysine (10μg/ml) coated tissue culture plastic. The growth medium was subsequently replaced with serum-free media (DMEM/F12 supplemented with N2 (Invitrogen)) containing EGF and bFGF (both at 10ng/ml). The majority of cells subsequently adhered to the tissue culture flask forming a monolayer. After 3 to 4 days, viable aggregations of cells started forming on the bottom of the flask. As these aggregates of cells became larger they detached from the flask and became free-floating spheres. Cells within these structures continued to proliferate, the spheres became larger in size, and at this point sub-culture was possible. These spheres were positive for NeuroD1 and nestin (Figure 3.22 e,f).

MSC-derived spheres will continue to proliferate and grow in size in response to the mitogens in the culture medium. Sub-culturing and continued propagation of the culture is necessary to prevent spheres of cells becoming too large and thus reducing the possibility of necrosis at the centre of the cell aggregate. Sub-culturing can be carried out by pelleting the aggregates and mechanically dissociating the spheres into single cells using a fire polished glass pipette. The cells are then re-seeded into new flasks in fresh medium. We have shown that our MSC-derived spheres can be continually propagated if sub-culturing is optimal.

Consistent with our previous results that suggested that nestin expression was a pre-requisite for the acquisition of a neural-like morphology we also found that nestin expression correlated with sphere formation (Figure 3.23). Spheres were originally generated in serum free media supplemented with EGF and FGF-2 +N2. We found however, that it was the removal of serum that was the critical factor for the formation of cellular spheres. As cellular spheres were formed in low attachment conditions in serum free conditions without addition of growth factors. Growth factors were however, required for cellular proliferation. Western blot analysis showed that nestin expression could only be following the removal of serum, however the level of

expression was higher and sphere formation greater in cultures supplemented with EGF and FGF-2 compared to serum deprivation alone. We also found that own attachment conditions were vital for the formation of spheres and that high attachment conditions inhibited sphere formation and nestin expression (Figure 3.24). Nestin expression therefore also appeared to linked to the ability of cells to undergo cell rounding and cytoplasmic retraction as well the removal of serum.

3.3.14 Neural Differentiation of MSC derived spheroids

To determine whether MSCs grown as free floating spheres produce neural derivatives, we assessed the expression of neural markers indicative of neuroprogenitors and differentiated neural derivatives. Immunocytochemical staining for nestin showed that small suspended aggregates of cells expressed nestin at high levels and that this expression was maintained as the aggregates increased in size to form solid free floating spheres (Figure 3.25a). These data indicate that the spheres contain a population of neuroprogenitor cells. To test the hypothesis that the cells in these spheres behave as neuroprogenitor cells, we differentiated MSC-derived spheres under conditions known to induce differentiation of embryonic neuroprogenitor cells (Svendsen et al., 1998). Seeding embryonic neuroprogenitor cells grown as neurospheres onto substrate coated surfaces and removing growth factors is sufficient to induce further neural differentiation resulting in the production neuronal and glial cells in endogenous NSC/progenitor (Svendsen et al., 1998). MSC cellular spheroids plated on PLO coated tissue culture attached and migrated from the site of attachment and expressed nestin on initial plating and after 3-4 days high levels of Tuj-1 expression were evident (Figure 3.25 b,c). However cells remained rounded and failed to spread. Our experiments have shown that when MSC-derived spheres were plated on laminin (25μg/ml) in the serum free conditions in the absence of growth factors, the cells attach and begin to migrate out from the sphere. Nestin expression was initially high on plating but was down regulated in adherent cells whilst markers of more mature neural cells were detectable, including the neuronal protein, β-III tubulin, and the astrocytic marker, GFAP. However, there was very little evidence of oligodendrocyte differentiation as indicated by the absence of O4 staining (data not shown). Cells acquired a more flat well spread morphology which in some cases still resembled a neuronal-like morphology. When cells were differentiated in the presence

of NGF (10ng/ml) and RA (0.5μM) in serum free media on Laminin coated tissue culture plastic cells positive for GAP-43, Tuj-1, GFAP, MAP-2 and NF-M (Figure 2.25 d-i) were detected after 12 days differentiation. However, in most cases the morphology was more consistent with a stromal morphology as opposed to a neuronal morphology.

Immuno-staining followed by flow cytometry was used to quantify the level of expression of neural and mesodermal markers in cellular spheroids maintained in serum free media supplemented with EGF and FGF-2 and in spheres differentiated in the presence of NGF and RA for 12 days following plating (Figure 3.26). Fibronectin expression was found to be reduced in cellular spheroids. However, this expression was restored to normal MSC levels following 12 days plating under differentiation conditions. This observation suggests that cellular spheroids revert back to a mesodermal phenotype when plated in adherent conditions regardless of the differentiation media. Consistent with this notion nestin was found to be highly expressed in cellular spheroids but down regulated after 12 days plating, although a minor subpopulation still expressed nestin at moderately high levels. Most cells (<98%) expressed Tuj-1 at high levels in cellular spheres and although after 12 days differentiation most cells still expressed Tuj-1 the intensity of this expression were reduced. NF-M and GFAP expression was also highly expressed in cellular spheroids, however following 12 days plating, the expression of these markers was confined to only a minor subpopulation of cells, which still express these markers at moderately high levels. MAP-2 expression shows little change following 12 days differentiation. Therefore rather than an up-regulation in the expression of more mature neural markers following differentiation we observed a reduction in the expression of neural markers and an increase in the expression of fibronectin indicates that MSC spheroids reverted to a mesodermal cell phenotype. In addition, cellular spheroids co-expressed a number of more mature neural markers and nestin. Analysis of the temporal expression of these markers was carried out by western blot analysis (Figure 3.27). Western blot analysis revealed no change in the expression of GFAP and MAP-2 during 12 day differentiation and although the expression of these markers higher in cellular spheroids than control MSCs no difference in expression was found after 12 days re-plating under differentiation conditions. GAP-43 and Tuj-1 expression however decreased progressively with re-plating. S100 expression was not detected in

cellular spheroids or cells replated under differentiation conditions. To see if BDNF supplementation resulted in the same expression profile cellular spheroids were replated in the presence of either RA+NGF or RA+BDNF for 12 days. No S100 expression was detected. However, GFAP, Tuj-1, GAP-43 and NF-M were expressed under both conditions at equivalent amounts 12 days following re-plating under these conditions.

In addition, comparison was made of the morphology and expression of key neuronal markers in MSC derived cellular spheroids and primary rat NPC when differentiated under identical conditions. Cells were plated on PLO coated tissue culture plastic and differentiated in the presence of RA+NGF for 6 days (Figure 3.28). Following two days differentiation MSC plated spheroids completely down regulate nestin expression, in contrast primary rat NPC remain nestin positive and produce nestin positive neurites, which extended and radiate from the primary site of attachment. After 6 days, Tuj-1 positive neurites can also be observed extending from the spheres original site of attachment. In contrast MSC plated spheres remained rounded without neurite production. Again at 6 days GFAP positive cells with an astrocyte morphology could be observed in NPC cultures in contrast MSC cellular spheroid cells highly expressed GFAP but were rounded and did not resemble atrocytes. In NPC cells MAP-2 positive neurite extension could be observed whereas MAP-2 expression in MSCs was very low. Also 12 day differentiated MSCs retained their mesodermal developmental potential with the ability to generate bone. These results are inconsistent with a permanent transition in cell fate of MSCs towards a neural lineage.

3.3.15 Link of PKC to neural-like morphology and protein expression

Following the observation that PKC is first up-regulated and then subsequently down-regulated following 6 days neural induction we next examined the effect of PKC activation on the morphology and neural protein expression of MSCs maintained in culture. In the case of MSC induction removal of serum was associated with nestin expression and an up-regulated expression of neural proteins and an increase in PKC levels as determined by a pan-PKC antibody. Therefore the increase in PKC levels was associated with high levels of neural protein expression and as PKC levels decreased, neural protein expression was reduced. We therefore determined a working

hypothesis that serum removal activated PKC, which resulted in a neural-like morphology. Therefore activation of PKC should result in the acquisition of a neural-like morphology despite the presence of serum within 5 hours (Figure 3.29). Consistent with this hypothesis, treatment of MSCs with 10μM PMA resulted in the formation of a neural-like morphology in MSCs maintained in serum containing media (Figure 3.29). The number of responding cells varied from approximately 25-90% depending on the concentration of PMA used. 10μM PMA resulted in >90% of cells assuming a neural morphology even in the presence of serum however treatment of cells with 0.1μM PMA resulted in only 25% of cells adopting a neural-like morphology. Therefore a dose dependent relationship was demonstrated (Figure 3.30).

We next examined the effect of PMA on neural protein expression in MSCs maintained in serum containing media. 5 hours treatment of cells with 10μM PMA resulted in a significant increase in the number of cells positive for NF-M, NeuN, GFAP, Nestin and Tuj-1 (Figure 3.30). By 24 hours however the number of cells expressing these markers had decreased and was not significantly higher than control cultures. This decrease in neural protein expression is consistent with the morphological data, which showed that effect of PMA on morphology was transient and spontaneously reversed by 24 hours. With cells returning to a stromal like morphology. Colcemid results in disruption of microtubules and prevents the acquisition of a neural-like morphology in MSCs (See chapter 4). Therefore we tested the whether the effect of PMA on neural protein expression was secondary to changes in the morphology induced by PMA or a direct effect of PMA. This was achieved by pretreating cells with colcemid to prevent the acquisition of a neural-like morphology in response to PMA. Cells were then treated with 10μM PMA in serum containing media. The level of protein expression in MSCs pre-treated with colcemid was not significantly different from those cells not treated with colcemid suggesting that induction of neural protein expression by PMA is a direct effect however colcemid itself induced neural protein expression and therefore this data is difficult to interpret. Staining for F-actin and microtubules revealed similar cytoskeleton rearrangements as seen DMSO induced cells. Immuno-staining was also used to confirm the expression of Tuj-1, GAP-43, NF-M and nestin (figure 3.32).

3.4 Discussion

Stem cells reside in specialised niches within specific adult tissues where they are subject to spatial and temporal regulation in respect to their developmental potential. Removal of certain stem cell populations from their normal microenvironment and their subsequent explanation in culture has been proposed to increase their developmental plasticity. Trans-differentiation *in vitro* is based on the idea that the developmental restrictions of tissue specific stem cells are dictated by the micro-environmental signals *in vivo* and by explanting these cells *in vitro* and providing new signals we may be able to manipulate the developmental potential of such cells. The aim of the current work was to determine if stem cells isolated from adult bone marrow could differentiate into neural derivatives when cultured under defined conditions used in neural cultures to maintain and differentiate neural precursor cells *in vitro* or neuroblastoma cells. In addition, if neural cells can be derived from bone marrow stem cells by trans-differentiation we aimed to examine whether such differentiation occurred via a conserved neurogenic pathway or a mechanism distinct to this process.

To investigate whether tissue specific stem cells have the capacity to generate neural derivatives by trans-differentiation we tested the neurogenic potential of Mesenchymal Stem Cells (MSCs) isolated from the adult bone marrow. Using two previously reported model systems of neural differentiation *in vitro* (Woodbury et al.,2000; Kobas et al., 2002); we evaluated the capacity of MSCs to differentiate into cells with properties indicative of neural derivatives.

We were first able to recapitulate the earlier observations of both Woodbury et al., 2000 and Deng et al., 2001 who showed that MSCs can adopt of neuronal-like phenotype in response to chemical induction using either forskolin, which increases intracellular cyclic AMP or antioxidant compounds. The neuronal-like phenotype was defined by the expression of pan-neural markers and morphological resemblance to primary neurons in culture.

3.4.1 MSCs are not capable of neuritogenesis or terminal differentiation following neural induction.

Neurons have a unique cytoarchitecture, which is characterized by axonal projections, which extend from the cell body and form numerous dendritic processes. These processes synapse with the axonal projections of other neurons. This pattern of neurite outgrowth results in the generation of neural networks, which convey the unique electrophysiological properties of neurons (Svendsen et al., 2001). To test for the ability of MSCs to form neuronal phenotypes capable of extensive neuritagenesis MSCs were first differentiated in neuronal induction medium and their neurite outgrowth evaluated.

MSCs isolated from adult BM were first exposed to varying concentrations of antioxidant compounds in serum free media previously reported to induce a neuronal phenotype in MSCs (Deng et al., 2001; Woodbury et al., 2000). Under these conditions, MSCs adopted a neural-like phenotype defined by the acquisition of morphology resembling neurons and expressed pan-neural markers (including Tuj-1, NF-M, MAP-2 and GAP-43etc). Neural-like cells were detected in response to all three antioxidant compounds. Responsive cells progressively assumed neuronal morphological traits over the first 3 hours. By 5 hours a maximal number of cells had responded.

A dose response curve was demonstrated for both forskolin and antioxidant compounds. As the concentration of these agents increased the total number of responding cells increased. In fact the number of neural-like cells observed was increased at each time when, the concentration of these agents was increased. This indicated that time was not the limiting factor but the concentration of the agent used. It is not known however, why some cells do not respond to induction, although we did observe that small spindle shaped cells respond first followed by larger flat cells indicating that cell size may play a role in determining which cells respond. In addition, we observed that serum removal alone was sufficient to result in a small proportion of cells (<20%) adopting a neural-like morphology. This suggested that serum removal alone can contribute to neural induction and is consistent with observations that serum removal is a critical parameter in the formation of cells from MSCs with a neurogenic potential. However, it also suggests that reports in which approximately 20% of cell adopts a neural-like morphology in serum free culture such as reported by Deng et al., 2001 may simply be the result of serum removal and not an

effect of the inductive agent. Our data also indicated that there was some degree of spontaneous reversion with small numbers of cells reverted back to a stromal cell phenotype by 48 hours. This observation of reversibility of MSC neural-like cells is consistent with previous reports (Bertani et al., 2005).

Neurons in culture have a unique architecture characterized by axonal projections, which extend from the cell body and form numerous dendritic processes terminating in actin rich growth cones (Charkravarthy et al., 1995). These neurites have the capacity to synapse with the axonal projections of other neurons a property, which conveys the unique electrophysiological properties. The neuroblastoma cell lines N2a and SHSY-5Y undergo exclusive neuronal differentiation in response to serum deprivation and/or 2% DMSO (Macleod et al., 2001; Evangelopoulos et al., 2005). Upon removal of serum and/or addition of 2% DMSO 52-91% of MSCs adopt morphology identical to that of N2a or SHSY-5Y cells induced to differentiate under identical conditions. Responsive MSCs rapidly (within 3-5hrs) lost their flat elongated morphology and as a result the cell body becomes increasingly spherical and highly retractile exhibiting a typical neuronal perikaryal appearance. Surrounding this cell body emerged a number of cytoplasmic processes initially interpreted as presumptive neurites.

In neuronal cells neurite induction involves the extension one or more actin rich lamellipodia surrounding the cell body, followed by the emergence of microtubule rich neurites which terminate in actin rich growth cones (Chierzi et al., 2005). Growth cones are highly motile structures, which function to extend the neurite along the axonal projection and at its tip which resulting in secondary and tertiary branching that is high in microtubule content. Immuno-cytochemistry revealed presumptive neurites on MSCs were also rich in microtubules and terminated in growth cone like structures, which stained negative for microtubules. However, in contrast to neurons these growth cone structures stained only weakly for F-actin and although secondary and tertiary branches along the presumptive axon were identified, these branches stained almost exclusively for F-actin with no microtubules detected.

In neurons neurite extension is microtubule dependent (Diaz-Nido et al., 1991) and growth cones are highly motile structures (Chierzi et al., 2005), which extend neurites

along their axonal projection and at its tip. Neurite outgrowth of N2a cells was found in the present study to be microtubule dependent. However presumptive neurite development in MSCs is not microtubule dependent and there was no evidence of actin rich growth cone activity. MSC derived neurites were unresponsive to MAG in contrast to N2a cells differentiated under identical conditions. In addition, a comparison of the localization of key neuronal proteins including Tuj-1 and GAP-43 showed that in MSCs the pattern of staining was not consistent with normal neuronal development. For example GAP-43 is a specific marker protein of growth cones (Avwenagha et al., 2003), although expressed in MSCs its expression was not confined to the terminal portion of developing processes, in fact expression was less intense in these regions. GAP-43 expression in MSCs was aberrant and mostly confined to the cell body in contrast to differentiating N2a and SHSY-5Y in rich intense expression was observed in terminal portions of developing neurites consistent with a growth cone structure. Jin et al., 2003 also reported that MSCs induced to differentiate into neural-like cells in response to various growth factors as defined by morpholology and marker expression had an intracellular distribution of neural protein expression not consistent with primary neuronal cells. We observed that Tuj-1, MAP-2 and microfilaments all were more densely expressed in the cell body and immuno-staining was weak in the presumptive neurites. Synaptic proteins such as synaptophysin and SNAP-25 were also not confirmed to synaptic sites and expression was observed through the cells. In contrast in differentiated SHSY-5Y these proteins were highly expressed in the terminal portions of developing neurites and only weak staining was observed in cell bodies. This pattern of staining is inconsistent with normal neural development since neurofilaments and MAP-2 as well Tuj-1 play key roles in neurite and axonal development and thus their pattern of staining in MSCs does not correlate with function and is more consistent with a aberrant pattern of expression.

Therefore in terms of development of a neural-like morphology and formation of neurites MSC presumptive neuritogenesis was not consistent with terminal differentiation but was more consistent a retraction in the cell cytoplasm forming cytoplasmic extensions that were non motile and did not constitute true neurites. This may simply be a reflection of the inability of MSCs to differentiate into mature neural derivatives or that observations reported constitute early neural development in which

the morphology of cells is not yet fully developed and protein expression and localization is not yet finalized. However it may also be that the neural morphology and protein expression is an artifact of the culture conditions. The observation that presumptive neurite development by MSCs was not microtubule dependent and were not MAG responsive is inconsistent with normal neuronal development and suggests that this morphology is an artifact.

The morphology of cells observed in DMSO treated cultures of MSCs was consistent however with a neuronal morphology and although rapid such structural alterations have been reported in fibroblasts (Bershadsky et al., 1990; Tint et al., 1991; Dugina et al., 1987) and neruobalstoma cells (Tint et al., 1992) differentiated in the presence of PMA, as observed in the present study. We observed that PKC was expressed at high levels whilst MSCs adopted a neural-like morphology and appeared to decrease as cells returned to a stromal-like morphology. We therefore treated MSCs with PMA. Protein kinase C activation resulted in morphological rearrangements consistent with that observed in DMSO cultures even in the presence of serum and these cells were neural-like in appearance. This therefore suggested that PKC activation may occur following serum withdrawal, which results in the acquisition of a neural-like morphology. The morphology of cells reverted to a stromal phenotype by 24 hours even in the continual presence of PMA. This is further evidence that activation of PKC results in a neural-like morphology since the reversal is likely to be the result of a down regulation in PKC because of the continual presence of PMA. It is important to note however, that PMA does not only activate PKC and is likely to activate a number of pathways.

We next investigated the potential of MSCs to adopt neural cell fates when cultured in conditions used to culture primary neural stem cells. We cultured MSCs as cellular spheres on non-adherent tissue culture dishes. These spheres were propagated for 3-4 weeks in NSC medium supplemented with 20ng/ml EGF and 40ng/ml bFGF with medium changes every 3 days. Cultures of rat E14 NPC were grown under identical culture conditions in parallel for comparison. MSC spheres or NPCs were subsequently plated undissociated on laminin, fibronectin or poly-L-ornithine coated plastic wells. Growth factors were removed and substituted with 0.5μM retinoic acid and 10ng/ml nerve growth factor or 10ng/ml brain derived neurotrophic factor. Under

these culture conditions rodent derived neurospheres attached with minimal dissociation to the substrate and formed elaborate neurites over 3-12 days. These neurites were highly positive for nestin, MAP-2, and TUJ-1 the same results were obtained for all substrates tested and for both conditions tested.

MSC derived spheres also attached to all substrates but behaved distinctly differently to neurospheres following the withdrawal of growth factors. On attachment cellular spheroids rapidly down regulated (within 24hrs) nestin but continually expressed high levels of TUJ-1 over 12 days. On all substrates in the absence of growth factors MSC derived spheres progressively dissociated forming a monolayer of cells. On laminin and fibronectin the cells flattened however, during this time there was no evidence of neurite development and cells remained rounded when plated on PLO coated tissue culture plastic. Cells expressing neural proteins were still identified in 12-day cultures but only at low numbers (5-10% approximately). GFAP and Tuj-1 positive cells were identified suggesting that spheres could differentiate into both neuron and glia.

3.4.2 Changes in the protein expression profile of MSCs in response to neural cues in vitro.

Neural development *in vivo* and *in vitro* is a highly specific process in which the temporal and spatial expression of neural genes is tightly regulated (For review see: Hagg, 2005). We examined the expression of proteins indicative of a neural phenotype in populations of both undifferentiated MSCs and MSCs following neural induction. We observed that undifferentiated MSCs express a subset of proteins usually associated with neuronal cell fate, but proteins specific for oligodendrocytes were absent. The proteins were expressed with varying degrees and in some cases were only expressed only by a subset of MSCs. The observation that MSCs spontaneously express neural proteins following prolonged culture under standard culture conditions is consistent with several recent reports with similar findings (Goolsby et al., 2003; Ratajczak et al., 2004; Tondreau et al., 2004). Woodbury et al., 2002 suggest that this neural protein expression represents a multidifferentiated state in which MSCs are capable of intrinsic neurogenic potential.

These neural proteins were up regulated following neural induction by exposure to 2% DMSO, serum deprivation or when cells were grown as cellular spheres in NSC

medium. Again, there was variation in the extent to which these proteins were up regulated. Analysis of neural protein expression after 5 hours chemical induction or the generation of cellular spheroids revealed both these MSC derived cell types to express high levels of neural proteins including NF-M, Tuj-1, Nestin, GAP-43 and reduced levels of vimentin or fibronectin. However prolonged induction of differentiation either in chemical induction for 6 days or plating of neurospheres in the presence of RA+NGF for 12 days resulted in a downregulation of neural proteins and up-regulation of mesodermal proteins. In addition, the number of neural-like cells decreased under both conditions as cells reverted to a stromal phenotype. GAP-43 expression in chemically induced cells did increase by 6 days. GAP-43 is a PKC substrate (Oestreicher et al., 1997) and the down regulation in PKC expression observed during 6 day chemical induction may lead to this increase in GAP-43. Depletion of GAP-43 markedly alters neurite and growth cone morphology (Aigner and Caroni 1993) and overexpression of GAP-43 in the CNS promotes neurite spouting an effect that is dependent on PKC phosphorylation (Aigner and Caroni 1995). This temporal pattern of neural protein expression is inconsistent with normal neural development in which neural protein expression is highly regulated. The conclusion from these observations is that either MSCs do not have an intrinsic neurogenic potential or that *in vitro* conditions are insufficient to promote terminal differentiation of these cells.

3.4.3 Developmental potential of MSCs cultured under neural inductive conditions.

Cellular differentiation involves the progressive restriction of cell fate as cells become increasing committed to a specific cell lineage. We hypothesized that if MSCs cultured under defined conditions could be instructed to adopt a neural fate then such reprogramming would involve the progressive loss of mesodermal differentiation capacity with a concomitant down regulation of mesodermal specific genes accompanied by a gene expression profile consistent with a neural specification. We observed that when MSCs were induced to differentiate into presumptive neural-like cells either by chemical induction or the formation of cellular spheroids and these cells assumed a neural-like morphology there was a down regulation in the expression of MSC proteins such as vimentin and fibronectin. On reversal of this morphology back to a stromal-like morphology the expression level of these proteins returned to normal levels. In addition, neural-like cells formed under both conditions did not lose

their ability to differentiate into the osteogenic lineage. This suggests that MSC neural like cells either do not fully committed to the neural lineage or are capable of rapid introversion between the two cell types. A number of investigators have reported this reversibility of the neural-like phenotype under similar conditions and the rapid interconvert ability between these two phenotypes (Hermann et al., 2004; Woodbury et al., 2002; Bertani et al., 2005). However, it was previously thought that increasing commitment of a stem cell towards a specific cell lineage is associated with a progressive restriction the differentiation potential of these cells.

3.4.4 Regulation of nestin expression

Consistent with a number of recent reports we found that expression of nestin was a pre-requisite for the formation of neural-like cells under both induction protocols used (Croft and Przyborski, 2004; Wislet-Gendebien et al., 2005). Nestin expression only occurred in responsive cells and unlike many of the markers used was not expressed in control or unresponsive cells. In addition, nestin expression was required for sphere formation. Conditions, which promoted nestin expression, were low attachment culture conditions and removal of serum. Although minimal differentiation potential of nestin positive cells was observed in our study under both conditions tested a recent study by Wislet-Gdebien et al., 2005 demonstrated that co-culture of nestin positive cells with cerebellar granule cells resulted in the formation of neurons with some proof of functional This indicates that nestin positive cells may require additional signals to differentiate fully. However numerous studies have claimed to demonstrate terminal differentiation *in vitro* without co-culture.

3.4.5 Link between PKC expression and neural induction

As discussed previously, protein kinase C activation by PMA is associated with morphological rearrangements in differentiating neruoblastoma cells (Tint et al., 1992) and down-regulation of PKC occurs during serum deprivation induced differentiation of SHSY-5Y cells (Macleod et al., 2001). It is thought that terminal differentiation and neural protein expression in these cells is associated with PKC down-regulation. Consistent with these observations activation of PKC by PMA resulted in structural rearrangements in MSCs, which adopt a neural-like appearance. This occurs in the presence of serum suggesting that serum removal may result in a neural-like morphology through the activation of PKC when serum is removed. In

differentiating SHSY-5Y cells in response to DMSO we observed that neural protein expression including MAP-2, GAP-43 and Tuj-1 all increased. This was associated with a concomitant reduction in PKC expression determined by a pan-PKC antibody. However, in induced MSCs, PKC expression initially increased and then decreased by 6 days. These changes in expression mirrored changes in morphology and protein expression in MSCs. Initially the expression of neural proteins increased 1-3 days following induction and then decreased by day 6. The number of cells with a neural-like morphology also increased by induction and then progressively decreased in number by day 12. This has led us to develop a working hypothesis that PKC activation occurs in response to serum withdrawal and DMSO treatment, which results in the acquisition of a neural-like morphology and neural protein expression. The gradual down-regulation in the expression of PKC is associated with a reversal in morphology of neural-like cells to a stromal phenotype and a reduction in neural protein expression. This is in contrast to the neuronal differentiation of neuroblastoma cells in response to DMSO in which PKC down-regulation is associated with commitment to differentiation as demonstrated in the present study.

3.4.6 Concluding remarks

Several recent independent studies have reported that non-hematopoietic MSCs isolated from the bone marrow appear to have the potential to form cells resembling those derived from the neuroectoderm. Here, we demonstrate, that MSCs with a consistent immunophenotype can generate neural-like cells under a range of experimental conditions. Most notably, MSCs form neural-like cells under culture conditions that are most commonly used to propagate and differentiate neural stem cells and neuroprogenitor cells. In addition, MSCs also adopt a neural-like morphology during chemical induction under conditions that differentiate neuroblastoma cells to mature neuronal derivatives. We refer to MSCs that respond to these culture methods as neural-like cells because they adopt morphology typical of primitive neurons and they express pan-neural markers. There remain, however, several important questions. First, we must provide more comprehensive evidence that terminally differentiated, functional neurons can be produced from MSCs. Second, we need to determine the molecular mechanisms that control the transition of MSCs into neural cells. Is this phenomenon a recapitulation of embryonic neurogenesis or is an alternative, yet to be described, developmental pathway

involved? Third, it is well known that bone marrow stroma consists of a heterogeneous population of cells. Which cells in particular respond to the growth conditions we have examined here, and is it possible to isolate such cells, expand them *ex vivo*, and induce them specially to from neural-derivatives? No doubt answering these points will generate many more questions, however, it is essential that we understand the molecular processes controlling the differentiation of MSCs if we are to fully realise their potential.

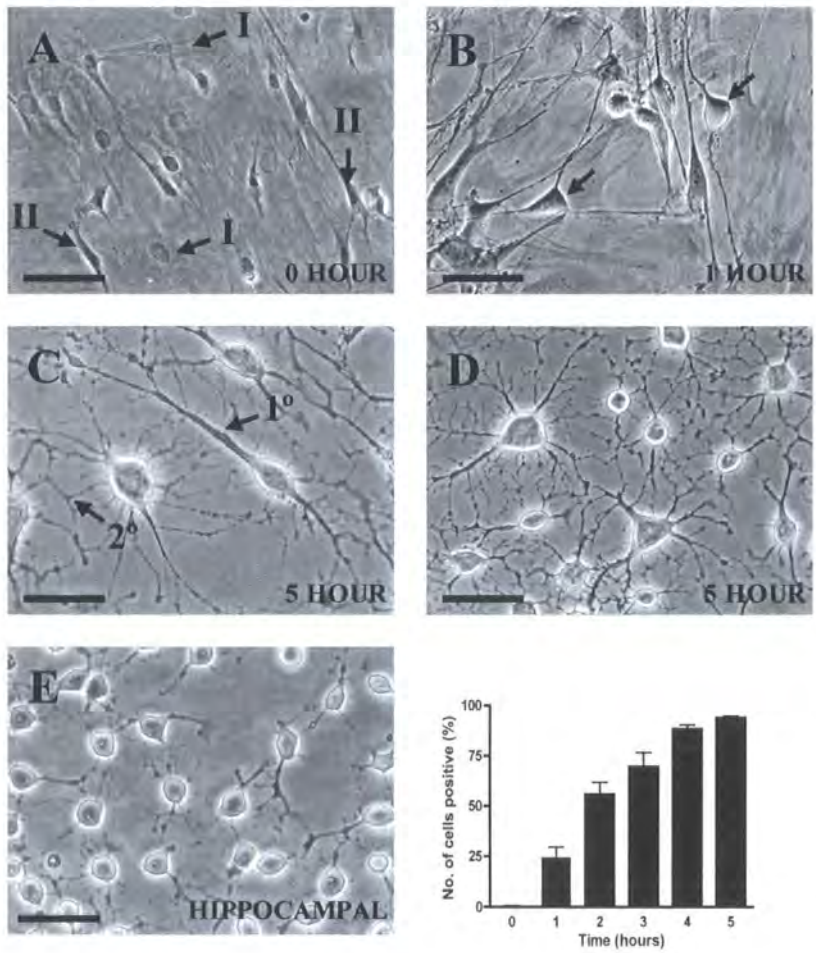


Figure 3.1: Exposure of rat MSCs to 2% DMSO in serum free media. Cultures of untreated BMSCs display a characteristic stromal cell morphology with the presence of type I, flattened elongated cells and type II spindle shaped cells (**A**). BMSCs were switched to serum-free DMEM in the presence of 2% DMSO at time zero. Morphological changes to cells were evident within 1 hour of treatment (**B**). Type II cells are the first to respond and display a more neuronal like morphology, in which the cytoplasm appears to retract towards the nucleus and processes elongate from the cell body (**B, arrows**). After 5 hours treatment (**C**), the vast majority of cells have responded and display extensive process like cytoplasmic extensions which include primary (**arrow 1°**) and secondary (**arrow 2°**) branching. This branching leads to the formation of extensive networks within the culture (**D**). Following 5 hours in the presence of 2% DMSO, BMSCs have adopted a morphology that resembles the structure of primary adult rat hippocampal progenitor cells (**E**). During this induction period, the percentage of cells expressing neuron specific enolase (NSE, Sigma) was determined by immunofluorescence using standard procedures. The inset plot shows the marked increase in expression of the neural marker, NSE, suggesting that BMSCs treated with DMSO adopt a neural-like phenotype. All data are represented as Mean values±SEM from 3 independent experiments in parallel cultures *Scale bars: 40μm* (**A,E**); *20μm* (**B-D**).

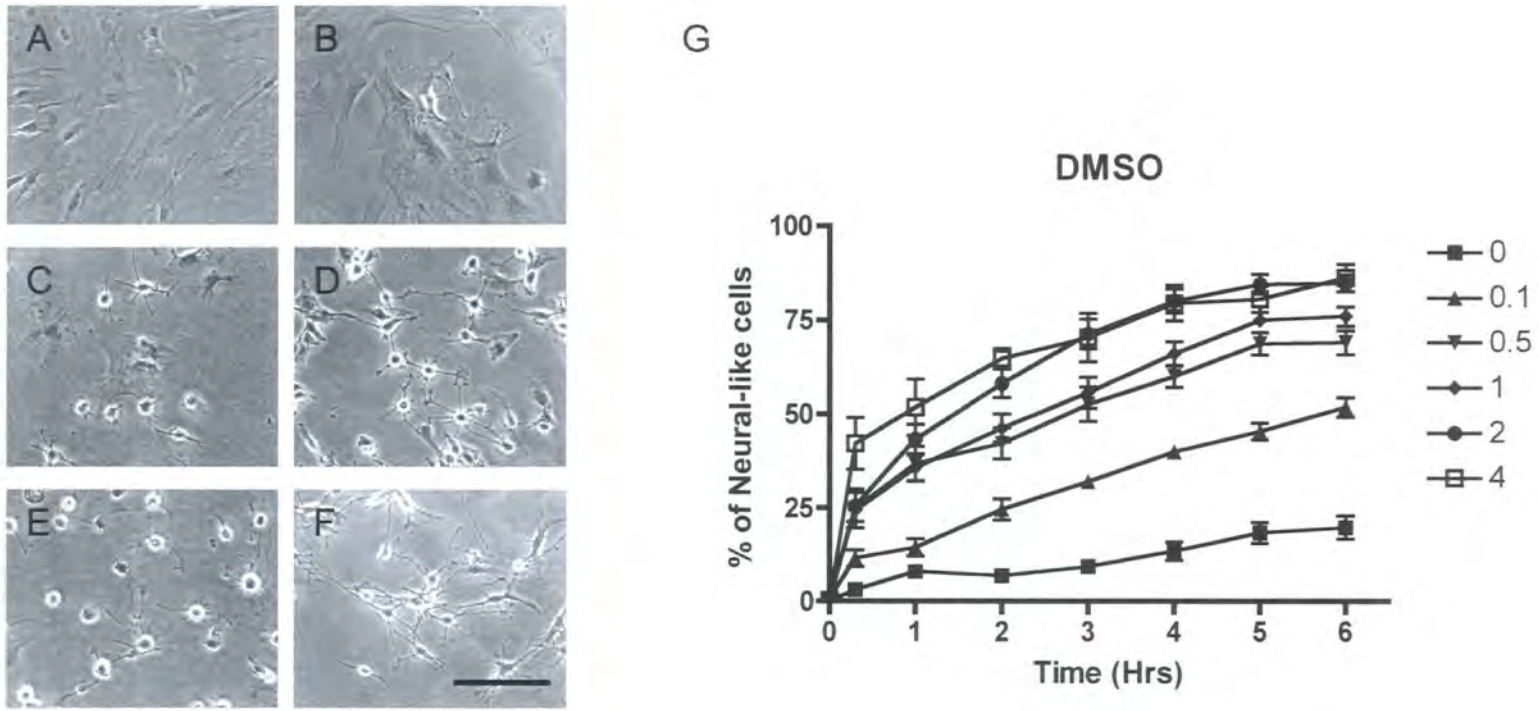


Figure 3.2a: Induction of neural-morphology following exposure to antioxidants. Photomicrographs show cultures of untreated MSCs displaying a characteristic stromal cell morphology (**A**). MSCs were then switched to serum free DMEM (at time zero) in the presence increasing concentrations of DMSO. Photomicrographs were obtained of cultures at 5 hours post induction (**B**) 0.1%, (**C**) 0.5%, (**D**) 1%, (**E**) 2% and (**F**) 4% DMSO. Cells that respond to treatment have perikaya, which are spherical, and highly retractile, with processes like extensions radiating from the cell body. At higher concentrations of DMSO the number of responding cells increases and cells are seen in which the processes display a high degree of branching resulting in the formation of an extensive network of neurite-like cell processes. (**G**) Quantification of the number of cells displaying a neural-like morphology in response to varying concentrations of DMSO. All data are represented as Mean values±SEM from 3 independent experiments in parallel cultures. *Scale bars: 40 μm.*

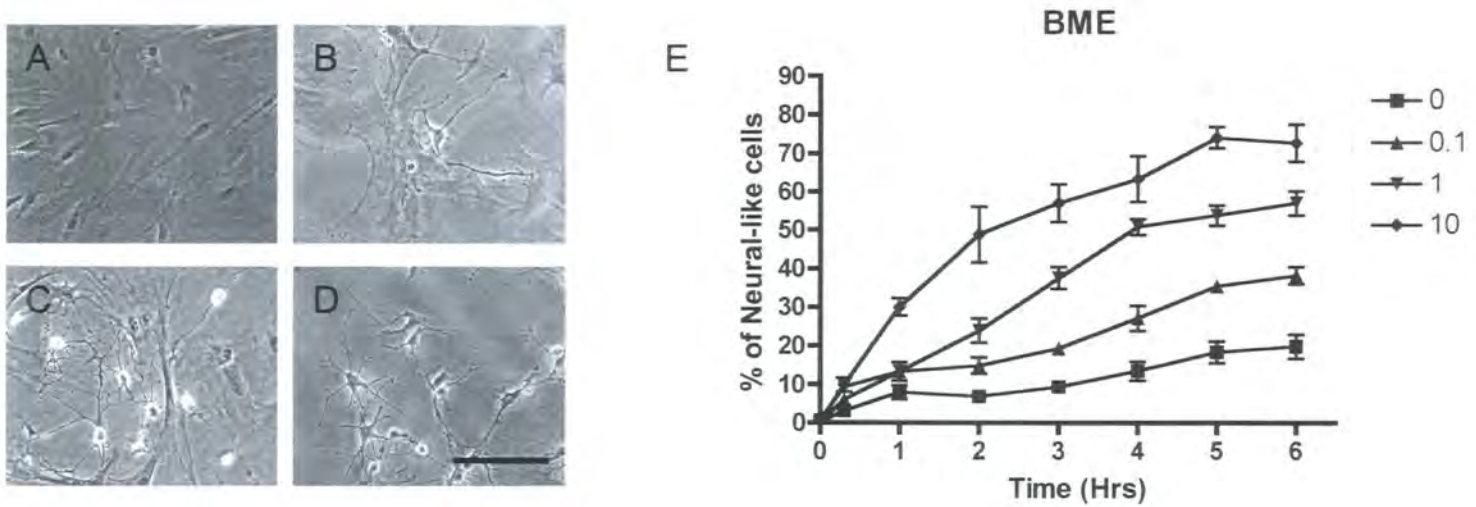


Figure 3.2b: Induction of neural-morphology following exposure to antioxidants. Photomicrographs show cultures of untreated MSCs displaying a characteristic stromal cell morphology (**A**). MSCs were then switched to serum free BME (at time zero) in the presence increasing concentrations of BME. Photomicrographs were obtained of cultures at 5 hours post induction (**B**) 0.1μM, (**C**) 1μM, and (**D**) 10μM BME. Cells that respond to treatment have perikaya, which are spherical, and highly retractile, with processes like extensions radiating from the cell body. At higher concentrations of BME the number of responding cells increases and cells are seen in which the processes display a high degree of branching resulting in the formation of an extensive network of neurite-like cell processes. (**E**) Quantification of the number of cells displaying a neural-like morphology in response to varying concentrations of BME. All data are represented as Mean values±SEM from 3 independent experiments in parallel cultures. *Scale bars: 40 μm.*

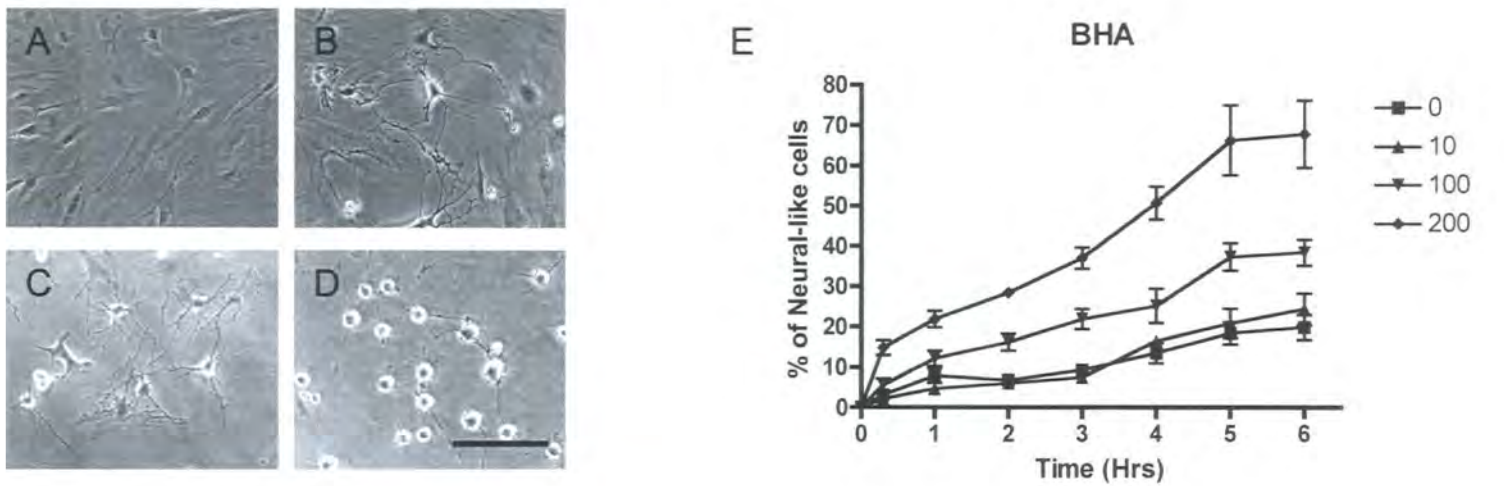


Figure 3.2c: Induction of neural-morphology following exposure to antioxidants. Photomicrographs show cultures of untreated MSCs displaying a characteristic stromal cell morphology (**A**). MSCs were then switched to serum free BHA (at time zero) in the presence increasing concentrations of BHA. Photomicrographs were obtained of cultures at 5 hours post induction (**B**) 10μM, (**C**) 100μM, and (**D**) 200μM BHA. Cells that respond to treatment have perikaya, which are spherical, and highly retractile, with processes like extensions radiating from the cell body. At higher concentrations of BHA the number of responding cells increases and cells are seen in which the processes display a high degree of branching resulting in the formation of an extensive network of neurite-like cell processes. (**E**) Quantification of the number of cells displaying a neural-like morphology in response to varying concentrations of BHA. All data are represented as Mean values±SEM from 3 independent experiments in parallel cultures. *Scale bars: 40 μm.*

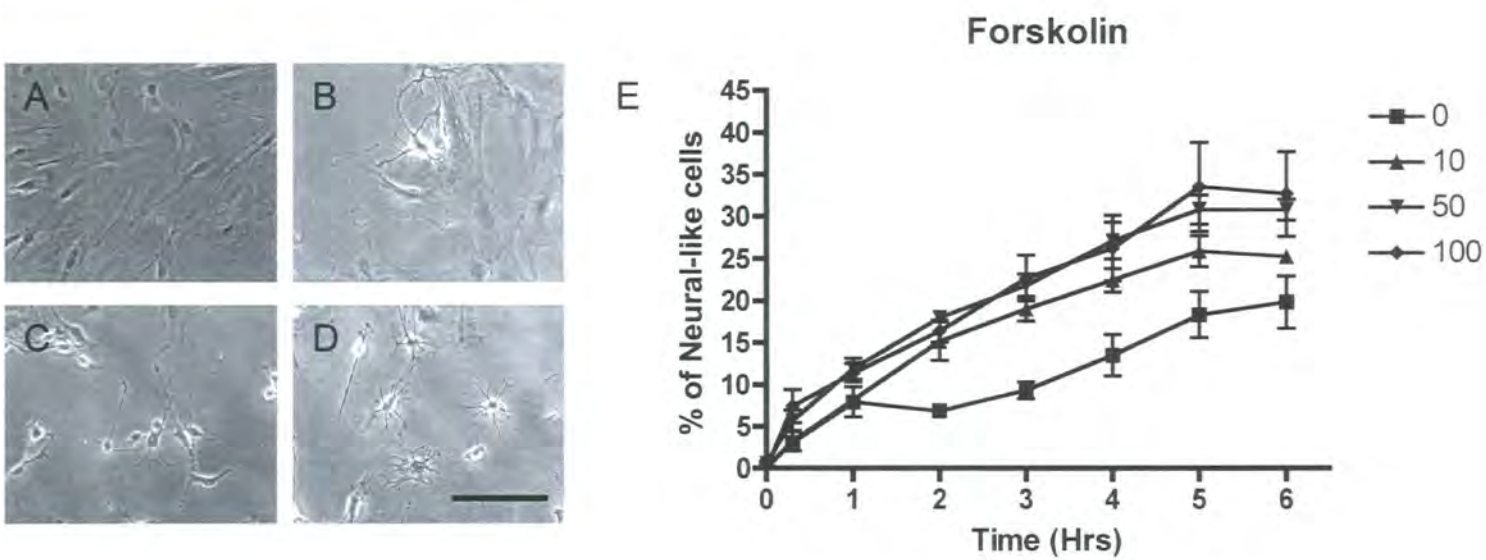


Figure 3.2d: Induction of neural-morphology following exposure to antioxidants. Photomicrographs show cultures of untreated MSCs displaying a characteristic stromal cell morphology (**A**). MSCs were then switched to serum free Forskolin (at time zero) in the presence increasing concentrations of Forskolin. Photomicrographs were obtained of cultures at 5 hours post induction (**B**) 10μM, (**C**) 50μM, and (**D**) 100μM Forskolin. Cells that respond to treatment have perikaya, which are spherical, and highly retractile, with processes like extensions radiating from the cell body. At higher concentrations of Forskolin the number of responding cells increases and cells are seen in which the processes display a high degree of branching resulting in the formation of an extensive network of neurite-like cell processes. (**E**) Quantification of the number of cells displaying a neural-like morphology in response to varying concentrations of Forskolin. All data are represented as Mean values±SEM from 3 independent experiments in parallel cultures. *Scale bars: 40 μm.*

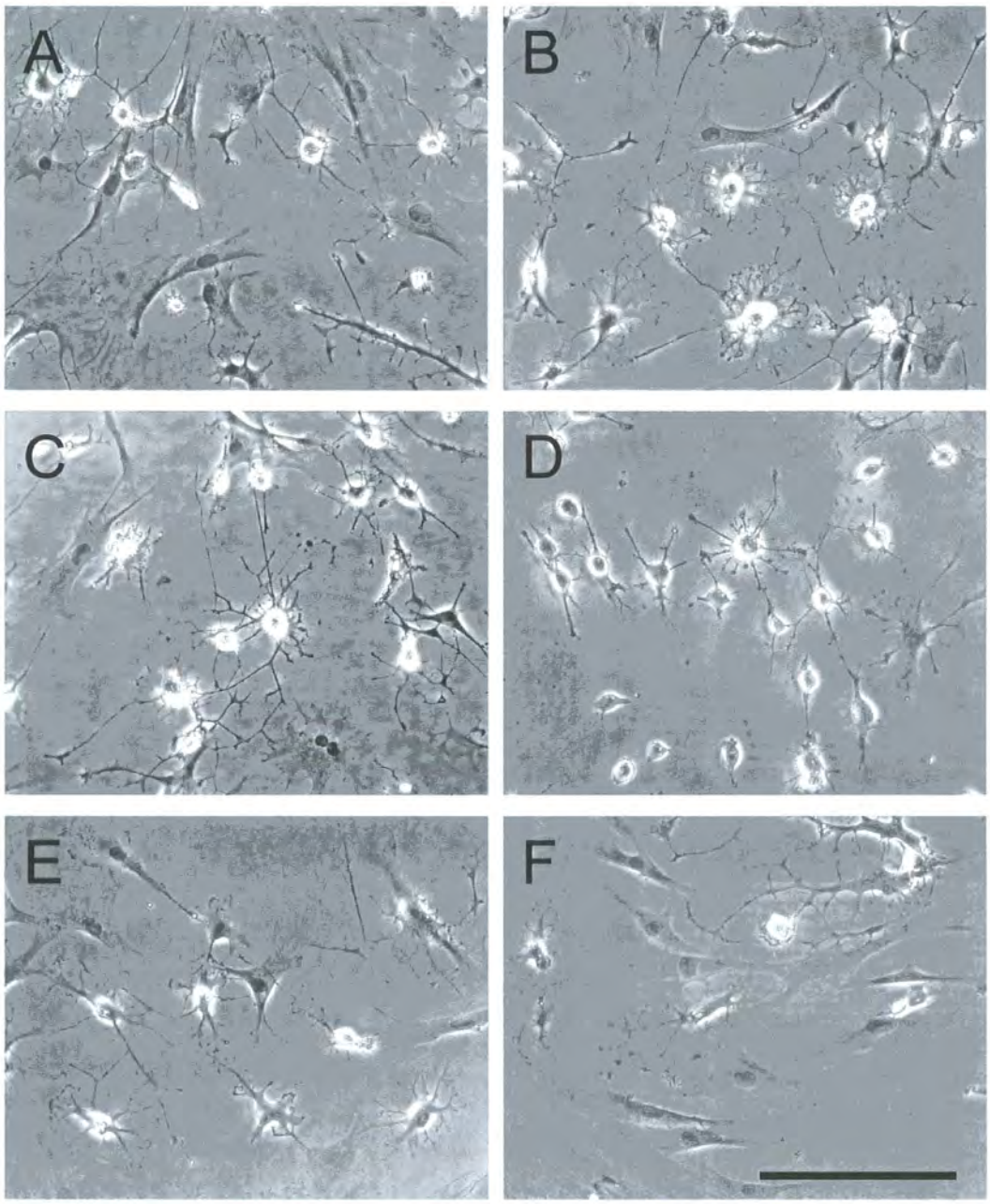


Figure 3.3a: Effect of 10μM BME on the acquisition of a neural-like morphology in cultured MSCs over 5 hours. Cells were transferred to a serum free basal media supplemented with 10μM BME for 5 hours. Phase contrast images were obtained at 1 hour (**B**), 3 hours (**C**), 5 hours (**D**), 24 hours (**E**) and 48 hours (**F**). These images were compared to MSCs cultured only in serum free media for 5 hours (**A**). *Scale bars: 50μm.*

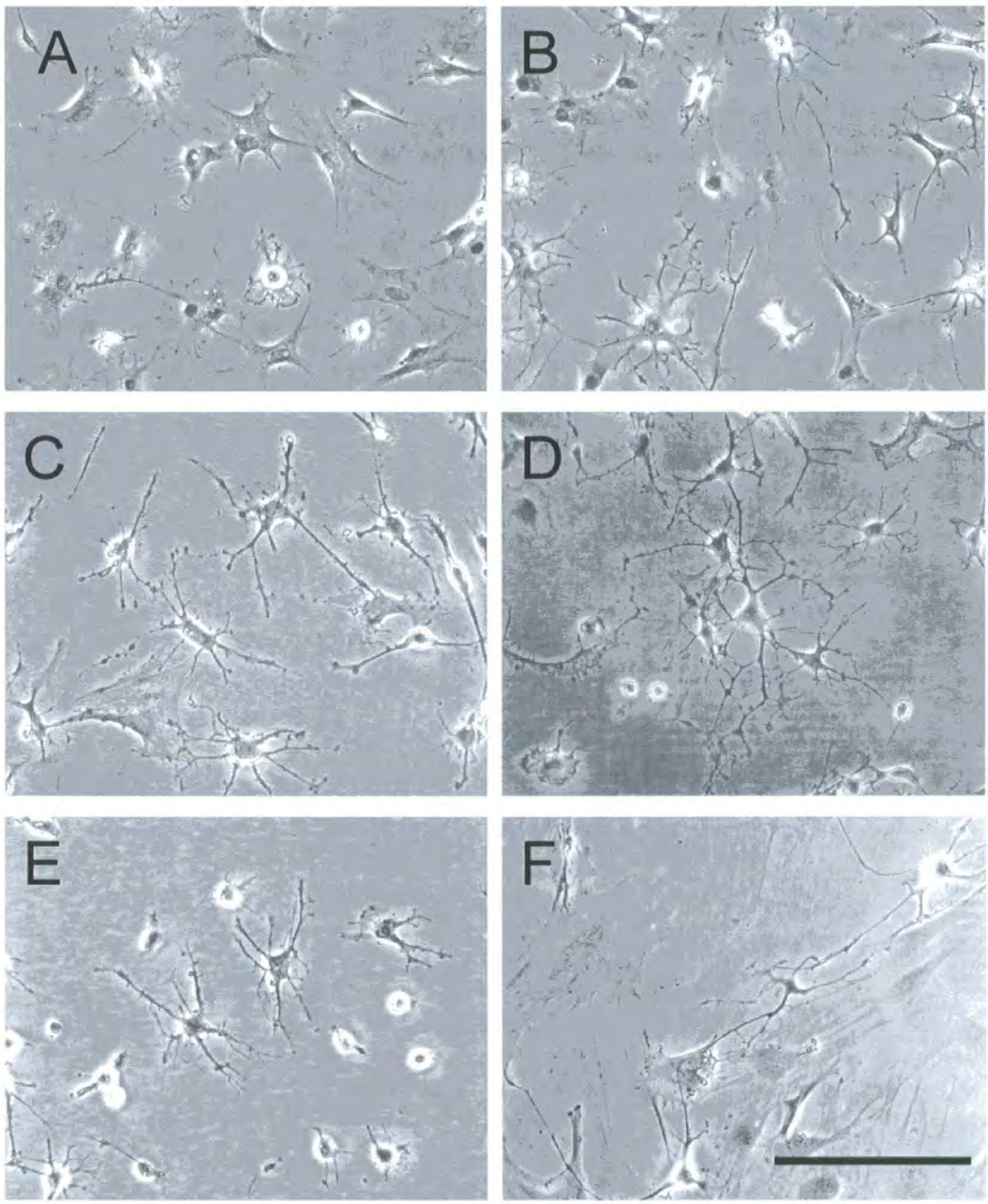


Figure 3.3b: Effect of 2% DMSO on the acquisition of a neural-like morphology in cultured MSCs over 5 hours. Cells were transferred to a serum free basal media supplemented with 2% DMSO for 5 hours. Phase contrast images were obtained at 1 hour (**B**), 3 hours (**C**), 5 hours (**D**), 24 hours (**E**) and 48 hours (**F**). These images were compared to MSCs cultured only in serum free media for 5 hours (**A**). *Scale bars: 50μm.*

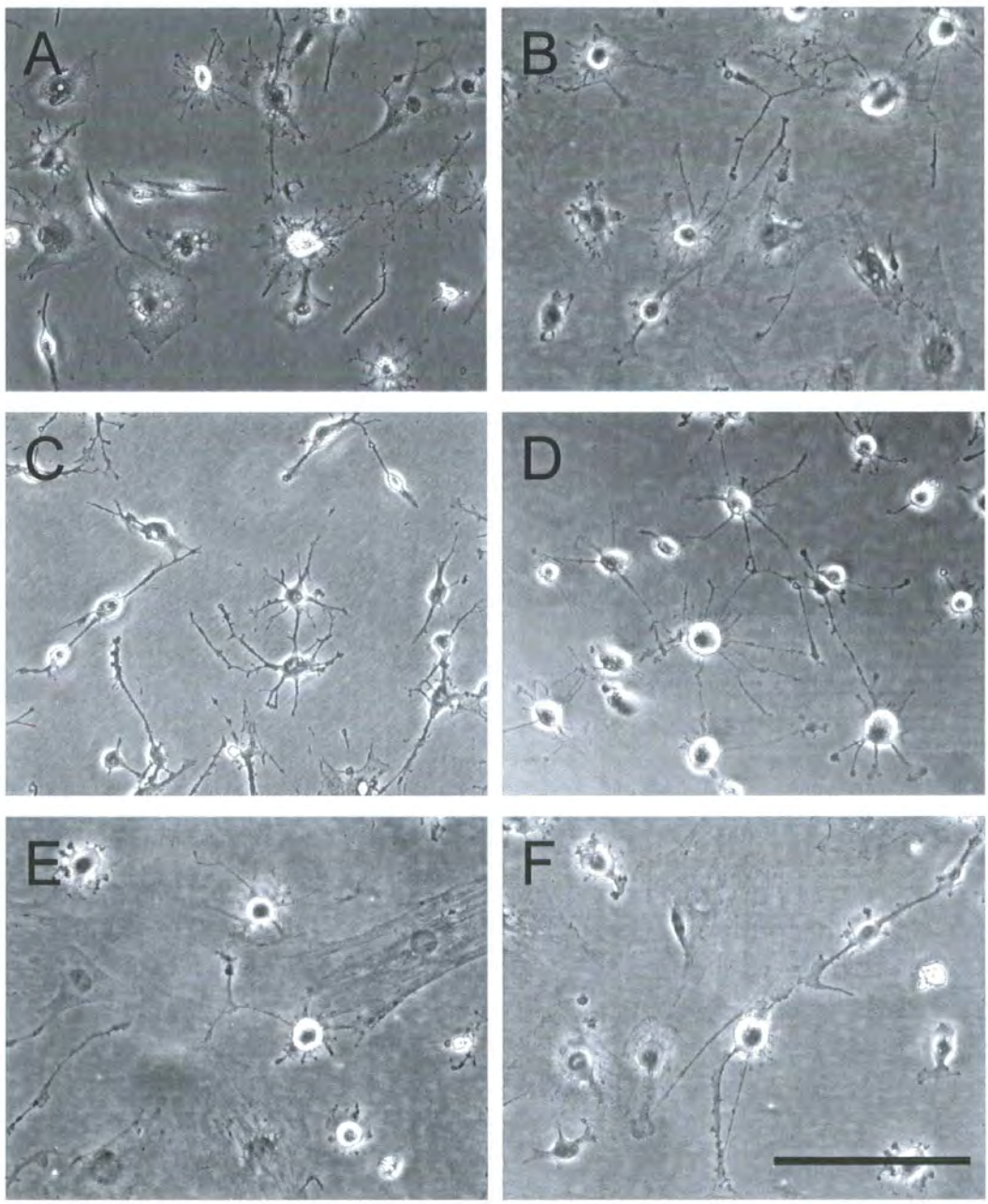


Figure 3.3c: Effect of 100μM BHA on the acquisition of a neural-like morphology in cultured MSCs over 5 hours. Cells were transferred to a serum free basal media supplemented with 100μM BHA for 5 hours. Phase contrast images were obtained at 1 hour (**B**), 3 hours (**C**), 5 hours (**D**), 24 hours (**E**) and 48 hours (**F**). These images were compared to MSCs cultured only in serum free media for 5 hours (**A**). *Scale bars: 50μm.*

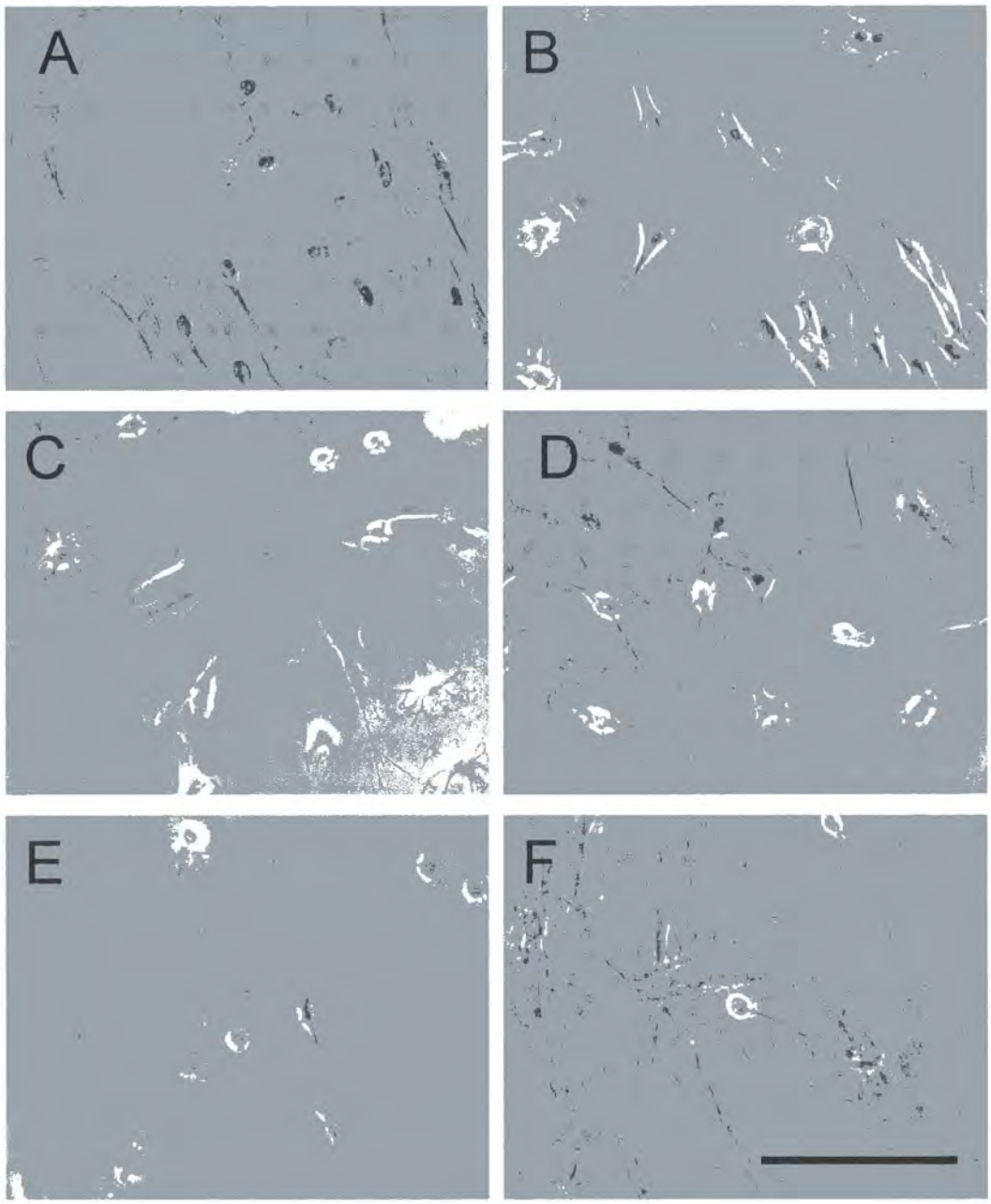


Figure 3.3d: Effect of 100μM Forskolin on the acquisition of a neural-like morphology in cultured MSCs over 5 hours. Cells were transferred to a serum free basal media supplemented with 100μM Forskolin for 5 hours. Phase contrast images were obtained at 1 hour (**B**), 3 hours (**C**), 5 hours (**D**), 24 hours (**E**) and 48 hours (**F**). These images were compared to MSCs cultured only in serum free media for 5 hours (**A**). *Scale bars: 50μm.*

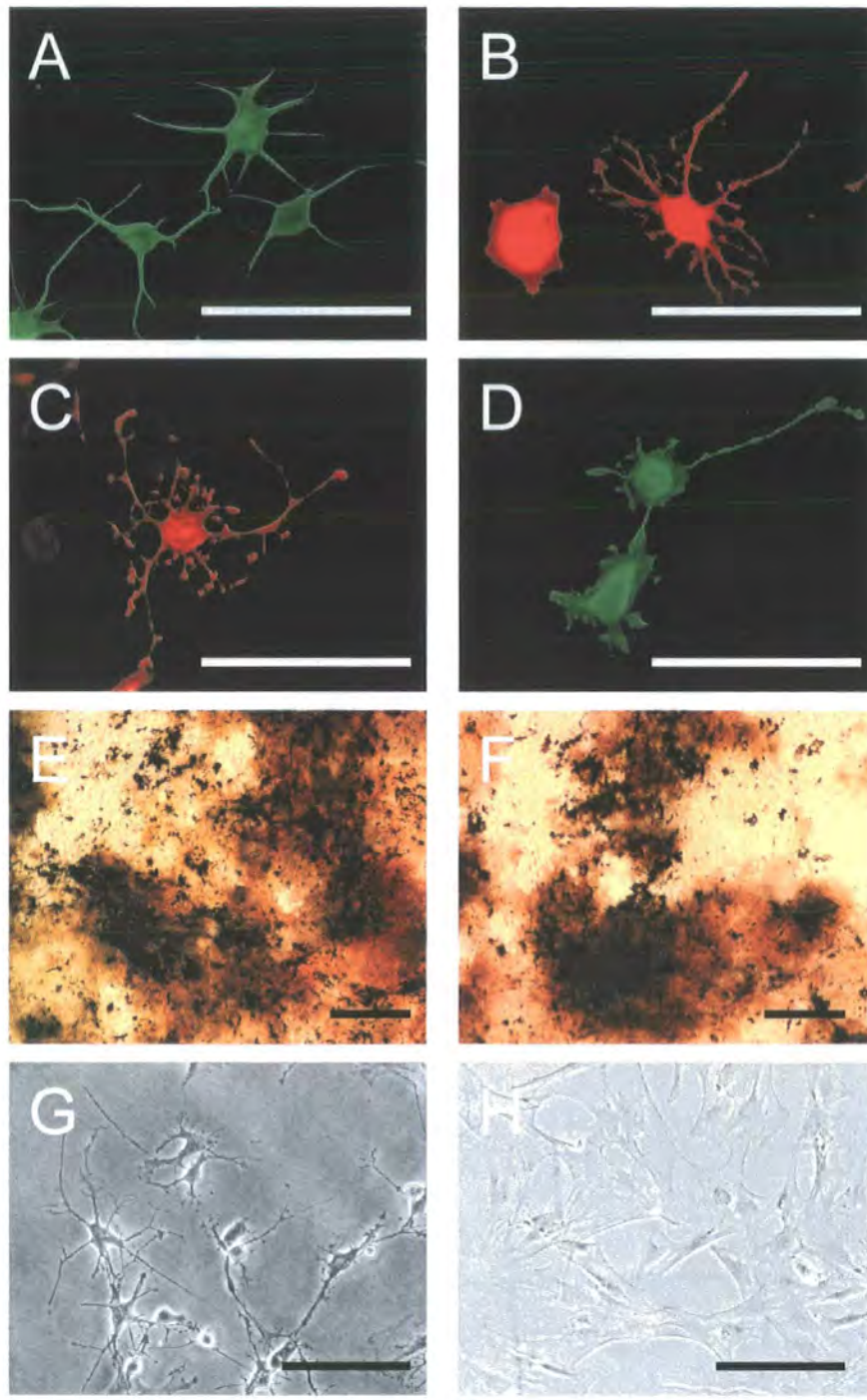


Figure 3.4: Effect of DMSO on MSCs in culture and effect of re-introduction of serum. (**A-D**) Cells were cultured for 5 hours in the presence of 2% DMSO and then fixed and processed for immuno-cytochemistry. (**A**) α-Tubulin, immuno-labeling of microtubules shows processes extending from the cell body containing microtubules. (**B**) In addition these processes as well as the cell body were found to contain the microtubule β-3 tubulin. (**C**) Visualization of the actin cytoskeleton using TRITC conjugated phalloidin. (**D**) Immunolabeling of GAP-43 protein, which shows concentrated staining in the cell body and the processes are only lightly stained. (**E**) Osteogenic differentiation of undifferentiated P8 MSCs in culture verified by Von Kossa staining. (**F**) Osteogenic differentiation of MSCs exposed to 2% DMSO. Re-introduction of serum into cultures previously exposed to 2% DMSO in serum free media (**G**) resulted in a reversion of the cell morphology back to a normal stromal morphology, 5 hours following re-introduction of serum (**H**). *Scale bar: 50μm.*

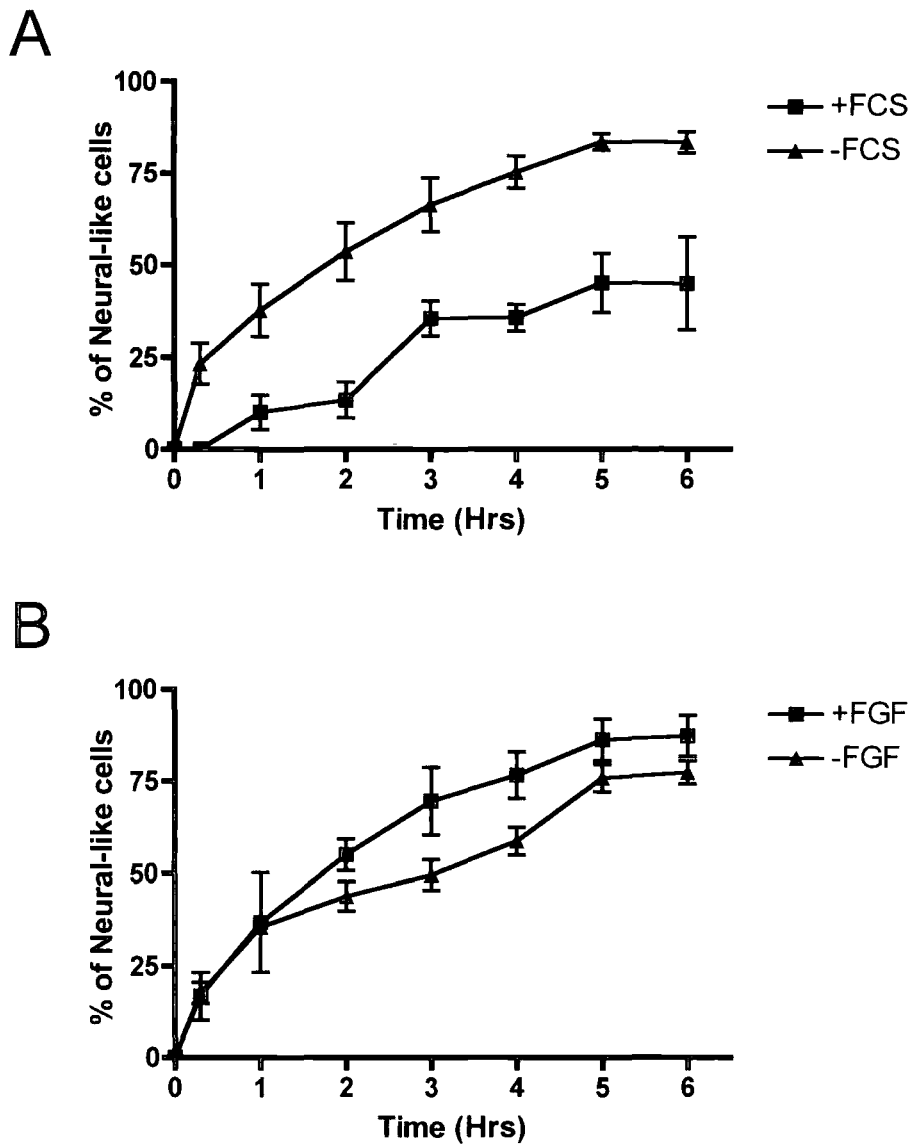


Figure 3.5: Effect of FGF-2 pre-induction and the presence of serum on the acquisition of a neural-like morphology by MSCs in response to 2% DMSO. (**A**) MSCs were transferred to a neural induction medium containing 2% DMSO in either the presence (+FCS) of serum or the absence (-FCS) of serum (10% FCS) for 6 hours. The number of neural-like cells was recorded every hour by phase contrast microscopy. (**B**) MSCs were pre-induced for 24 hours in normal growth media supplemented with 5ng/ml bFGF before been transferred to serum free media supplemented with 2% DMSO (+FGF). The number of neural-like cells was recorded every hour for 6 hours by phase contrast microscopy and compared to cells not pre-induced (-FGF). All data are mean values±SEM from 3 independent experiments in parallel cultures.

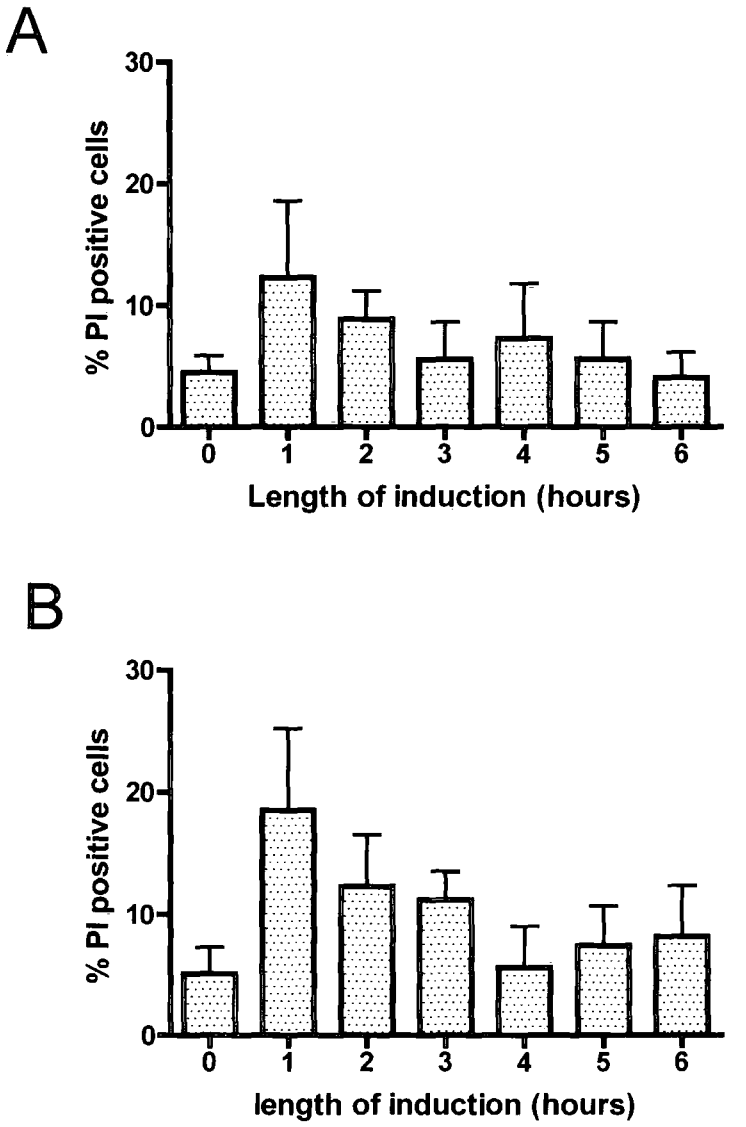


Figure 3.6: Effect of short-term neural induction on the viability of induced cells. Cell death following neural induction was accessed hourly for 6 hours by live staining with PI (5μg/ml) and quantified by flow cytometry. (**A**) Exposure of MSCs to 2% DMSO in the presence of serum. (**B**) Exposure of cells to the presence of 2% DMSO in the absence of serum. All data are mean values±SEM from 3 independent experiments in parallel cultures.

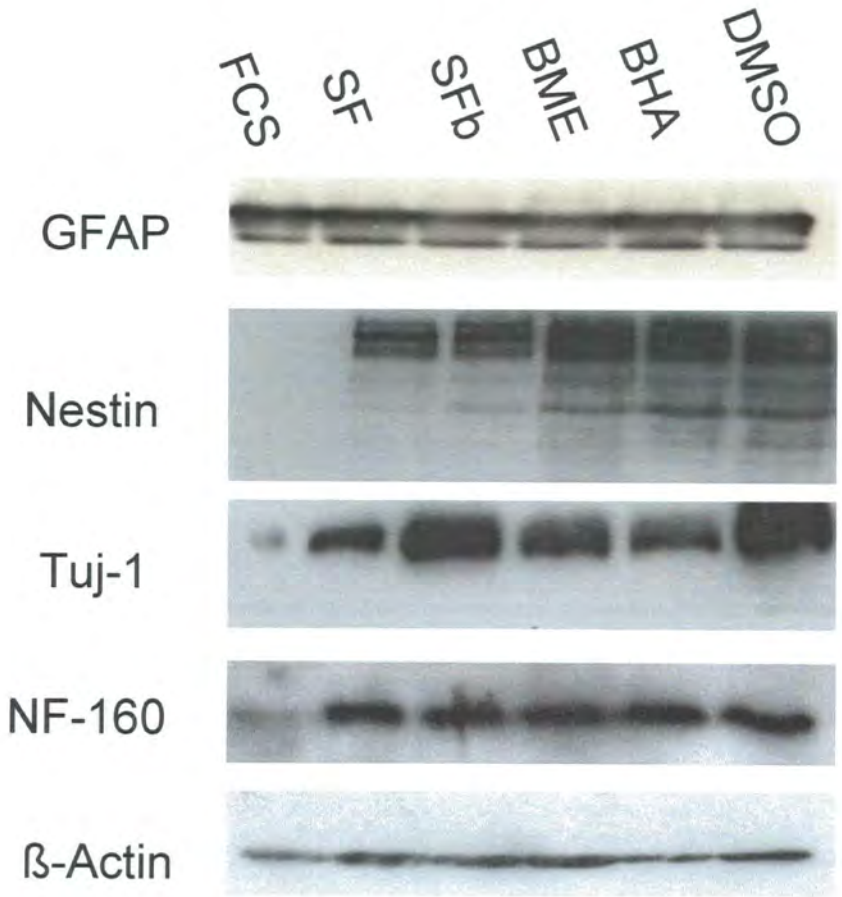


Figure 3.7: Western blot analysis of protein expression following 5 hour neural induction. Cells were cultured for 5 hours in either: serum media (control, FCS), serum free media (SF), serum free media following pre-induction for 24 hours with bFGF (SFb) or pre-induction followed by exposure to 10 μM BME, 100μM BHA or 2% DMSO in serum free media. Cells were harvested after 5 hours and processed for western blot analysis (n=3).

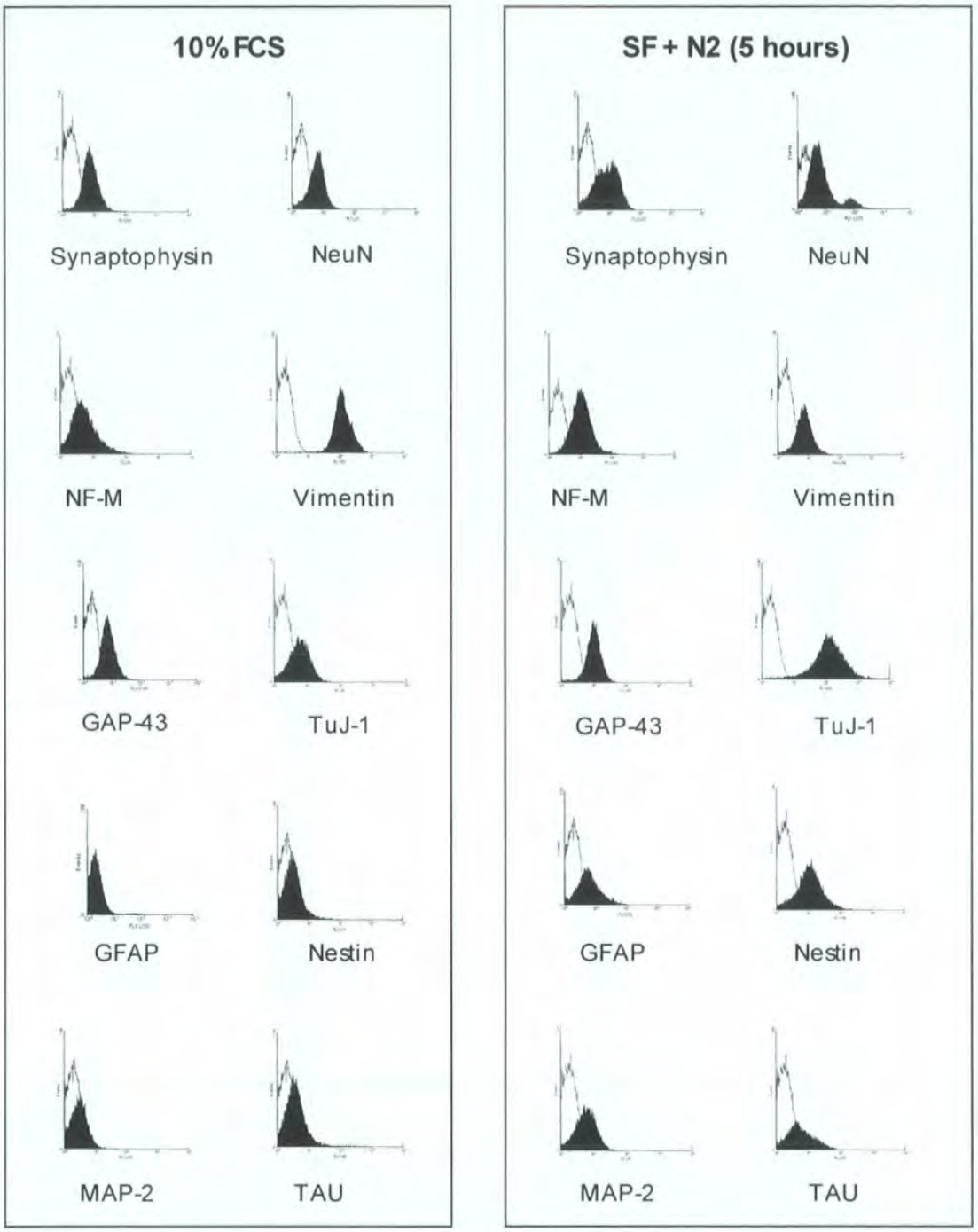
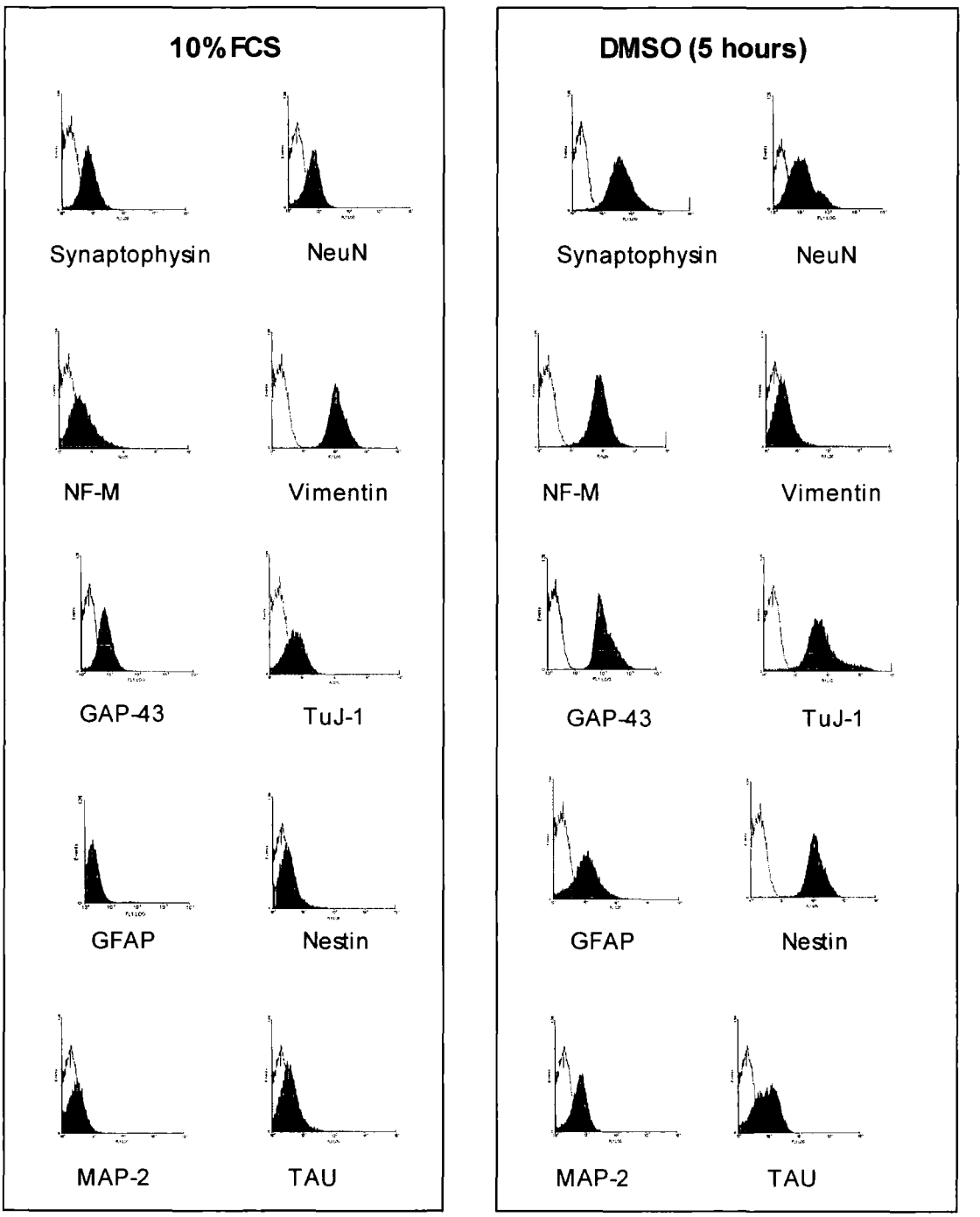


Figure 3.8: Expression of neural and mesodermal proteins following serum withdrawal or neural induction with 2% DMSO. Flow cytometry measurement of neural (Nestin, GAP43, TuJ1, NeuN, Synaptophysin and NeuroD1) and mesodermal-associated protein markers (Vimentin and Fibronectin) in rMSCs following either 5-hour serum-free culture (supplemented with N2, SF+N2) or 5 hours exposure to 2% DMSO in serum free media compared to cells maintained in the continued presence of 10% FCS. Flow cytometry data from representative experiments are shown as histograms with fluorescence intensity on the x-axis (solid peaks) and cell count on the y-axis. Background fluorescence was excluded by the use of isotype matched control antibodies (open peaks).



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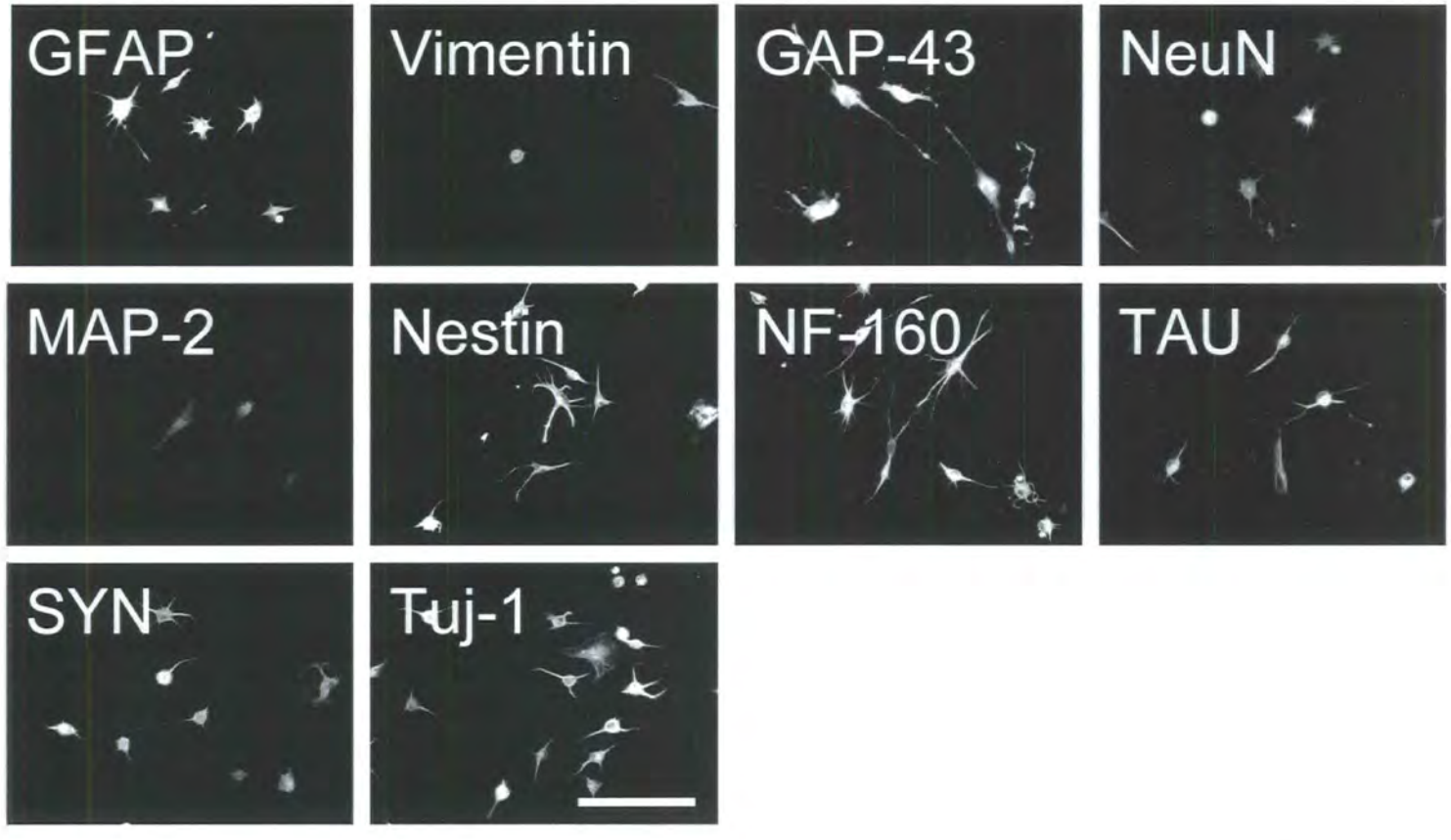


Figure 3.9a: Expression of pan-neural markers as detected by immuno-cytochemistry following 5 hours exposure to 2% DMSO in serum free media. Expression of pan-neural markers in MSCs following 5 hours growth in serum free media supplemented with 2% DMSO. The expression of these markers and their localization was determined by immuno-labeling with specific antibodies raised against these protein markers. Localization of primary antibody binding was determined by incubation with a FITC-conjugated secondary antibody. Images are shown in grey scale for clarity. *Scale bar: 50μm.*

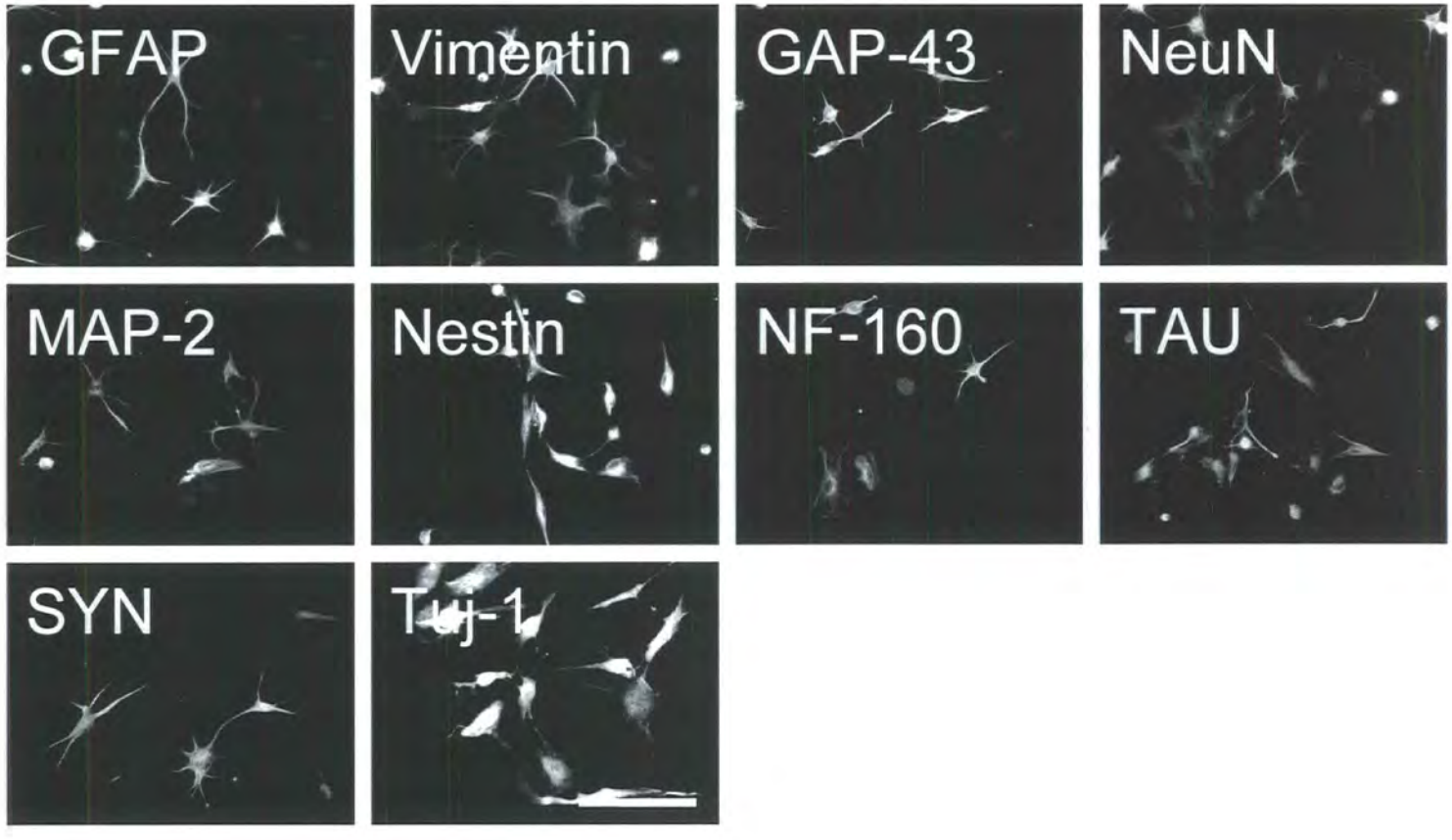


Figure 3.9b: Expression of pan-neural markers as detected by immuno-cytochemistry following 5 hours serum withdrawal. Expression of pan-neural markers in MSCs following 5 hours exposure to serum free media. The expression of these markers and their localization was determined by immuno-labeling with specific antibodies raised against these protein markers. Localization of primary antibody binding was determined by incubation with a FITC-conjugated secondary antibody. Images are shown in grey scale for clarity. *Scale bar: 50μm.*

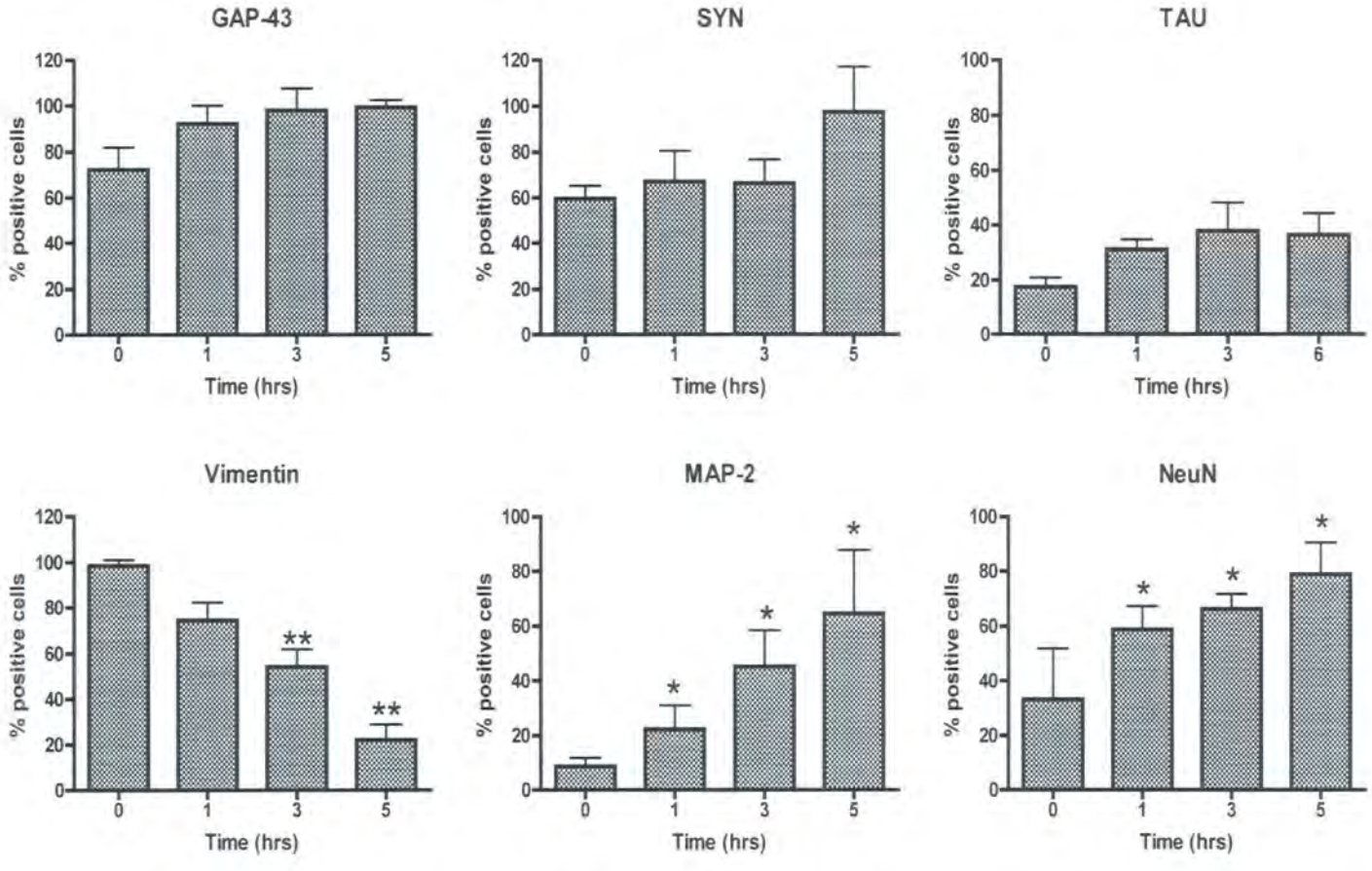
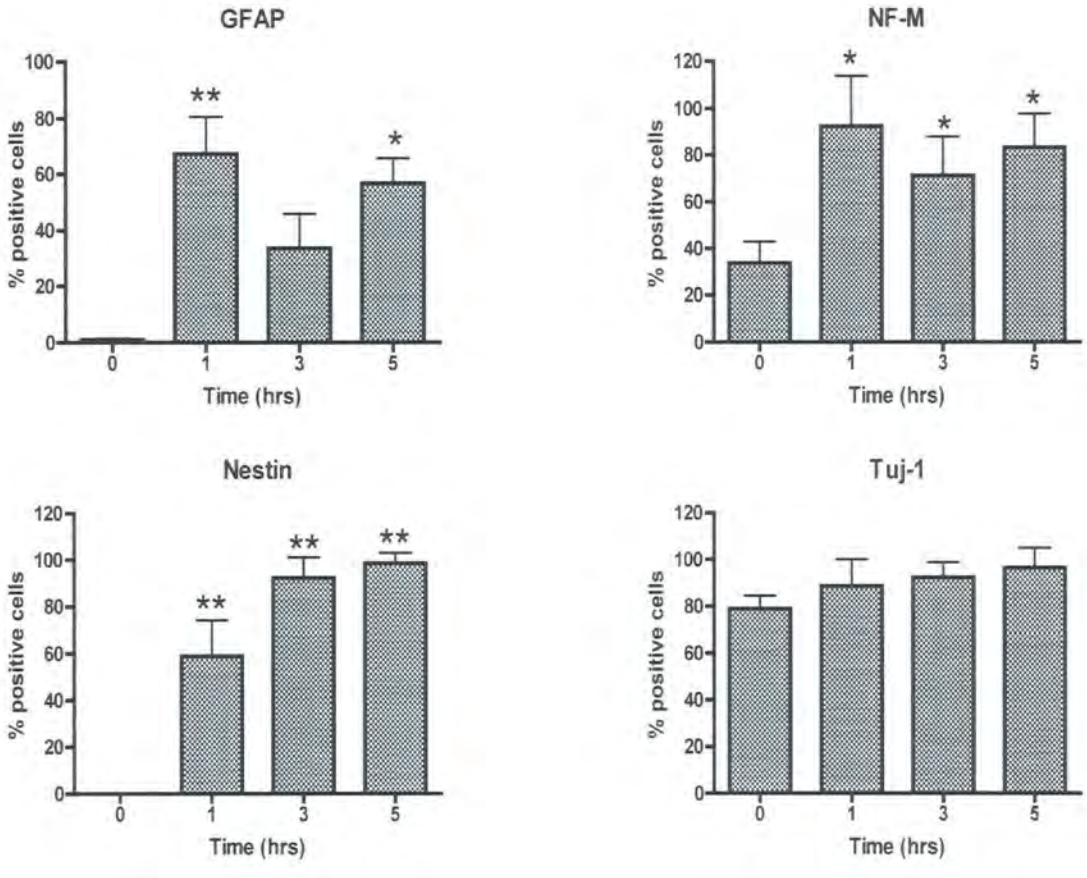


Figure 3.10: Temporal expression of pan-neural markers over 5 hours following neural induction in 2% DMSO in serum free media. Flow cytometry analysis of pan-neural marker expression. P8 MSCs were cultured in the presence of 2% DMSO in serum free media for 5 hours. Samples were obtained from cultures at 1, 3 and 5 hours post induction and processed for intra-cellular staining followed by flow cytometry. Expression levels (% positive cells) is indicated and compared to the expression level in non-induced cells maintained under standard growth conditions (time point 0). Data represents Mean±SEM from three independent experiments. (*P<0.05 **P<0.01 1 way ANOVA followed by Dunnetts post hoc analysis to compare to day 0, control).



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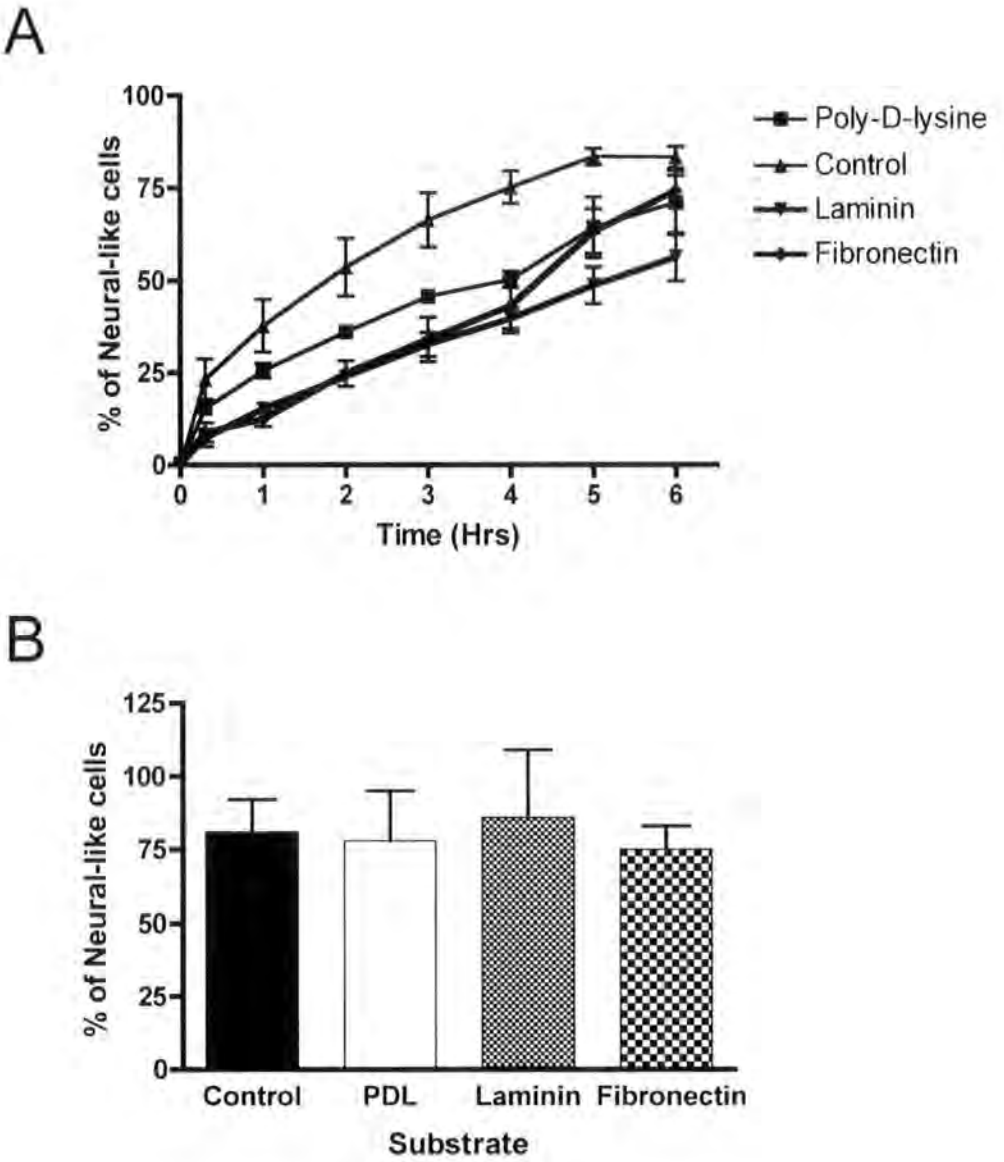


Figure 3.11: Effect of culture substrate on the acquisition of a neural-like morphology in MSCs in response to 2% DMSO in serum free media. Cells were cultured on either Laminin, Poly-D-lysine, fibronectin or normal tissue culture plastic and then transferred to neural induction medium. The number of neural-like cells was recorded every hour for 6 hours (**A**) and again at 12 hours (**B**). All data represents Mean±SEM from three independent experiments.

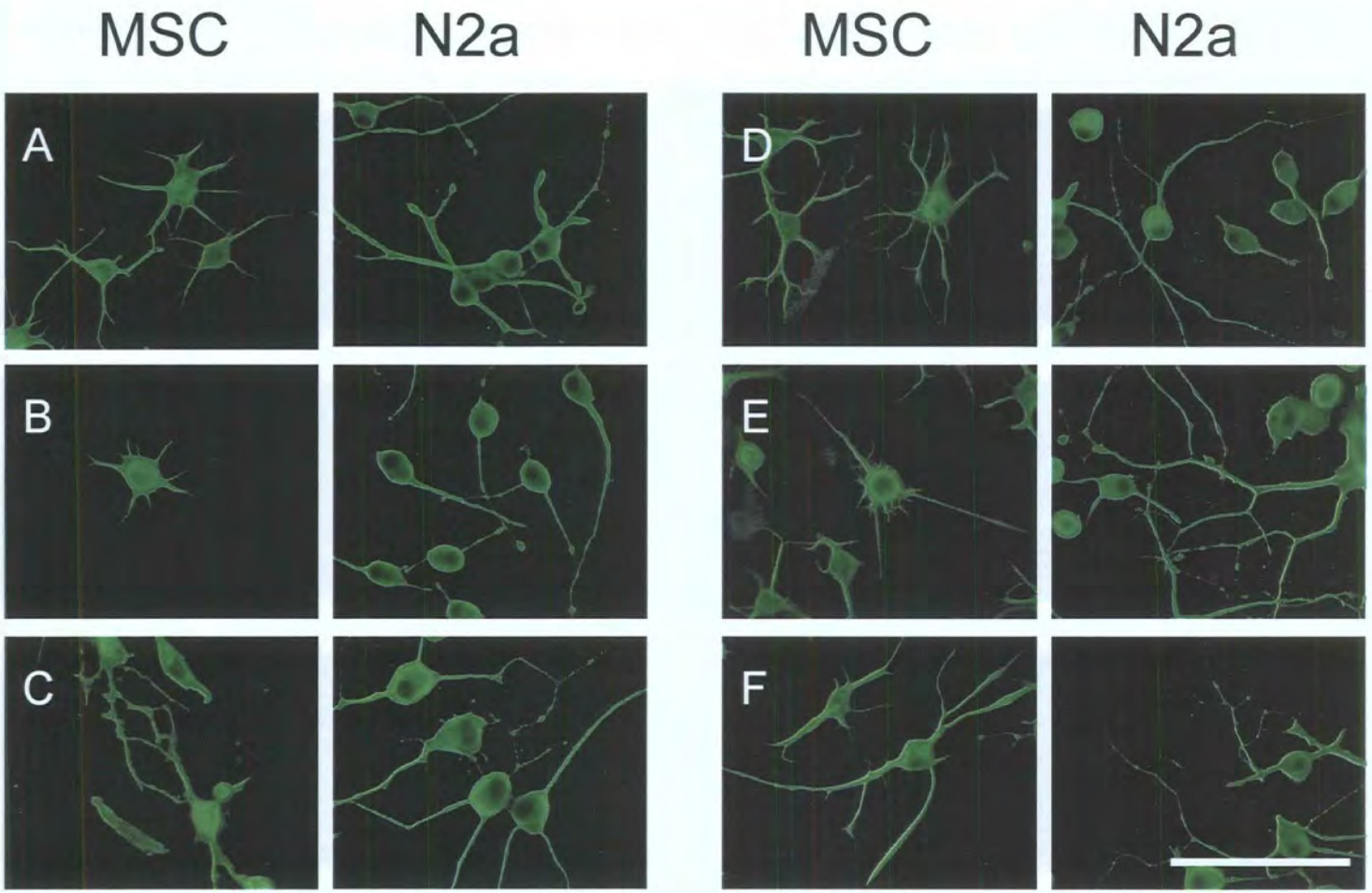


Figure 3.12: Immuno-staining of 5-hour chemically induced MSCs compared to N2a cells induced to differentiate under identical culture conditions. MSCs and N2a cells plated on PLO coated tissue culture plastic were induced to differentiate in the presence of 2% DMSO in serum free for 5 hours. The expression of neuronal markers was localised by immuno-labeling with specific mouse mono-clonal antibodies against: GAP-43 (**A**), NeuN (**B**), Tuj-1 (**C**), NF-M (**D**), MAP-2 (**E**) and NF-L (**F**). *Scale bars: 50μm.*

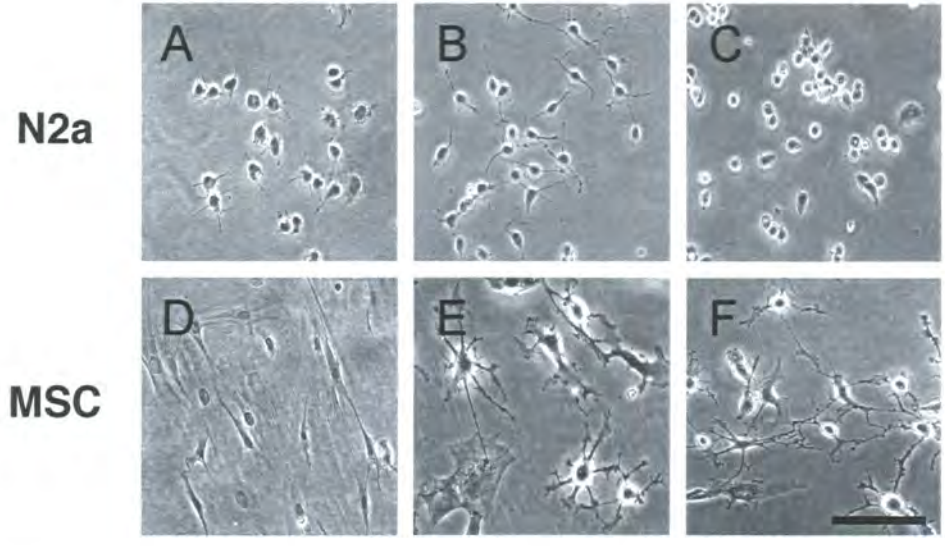


Figure 3.13a: Structural features of N2a cells versus MSCs following exposure to 2% DMSO in serum free media. Process extension in N2a cells but not MSCs is microtubule dependent. (**A**) Control N2a cells (**B**) N2a cells treated with 2% DMSO for 3d. (**C**) DMSO treated N2a cells following 3 hours incubation with the microtubule destabilizing agent colcemid. Treatment results in retraction of processes towards to the cell body. Processes derived from MSCs are not microtubule dependent. (**D**) Control MSC. (**E**) 3d DMSO treated cells. (**F**) 3d DMSO treated cells followed by 3 hours colcemid treatment. Addition of colcemid has no apparent effect on process morphology in MSCs but because N2a process formation and extension is microtubule dependent, colcemid treatment causes the retraction of processes. *Scale bar: 40μm.*

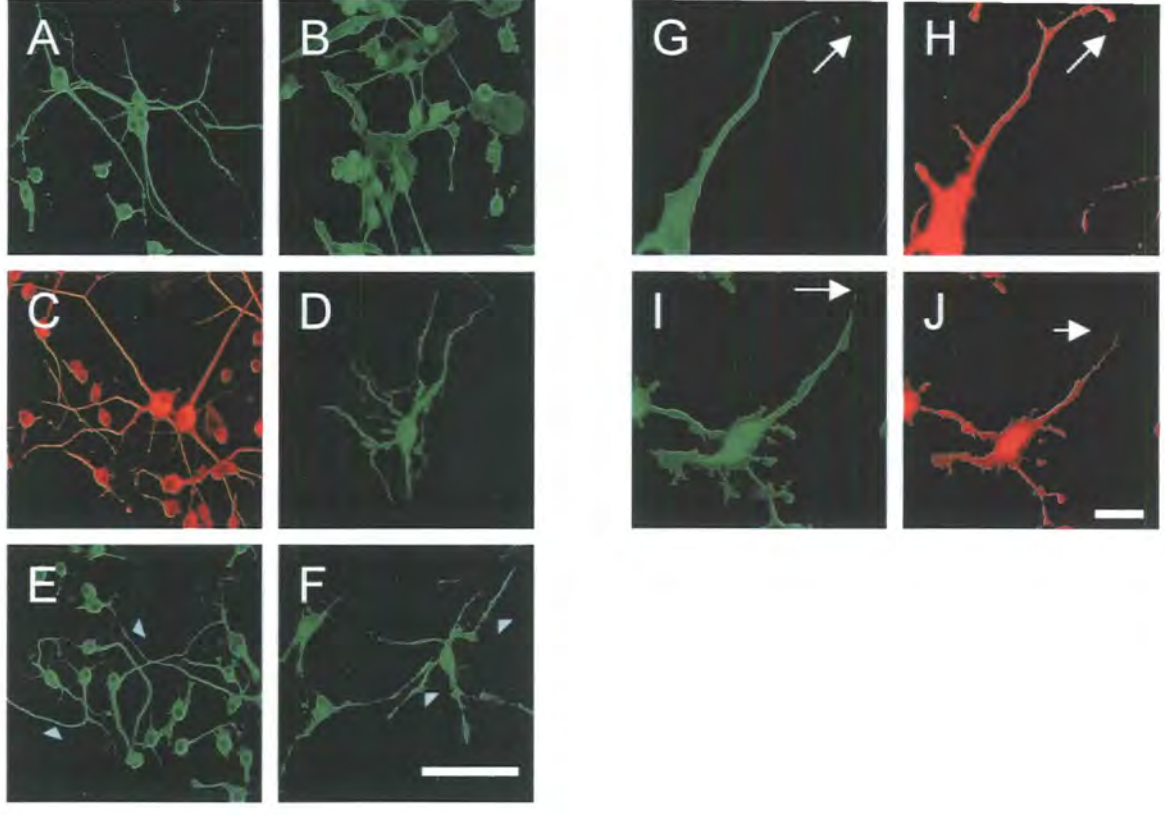


Figure 3.13b: **Structural features of N2a cells vs MSCs following exposure to 2% DMSO in serum free media.** N2a cells (**A**) and MSCs (**B**) possess microtubule containing presumptive neurites (alpha-tubulin staining, green). In N2a cells these processes stain highly positive for TUJ-1 (**C**). However MSC derived processes stain only weakly for TUJ-1, with expression principally localized to the cell body (**D**). Neuroblastoma derived neurites continually undergo reorganization by a process of actinoplast-tubuloplast segregation (Tint et al., 1992). These segregations appear as bulb like structures along the axon (**E, triangles**). Similar structures are evident on MSC derived processes (**F, triangles**). **A, B, E, F**: alpha tubulin staining. **C,D** TUJ-1 staining. Microtubule containing processes (**G**) terminate in an actin rich growth cone (**H**) in N2a cells. Although similar structures are evident on MSCs (**I,J**) these are not actin rich and stain weakly for both tubulin (**I**) and actin (**J**). Alpha-tubulin (green) and Actin (red). *Scale bar: 40μm*

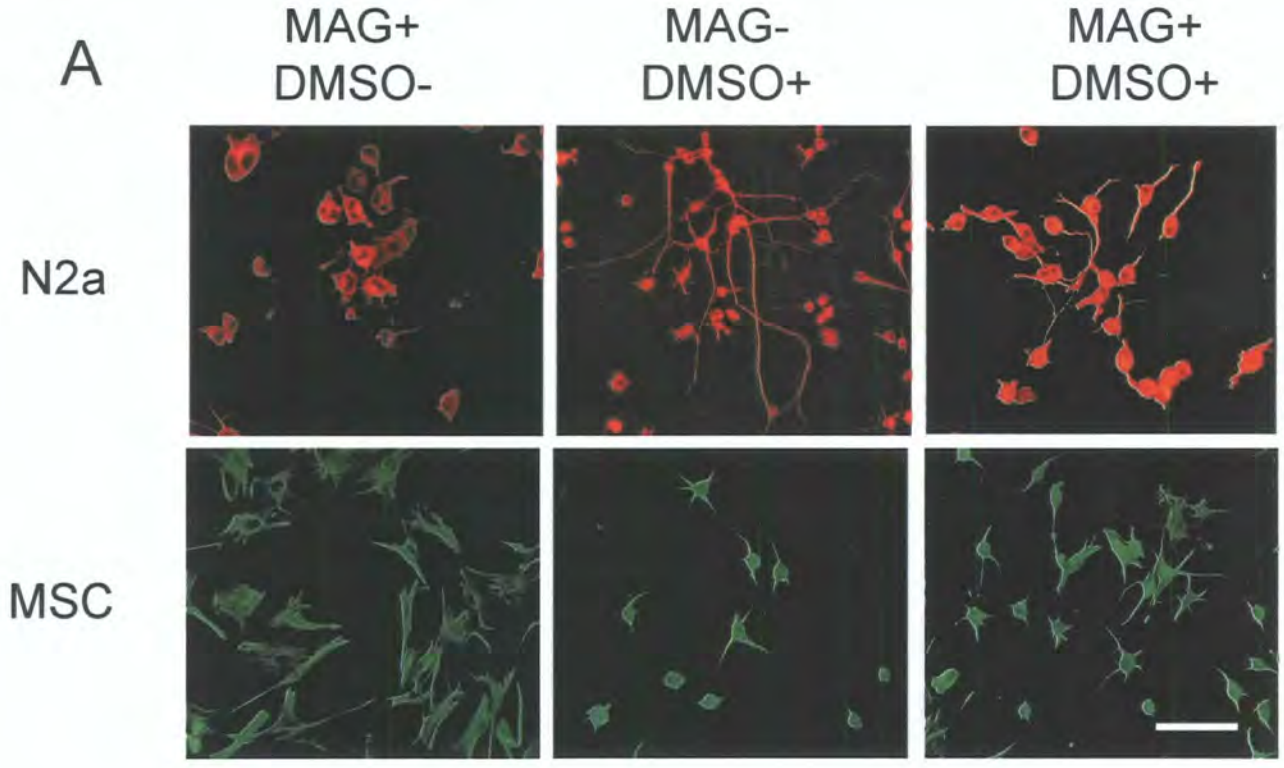
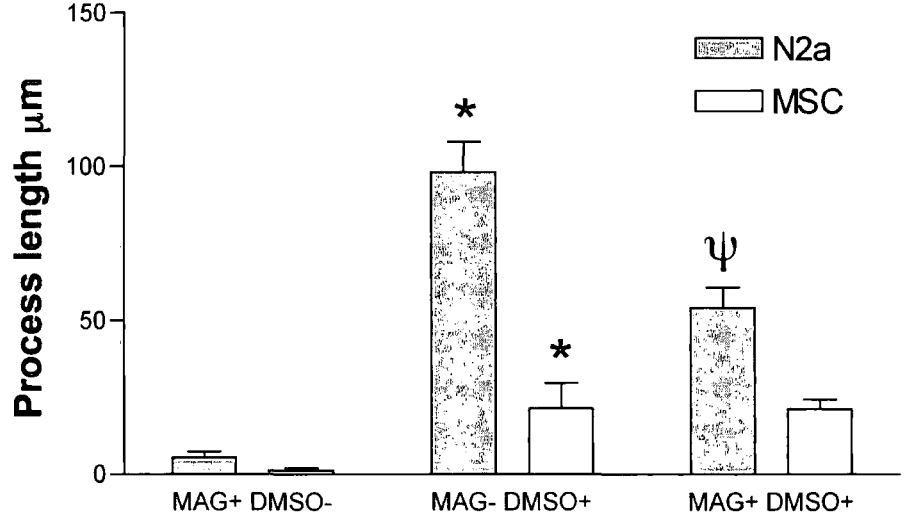


Figure 3.14: **Effect of MAG on the neurite outgrowth of N2a cells versus MSCs following exposure to 2% DMSO in serum free media.** N2a and MSCs were grown on MAG coated tissue culture plastic in standard basal media. Cells were subsequently transferred to neural induction media (DMEM supplemented with 10% FCS) for 6 days (MAG+ DMSO+). Cells were then fixed and stained with anti-Tuj-1 to identify differentiated cells. Controls include: cells induced to differentiate on normal uncoated tissue culture plastic (MAG- DMSO+) and cells grown to MAG but not induced (MAG+ DMSO-). (**A**) Immuno-florescent images of Tuj-1+ N2a and MSC cells following 6-day neural induction. (**B**) Quantification of neurite outgrowth in control or induced conditions following image analysis. Average neurite length is indicated and was quantified from 10 randomly selected fields of view for each experiment. All data are mean values±SEM from 3 independent experiments in parallel cultures. Significant differences are indicated (*P<0.001 ΨP<0.05, 1 way ANOVA followed by Dunnetts post hoc analysis to compare the neurite length of each cell type to control (MAG-DMSO-). Scale bar: 40μm

B



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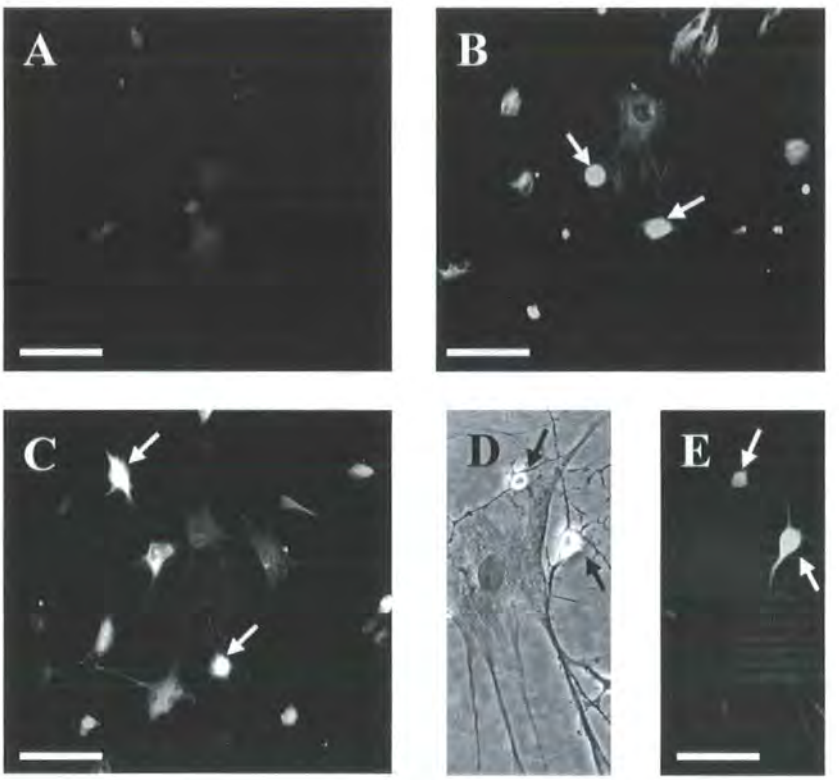


Figure 3.15: Expression of nestin in response to withdrawal of serum and in response to the addition of basic fibroblast growth factor. Under normal culture conditions, untreated BMSCs express only very low levels of nestin protein (**A**). The expression of nestin was examined by immunofluorescence staining under the following growth conditions: (**B**) removal of serum from the growth medium for a period of 24 hours; (**C**) addition of bFGF (5ng/ml); (**D,E**) 5 hours treatment with 2% DMSO. Upon removal of serum for 24 hours, there was a clear up-regulation in nestin expression which appears to be higher in type II cells, with some cells beginning to display morphological changes typical of neuroprogenitor cells (**B, arrows**). Addition of bFGF to serum containing media for a period of 24 hours (pre-induction conditions) also dramatically increases the expression of nestin with higher number of cells adopting a neural-like morphology (**C, arrows**). It should be noted that non-responsive cells possess a stromal, flat elongated morphology and continue to express only low levels of nestin. Following addition of 2% DMSO, only cells which have the ability to up-regulate nestin displayed a neural-like morphology and appear capable of further differentiation (**D,E arrows**). *Scale bars: 40μm* (**A-E**).

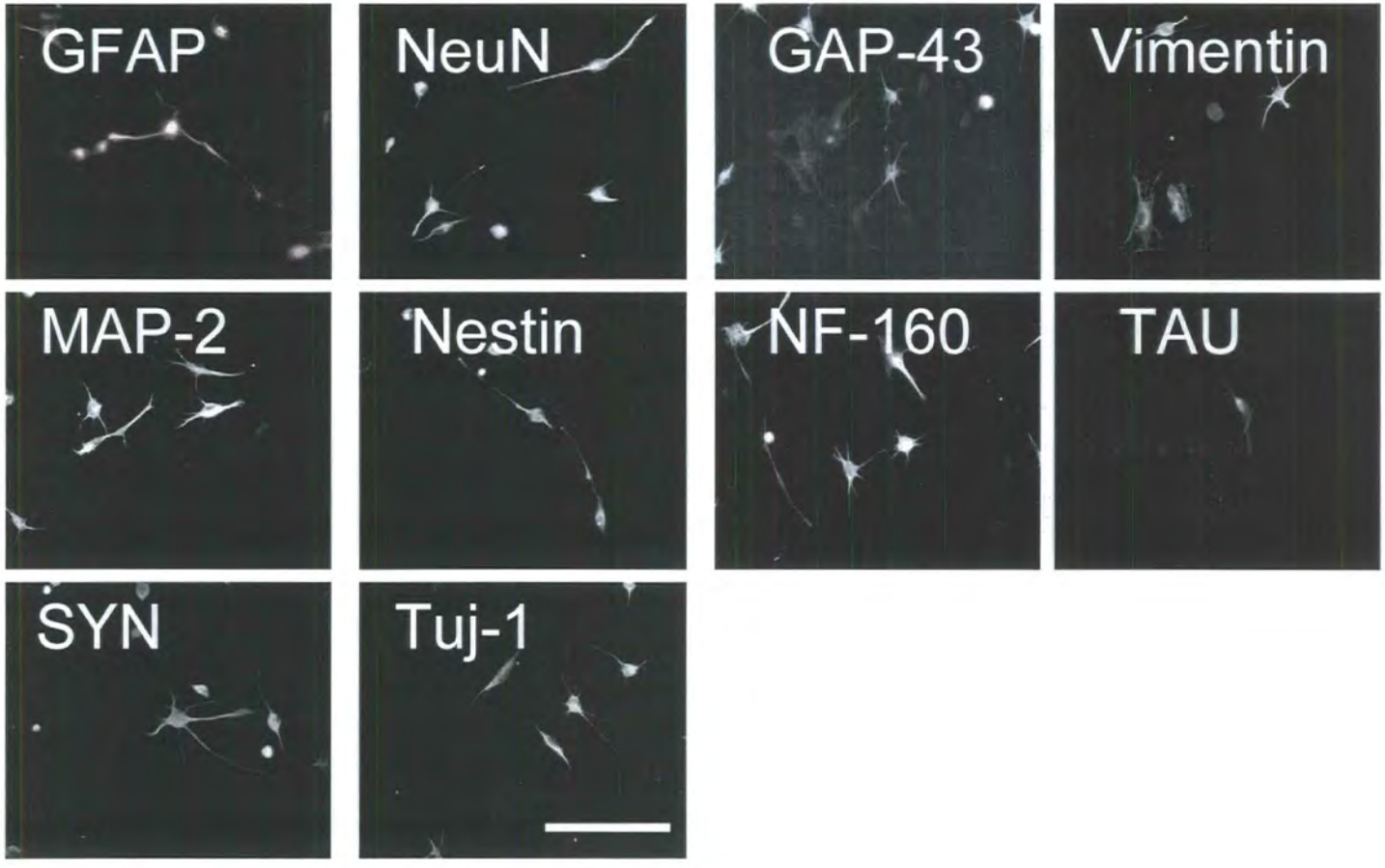


Figure 3.16: Expression of neural and mesodermal proteins in 6 day induced MSCs as determined by immuno-cytochemistry. Cells were incubated in serum free media supplemented with 2% DMSO and N2. Cells were fixed after 6 days and processed for immuno-cytochemistry. *Scale bars: 50μm.*

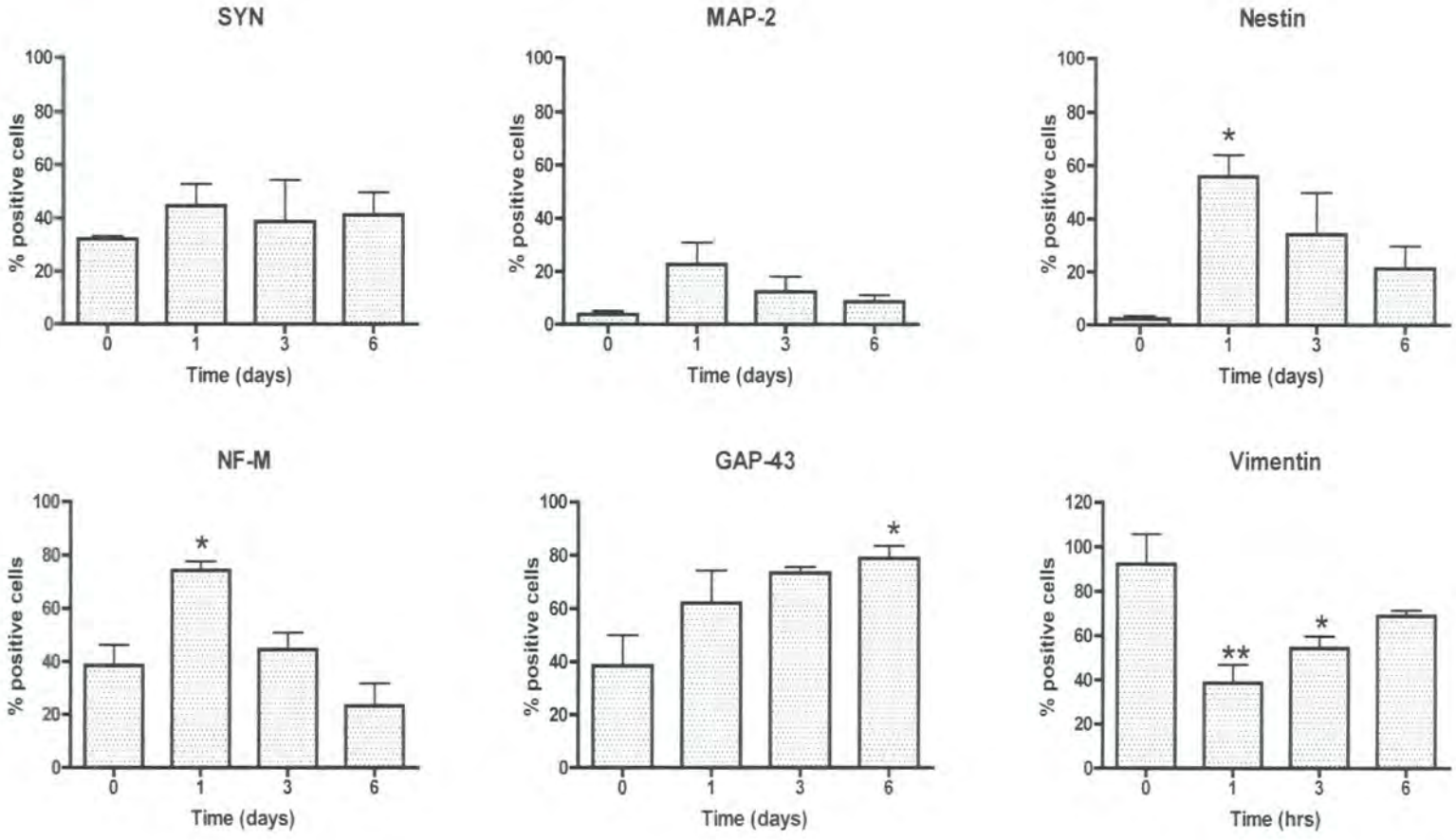


Figure 3.17: Analysis of protein expression in MSC derived presumptive neurons following 6 days differentiation in the presence of 2% DMSO in N2 supplemented media. Cells were grown in the presence of 2% DMSO in serum free media supplemented with N2 and maintained for either 1d, 3d, or 6d after which cells were harvested and processed for intracellular staining and flow cytometry. The graphs illustrate the number of positive cells for each marker at each time point as determined by flow cytometry. All data are mean values±SEM from 3 independent experiments in parallel cultures. Significant differences from the control group (time point 0) are indicated with an asterisk (*P<0.05**P<0.01).

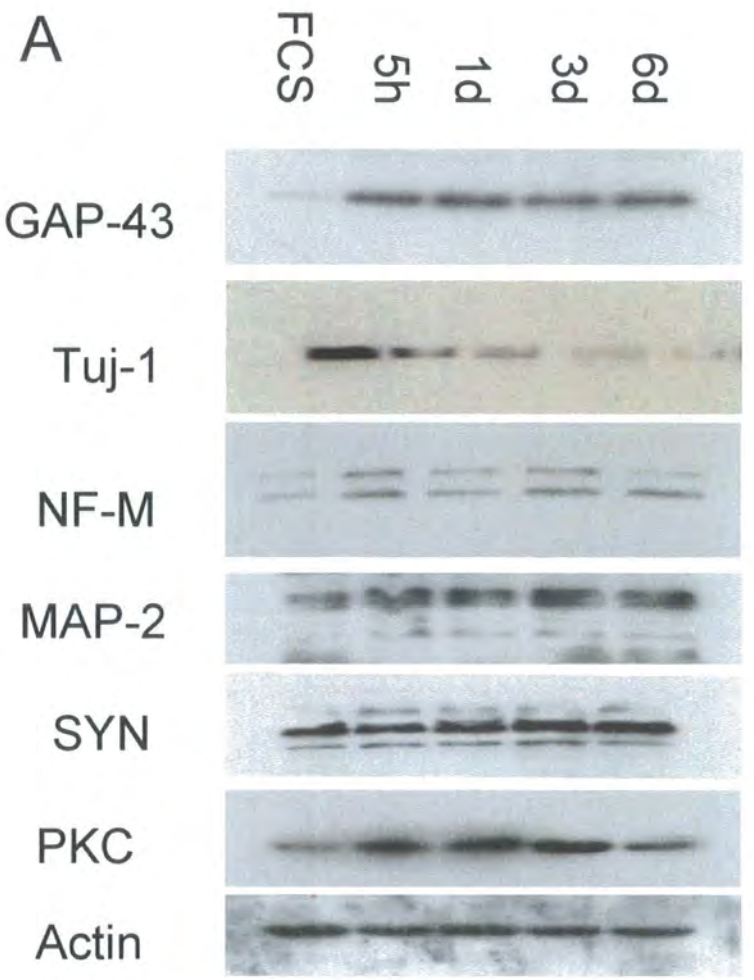
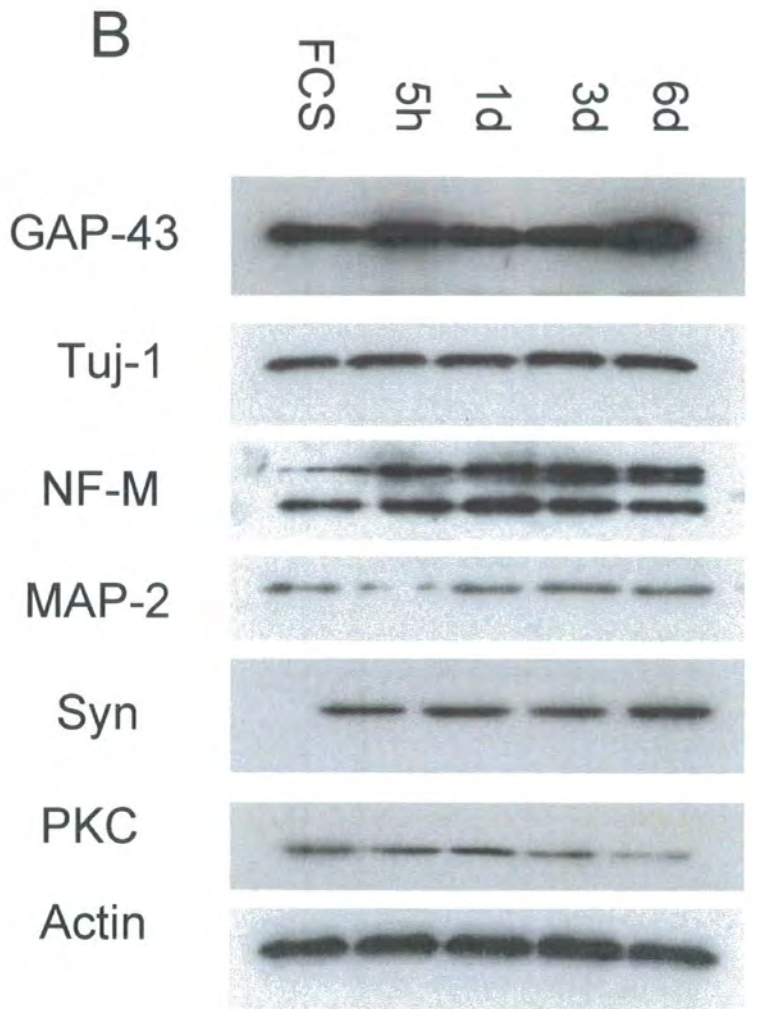


Figure 3.18: Western blot analysis of neuronal protein expression in MSC derived presumptive neurons versus SHSY-5Y cells induced to differentiate under identical conditions. Cells were incubated in serum free media supplemented with 2% DMSO and N2 for 6 days. Cells were harvested and processed for western blot analysis at intervals 5 hours, 1d, 3d, and 6d. (**A**) MSCs, (**B**) SHSY-5Y cells.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Tuj-1
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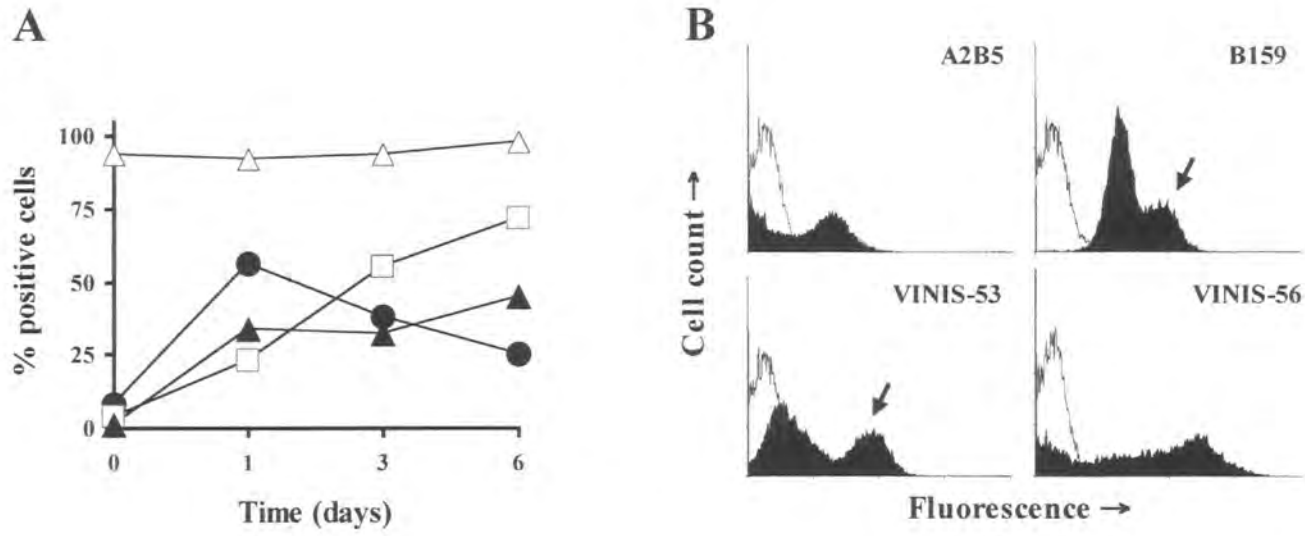


Figure 3.19: Regulation of cell surface antigen expression by MSCs treated with antioxidants in serum free media. Flow cytometry and immunofluorescence detection was used to monitor the expression of various cell surface antigens known to be associated with the neural lineage. BMSCs were treated with 2% DMSO and maintained for 6 days. Plot **A** illustrates the expression profile of the cell surface antigens A2B5 (●), VINIS-53 (▲), VINIS-56 (□) and B159 (△) during the 6-day treatment period. Flow cytometric traces (**B**) show the distribution of antigen expression in BMSCs exposed to antioxidants for 6 days. Neuron cell adhesion molecule (NCAM) expression was examined using the two monoclonal antibodies B159 and VINIS-53 that recognize distinct epitopes of the NCAM protein. B159 staining was consistently high (93-97% positive cells) without significant time dependent changes, however, in older cultures although the total number of B159-positive cells was unchanged, an antigen bright population became evident (**B, arrow**). The number of cells staining positive for VINIS-53 did increase subsequent to antioxidant treatment and the level of VINIS-53 expression remained reasonably stable over the six day period. In addition, a sub-population of VINIS-53 antigen bright cells (approximately equivalent to the number of B159 antigen bright cells) was identified in day six cultures (**B, arrows**). Together, these findings suggest that antioxidant treatment increases the number of BMSCs expressing NCAM and up-regulates the concentrations of NCAM expressed on the cell surface. Increases in the neural associated cell surface antigens A2B5 and VINIS-56 further indicate that BMSCs exposed to antioxidants adopt neural-like characteristics.

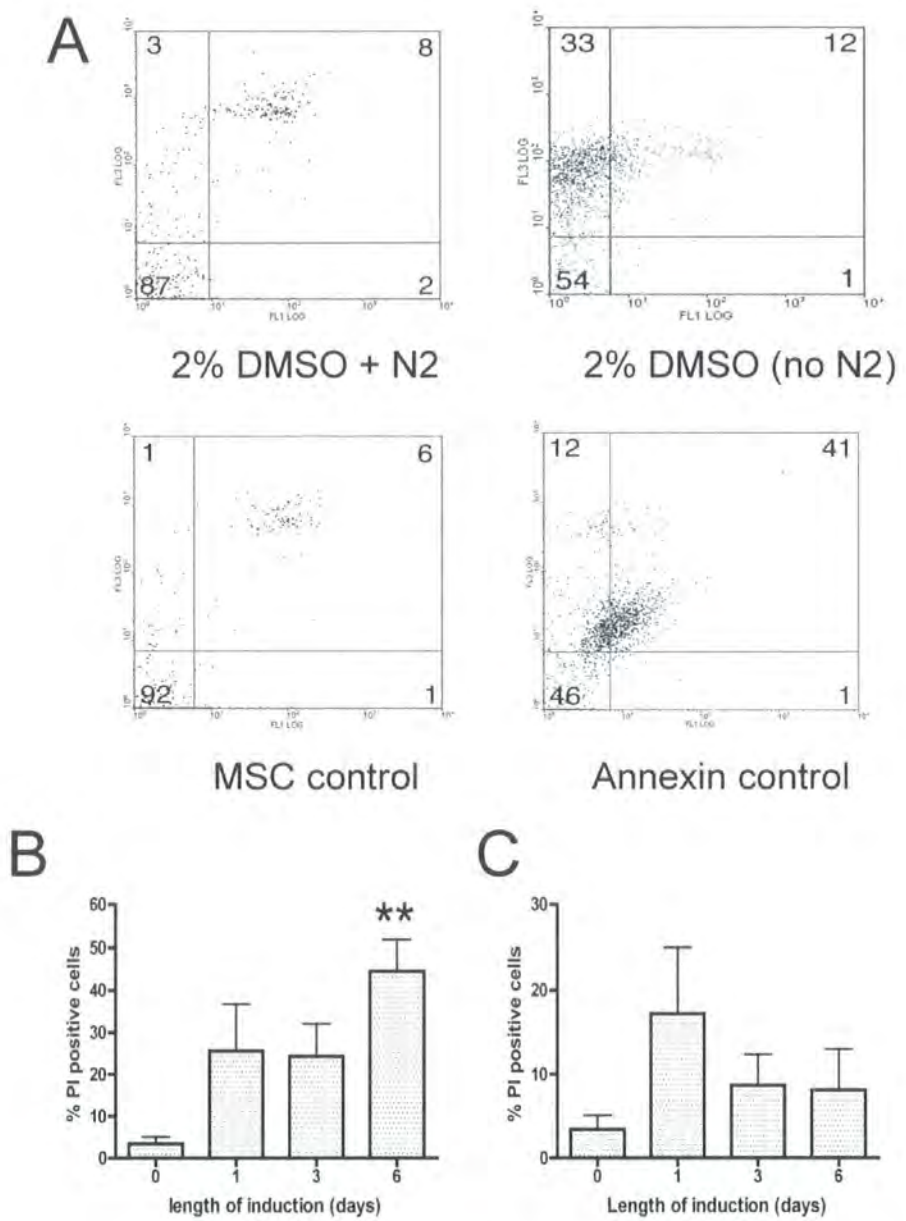


Figure 3.20: Analysis of cell viability during long-term differentiation. Cell viability was determined by either annexin V staining (**A**) or PI staining (**B**) and analysis of the number of positive cells was determined by flow cytometry. (**A**) Cells were cultured in 2% DMSO in serum free media in either the presence or absence of N2 supplement. Cells were harvested and processed for flow cytometry after 6 days. Cells were dual stained with Annexin V (FITC) and PI. The histograms (**A**) indicate the number of cells positively stained. The upper right quadrant indicates the number of cells positive for both annexin V and PI. In contrast, the upper left quadrant indicates the number of cells positive only for PI. The analysis was compared the an MSC control in which cells were maintained under standard culture conditions and an annexin V control. (**B**) indicates the number of cells positive for PI staining 1,3,6 days following exposure to DMSO either in serum free media (**B**) or in N2 supplemented serum free media (**C**). All data are mean values±SEM from 3 independent experiments in parallel cultures. Significant differences from the control group (time point 0) are indicated with an asterisk (**P<0.01).

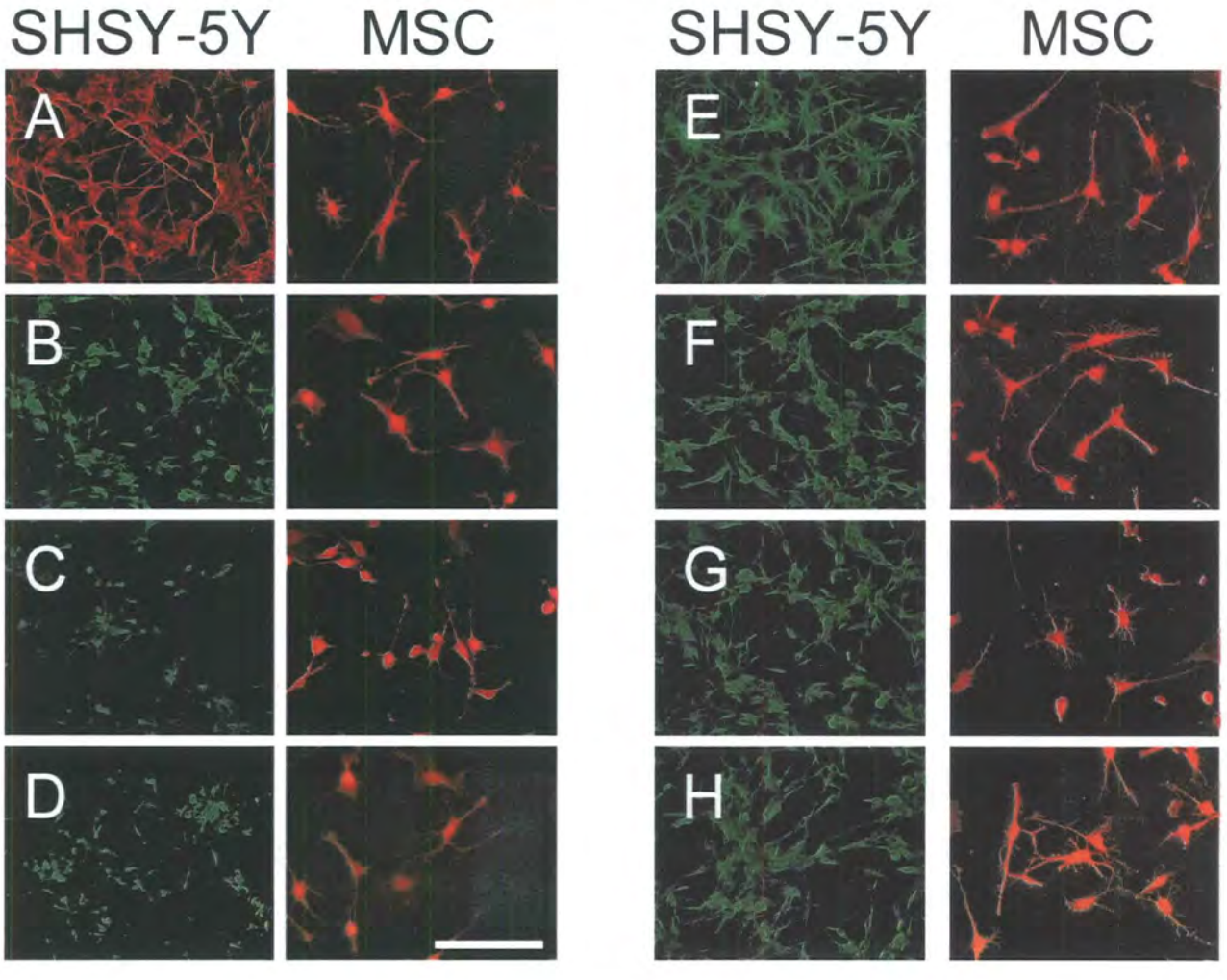


Figure 3.21: Immuno-staining of 6-day chemically induced MSCs compared to SHSY-5Y cells induced to differentiate under identical conditions. MSCs and SHSY-5Y cells plated on PLO coated tissue culture plastic were induced to differentiate in the presence of 2% DMSO in serum free, N2 supplemented media for 6 days. The expression of neuronal markers was localised by immuno-labeling with specific mouse monoclonal antibodies against Tuj-1 (**A**), synaptophysin (SYN, **B**), GAP-43 (**C**), SNAP-25 (**D**), Vimentin (**E**), NF-M (**F**), MAP-2 (**G**) and NF-L (**H**). *Scale bars: 50μm.*

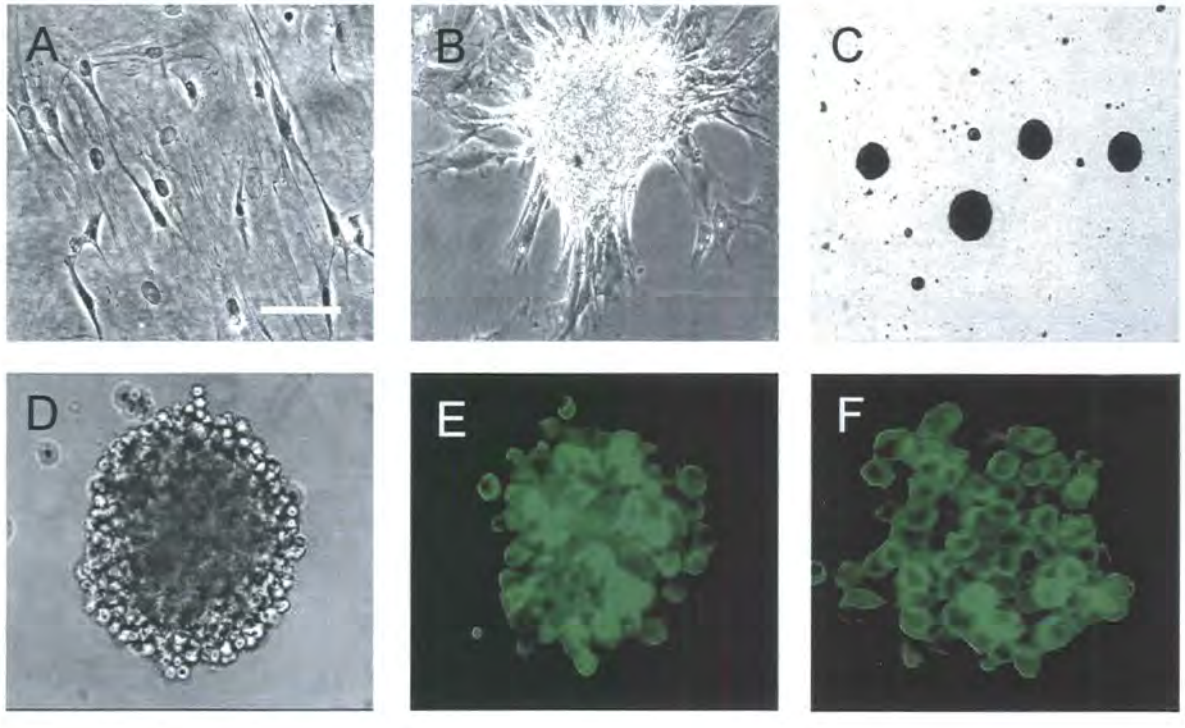


Figure 3.22: **Generation of neuro-sphere-like structures in serum-free media.** To generate spherical aggregations of cells from monolayer cultures, MSCs were plated on tissue culture plastic at 8,000 cells/cm^2 and grown to >80% confluence in DMEM/10%FCS/200mM L-glutamine (**A**). MSCs were then transferred to a serum-free culture medium consisting of DMEM/F12 supplemented with 200mM L-Glutamine, N2 supplement, bFGF (10ng/ml) and EGF (10ng/ml). Under these culture conditions small aggregations of cells began to develop on the bottom of the culture flask within 3-5 days (**B**). As these aggregations became larger they detached from the flaks and became free floating spheres within the media (**C**). At 7 days, these spheres were harvested by removing the media and centrifuging at 500 rpm for 3 minutes. Cell aggregates were then gently re-suspended and sub-cultured under the same growth conditions. Spheres of cells continue to proliferate and enlarge in response to EGF and bFGF (**D**). When cell aggregates become greater than 100μm in diameter, they were further sub-cultured by mechanical dissociation using a fire polished Pasteur pipette. When grown in suspension, cell aggregates stained positive for nestin (**D**), suggesting the presence of neuroprogenitor-like cells, they also stained positive stain positive for neuroD1 (**F**). *Scale bars: 50μm (**A**); 300μm (**B**); 100μm (**C**); 30μm (**D**) ; 500μm (**E-F**).*

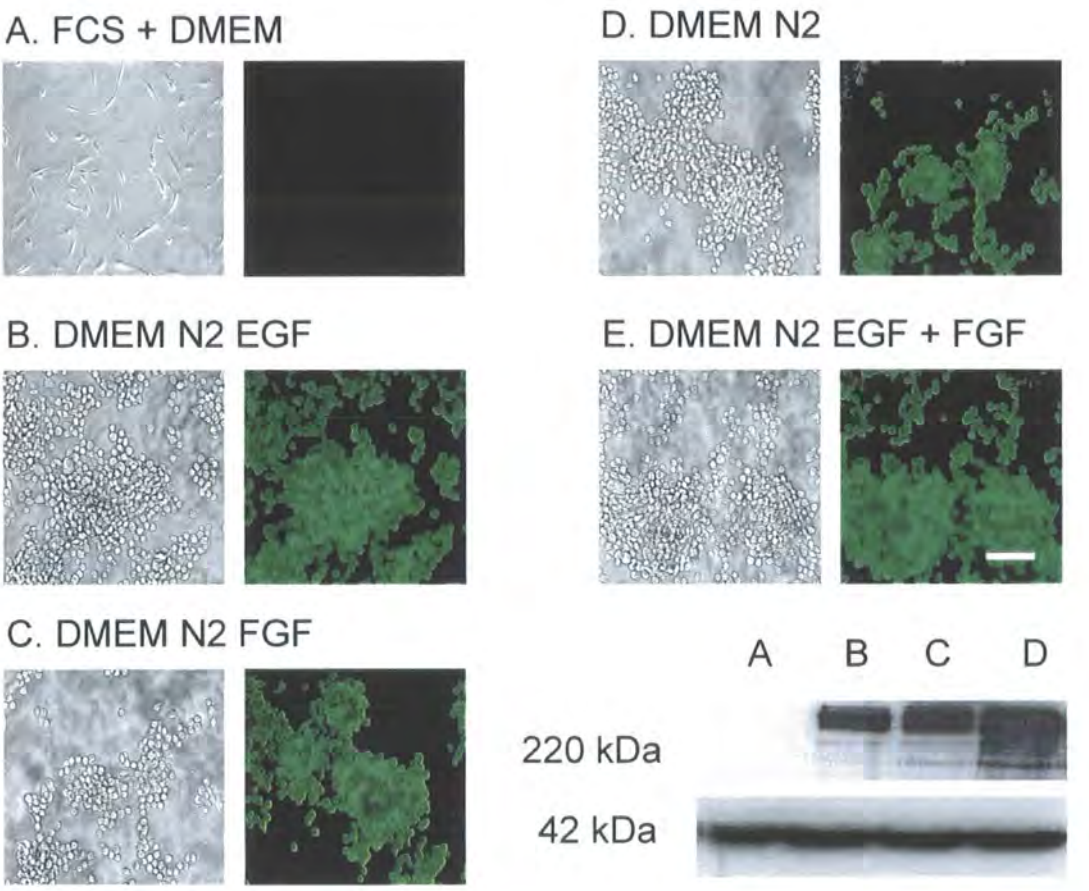
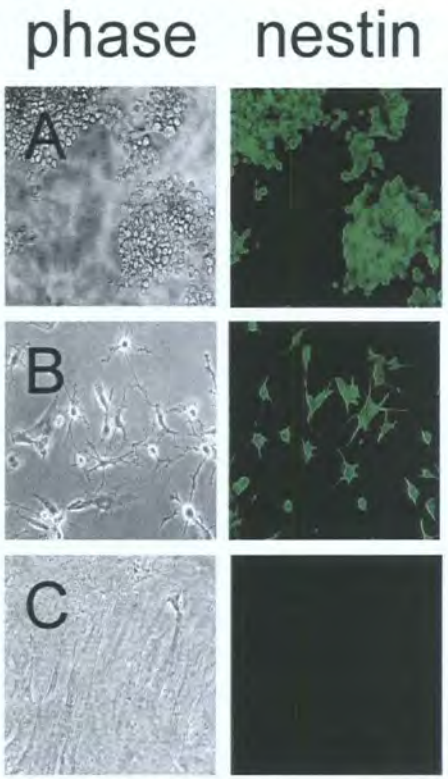
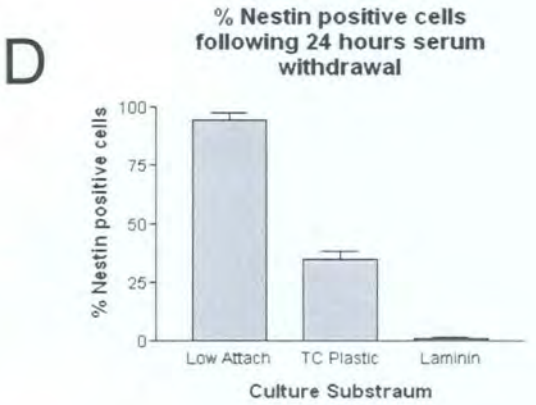


Figure 3.23: Effects of the removal of serum on the sphere forming capacity of MSCs in culture. Removal of serum is essential for both spheroid formation and expression of nestin. rMSCs were maintained in low attachment culture dishes under defined media conditions: - **(A)** 10% FCS DMEM **(B)** DMEM F12 + N2 + EGF **(C)** DMEM F12 + N2 + FGF **(D)** DMEM F12 + N2 **(E)** DMEM F12 + N2 + EGF + FGF. After 24 hours, cells were fixed in 4% paraformaldehyde and labelled with an anti-nestin antibody (green). *Scale bar: 40μm* **(A-E)**. Nestin expression correlates with spheroid formation which occurs in cells grown in serum free conditions. Expression of nestin in serum free culture conditions was confirmed by western blot analysis. ß-actin was used to control for protein loading.





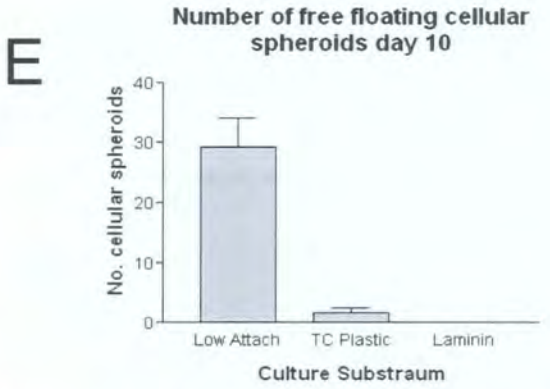


Figure 3.24: Low attachment culture conditions are critical for nestin induction, cell rounding and sphere formation. Cells cultured on low attachment substratum undergo cell rounding and aggregate to form nestin positive cell clusters following 24 hours culture in serum-free media (**A**). Cells cultured on Laminin coated tissue culture plastic (high attachment substratum) do not express nestin or undergo cell rounding and therefore do not aggregate in serum free media (**C**). As a result, no free-floating cellular spheroids were detectable in cultures maintained in serum free media for 10 days (**E**). Cells grown on normal tissue culture plastic acquire an intermediate-like morphology in response to serum withdrawal (**B**). 27-42% of cells have a cytoplasm, which is collapsed around the nucleus, but unlike cell rounding on low attachment substratum this retraction leaves several cytoplasm extensions radiating from the cell body. (**D**) % of nestin positive cells under each condition after 24 hours and (**E**) the number of free-floating cellular spheres. All data is represented as Mean±SEM from 3 independent experiments in parallel cultures. *Scale bars: 50μm.*

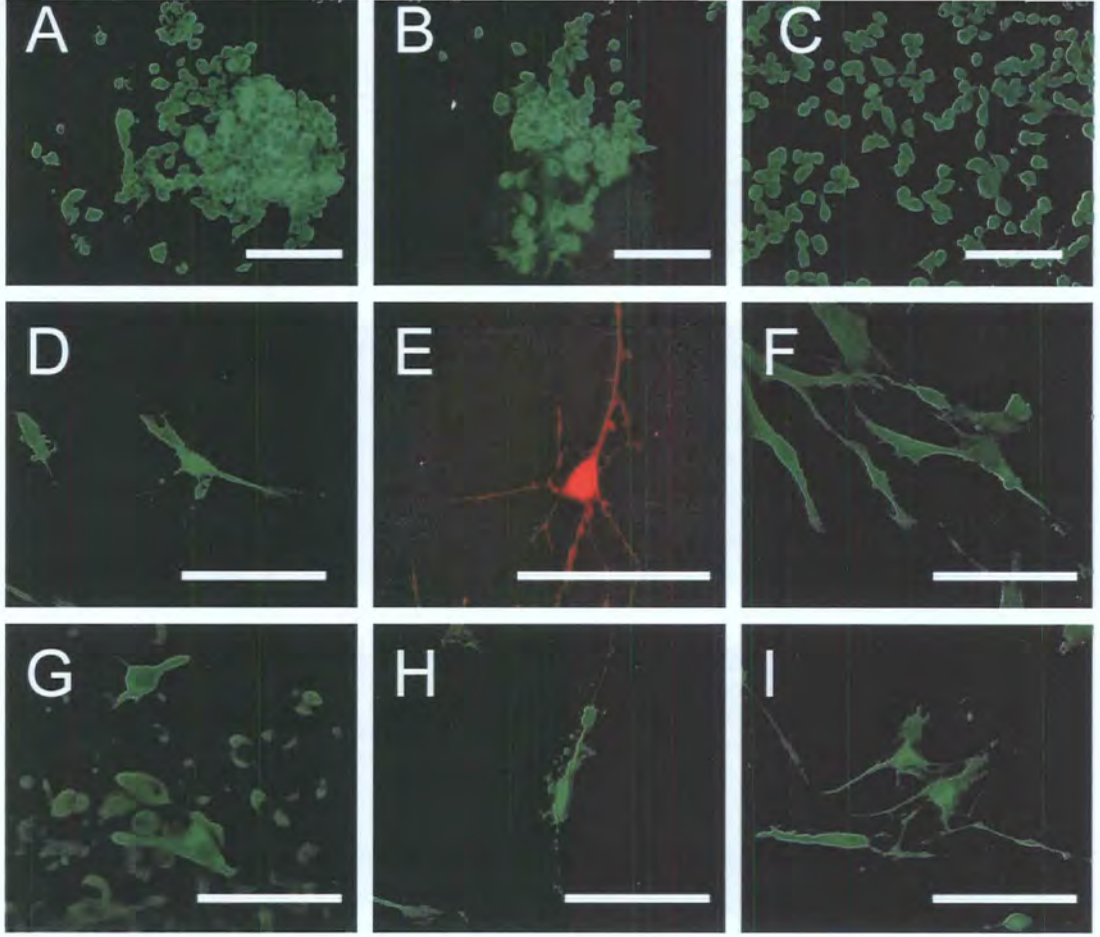


Figure 3.25: MSC derived cellular spheroids and presumptive differentiated cells. (**A**) MSC derived cellular spheroid expressing nestin 12 hours following plating on PLO coated tissue culture plastic. (**B**) Spheroid expressing Tuj-1 at high levels 24 hours following plating. (**C**) Spheroid derived cells 4 days following plating on PLO tissue culture plastic expressing Tuj-1. In an attempt to induce differentiation of MSC spheroids, cells were plated on laminin coated tissue culture plastic in the presence of RA (0.5μM) and NGF (10ng/ml) in serum free media for 12 days. Cells expressing neuronal proteins were detected by immunolabeling with antibodies directed against: GAP-43 (**D**), Tuj-1 (**E,F**), GFAP (**G**), MAP-2 (**H**), NF-M (**I**). *Scale bars: 50μm.*

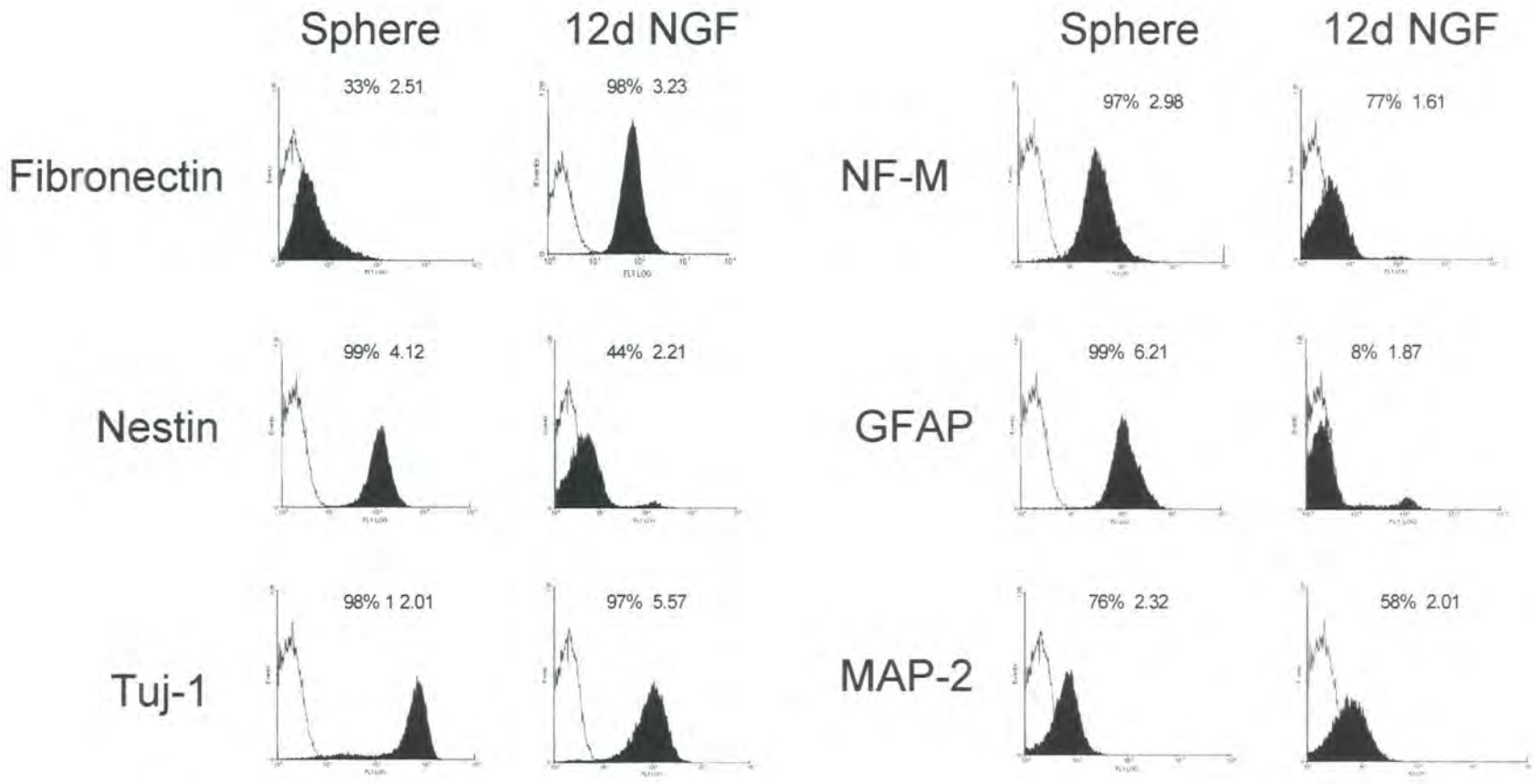


Figure 3.26: Expression of neural and mesodermal proteins following differentiation of MSC derived cellular spheroids. Flow cytometry measurement of neural (Nestin, GFAP, TuJ1, NF-M and MAP-2) and mesodermal associated protein markers (Fibronectin) in rMSCs following either culture as cellular spheroids or plating on laminin coated tissue culture plastic in the presence of 10ng/ml NGF and 0.5 μM RA for 12 days in serum free media. Flow cytometry data from representative experiments are shown as histograms with fluorescence intensity on the x-axis (solid peaks) and cell count on the y-axis. Background fluorescence was excluded by the use of isotype matched control antibodies (open peaks). The percentage of positively labelled cells (left) is given with the geometric mean fluorescence intensity (MFI) (right) for each antigen.

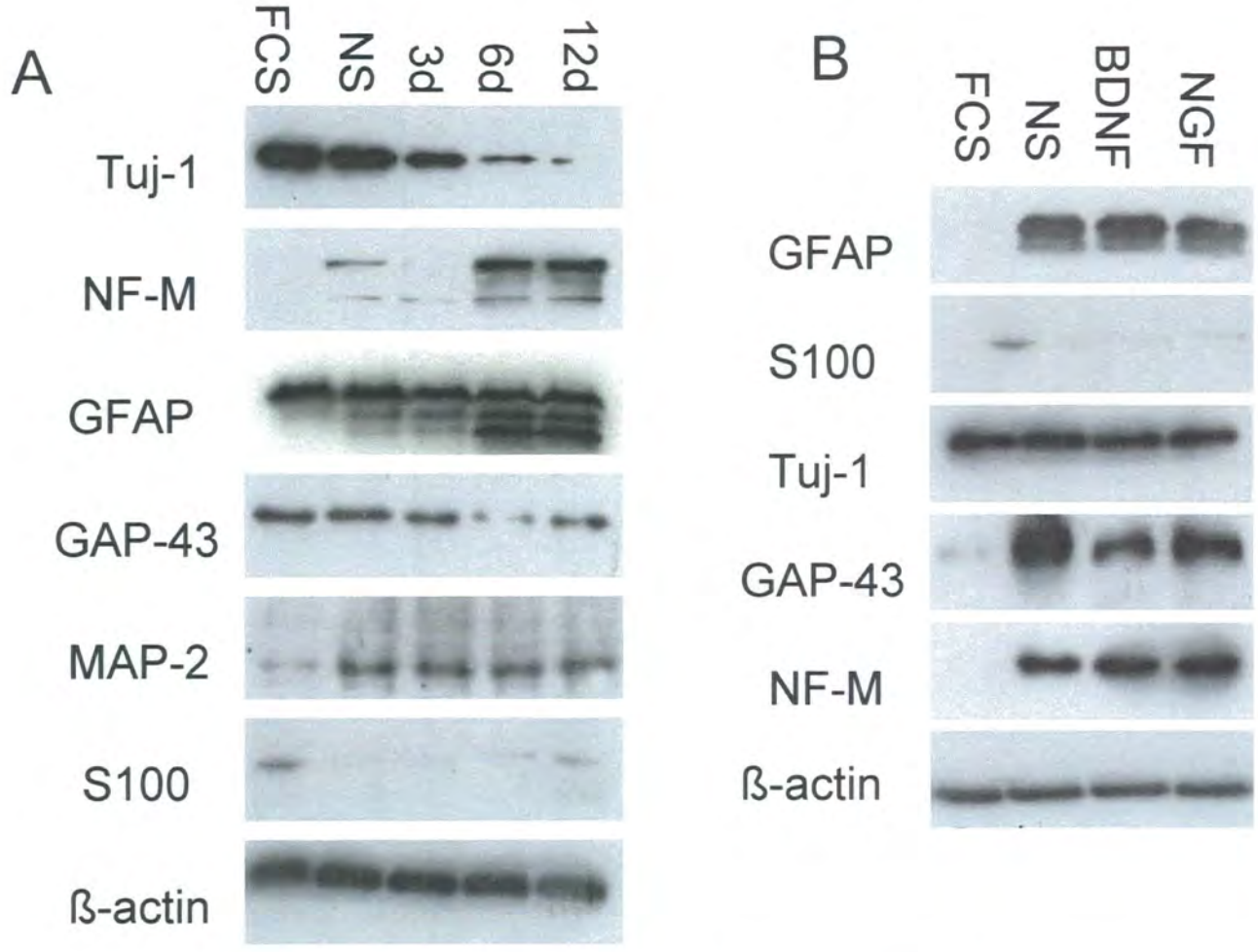


Figure 3.27: WB analysis following differentiation of MSC cellular spheroids in the presence of NGF/BDNF and RA in serum free media for 12 days. (**A**) Time course of neural protein expression in response in RA and NGF for 12 days on Laminin coated tissue culture plastic. (**B**) Comparison of expression of neural proteins in cultures treated with RA+BDNF for 12 days compared to RA+NGF for 12 days. NS=MSC spheroids cultured in DMEMF12 supplemented with EGF and FGF-2 for 3 days.

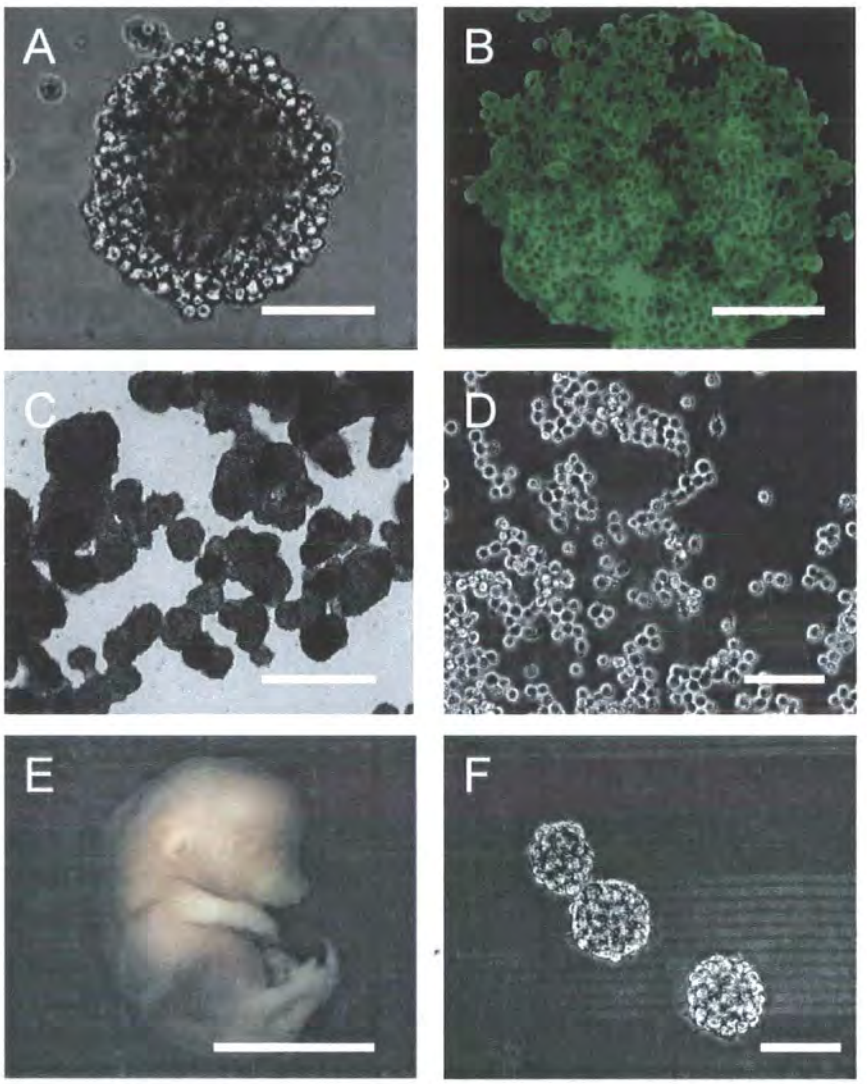


Figure 3.28: **Comparison of neural-like cells derived from cellular spheres with the neural progeny of differentiating NSCs.** (**A**) MSC derived cellular spheroid following 2 weeks subculture in DMEM/F12 media supplemented with EGF 10 ng/ml and FGF-2 10 ng/ml. These cellular spheroids were nestin positive (**B**). The cellular spheroids were propagated in culture and proliferated in response to growth factors in non-adherent culture conditions (**C**). On plating these cellular spheroids on PLO coated tissue culture plastic the cells dissociated and adhered (**D**). Embryonic striatal NSCs, were derived from day 14 rat embryo's (**E**) and grown under identical culture conditions as MSC derived cellular spheroids. Neurospheres formed from day 14 ventral midbrain had a similar morphology in culture to MSC cellular spheroids (**F**). *Scale bars: 50μm*

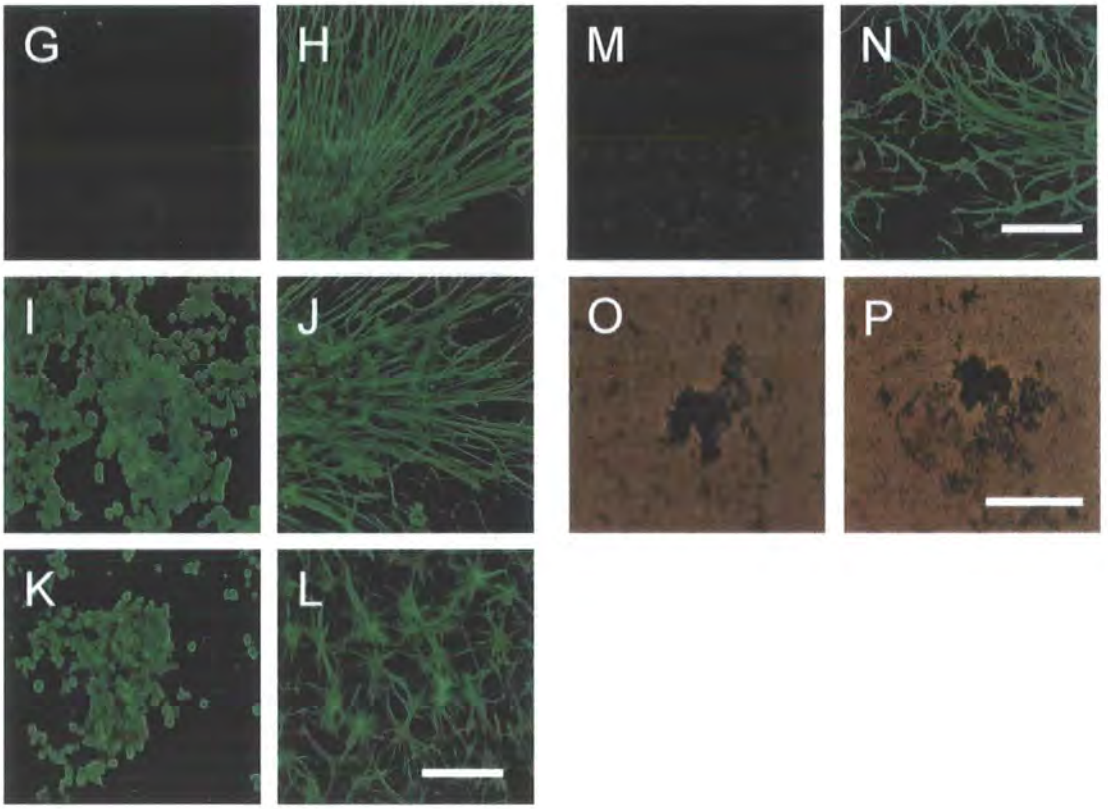


Figure 3.28 continued: (**G**) Nestin is down regulated 2 days following plating of MSC cellular spheroids on Laminin coated tissue culture plastic. (**H**) Un-dissociated rat neurospheres plated for 2 days on Laminin coated tissue culture plastic still express nestin, which is highly expressed in developing neurites. (**I**) Un-dissociated MSC spheroids plated on PLO coated tissue culture plastic for 6 days express high levels of Tuj-1 but there is no evidence of extensive neurite outgrowth as observed in Tuj-1+ cell progeny of rat NSC/progenitor cells cultured under identical conditions for 6 days (**J**). (**K**) Expression of GFAP in MSC spheroids 6 days following plating on PLO coated tissue culture plastic compared to NSC/progenitor cells 6 days following plating under identical conditions (**L**). (**M**) No MAP-2 expression was detected in MSC spheroid progeny 6 days following plating in contrast to rat NSC/progenitor cells in which high levels of expression were evident in developing neurites (**N**). Both undifferentiated MSCs and MSC cellular spheroids were capable of forming bone following osteogenic differentiation confirmed by Von Kossa staining. *Scale bars: 50μm*

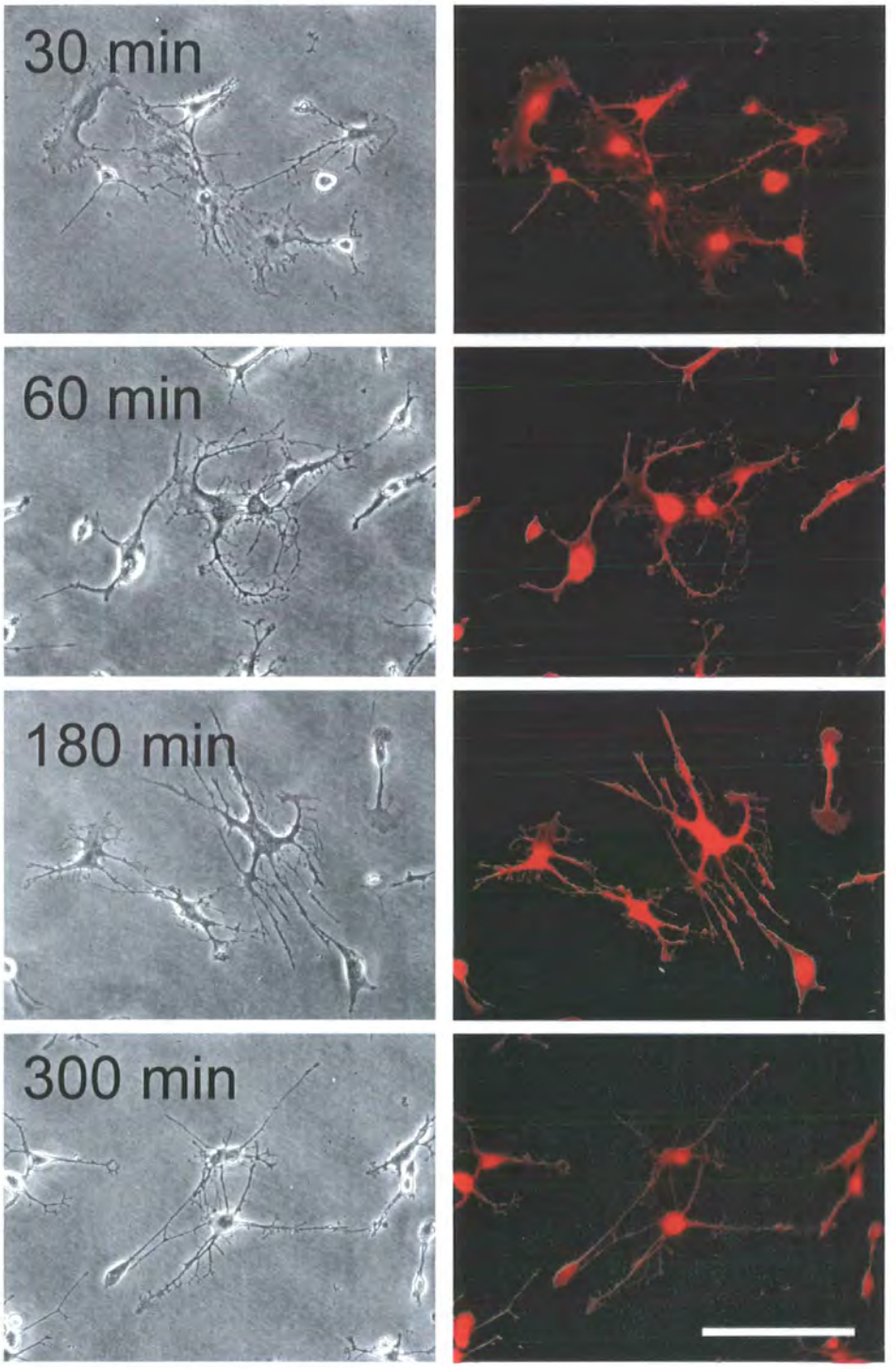


Figure 3.29: Effect of PMA on the morphology of MSCs in culture. MSCs were incubated with 10μM PMA for 300 minutes in serum containing media. Cultures were fixed and stained at 30, 60, 180 and 300 minutes post exposure and phase contrast images obtained. To visualise the F-actin cytoskeleton cells were labelled with TRITC conjugated phalloidin. *Scale bars: 50μm.*

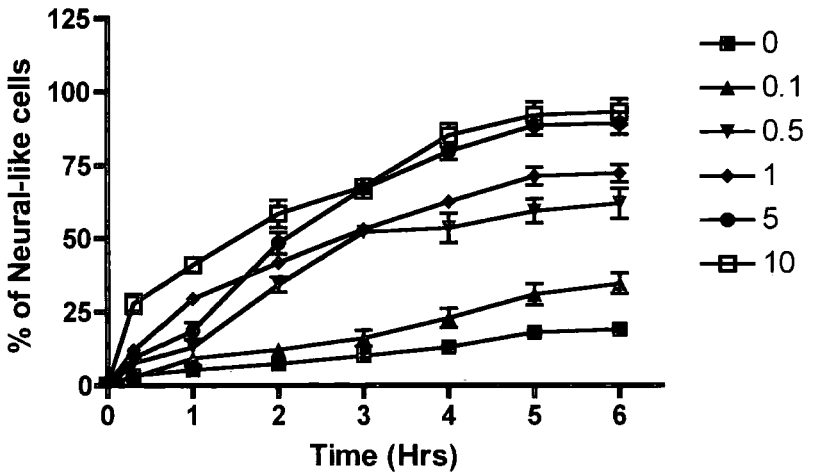


Figure 3.30: Effect of PMA at various concentrations on the acquisition of a neural-like morphology in MSCs. Cells were incubated in various concentrations of PMA in serum containing media for 6 hours. The number of neural-like cells was assayed every hour for 6 hours. All data are Mean±SEM from 3 independent experiments in parallel cultures.

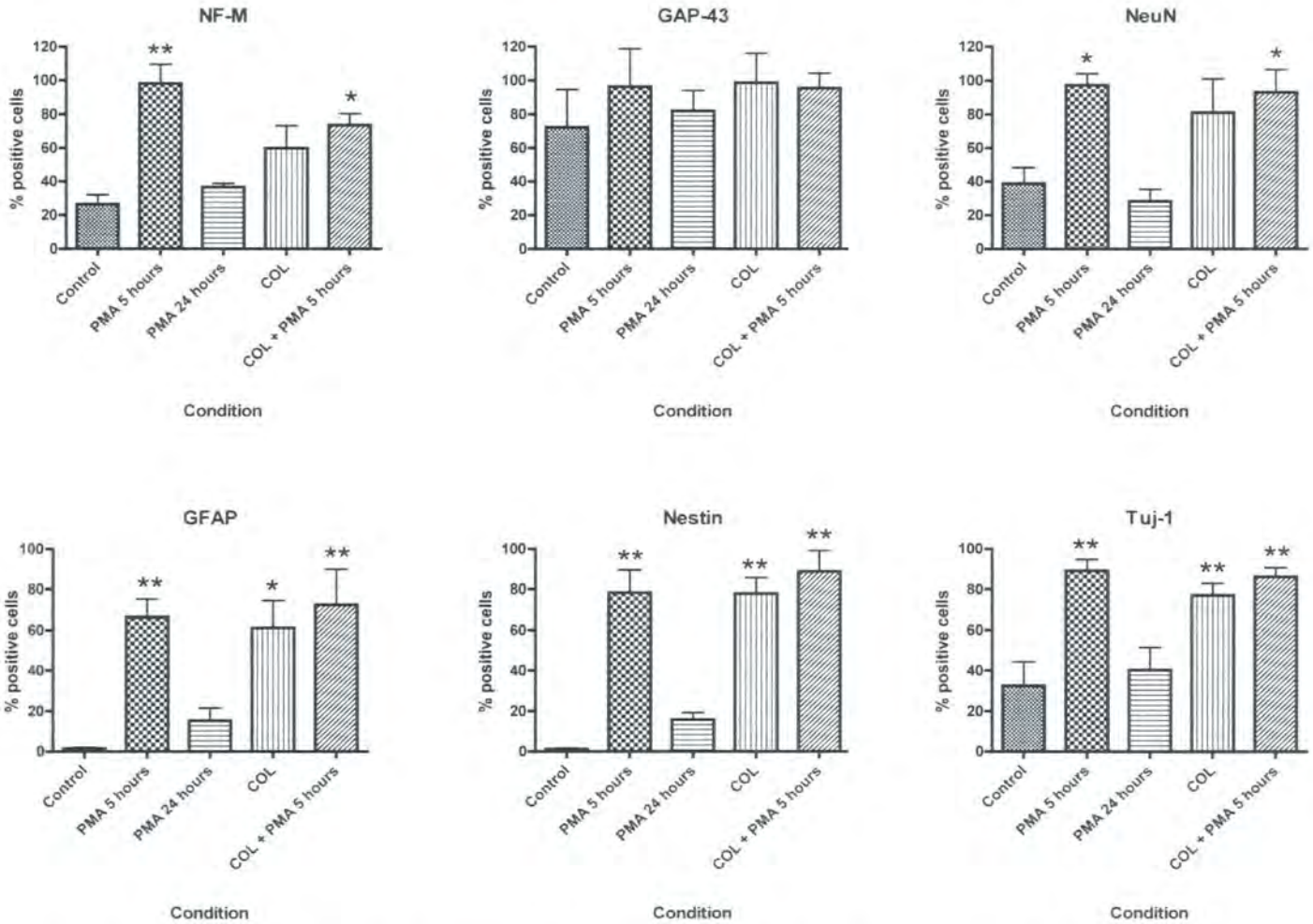


Figure 3.31: **Expression of neural proteins in cells cultured in the presence of 10μM PMA and the effect of PMA on COL pretreated cells.** Flow cytometry was used to quantify the number of positive cells for each marker under each culture condition. Cells were treated either with PMA (10μM) in serum containing media for 5 hours or 24 hours. To examine the role of microtubules in the response of cells to PMA, cells were pre-treated for 20 hours with colcemid (COL) prior to PMA treatment. All data are Mean values±SEM from 3 independent experiments in parallel cultures. Significant differences compared to control are indicated (*P<0.05, **P<0.01).

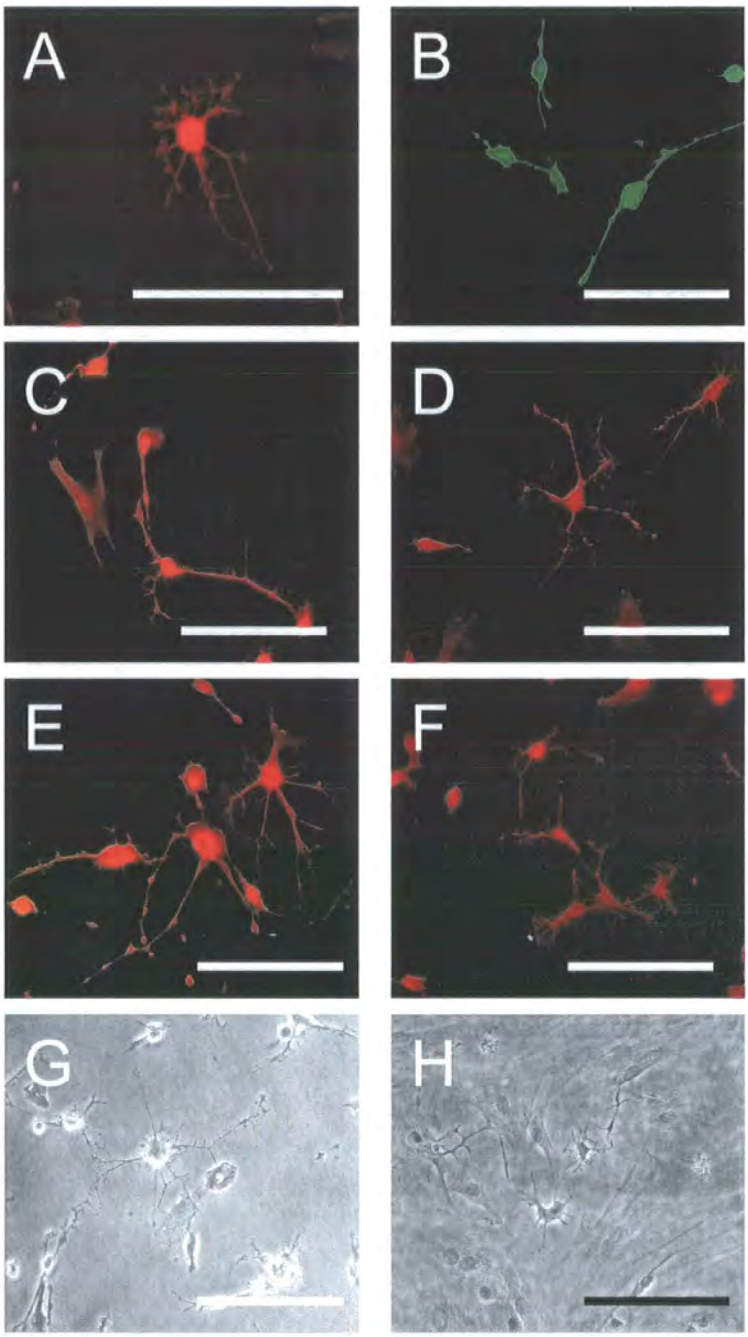


Figure 3.32: The effect on 10μM PMA in serum containing media on the morphology and neural protein expression profile of MSCs. (**A**) F-Actin cytoskeleton of 5 hour PMA treated MSC labeled with TRITC conjugated phalloidin. (**B**) α-Tubulin labeling of 5 hour PMA treated cells to visualize the microtubule system. Expression of Tuj-1 (**C**), GAP-43 (**D**), nestin (**E**) and NF-M (**F**) in 5 hour PMA treated cells. Phase contrast image of PMA treated cells (**G**) and phase contrast image of PMA treated cells pre-treated with colcemid for 20 hours (**H**). *Scale bars: 50μm.*

CHAPTER 4

Formation of Neurons by Non-Neural Adult Stem Cells: Potential Mechanism Implicates an Artifact of Growth in Culture

4.1 Introduction

Adult stem cells which have been isolated from numerous tissue sources, including the central nervous system (Gage, 2000; Reynolds and Weiss, 1992), BM (Pittenger et al., 1999; Weissman, 2000) and skin (Watt et al., 1998) are thought to have a more restricted developmental potential, generating only differentiated cell types of the same cell lineage as the organ in which they reside. Cell fate is determined by a variety of factors that regulate epigenetic changes during embryonic development and in normal adult physiology. Traditionally, cell commitment has been viewed as consisting of a series of irreversible steps which involve increasing commitment to particular cell lineage (Anderson et al., 2001).

However, this model of irreversible and restricted differentiation has been challenged by several recent experimental findings. Nuclear transfer experiments showed that the nuclei of adult cells could be reprogrammed to ES-like nuclei with corresponding pluripotency by the cytoplasmic factors of the oocyte (Munsie et al., 2000; Wakayama et al., 2001). This demonstrated that previously silent genes could be activated in the adult nuclei. Formation of stable heterokaryons following the fusion of terminally differentiated, disparate cell types, results in the expression of previously inactive genes through exposure to a novel cytoplasmic environment (Blau et al., 1985; Blau et al., 1983). These studies collectively demonstrate that the differentiated state of a cell requires continual regulation within the cell (Blau, 1992; Blau and Baltimore, 1991). This remarkable plasticity was however only achieved following considerable experimental manipulation.

Recent transplant studies suggested this plasticity may occur under certain physiological conditions in which at least a subpopulation of adult stem cells are capable of generating cells of a different embryonic germ layer, a process referred to as 'trans-differentiation'. This term demotes an alternation in the differentiation potential of a cell already programmed to a given cell lineage (Eguchi G, 1993; Weissman et al., 2000). For example, when donor bone marrow cells are transplanted into lethally irradiated recipients, genetic markers of the donor cell (Such green

florescent protein, GFP or Y chromosome) can be detected in various adult tissues outside of the haematopoietic cell lineages including skeletal muscle (Gussoni et al., 1999; Ferrari et al., 1998), liver (Petersen et al., 1999; Lagasse et al., 2000; Theise et al., 2000), heart (Orlic et al., 2001a; Orlic 2001b) and brain (Eglitis and Mezey, 1997; Brazelton et al., 2000; Mezey et al., 2000). HSCs have been found to contribute to the epithelia of various organs of ectodermal and endodermal origin following transplantation of a single HSC into a lethally irradiated host (Krause et al., 2001). In addition, reports of a high degree of plasticity have not been confined to BM derived cells. NSCs were shown to differentiate into virtually all cell types when injected into blastocysts (Clarke et al., 2000).

These findings have been met with considerable controversy with reports of conflicting results and low reproducibility. Some investigators have found little or no evidence to support the trans-differentiation of adult stem cells using similar or identical experimental paradigms to those described above (Massengale et al., 2005; Morshead et al., 2002; Wagers et al., 2002). Others argue that cell fusion and not trans-differentiation is the explanation for unexpected cell fate changes in vivo (Ying et al., 2002; Terada et al., 2002; Wang et al., 2003). The studies of Alvarez-Dolado et al., 2003 using transgenic markers to monitor the fate of single cells following transplantation or co-culture found no evidence of trans-differentiation but found stem cells from one germ layer could acquire the attributes of cells of other tissues by the formation hybrid cells as a result of spontaneous cell fusion.

Stem cells reside in specialized niches within specific adult tissues where they are subject to spatial and temporal regulation in respect to their developmental potential. Removal of certain stem cell populations from their normal microenvironment and their subsequent explants in culture has been proposed to increase their developmental plasticity. Multilineage adult progenitor cells (MAPCs) have been isolated from mammalian BM (Jiang et al., 2002a) and shown to differentiate at the single cell level into ectoderm and endoderm derived tissues, regenerate the blood system and repopulate embryonic tissues in vivo, on implantation into a blastocyst (Jiang et al., 2002a). In order to achieve these differentiation capabilities the cells are removed from their niche, explanted ex vivo under highly defined and selective culture conditions. These studies and others (D'Ippolito et al., 2004; Pochampally et al.,

2004) demonstrate that certain cell populations in situ may seldom exhibit their full differentiation repertoire and the in vivo microenvironment may have a profound role in this repression.

A multitude of studies have now been published which demonstrate an increase in adult stem cell plasticity following in vitro cultivation (Toma et al., 2001; Kabas et al., 2001; Hermann et al., 2004) and in cases where co-culture has been utilised to promote differentiation towards a particular cell lineage, some investigators have been able to demonstrate that differentiation has been independent of cell fusion (Sato et al., 2005, Newsome et al., 2004; Muasawa et al., 2005; Kajstura et al., 2005). These results imply either that certain adult stem cells have an intrinsic capacity for differentiation beyond their normal repertoire (with such plasticity suppressed in situ) or that adult such cells are reprogrammed in the their differentiation potential towards specific cell lineages by extracellular cues. Reprogramming of a previously specialised adult stem/progenitor cell in such a way as to enable a cell to differentiate towards a different cell fate would require the expression of a significantly different array of genes. The question is can this be achieved through the induction of epigenetic changes in vitro?

MSCs are one adult stem cell population proposed to demonstrate increased plasticity following cultivation in vitro (Woodbury et al., 2000; Lange et al., 2005; Choi et al., 2005). These cells are particularly good candidates for cell therapy because of their accessibility and capacity, for ex vivo expansion (Di Girolamo et al., 1999). Whilst retaining their capacity for mesoderm differentiation, MSCs have been shown to differentiate into endodermal and ectodermal derivatives under defined culture conditions independent of cell fusion (Lange et al., 2005; Hermann et al., 2004). One of the most striking examples of this has been the demonstration that MSCs can form neuroectodermal derivatives in vitro (Deng et al., 2001; Jin et al., 2003; Kohymama et al., 2001; Hermann et al., 2004; Wislet-Grenbien et al., 2003) or following transplantation into the CNS (Brazelton et al., 2000; Corti et al., 2002; Mezey et al., 2000; Nakano et al., 2001). Although such differentiation is reportedly limited, MSCs preconditioned by exposure to the mitogen basic fibroblast growth factor (bFGF) in culture have been shown to engraft and differentiate in a site-specific manor

following transplantation in the embryonic brain without evidence of fusion (Munoz-Elias et al., 2004).

To investigate whether non-neural tissue specific stem cells have an intrinsic capacity to generate neural derivatives by trans-differentiation we tested the neurogenic potential of MSCs isolated from the adult bone marrow. In contrast to recently reported findings we found that the formation of presumptive neural cells (defined by morphology and gene expression) were the result of a stress response to a change in the culture conditions, an event previously interpreted by other investigators as trans-differentiation.

4.2 Materials and Methods

4.2.1 Materials

Tissue culture reagents and other materials were acquired from Sigma (Sigma-Aldrich, Poole, UK) unless otherwise stated. All substances were of the appropriate chemical, molecular biological or tissue culture grade. Cytochalasin B (CB) and colcemid (COL) were dissolved in ethanol and used at a final concentration of 10 μg/ml and 1 μg/ml, respectively, unless otherwise stated. The broad spectrum protein kinase C inhibitors Staurosporine (Str) and Chelerythrine chloride (ChCl) were dissolved in dimethylsulfoxide (DMSO) and used at final concentrations of 10 μM and 50 μM, respectively. The mitogen activated kinase (MAPK) inhibitors were purchased from Calbiochem (UK), reconstituted in DMSO and used at the following concentrations: PD98059 (75 nM, selective MEK inhibitor), SB 202190 (10 nM, a potent inhibitor of p38), SB 203580 (10 nM, a highly selective inhibitor of p38) and SP 600125 (10 nM, a potent inhibitor of c-Jun N-terminal kinase, JNK) were reconstituted in DMSO. MAP Kinase and PKC inhibitors were added to the culture 30 minutes prior to any further treatment.

4.2.2 Cell Culture

Rat MSCs (rMSCs) were isolated from the femurs and tibiae of 6-8 month old Wistar rats. The bone marrow (BM) was aspirated with 20 ml collection media (RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml

streptomycin, and 12 μM L-glutamine) into a T75cm^2 flask to allow stromal cells to adhere to the culture surface. Adherent cells were then washed and maintained in complete culture medium (CCM: Dulbecco's Modified Eagles Medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 12 μM L-glutamine and 1x non-essential amino acids) at 37°C in 5% CO_2. Isolation of rMSCs was verified by their capacity to differentiate into mesodermal derivatives (bone, fat) and their cell surface expression of MSC markers CD44, CD29 and CD90 and the absence of heamatipoetic markers CD45 and CD11b (data not shown). Passage 8 (P8) (approximately 25 population doublings) cells were used in the experiments described herein. To induce a presumptive neural phenotype, rMSCs were grown to >80% confluence in CCM. Unless stated otherwise, rMSCs were subsequently incubated in serum free DMEM/F12 media supplemented with N-2 supplement on tissue culture plastic. N-2 supplement is a media growth supplement used for growing post mitotic neurons in serum free media conditions. It contains insulin, human transferrin, sodium selenite, putrescine and progeste-rone. For some experiments the media was also additionally supplemented with bFGF at a final concentration of 20 ng/ml. Rat dermal fibroblasts were cultured in alpha-MEM, containing 10% FCS and 2mM L-glutamine.

4.2.3 Intracellular staining and analysis by flow cytometry

A single cell suspension of cells was obtained by incubating cells with trypsin/EDTA at 37°C. Cells were then washed with PBS x2 and pelleted in a 12 x 75 mm culture tube. The pellet was then resuspended in cold 2% PFA-PBS solution. The suspension is incubated for 24 hours at 4°C, centrifuged for 5 minutes at 250g, and then the supernatant is removed. The pellet was then permebiised in 1 ml of RT triton X-100 (0.2-1% in PBS) and the mixture was incubated for 15 minutes at 37°C. Non-specific binding was blocked by incubation with 5% goat serum. Cells were incubated with primary antibody (TuJ-1 (Convance, 1:500); Nestin (Chemicon, 1:100); Vimentin (Chemicon, 1:200); Synaptophsin (Sigma, 1:100); GAP-43 (Sigma, 1:200); NeuN (Chemicon, 1:50); NeuroD1 (Abcam, 1:100); Fibronectin (Sigma, 1:200)) for 60 minutes 4°C in antibody buffer (PBS, 1% goat serum, 0.1% bovine serum albumin,

BSA), followed by incubation with fluorescein isothiocyanate (FITC) conjugated goat antibodies raised against mouse IgG (1:100).

4.2.4 Flow cytometry analysis

All samples were maintained on ice prior to analysis. Analysis was performed using a coulter EPICS XL flow cytometer. FITC and Cy-3 were identified by using a 530 and 585 band pass filter respectively, and quantification was completed using CellQuest Software (Becton Dickinson). Ten thousand events were acquired per sample with fluorescence measured on logarithmic scales. Forward and side light scatter gates were set to exclude dead debris and clumps of cells. To calculate the percentage of positive cells, linear gates were set at 0.1%, on samples stained only with secondary antibodies, and expression corresponding to a fluorescence signal exceeding this percentage was measured.

4.2.5 Cell morphology assays

Digital images of phase contrast microscopy were acquired using a Nikon inverted microscope and camera (Nikon CM200). Measurements of cell footprint area (surface area occupied by the cell defined by its outer periphery of the cell) were determined by examining 10 non-overlapping microscopic fields (>20 cells/field) for three independent experiments using Image J software (NIH). The number of arborized cells was also counted for each visual field and treatment condition. For time-lapse microscopy, phase images of the same field of view were recorded at 15-minute intervals using a Nikon inverted microscope equipped with a temperature-controlled stage.

4.2.6 Time-lapse microscopy

MSCs were grown in specialised 60-mm glass chamber slide provided by the manufactor. Images were acquired using a Nikon inverted differential interference contrast microscope equipped with a temperature-controlled stage at 400x. Images were acquired at 15 min intervals. Images were prepared for display using Adobe Photoshop software.

4.2.7 Immuno-fluorescence microscopy

To preserve the structure of the microtubule cytoskeleton, cells were washed in PBS, extracted with 1% Triton X-100 in microtubule stabilising buffer (PEM: 1 mM $MgCl_2$, 5 mM EGTA, 80 mM K-pipes, pH 6.8). After permeabilisation cells were fixed with 0.5% gluteraldehyde in PBS. Free aldehyde groups were blocked by sodium borohydride (10 minutes) and lysine (2% solution) for 1 hour. For Nestin staining, cells were washed with PBS and fixed in 4% PFA solution for 30 minutes followed by post fixation and permeabilisation in 0.5% Triton X-100 in PBS for 15 minutes. All samples were subsequently rinsed three times in blocking/wash buffer (2% PFA in PBS), incubated with monoclonal mouse antibody directed against either α-Tubulin DMIA (Sigma, 1:100) or Nestin (Chemicon, 1:200). FITC-conjugated goat antibodies against mouse IgG (Sigma, 1:100) were used as secondary antibodies. To visualise the F-Actin cytoskeleton, cells were stained with TRITC-labelled phalloidin. Labelled cells were cells visualised using an inverted fluorescent microscope (model E660 Nikon) and a CCD camera (Spot RT; diagnostic instruments) with individual filter sets for each channel. All images were captured using the same collection parameters for quantitative comparisons. The fraction of positive cells was determined for each culture condition by counting 10 non-overlapping microscopic fields (>20 cells/field) for each condition in at least three independent experiments. Colour images were generated using Adobe photoshop (Adobe systems, mountain view, CA)

4.2.8 Western Blot Analysis

Protein extracts (30 μg per lane) were separated by electrophoresis and transferred onto PDVF membrane (Amersham). For immunoblotting, membranes first incubated in blocking solution (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) containing 5% milk powder, 0.2% Tween 20) for 1 hour followed by primary (TuJ1 (Convance), 1:5000; Nestin (Chemicon), 1:1000; β-Actin (Sigma), 1:5000) and secondary (mouse or rabbit IgG-HRP (Amersham, 1:1000) antibody. Protein-antibody binding was detected on film (Hyperfilm ECL, Amersham) using chemiluminescence (Amersham). Densitometry following western blot analysis was carried out on blots obtained from three independent experiments and where analysed by ImageJ.

4.2.9 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism Software version 4. Results were analysed for statistical significance using ANOVA and all error bars are expressed as standard error +/- mean. All data unless stated otherwise is expressed as Mean±SEM. Post hoc analysis was done using Bonferroni corrected planed comparison.

4.3 Results

4.3.1 Effect of serum on the induction of Nestin expression by rMSCs

To identify conditions that may promote the differentiation of MSCs toward a neural lineage, we cultured rMSCs for 24 hours in bFGF (10 ng/ml) supplemented media in either the presence of 10% FCS in DMEMF12, or under serum free (SF) conditions using DMEMF12 + N2 supplement or DMEMF12 alone (Figure 4.1). We examined these cultures for the expression of Nestin, a class III intermediate filament protein commonly used as a marker of neural progenitors (Lendahl et al., 1990). In the present study we found that addition of 10 ng/ml bFGF for 24 hours was sufficient to induce Nestin expression in rMSC cultures. However, growth factor treatment in the presence of serum (10% FCS) resulted in only minimal induction of Nestin expression (9-10%) compared to induction following growth factor treatment in serum free cultures, DMEMF12 + N2 and DMEMF12 (28% and 34%, respectively) (Figure 4.1a). This finding suggests that serum removal is required for increased induction of Nestin expression and implied that serum may itself have a regulatory role in the acquisition of a neural phenotype.

Consistent with this observation, Nestin expression was virtually undetectable in cells continually maintained in serum supplemented media (9-10% Nestin positive cells, low expression) and removal of serum from the culture was sufficient to induce Nestin expression in a fraction of rMSCs (24% nestin bright) (Figure 4.1b). No significant difference was found between the level of Nestin expression in serum free cultures and cultures in which serum had been substituted by N2 supplement (DMEMF12 + N2, 31%) indicating that serum removal was a critical determinant in the level of Nestin expression within the culture.

The intensity of Nestin expression was heterogeneous during growth factor treatment but correlated with the acquisition of a neural-like morphology (Figure 4.1c). Cells maintained in media supplemented with 10% FCS had a fibroblastic morphology consistent with a stromal phenotype. These cells stained only very weakly for Nestin protein and within the confines of this study counted as Nestin negative. However, 31-35% of cells expressed high levels of Nestin following 24 hours serum free culture, and all these Nestin positive cells developed a neural-like morphology. The same pattern of Nestin expression was observed for growth factor treatment in the presence or absence of serum (Figure 4.1c). Western analysis confirmed that the induction of Nestin was significantly higher in serum free cultures and that such regulation in expression was also evident for the neuronal marker TuJ-1 (Figure 4.2).

4.3.2 Changes in protein expression profile by rMSCs cultured in serum free media

Neural development is a controlled process in which the temporal and spatial expression of neural genes is tightly regulated (Gage et al., 2000). We examined the expression of neural proteins to determine whether serum withdrawal induced a true transition towards the neural lineage with the concomitant down regulation of mesodermal markers commonly expressed by MSCs (Figure 4.3). Cells cultured in serum containing media were negative for Nestin and NeuroD1 but a fraction of rMSCs did express TuJ-1, NeuN and Synaptophysin at low levels. GAP-43 was expressed on 95% of cells but also at relatively low levels. Culturing rMSCs in serum free media for 24 hours resulted in an up-regulation in the expression of the neural proteins tested, namely Synaptophysin, NeuN, GAP-43, TuJ-1, Nestin and NeuroD1 (Figure 4.3).

4.3.3 Changes in the morphology of rMSCs in response to serum free culture

The morphology of rMSCs grown in serum free conditions was recorded at regular time intervals using phase imaging (Figure 4.4). Cells were measured for footprint area and whether they resembled neurons (Figure 4.6). Upon removal of serum 23-25% of rMSCs adopted a neural-like morphology within 5 hours (Figure 4.4, 4.6a). Such a response was significantly enhanced when rMSCs were exposed to 2% DMSO

in serum free culture, where 91% of cells displayed a neural-like morphology (Figure 4.4, 4.6a). The structural features that underlie the change in cell morphology from a flat fibroblastic morphology to a neural-like morphology were demonstrated by time-lapse imaging (Figure 4.5). The cytoplasm of responsive cells shrank toward the nucleus and this retraction left behind cytoplasmic extensions radiating from the nucleus to where the cell periphery had originally been located. Not all cells responded in the same fashion and there were varying degrees of cell shrinkage in response to serum withdrawal. Responsive rMSCs became increasingly spherical and highly refractile resembling typical neuronal perikarya.

4.3.4 Organisation of the rMSC cytoskeleton during the formation of neural-like cells

We examined the structure of the cytoskeleton in rMSCs during their transformation into neural-like cells (Figure 4.7). Rat MSCs were cultured in the presence of 2% DMSO in the absence of serum to induce a high percentage of cells to undergo cellular collapse. Staining for F-Actin and α-Tubulin over a 5 hour period showed that these cytoskeletal elements appeared to collapse toward the nucleus. Cells were then fixed at various time points from 0 to 5 h ours post treatment. Figure 4.7 shows representative images of cells at each time point. We observed that both cytoskeletal systems appeared to collapse during cytoplasmic retraction. This collapse of the F-actin and micrtubule systems towards the nucleus results in a rounded, cell morphology with numerous membraneacous extensions radiating from the centre of the cell. Staining shows these processes contained both actin and tubulin and we were therefore unable to determine which cytoskeletal system was the principal cause of this morphology. However, it was evident that this morphology was the result of cytoskeletal retraction and not the active process like extension, which is characteristic of authentic neuronal development. The membranueous extensions that remained following cytoskeletal collapse were merely an artefact of this retraction, a morphological characteristic previous interpreted as a neuronal morphology.

Together with the time lapse imaging data (Figure 4.5), we propose that rMSCs acquire neuronal morphologies through the collapse of the cytoskeleton and not through the active process of cytoplasmic extension and neurite outgrowth as

traditionally observed in a growing neuron. Although the cytoplasmic processes in responsive cells contained both Actin and Tubulin, we were unable to determine which cytoskeletal system was principally the cause of this morphological change. To determine the cytoskeletal compartment which is the principal cause of cellular collapse, we selectively disrupted F-Actin and the microtubule systems independently using cytochalasin B (CB) and colcemid (COL), respectively (Figure 4.8, 4.9). Rat MSCs treated with CB in serum supplemented media resulted in a characteristic arborized morphology in >90% of cells (Figure 4.8). Disruption of the Actin cytoskeleton in this way resulted in cells with morphology closely resembling the structure of cells treated with DMSO or those cells that underwent collapse following serum withdrawal. In contrast, the selective disruption of the microtubule system using COL (a microtubule depolymerising agent) did not result in an arborized morphology. Although no polymerised α-Tubulin was detected, staining for F-Actin appeared to remain unaffected (Figure 4.9). These data suggest that F-Actin is principally disrupted in response to serum withdrawal and DMSO treatment and that the structure of the microtubule system changes as a result of F-Actin retraction.

To verify this conclusion we treated rMSCs with CB for 5 hours to induce cells with neural-like morphologies and then subsequently removed serum from the culture for 5 hours (Figure 4.11). No additional morphological response was apparent as a result of serum removal. Similarly, the addition of COL for 5 hours post-CB treatment did not result in any further morphological changes despite an intact polymerised tubulin system still detectable following CB treatment. This supported the notion that F-Actin disruption was the principal cause of morphological changes following serum withdrawal. Moreover, F-Actin alone was sufficient to maintain the neurite-like processes, whereas the growth and extension of true neurite is dependent on a dynamic microtubule cytoskeleton (Roisen et al., 1975). Interestingly removal of serum or addition of CB following COL treatment also did not result in any cell shape changes despite an intact F-Actin system being present (Figure 4.11). This suggests that microtubules provide the retractile force for F-Actin collapse and α-tubulin is required for F-actin mediated collapse of the cytoskeleton.

4.3.5 Re-organisation of the cytoskeleton and the expression of neural genes

We next investigated whether the increased expression of neural proteins that occurs subsequent to induction of a neural-like phenotype is related to the collapse of the cytoskeleton. We chose to examine the expression of Nestin since this protein was not found in untreated rMSCs and its increased expression appeared to correlate with the formation of neural-like cells in response to serum withdrawal (Figure 4.12). Varying degrees of F-Actin disruption were induced by treatment with CB over a range of concentrations (Figure 4.12b). Merged images show that it was the arborised cells that expressed Nestin. A direct correlation existed between the extent of disruption and the number of Nestin expressing cells (Figure 4.12b). In addition, as amount of Actin disruption increased (as a consequence of increased CB concentration), the cellular footprint area reduced together with an increase in the number of arborised cells. Although microfilament disruption was slightly evident at 0.1μM CB treatment, no significant up regulation in Nestin expression was detected by immunocytochemistry. Accordingly, disruption of the Actin cytoskeleton by treatment with CB resulted in neural-like cells that express high levels of Nestin in a concentration dependent manner. Western analysis performed on samples of cells treated with different concentrations of CB, ruled out the possibility that the appearance of Nestin bright cells was an artefact of cellular collapse and not simply the result of an increased amount of antigen per surface area (Figure 4.13). We also recorded that the effect of CB on Actin disruption and the induction of expression for certain other neural proteins appeared to be related to cytoskeleton disruption and reached maximal levels after 5 hours treatment (Figure 4.14c).

Treatment of cultures with 10μM CB resulted in cellular arborisation in which 92% of cells possessed a fully arborized phenotype by 5 hours (Figure 4.14a), which correlated with a reduced cell footprint area over the same period (Figure 4.14b). Using flow cytometry we found that percentage of cells expressing NeuroD1 and Nestin increased in a similar fashion to the number of cells changing shape over time, whereas levels of Vimentin decreased over this period (Figure 4.14c). Intracellular staining and flow cytometry was used to quantify neural protein expression (Tuj-1, NeuroD1 and Nestin) in response to 5 hours treatment with CB or COL in serum containing media (Figure 4.15). Both CB (Figure 4.15b) and COL (Figure 4.15c)

result in an up-regulation of Tuj-1. Both treatments also induce NeuroD1 and nestin expression in cells with a concomitant reduction in vimentin expression. Therefore, the pattern of response is consistent between CB and COL treatment indicating that it is the disruption overall of the cytoskeleton which results in the neural protein expression and not the acquisition of a neural-like morphology per se which is not evident in COL treated cells.

4.3.6 Cell signalling molecules that mediate the cytoskeletal regulation of neural protein expression in rMSCs

Perturbation of cell shape by compounds that disrupt the cytoskeleton has been shown to alter the activity of several signalling molecules (Ren et al., 1999; Yujiri et al., 1999; Subbaramaiah et al., 2000). For example, elements of the Ras, Raf, MAPK cascade are understood to associate with a microfilament signalling particle which is thought to mediate MAPK activation by the cytoskeleton (Carraway et al., 1999; Li et al., 1999). To investigate the molecular mechanism(s) underlying the induction of neural-like phenotype and expression of neural proteins by rMSCs, we used selective inhibitors to antagonise particular signalling molecules during this process. The protein kinase C (PKC) signalling pathway has previously been implicated in the regulation of both cell morphology and neural gene expression. We found that the up-regulated expression of NeuroD1 and Nestin by rMSCs cultured in SF media or treated with CB in serum containing media for 24 hours was significantly inhibited by broad-spectrum PKC inhibitors (Table 4.1). However, this attenuation may not be a direct effect on the inhibition of signalling molecules involved in neural protein induction since the presence of the inhibitors also reduced the morphological response to SF and CB treatments (Figure 4.16), an effect that may account for the reduced expression of neural protein in these cultures.

In contrast, MAPK inhibitors did not affect the morphological response of rMSCs to Actin disruption (Figure 4.17). The induction of neural proteins in response to cytoskeleton disruption did not appear to involve MEK-ERK signalling but was dependent on both JNK and p38 signalling (Table 4.1, Figure 4.18, 14.19). Inhibition of either the JNK or p38 pathways significantly reduced the level of neural protein

expression, however, this reduction was partial in both cases. This may be because other signalling molecules operate that substitute for JNK and p38 signalling.

4.3.7 Reversal of morphological and protein expression responses to serum removal or Actin disruption

The differentiation of multipotent stem cells involves a progressive restriction in cell fate with increasing commitment to a specific cell lineage. We hypothesised that if rMSCs cultured under defined conditions could be instructed to adopt a neural fate then such reprogramming would involve the progressive loss of mesodermal specific genes accompanied by a gene expression profile consistent with a neural lineage. To test whether the expression of neural proteins represented a firm commitment to the neural lineage or simply a transient reversible expression, we examined the levels of neural and mesodermal proteins following the re-introduction of serum or removal of CB (Figure 4.22). Cells cultured in serum free media or in the presence CB up regulated their expression of Nestin, NeuroD1 and TuJ-1 with the concomitant down regulation of the mesoderm marker, Vimentin. This change in the protein expression profile in both CB treated cells and cells cultured in serum free media was completely reversible following the removal of CB or re-introduction of serum to serum free cultures. The protein expression profile of these previously treated cells was comparable with rMSCs maintained in serum containing media. This reversal in the expression of neural proteins was accompanied by a reversal of the morphology of the cells from an arborized phenotype to a flat fibroblastic morphology (data not shown). This reversible behaviour suggested that rather than a differentiation response, the expression of neural proteins may be a cellular stress response to cytoskeleton disruption. Aberrant expression of proteins in response to cellular stress is often transient (Kulka, 1989). We have also shown that the expression level of the neural marker, TuJ-1, was initially increased in response to serum free culture conditions but subsequently returned to lower levels over the next 5-6 days when maintained in the same type of growth media (Figure 4.20c,d) as determined by western blot analysis of protein expression following 6 days induction. This reduction in TuJ-1 expression was also associated with a progressive reduction in the number of cells displaying an arborised phenotype (Figure 4.20a,b). Therefore the morphological and protein

response of rMSCs to serum free culture is a transient effect with cells progressively recovering over time.

4.3.8 Role of protein synthesis in the expression of neural proteins and acquisition of neural-like morphology in response to inductive agents

To determine whether new protein synthesis is required to produce the morphological and protein responses observed in serum free and DMSO treated cells, MSCs were treated with the protein synthesis inhibitor CHX at concentrations sufficient to inhibit protein synthesis in murine BM stromal lines by >95% (Gimble et al., 1989, Gautam et al., 1995) Treatment of MSCs with CHX at a concentration of 10μg/ml for 24 hours arrested cell proliferation, indicating the effectiveness of CHX of inhibiting protein synthesis. MSCs pre-treated with CHX and then cultured in the presence of DMSO displayed neural-like morphologies. Therefore CHX treatment had no significant effect on the acquisition of a neural-like morphology indicating that protein synthesis was not required for cells to assume these neural-like morphologies (Figure 4.21). CHX treatment itself did result in a small proportion (<20% approx) assuming a neural-like morphology, however further treatment with DMSO resulted in much higher numbers of neural-like cells (>75% approx). CHX pre-treatment in addition, did not significantly inhibit expression of nestin or Tuj-1, which were detectable at high levels both in CHX+DMSO and DMSO, treated samples as detected by western blot analysis (Figure 4.21). Therefore protein synthesis is not required the expression of neural proteins in response to DMSO or serum deprivation. This is inconsistent with a differentiation response, but consistent with a stress response and an inhibition in the breakdown of aberrant proteins, the concentration of which subsequently rises in the cell.

4.3.9 Expression of neural proteins by dermal fibroblasts in response to Actin disruption

We examined the behaviour of rat dermal fibroblasts under identical growth conditions as those used with rMSCs, to determine whether the induction of neural protein expression in response to Actin disruption was specific to rMSCs. Rat dermal fibroblasts adopted an arborized morphology and underwent cytoskeletal collapse in

response to CB treatment in serum containing media (Figure 4.23) and serum free media. This morphology was highly comparable to that observed following such treatment in rMSCs. Fibroblasts also showed up-regulated expression of Nestin and TuJ-1 in response to CB, expression that increased from 5 hours to 24 hours (Figure 4.23b). These effects on rat fibroblasts were completely reversible (data not shown).

4.3.10 Rat MSCs express mesodermal, endodermal and ectodermal cell lineage markers

If the induction of neural protein expression is a consequence of a stress response, it is likely that such a response would also result in the aberrant expression of other proteins not necessarily associated with neural development. Therefore, we examined the expression of several markers indicative of particular primary germ layers in untreated rMSCs and cells treated with CB (Table 4.2). We also tested the expression of various neural proteins representative of different stages of neural development. Rat MSCs expressed a range of neural proteins, however, these were not expressed in a pattern consistent with the sequential stages of authentic neural development. For example, Nestin, a reputed marker of neuroprogenitor cells, continues to be expressed in rMSCs at such at time when these cells also express neural proteins found in mature neurons and other lineage specific markers. In general the protein expression profile exhibited by rMSCs exposed to CB does not appear to conform to any particular pathway of development. This expression pattern suggests that the formation of the neural-like phenotype by rMSCs is unlikely to be a specific response to differentiate along the neural lineage but rather a typical stress response by the cell.

4.4 Discussion

Stem cells reside in specialized niches in adult tissues where they are subject to spatial and temporal regulation in respect to their developmental potential. Removal of certain stem cell populations from their normal microenvironment and their subsequent culture may be a potential explanation for their increased developmental plasticity. Multilineage adult progenitor cells have been isolated from mammalian bone marrow and shown to differentiate into tissues representative of all three germ

layers (Jiang et al., 2002a). In order to achieve these differentiation capabilities the cells are removed from their niche and maintained ex vivo under highly defined and selective culture conditions. Recent studies have reported that MSCs isolated from adult BM have the capacity to differentiate into neuro-ectodermal derivatives independent of cell fusion following cultivation ex vivo (Wislet-Gendebien et al., 2003, 2005; Hermann et al., 2004). This implied that although such adult stem cells are predisposed to differentiate into cells of a particular lineage they may be much more 'plastic' than previously appreciated and that the developmental potential of these cells may be dictated by niche specific signals.

These findings have profound implications for both cell replacement therapy and stem cell biology, and as a result must be extensively validated. Cell culture has the advantage of removing cells from their normal micro-environment and providing signals to direct differentiation towards specific cell fates. This approach has obvious benefits for investigating the mechanisms of cell plasticity and differentiation but tissue culture artefacts are major concerns.

Despite the importance of these observations little has been reported on the mechanisms that underlie the unexpected potency of MSCs in vitro. MSCs may have an intrinsic capacity to differentiate into neural cell fates, a property which could be suppressed in the BM niche but not in culture, or following localisation of MSCs in other tissues in vivo. Untreated, cultured MSCs have been found to express genes representative of all three germinal layers (Woodbury et al., 2002). The expression of neural genes by undifferentiated MSCs has been confirmed and extended by several other investigators (Tondreau et al., 2004; Bossolasco et al., 2005). Expression of neural proteins has been found to change as a function of passage in culture (Tondreau et al., 2004) and in response to exposure of MSCs to certain growth factors (Sanchez-Ramos et al., 2001; Jin et al., 2003, Hermann et al., 2004) and chemical agents (Woodbury et al., 2000; Deng et al., 2001). Such changes could also result from the emergence of transformed cell lines with aberrant properties. These cells could then be misinterpreted as trans-differentiation events following prolonged culture (Morshead et al., 2002).

In the current study we attempted to elucidate the mechanism by which cultured MSCs acquire a presumptive neuronal phenotype. Culturing rMSCs in the absence of serum was a critical determinant of the number of nestin positive cells that formed. Serum had an inhibitory effect on the expression of neural proteins by MSCs and maximal expression of neural proteins in response to growth factors was only achieved in serum free media. Moreover, cells with neural-like morphology were only detected in serum free media and these cells represented the Nestin positive population. Previous studies have explored what may be involved in the regulation of neural protein expression by cultured MSCs (Suon et al., 2004; Jori et al., 2005). However, these investigations are compounded by the diversity of procedures and inductive agents used to induce phenotypic changes in these cells. It is therefore of significance that we found that serum played a crucial regulatory role regardless of other inductive factors (i.e. growth factors) being present.

Cultured neurons have a unique architecture characterised by neurite outgrowth and axonal projections (Svendsen et al., 2001). However, we have shown that the formation of neural-like cells by rMSCs was not the result of typical neuronal development but the result of cellular shrinkage. Targeted disruption of individual cytoskeletal compartments revealed that this morphological response was principally the result of F-Actin disruption although an intact microtubule system is required to provide the retractile force for Actin collapse. Furthermore, CB-induced depolymerisation of Actin was completely reversible and this dynamic response was consistent with the reversibility of the morphological response to serum. A correlation between the extent of cytoskeletal disruption and the level of neural protein expression was first evident when cells cultured in serum free media were examined for their expression of Nestin. Only nestin bright cells possessed a fully arborized phenotype (most neural-like cells) whilst Nestin negative or Nestin dim cells showed an apparently unaltered fibroblastic morphology. Although these data agree with early reports relating to the acquisition of a neural-like morphology (Sanchez-Ramos et al., 2000), they do not support appear to represent authentic neural development. Furthermore, we found that induced rMSCs expressed only a limited repertoire of neural genes and all of these except Nestin and NeuroD1 were

constitutively expressed in untreated cells, which is consistent with earlier reports (Woodbury et al., 2002; Tondreau et al., 2004; Bossolasco et al., 2005). In addition, the concentration of neural proteins in rMSCs, which formed neural-like cells, was also significantly lower than the equivalent expression in primary neuronal cells cultured under identical conditions (data not shown).

Alteration to the architecture of a cell has previously been linked to changes in gene transcription. For example, genes involved in tissue remodelling are closely associated with dynamic changes in cell morphology induced by stress and shape-changing physiologic processes (Higgins et al., 1992; Seebacher et al., 1992; Eckstein and Bade, 1996; Ryan et al., 1996, Feng et al., 1999; Coats et al., 2000; Providence et al., 2000; Kutz et al., 2001). These findings implicate a direct involvement of the cytoskeleton in the cell signalling apparatus. Consistent with this hypothesis, targeted re-organisation of cell morphology with microfilament disrupting agents can activate the transcription of shape responsive genes (Higgins et al., 1992; Lee et al., 1993). Drug-induced alterations in both the microfilament and microtubule networks, mobilise intracellular signalling elements activating the ERK, JNK and p38 MAPKs that have been shown to result in changes in gene transcription (Irigoyen et al., 1997; Rijken et al., 1998; Schmid-Alliana, 1998; Sotiropoulos et al., 1999; Ren et al., 1999; Irigoyen and Nagamine, 1999; Yujiri et al., 1999). In the current study we have provided evidence that PKC signalling is potentially involved in mediating the changes in morphology and protein expression by rMSCs during the formation of neural-like cells in response to serum withdrawal and disruption of the Actin cytoskeleton.

Neuroblastoma cell lines undergo neuronal differentiation in response to serum withdrawal (Evangelopoulos et al., 2005). Expression of neural genes in these cells requires the nuclear accumulation of ERK, a process that is PKC dependent (Olsson et al., 2000). Continued differentiation and expression of neural genes is, however, associated with a down regulation of PKC (Carlson et al., 1993). In contrast, inhibition of PKC signalling in rMSCs attenuated the induction of Nestin and NeuroD1 proteins following serum free culture or F-Actin disruption. However, this

antagonism was associated with a significant inhibition of the morphological response of MSCs, implicating a central role for PKC in orchestrating F-Actin collapse in response to selective culture conditions. Signalling through JNK and p38 were required for induction of Nestin and NeuroD1 expression but not MEK-ERK signalling. The regulation of the signalling pathways studied in relation to neural protein expression by rMSCs is not consistent with neural differentiation by neuronal cell lines but is consistent with a pattern of cell stress induced activation of protein expression.

The finding in the current study, that primary rat dermal fibroblasts express TuJ-1 and Nestin at very low levels and up regulate these proteins in response to CB induced Actin disruption, suggested that neural cell marker proteins may not be exclusively expressed by cells of the neural lineage. Nestin and NSE are two neural marker proteins, which have now been detected in a number of non-neural tissues (Lendahl et al., 1990; Sjoberg et al., 1994; Reeve et al., 1986). Since fibroblasts have no known stem cell-like properties, the up regulation of neural proteins in response to Actin disruption may be further evidence that such changes are stress related and not a differentiation response.

Evidence for the trans-differentiation of cultured MSCs into neurons has replied primarily on changes in cell shape and protein expression. We propose based on the evidence presented herein, that these criteria are not reliable indicators of true neural development. Definitive proof of neural differentiation will require the use of in vitro assays to test basic neurogenic properties such as synapse formation, neuronal polarity and electrophysiological properties. A small number of studies have analysed the electrophysiological properties of presumptive neurons derived from BMDSCs. Some investigators reported a lack of Na^+ and K^+ channels and functional neurotransmitter receptors (Hofstetter et al., 2002; Padovan et al., 2003). Others have shown that electrophysiological properties of such cells were atypical when compared to primary neurons (Kohyama et al., 2001; Hung et al., 2002). No study has yet found any evidence of neuronal polarity or synapse formation, both of which are essential characteristics of functional neurons. Some investigators claim that the lack of

maturity of MSC derived neurons is the result of restrictive culture conditions (Koshizuka et al., 2004; Walczak et al., 2004). However functional neurons have been generated from embryonic (Miles et al., 2004) and neural stem cells in vitro (Song et al., 2002) under similar growth conditions.

Consistent with results in the present study, others have demonstrated that changes in cell morphology do not provide a reliable indicator of neural differentiation since such changes can result from Actin collapse and not neural development (Neuhuber et al., 2004; Lu et al., 2004). In addition, micro-array analysis of MSCs induced to differentiate using the protocol devised by Woodbury et al. (2000) was shown not to represent a definitive program of neural differentiation since several genes are induced that are not associated with a neural fate (Bertani et al. 2005). Here we describe a potential mechanism by which Actin collapse in MSCs results in the expression of neural proteins. This expression pattern does not compare with neural differentiation but is likely to be a response to cell stress induced by the culture conditions. Importantly, we have found that serum withdrawal alone is sufficient to induce alternations to cell shape and protein expression and we propose that these changes have been misinterpreted as trans-differentiation on several recent occasions in which serum free media has been used for induction of MSC differentiation (Sanchez-Ramos et al., 2000; Hermann et al., 2004; Wislet-Gendebien et al., 2005; Magaki et al., 2005).

We conclude that morphological and gene expression data are not sufficient indicators of trans-differentiation and as a result the findings of this study have profound implications for the interpretation of trans-differentiation events in vitro. The findings of the present study challenge the conclusions of several other authors and highlight the need for greater caution with the interpretation of findings, and the need for more appropriate experimental controls.

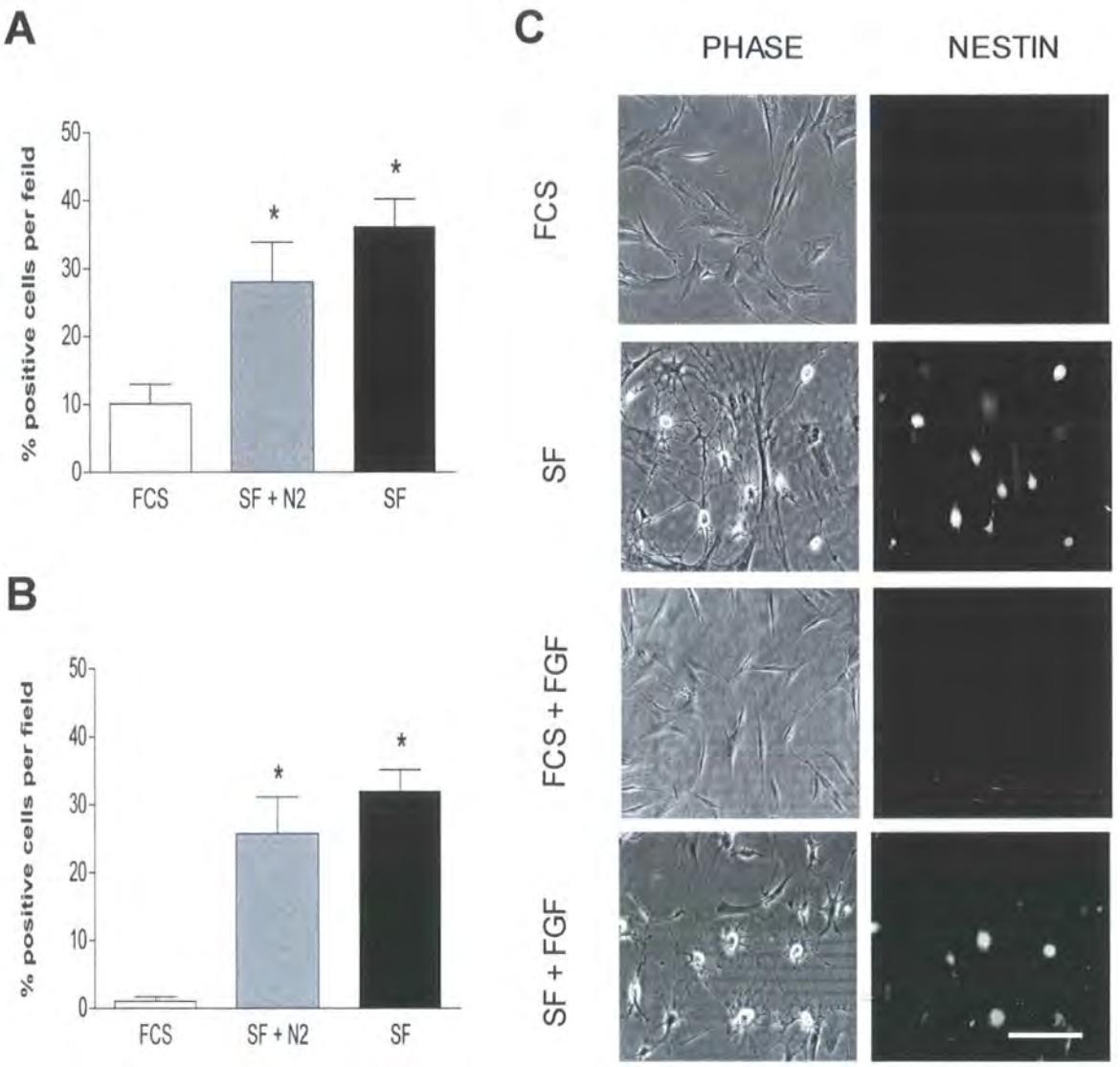


Figure 4.1: Expression of neural proteins by rMSCs is regulated by the presence of FCS. **(A)** Effect of FCS on FGF-induced Nestin expression. Cells were grown with the inclusion of FGF (10ng/ml) for 24 hours in the presence (white bars) or absence (grey bars: SF (serum free) + N2; or black bars: SF media) of 10% FCS and the proportion of Nestin positive cells recorded (*$P<0.05$ compared to FCS treated cells, n=3, mean±SEM). **(B)** Effect of serum (FCS) withdrawal alone on Nestin expression. Cells were grown in the presence (white bars) or absence (grey bars: SF + N2; or black bars: SF) of 10% FCS and the proportion of Nestin positive cells recorded (*$P<0.05$ compared to FCS treated cells, n=3, mean±SEM). **(C)** Immuno-staining and corresponding phase images showing cells maintained in FCS-supplemented media possessed a flat fibroblastic morphology, which was apparently unchanged following the addition of FGF. However, withdrawal of serum induced the appearance of Nestin bright cells with a neural-like morphology (*Scale bar: 10μm*).

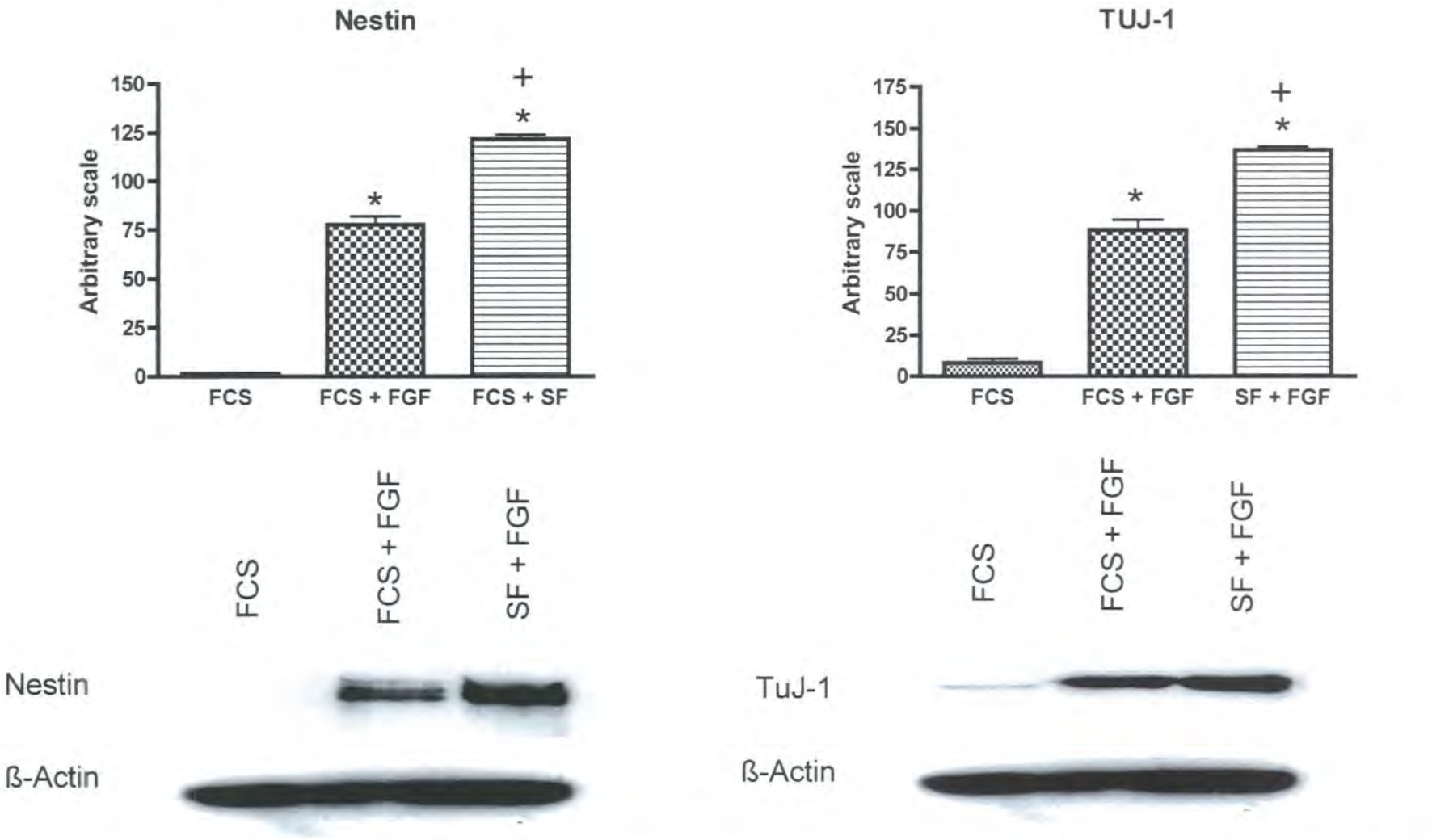


Figure 4.2 Quantification of Nestin and Tuj-1 expression in response to serum withdrawal and FGF treatment. Western analysis showing significant increased levels of the neural antigens TuJ1 and Nestin in response to FGF in the presence of serum (FCS+FGF), but particularly in response to serum withdrawal (SF+FGF). All data are mean values ± SEM from 3 independent experiments in parallel cultures. Significant differences from the control group (FCS) are indicated with an asterisk (*P<0.05 +P<0.01, 1-way ANOVA+post hoc analysis)

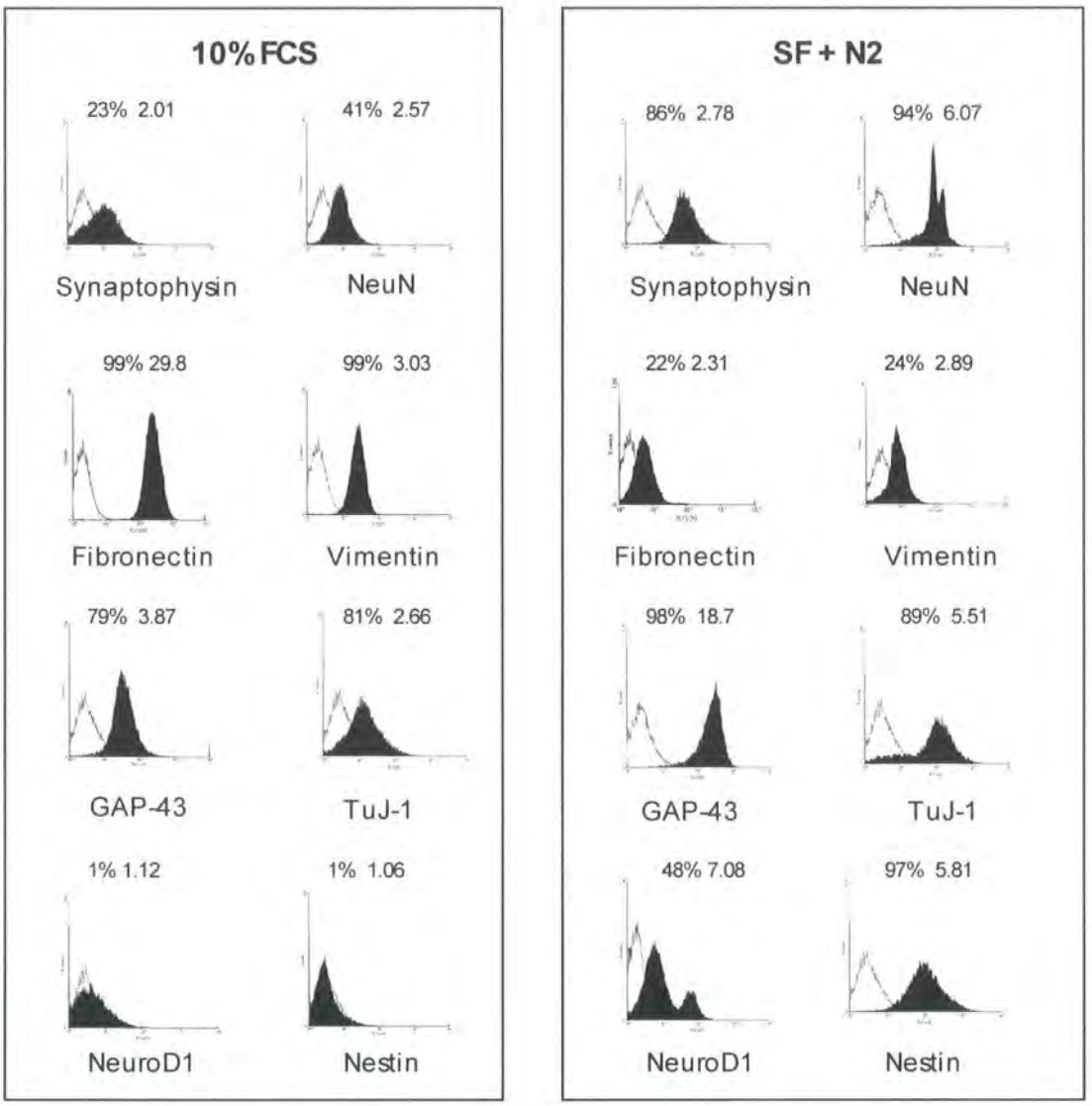


Figure 4.3: Expression of neural and mesodermal marker proteins following serum withdrawal as determined by flow cytometry. Flow cytometry measurement of neural (Nestin, GAP43, TuJ1, NeuN, Synaptophysin and NeuroD1) and mesodermal-associated protein markers (Vimentin and Fibronectin) in rMSCs following 24-hour serum-free culture compared to cells maintained in the continued presence of 10% FCS. Flow cytometry data from representative experiments are shown as histograms with fluorescence intensity on the x-axis (solid peaks) and cell count on the y-axis. Background fluorescence was excluded by the use of isotype matched control antibodies (open peaks). The percentage of positively labelled cells (left) is given with the geometric mean fluorescence intensity (MFI) (right) for each antigen.

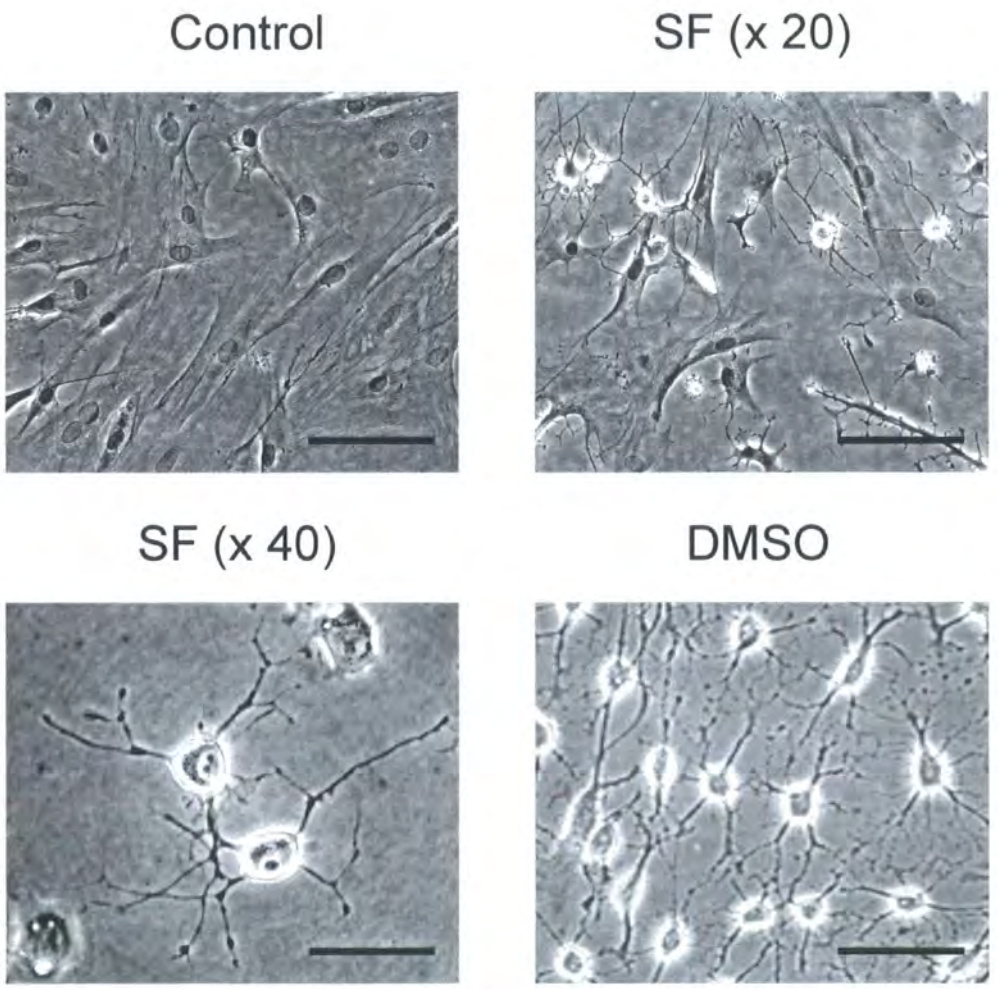


Figure 4.4: Morphological response of rMSCs following withdrawal of serum. Phase images showing the morphology of rMSCs under various growth conditions. Cells maintained in 10% FCS supplemented media have a characteristic stromal morphology (control, scale bar: 80μm). Removal of FCS from the culture for 5 hours results in 25% (approx) of cells adopting a neural-like morphology (Scale bars: x 20 50μm; x 40 25μm). Other cells in culture displayed extensive membrane ruffling or cell rounding. Addition of 1% DMSO to SF cultures resulted in the majority of cells forming neural-like morphologies (*Scale bar: 40μm*).

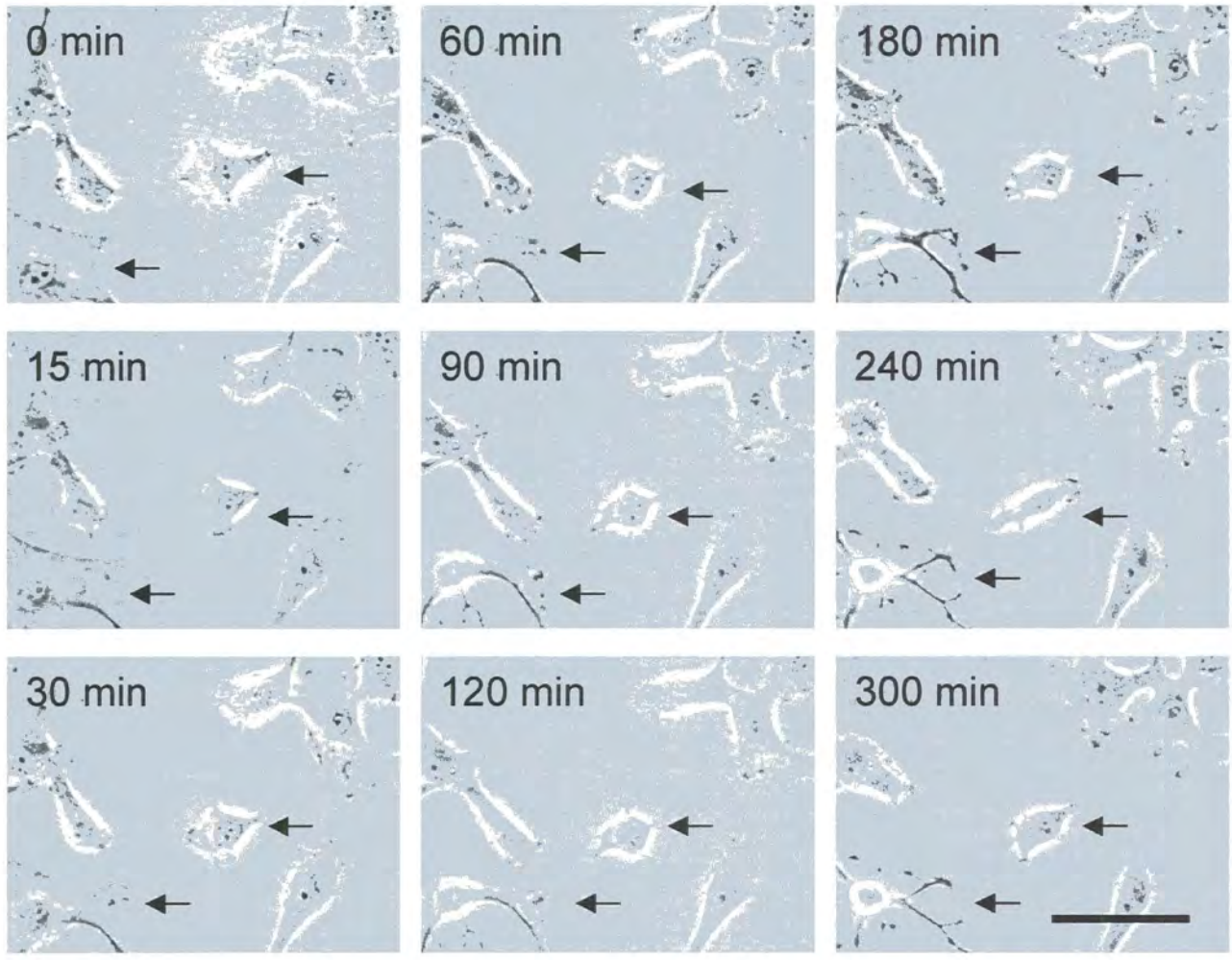
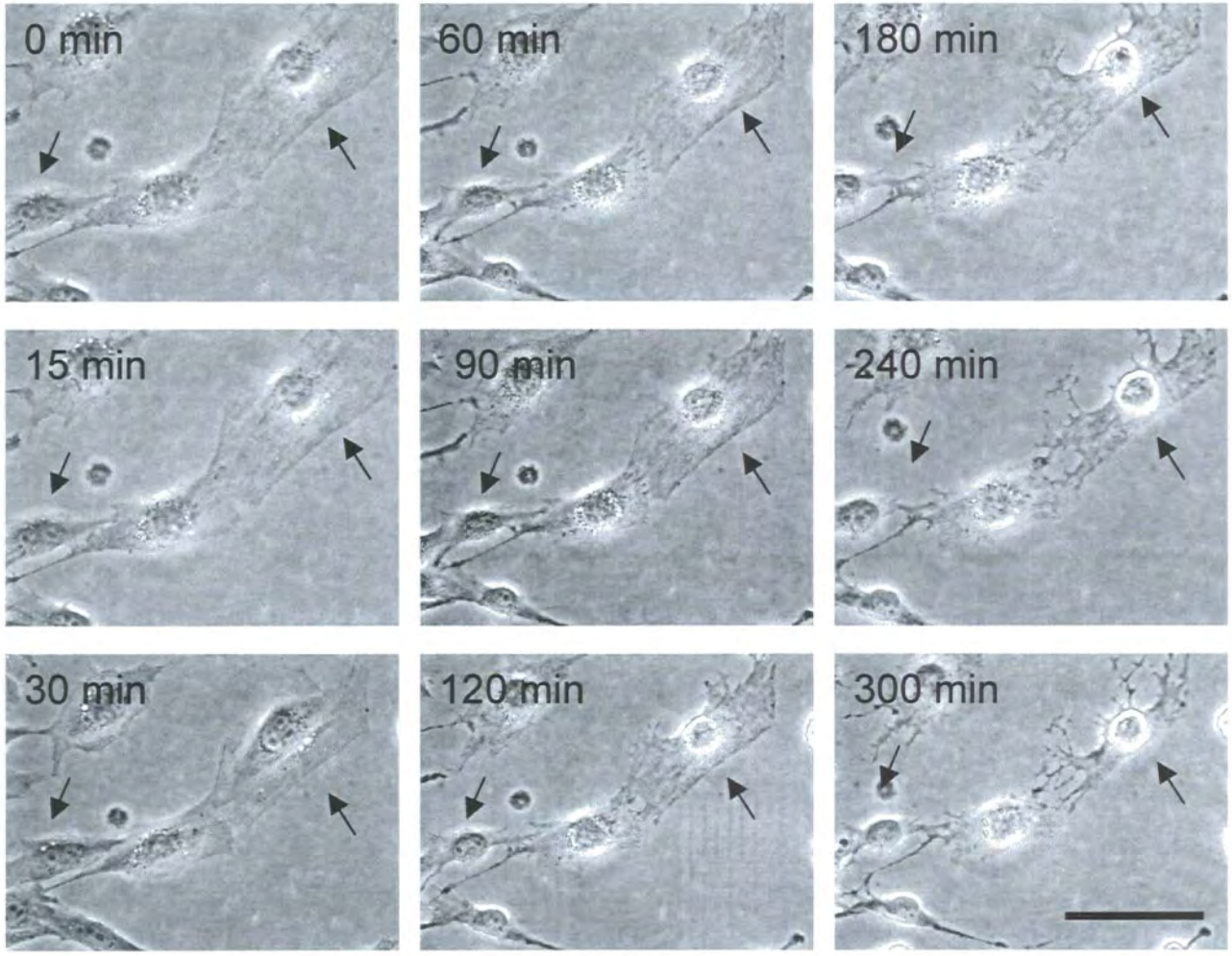


Figure 4.5: Time lapse imaging of rMSCs during the first 5 hours of serum free culture. Images from two representative experiments (Example 1 and Example 2) are shown at 9 time points 0, 15, 30, 60, 90, 120, 180, 240 and 300 min; *scale bar: 50μm*).

Example 2



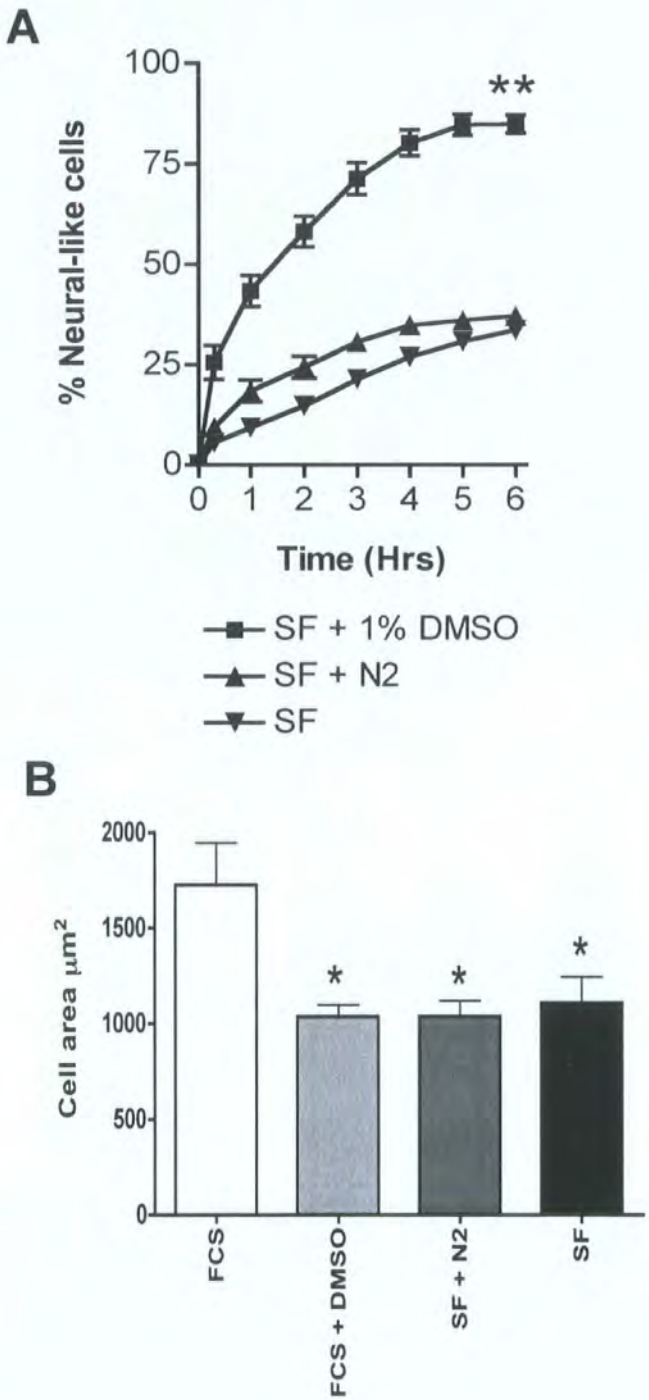


Figure 4.6: Quantification of neural morphology and cellular footprint area. **(A)** The percentage of cells displaying a neural-like morphology progressively increased following the removal of serum until reaching a maximal response between 3-5 hours. This response is unaffected by the presence of N-2 supplement and was poteniated by the addition of 1% DMSO (**P<0.01 when compared to cells maintained in SF media, n=3, mean±SEM). **(B)** The footprint area of individual cells was significantly reduced 5 hours after serum withdrawal (white bars) and to an equivalent extent following addition of 1% DMSO (5 hours) in SF media (light grey bar) and SF media plus N2 (dark grey bar) compared to control (10% FCS, black bar). *P<0.05 when compared to control cells (n=3, mean±SEM).

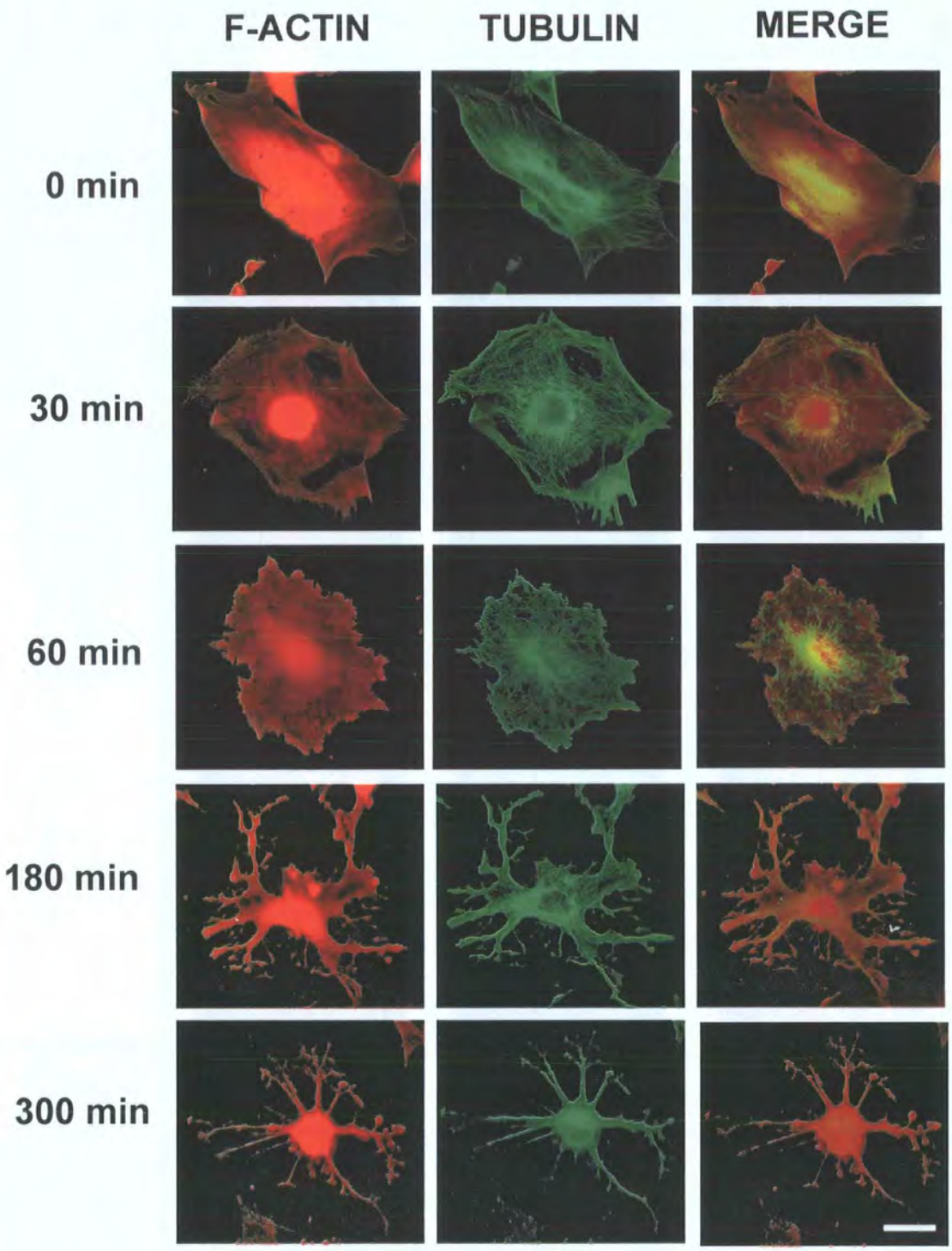


Figure 4.7: Changes in the organization of the cytoskeleton that leads to the formation of the presumptive neural-like structure. Cells were cultured under standard serum conditions until >70% confluence at which time cells were then switched to serum free media in the presence of 1% DMSO to increase the number of responsive cells. The cells were then fixed at several time points ranging from 0 to 300 minutes in 0.5% gluteraldehyde. The F-Actin cytoskeleton was visualized using the actin binding protein phallodin directly conjugated to a TRITC fluochrome. The microtubule system was visualized using a monoclonal alpha-tubulin antibody directed against tubulin in which binding was detected by an FITC conjugated secondary antibody. Each image is of a representative single cell within the culture at the time point specified. *Scale bar: 10 μm.*

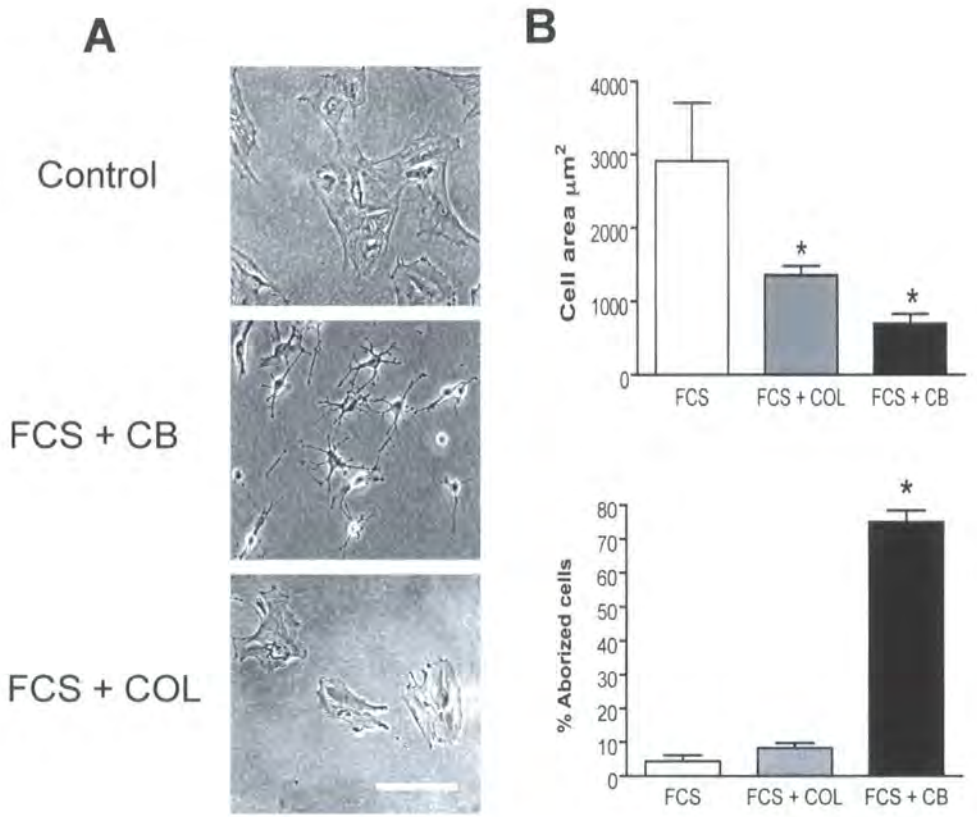


Figure 4.8: Effect of selective cytoskeleton disrupting agents on the morphology and cytoskeleton of rMSCs maintained in media containing 10% FCS. (**A**) Phase images showing the morphology of cells following exposure to 10µM cytophasin-B (CB) or 10µg/ml colcemid (COL) for 5 hours. CB treatment resulted in a characteristic arborized phenotype in 75-81% of cells whilst exposure to COL resulted in only partial cell shrinkage and extensive membrane ruffling. (**B**) The cellular footprint area (μm^2) and number of fully arborized cells (% total cells / field) was determined (n=10 fields of view, +20 cells per field; mean $\pm$ SEM). Treatment with either CB or COL resulted in a significant reduction in the cellular footprint area compared to control cells (10% FCS alone) but only CB treatment resulted in an aborized phenotype ($^*P<0.05$ compared to control cells (FCS), n=3, mean±SEM).

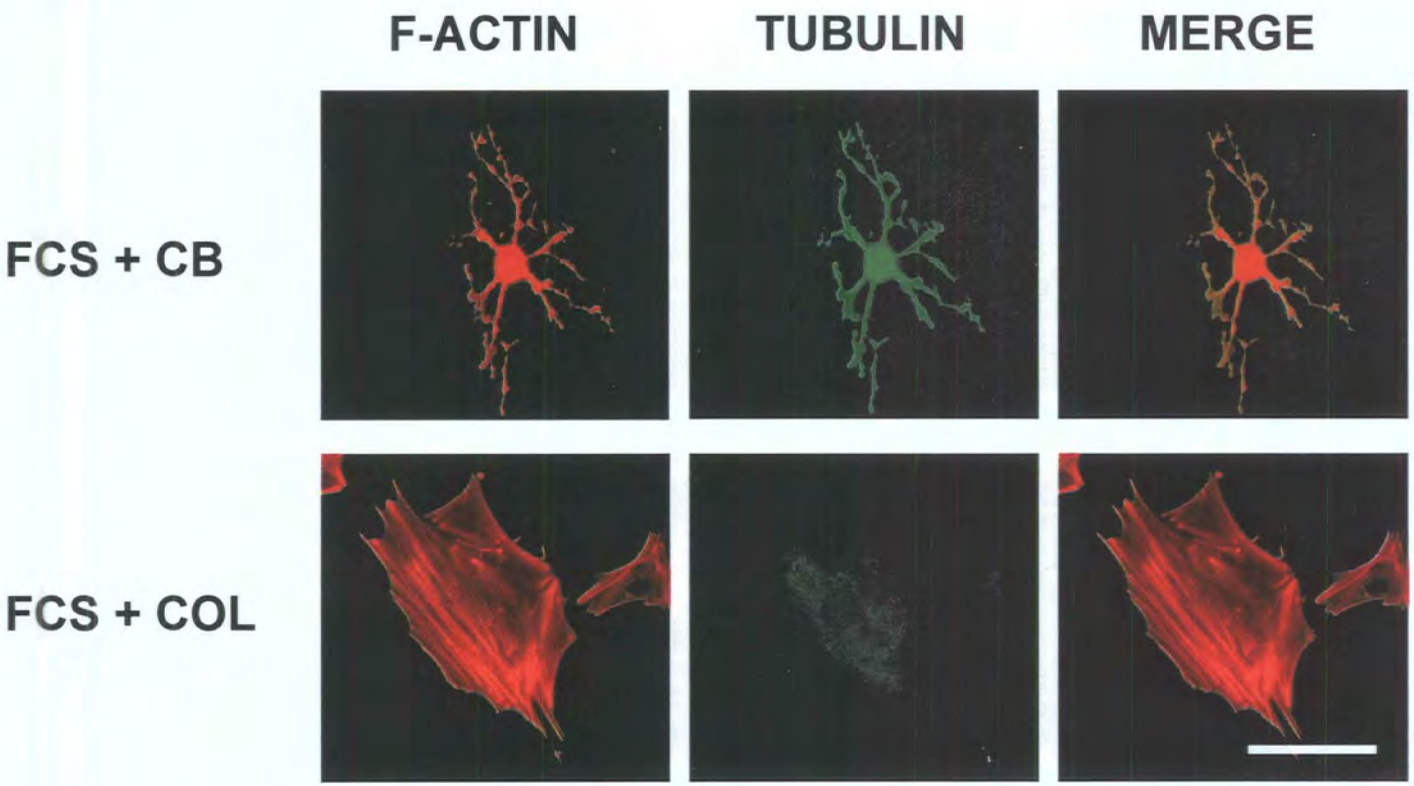


Figure 4.9: Effect of CB and COL treatment on the organization of the F-Actin and microtubule cytoskeleton. Disruption of F-Actin gave rise to a highly arborized phenotype whereas de-polymerisation of the microtubule system with COL for 20 hours did not appear to affect the F-Actin cytoskeleton and these cells maintained their fibroblastic cell shape. Scale bar: 10μm.

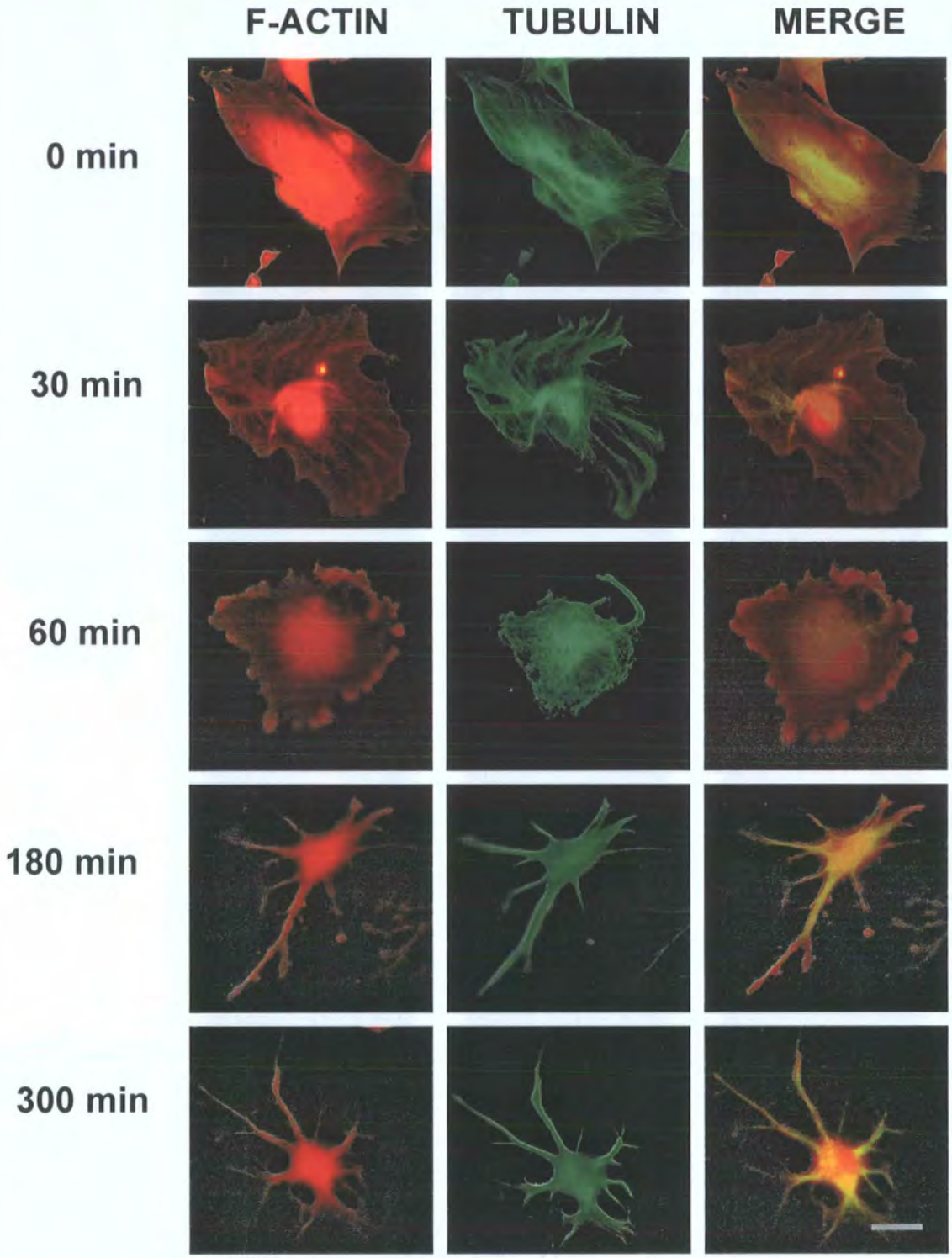


Figure 4.10: Changes in the organization of the cytoskeleton in response to CB treatment. Cells were cultured under standard serum conditions until >70% confluence at which time cells were exposed to 10μM CB for 5 hours in the presence of serum. The cells were then fixed at several time points ranging from 0 to 300 minutes in 0.5% gluteraldehyde. The F-Actin cytoskeleton was visualized using the actin binding protein phallodin directly conjugated to a TRITC fluochrome. The microtubule system was visualized using a monoclonal alpha-tubulin antibody directed against tubulin in which binding was detected by an FITC conjugated secondary antibody. Each image is of a representative single cell within the culture at the time point specified. *Scale bar: 10 μm.*

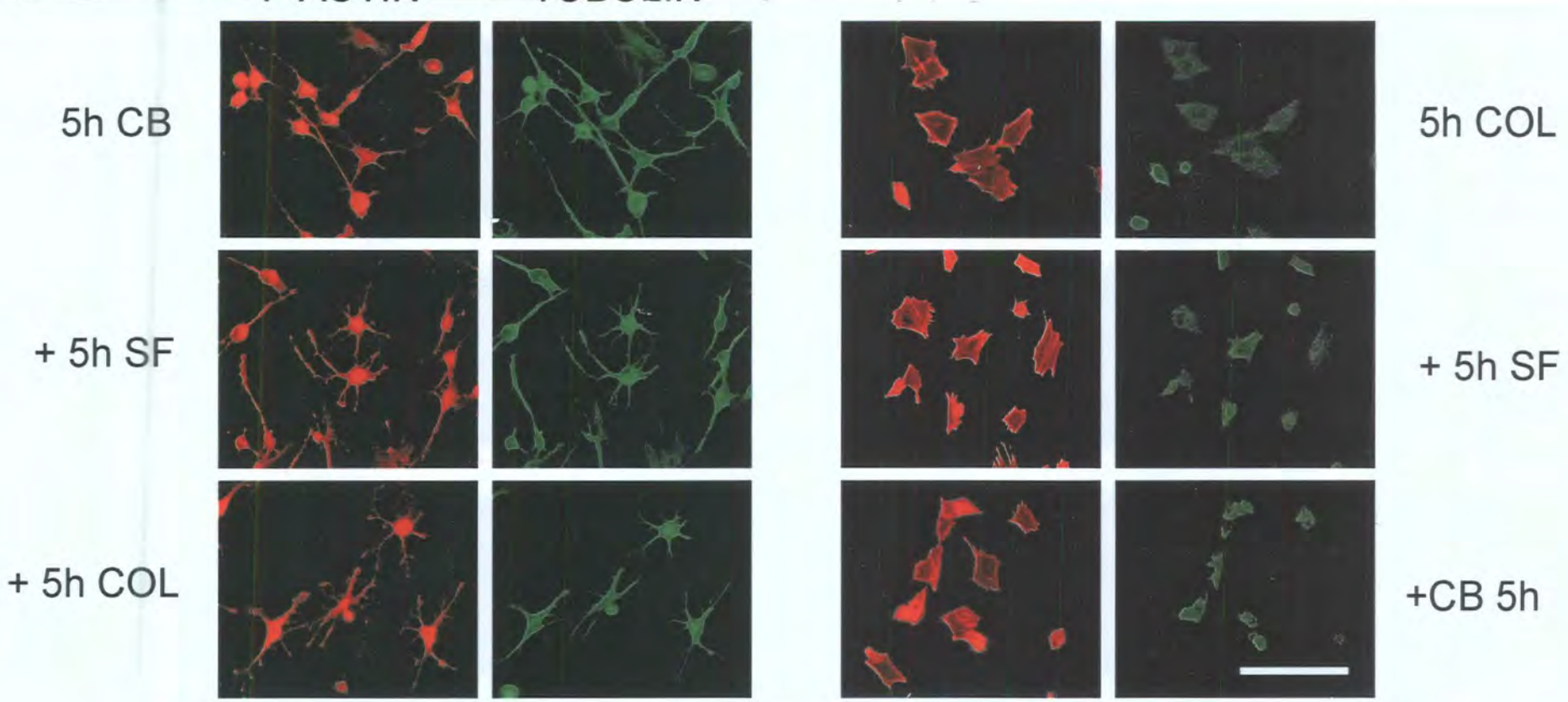


Figure 4.11: Role of microtubules in F-Actin induced cytoskeleton collapse. F-Actin and the microtubule network were visualized as previously described. Cellular arborisation was induced in virtually all cells (>90%) by the addition of CB (10μM) to FCS supplemented media, in order to mimic the serum withdrawal induced cytoskeleton collapse. In addition, the role of microtubules in F-Actin collapse was examined by pre-treating cells with COL (1 μM) for 20h before serum withdrawal or prior to CB treatment. Removal of serum (SF) or addition of COL for a further 5 hours to CB treated cells did not induce any further apparent morphological changes. Indicating that F-Actin collapse alone was responsible for the morphological response to CB induced actin disruption or serum withdrawal. Neither removal of serum or addition of CB for 5 hours has any additional affect on the morphology or cytoskeleton of COL treated cells despite an intact F-Actin system in these cells. Therefore microtubules provide the retractile force for F-Actin skeletal collapse following removal of serum or CB treatment as an intact microtubule system is an absolute requirement for the formation of an arborized phenotype in response to actin disruption. *Scale bars: 50μm.*

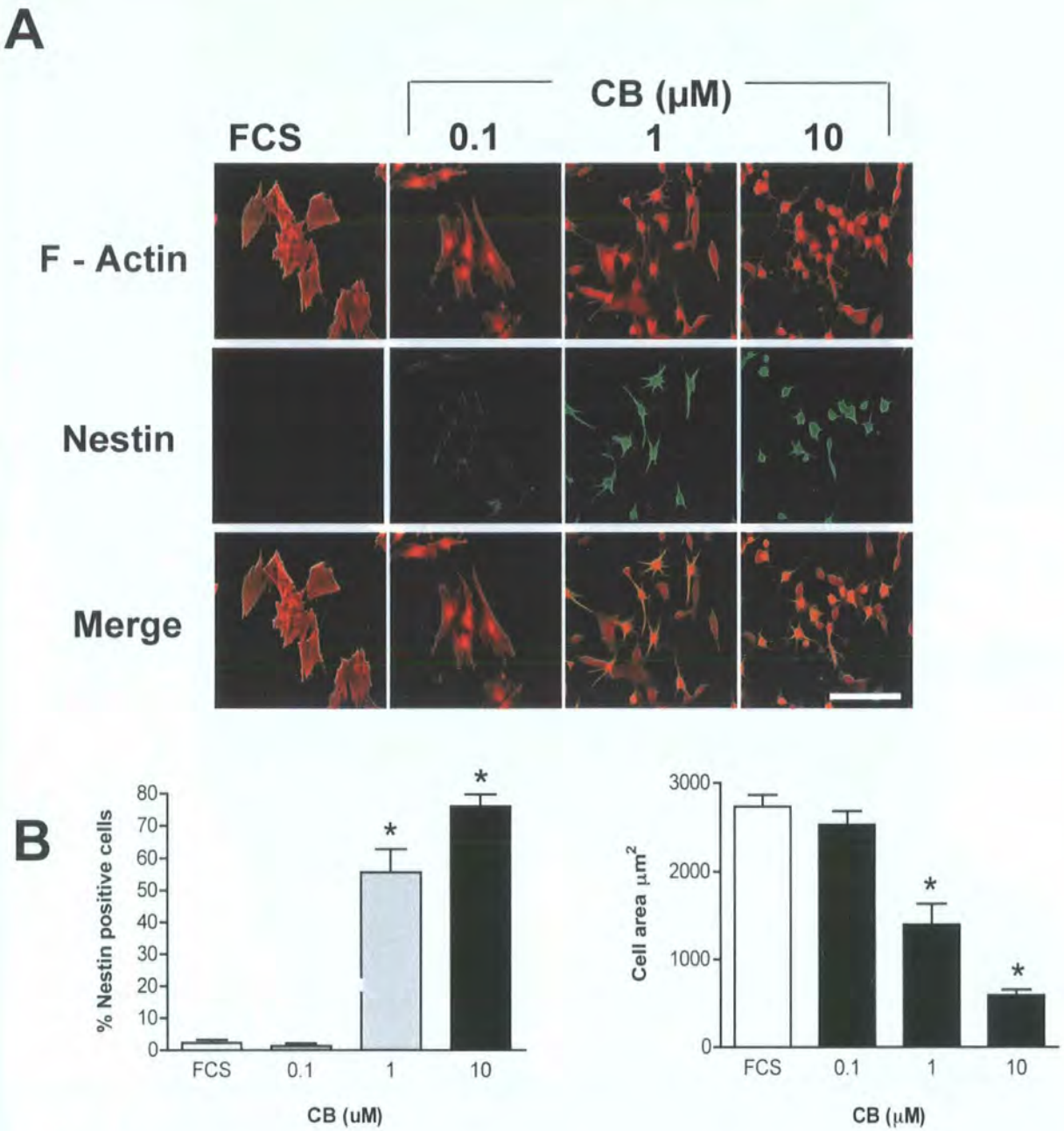


Figure 4.12: Regulation of Nestin expression in response to treatment with cytophasin-B (CB, 0.1-10μM) for 5 hours. **(A)** Cells incubated in 10% FCS had a normal fibroblastic morphology and Actin filaments organized in classical stress fiber patterns. The F-Actin microfilaments became progressively more disrupted as CB concentration increased and as cells progressively acquired an aborized phenotype. High levels of Nestin expression were confined only to those cells with a fully arborized phenotype (see merge images) (Scale bar: 80μm). **(B)** Measurement of cell footprint area (μm^2) and percentage of Nestin expressing cells (number of Nestin bright cells / total cells per visual field) further demonstrated that the expression of Nestin only occurred at higher concentrations of CB (1-10μM) correlating with the proportion of fully arborized cells and the disruption of the F-Actin cytoskeleton. Data represented as mean±SEM (n=10, +20 cells/field, n=3 independent experiments, *P<0.01 compared to control cultures, FCS).

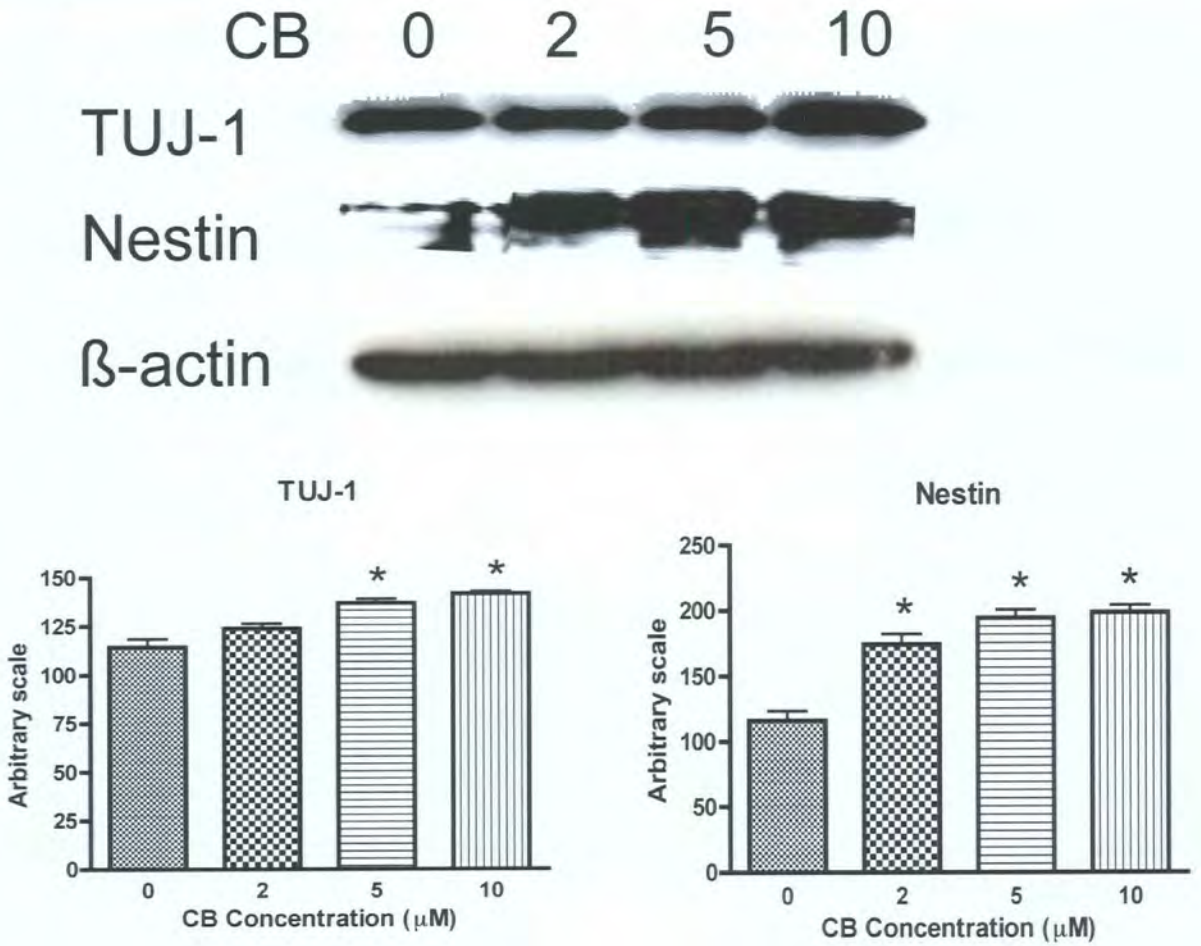


Figure 4.13: Regulation of Nestin and Tuj-1 expression in response to treatment with cytophasin-B (CB, 2-10μM) for 5 hours. Western blot analysis of Tuj-1 and Nestin expression in response to increasing concentrations of CB for 5 hours. Densitometry results of protein expression following western blot analysis. Data represents Mean±SEM from 3 independent experiments (*P<0.05 compared to non-CB treated control cells, n=3).

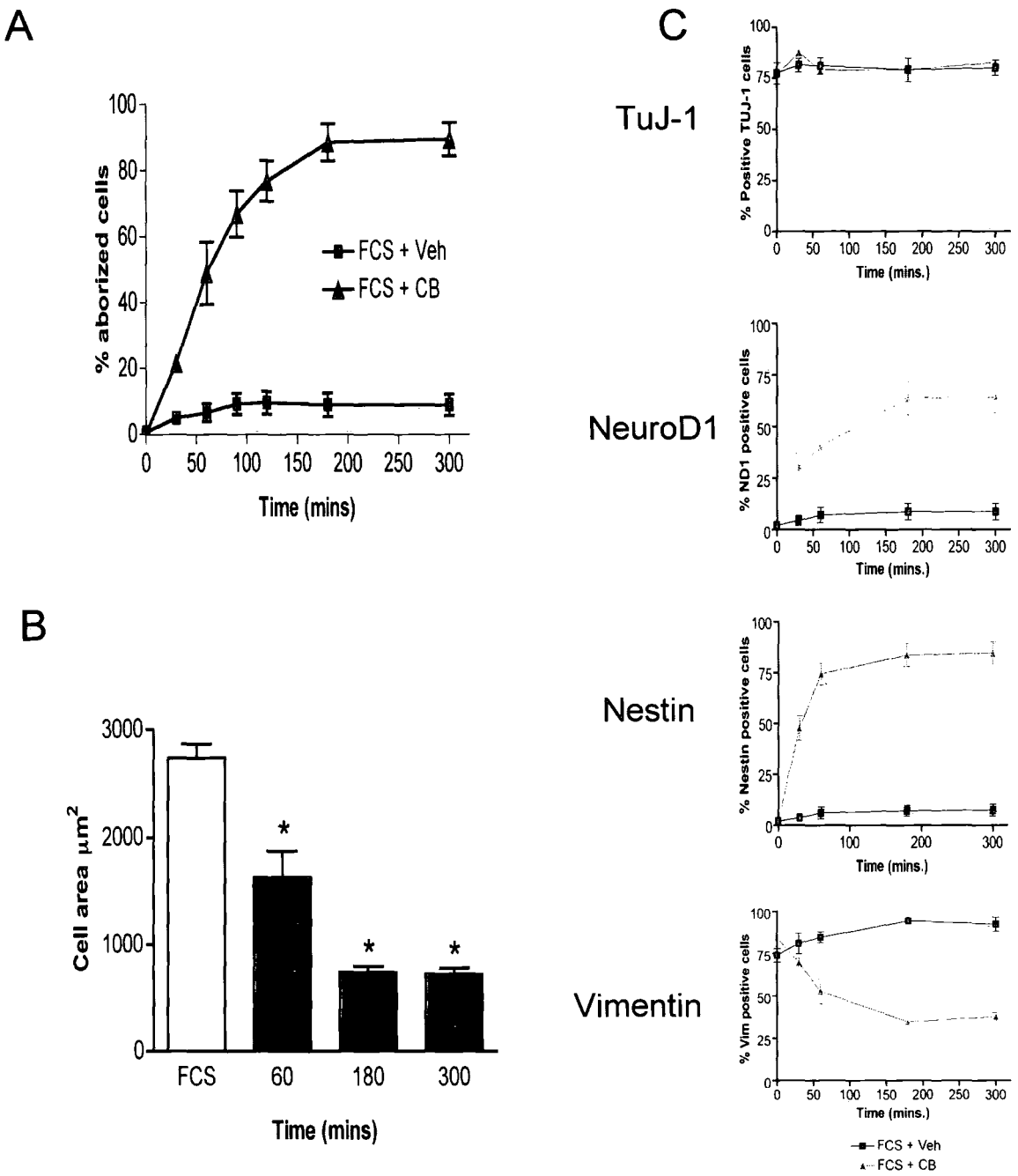


Figure 4.14: Rat MSCs form neural-like cells and express neural proteins in response to cytophasin-B (CB) over time. Cells were treated with CB (10µM) to disrupt F-Actin and subsequently fixed at various time points ranging from 0 to 300 minutes. The percentage of arborized cells (**A**, data represented as mean±SEM (n=3)) and mean cellular foot print area (**B**, for 10 fields of view, +20 cells/field; $^{*}P<0.05$ when compared to vehicle treated cells) were measured at each time point. These data show that the morphological response to CB treatment occurred progressively during the 5 hour treatment period. (**C**) Expression of neural and mesodermal proteins was determined by flow cytometric analysis of fixed and permeabilised at each time point. The number of cells positive for neural proteins, NeuroD1 and Nestin, correlated with the increased number of arborized cells over time. In contrast, expression of the mesodermal-associated marker, Vimentin, progressively decreased over time. The percentage of cells positive for TuJ-1 did not change in response to CB. Data shown represent mean±SEM, n=3.

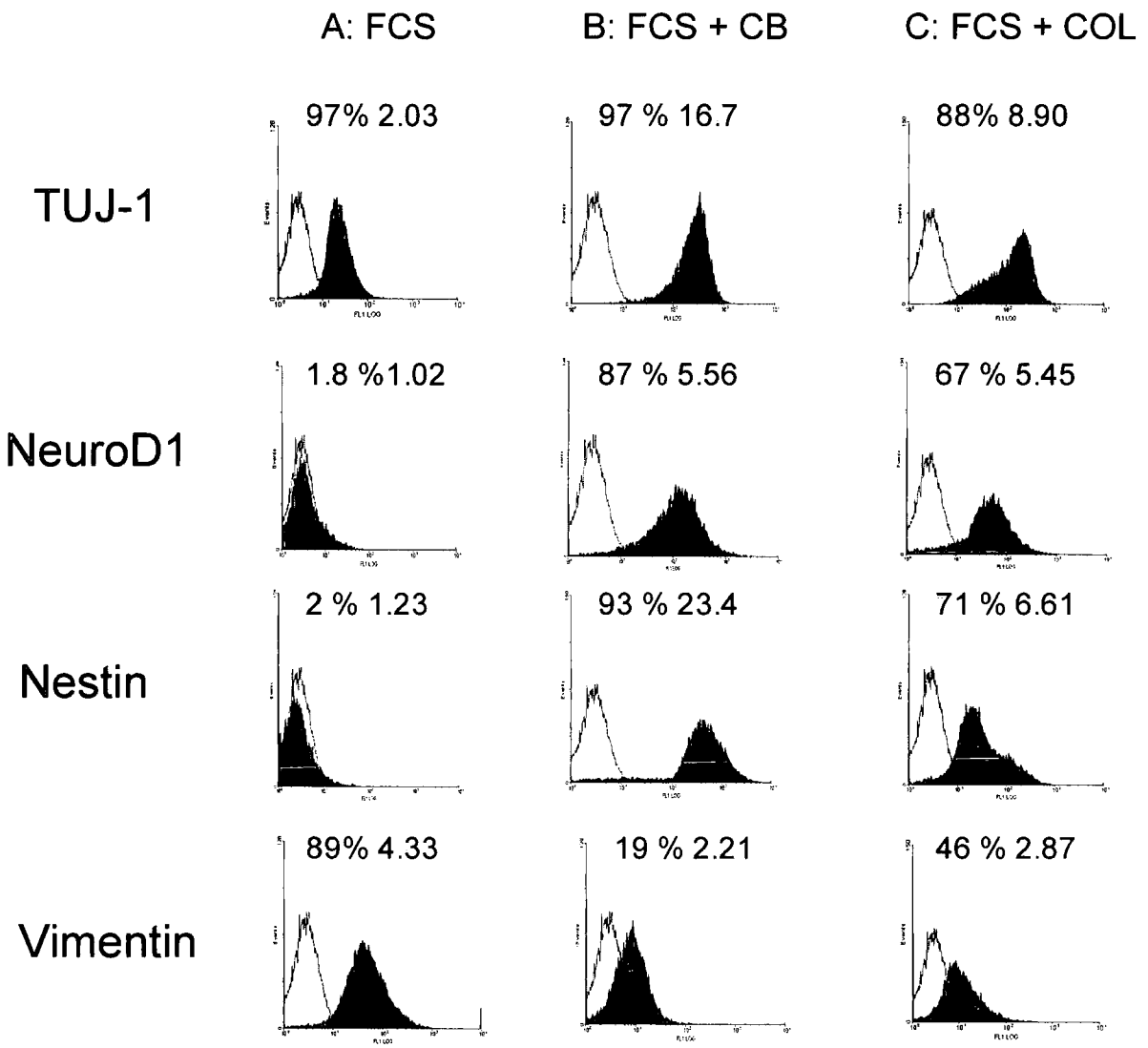


Figure 4.15: Expression of neural and mesodermal antigens in response to CB or COL treatment as determined by flow cytometric analysis. Flow cytometric analysis of neural and mesodermal cell markers. P8 rMSCs were cultured under standard conditions (**A,** control) or following 5 hours treatment with CB 10μM (**B**) or COL 10 μg/ml (**C**). Cells were subsequently fixed, permeabilised and labeled with antibodies directed against intracellular neural (Tuj-1, NeuroD1, nestin) and mesodermal (vimentin) antigens. FITC conjugated secondary antibodies detected binding of the primary antibodies. The open peaks indicate IgG isotype control corresponding to the antibodies in which they were generated. The solid peaks indicate are counts of the cell population that is positive for the antibody indicated in each individual histogram. The number of positive cells is shown on the y-axis and the mean fluorescence intensity (MFI) of staining on the x-axis. Values of percentage positive cells and MFI are also indicated in each histogram.

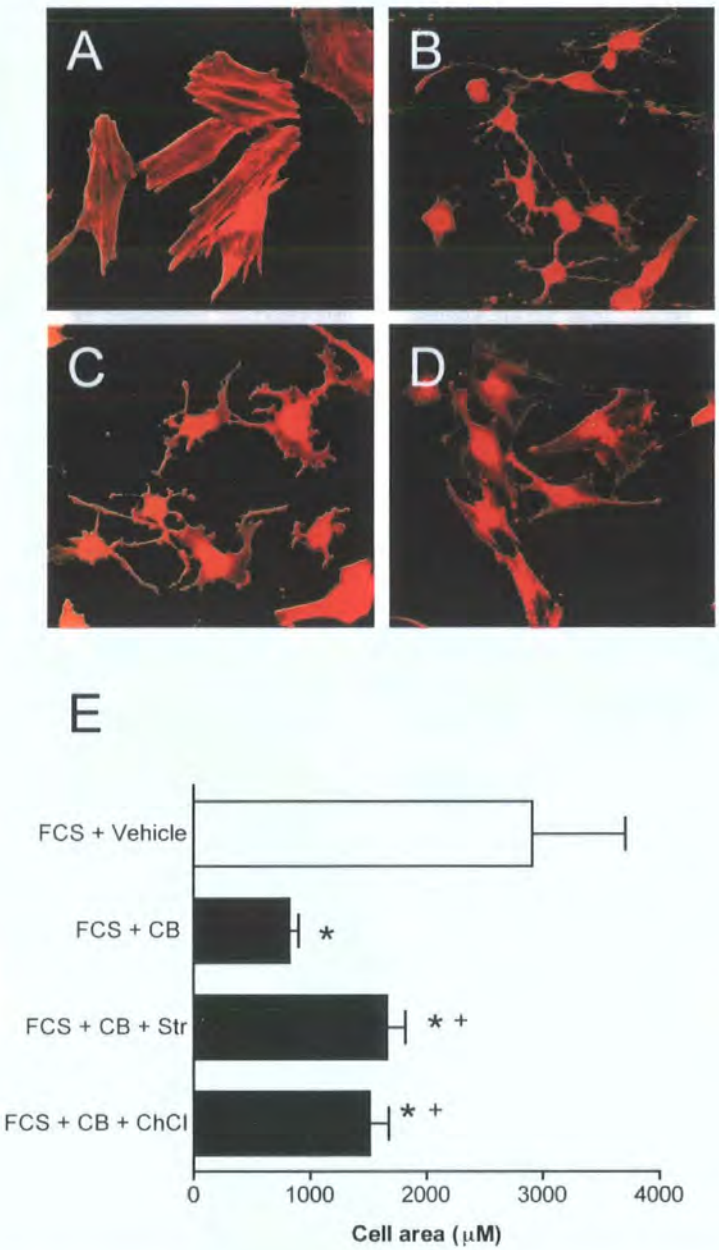


Figure 4.16: Effect of broad-spectrum PKC on changes in cell shape induced by CB treatment. Cells were treated with 10μM CB in serum containing media (FCS) in the presence or absence of broad-spectrum PKC inhibitors. After 5 hours cells were fixed and permeabilised for immunocytochemistry. F-actin cytoskeleton was visualised using TRITC conjugated Phalloidin. (**A**) FCS + Vehicle, (**B**) FCS + CB, (**C**) FCS + CB + Str, (**D**) FCS + CB + ChCl. (**E**) The mean cellular footprint area was determined for cells cultured under each condition (mean±SEM, 10 fields of view, +20 cells per field). The footprint area of CB treated cells was significantly reduced compared to vehicle treated cells (FCS + vehicle (control)). The PKC inhibitors straurosporine (Str) and Chelerytrine (ChCl) partially inhibited the CB-induced reduction in the footprint area in response to CB treatment, however, the reduction was still significant compared to vehicle treated controls (*$P<0.05$ compared to vehicle treated cells , +$P<0.01$ when compared to FCS+CB treated cells) *Scale bars: 50μm.*

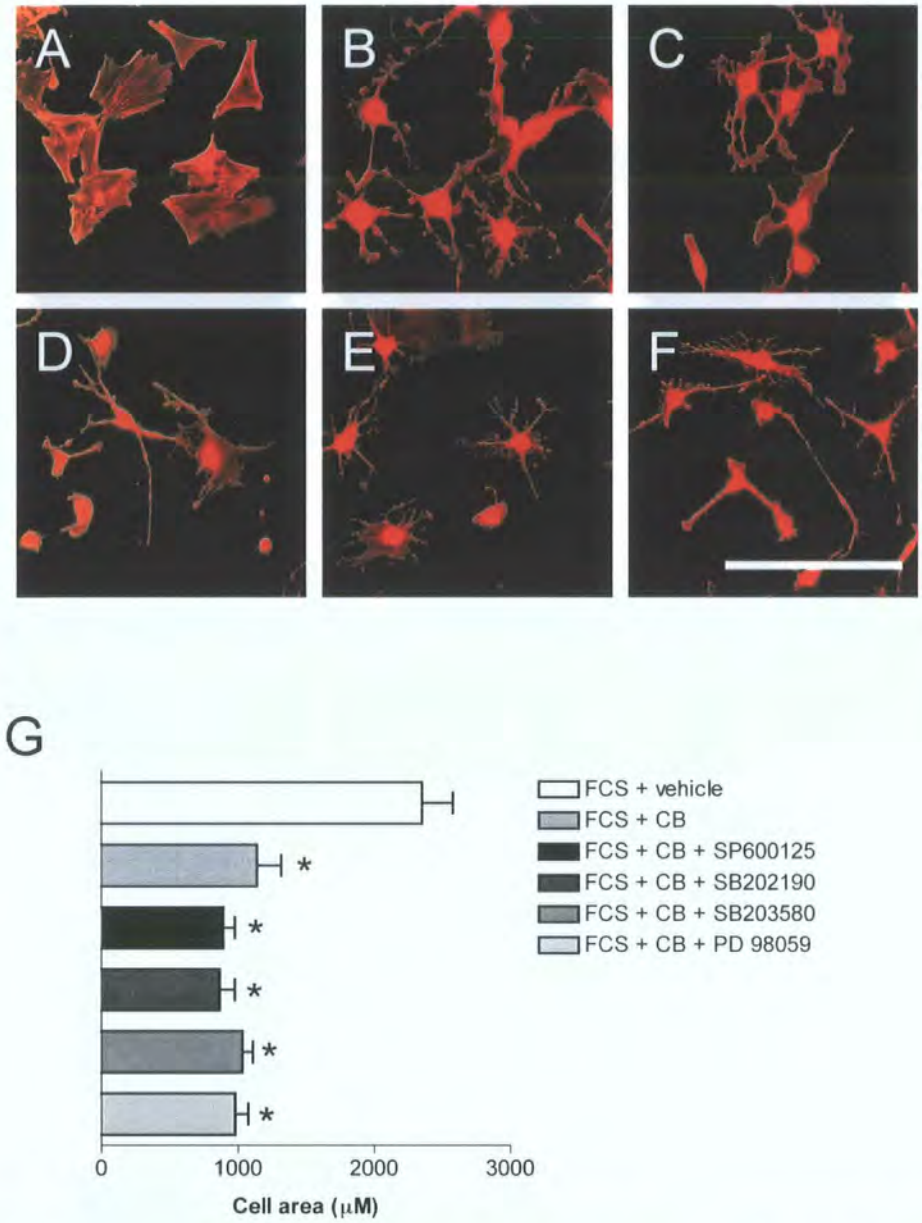


Figure 4.17: Effect of MAPK inhibitors on changes in cell shape induced by CB treatment. Cells were treated with 10μM CB in serum containing media (FCS) in the presence or absence of specific MAPK inhibitors. After 5 hours cells were fixed and permeabilised for immunocytochemistry. F-actin cytoskeleton was visualised using TRITC conjugated Phalloidin. (**A**) FCS + Vehicle, (**B**) FCS + CB, (**C**) FCS + CB + SP600125, (**D**) FCS + CB + SB202190, (**E**) FCS + CB + SB203580 and (**F**) FCS + CB + PD98059. (**G**) The mean cellular footprint area was determined for cells cultured under each condition (mean±SEM, 10 fields of view, +20 cells per field, data from 3 independent experiments). The footprint area of CB treated cells was significantly reduced compared to vehicle treated cells (FCS + vehicle (control)). Addition of MAPK inhibitors had no significant effect on the CB-induced reduction in the cellular footprint area, which remained significantly reduced compared to control for all CB treated cultures. (*$P<0.05$ compared to vehicle treated cells). *Scale bars: 50μm.*

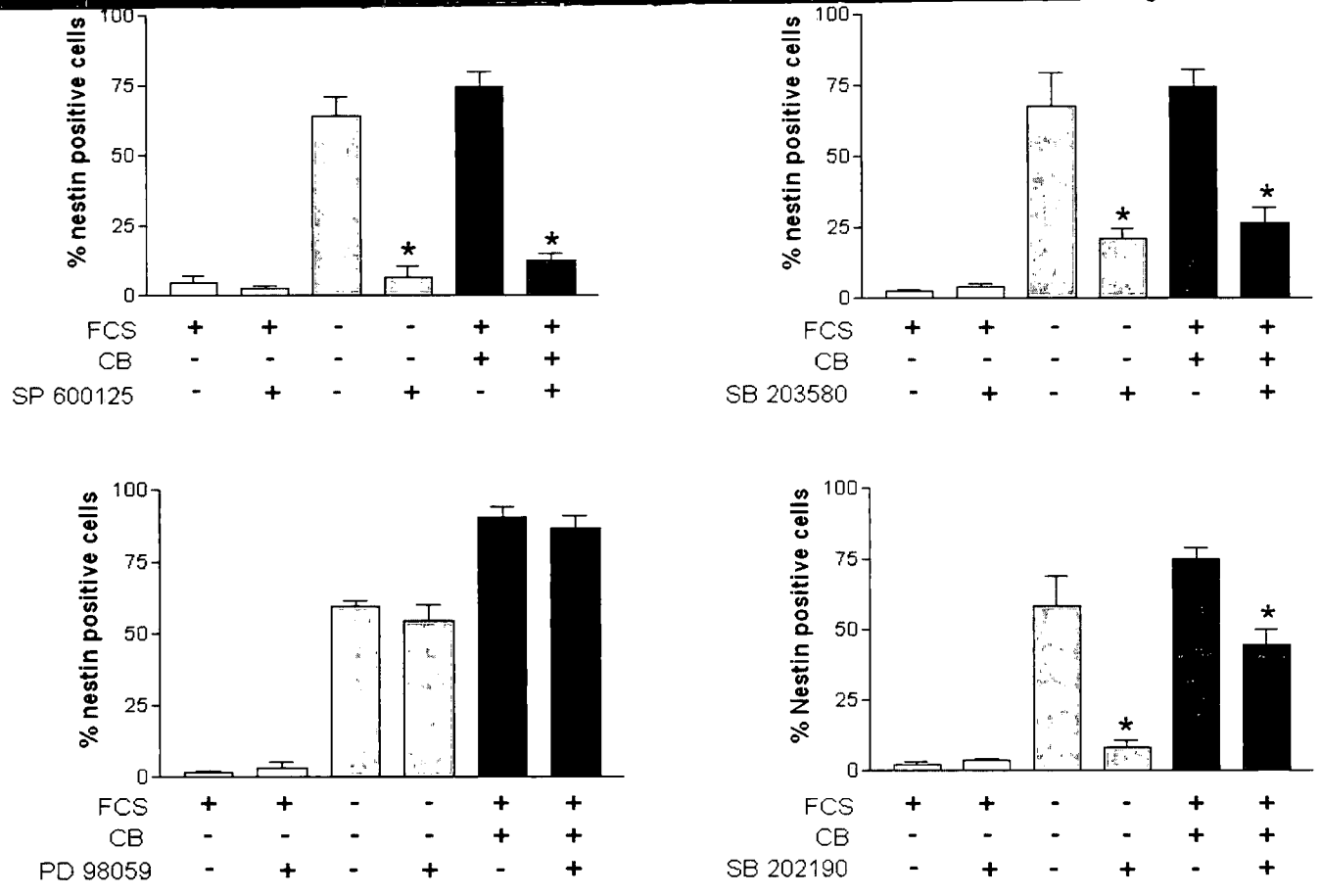


Figure 4.18: Expression of Nestin in response to CB or COL treatment as determined by flow cytometric analysis. Flow cytometric analysis of Nestin expression. P8 MSCs were cultured in either the presence or absence of FCS or the presence or absence of CB 10μM. Both these inductive agents were tested in the presence or absence of specific MAPK inhibitors. The level of nestin expression under these culture conditions was determined by flow cytometry. Data represents Mean ± SEM from three independent experiments. (*P<0.05 Students t-test for paired comparisons).

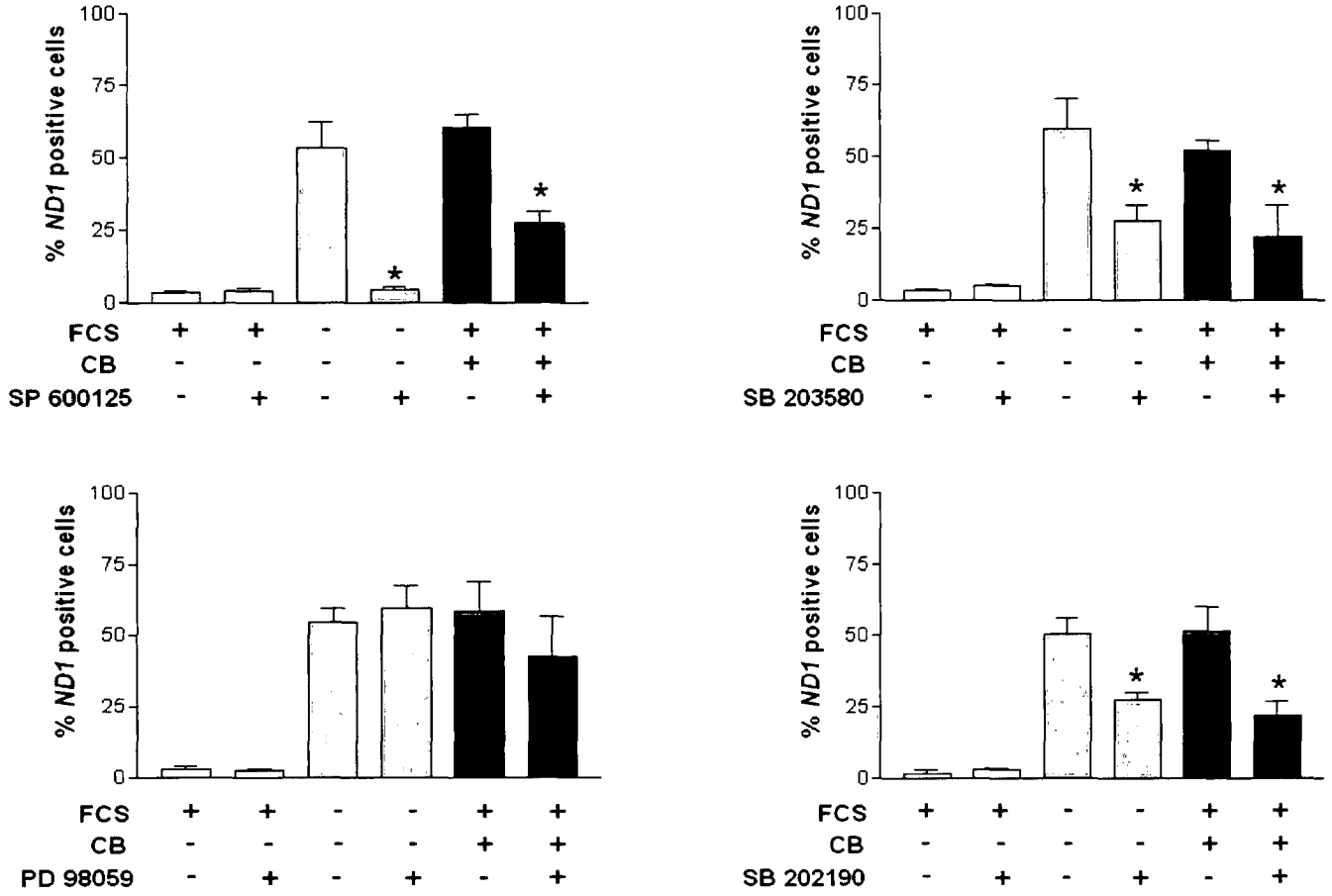


Figure 4.19: Expression of NeuroD1 (ND1) in response to CB or COL treatment as determined by flow cytometric analysis. Flow cytometric analysis of NeuroD1 expression. P8 MSCs were cultured in either the presence or absence of FCS or the presence or absence of CB 10μM. Both these inductive agents were tested in the presence or absence of specific MAPK inhibitors. The level of nestin expression under these culture conditions was determined by flow cytometry. Data represents Mean ± SEM from three independent experiments. (*P<0.05 Students t-test for paired comparisons).

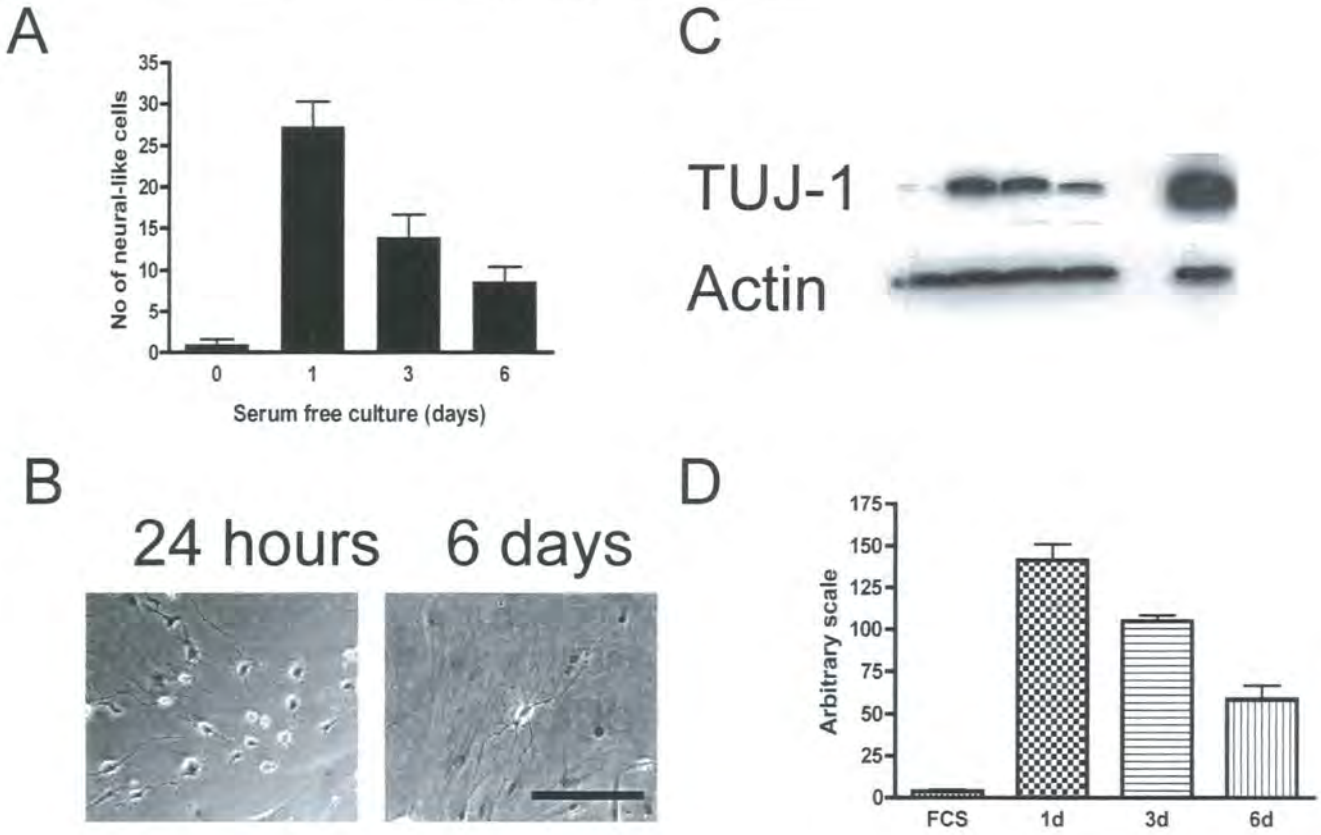


Figure 4.20: Expression of Tuj-1 and percentage of neural-like cells over 6 days of differentiation. Rat MSCs were maintained in serum free culture for 6 days and the number of arborized cells was determined at various time points (1, 3 and 6 days) compared to control cells which remained in serum containing media (**A**) (number of arborized cells/total number of cells per field, n=10 fields, +20 cells/field). Phase contrast images of 1% DMSO + N2 treated cells shows cells with a neural-like morphology at 24 hours and 6 days (**B**). The expression of the neuronal protein TuJ-1 was examined over 6 days by western blot analysis (**C**) showing that TuJ-1 levels progressively decrease over 6 days with expression at its maximum following 24 hours serum free culture. The decrease in expression correlated with a significant decrease in the number of arborized cells following 6 days serum free culture (**A**). (**D**) Densitometry results of protein expression following western blot analysis. Data represents Mean±SEM from 3 independent experiments. *Scale bars: 50μm.*

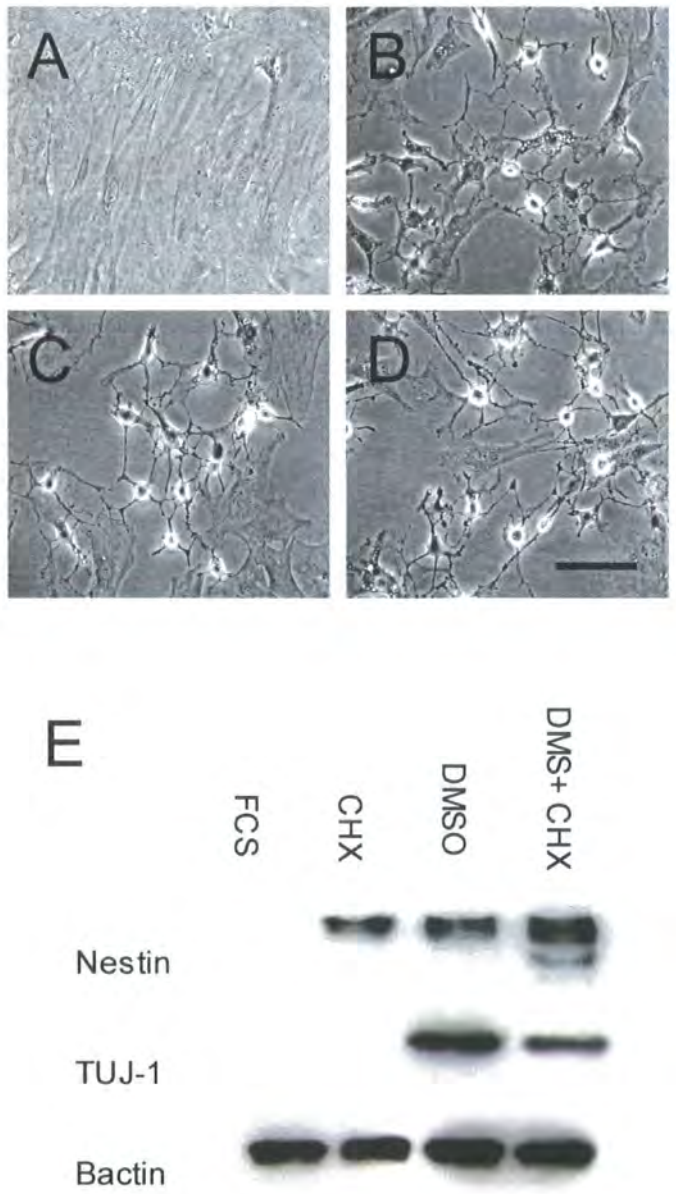


Figure 4.21: Effect of protein synthesis inhibition on the acquisition of a neural-like morphology and expression of nestin and Tuj-1 in response to 1% DMSO. Control MSCs (**A**) were pre-treated for 24 hours with CHX at a concentration of 10 μg/ml which arrested cell proliferation (**B**). CHX pre-treated cells were subsequently incubated with 1% DMSO in serum free media for 5 hours (**C**) and compared to DMSO treated cells that were not previously incubated with CHX (**D**). *Scale bars: 50μm.* Western blot analysis was used to analyze the expression of nestin and Tuj-1 in response to 1% DMSO in CHX treated MSCs (**E**).

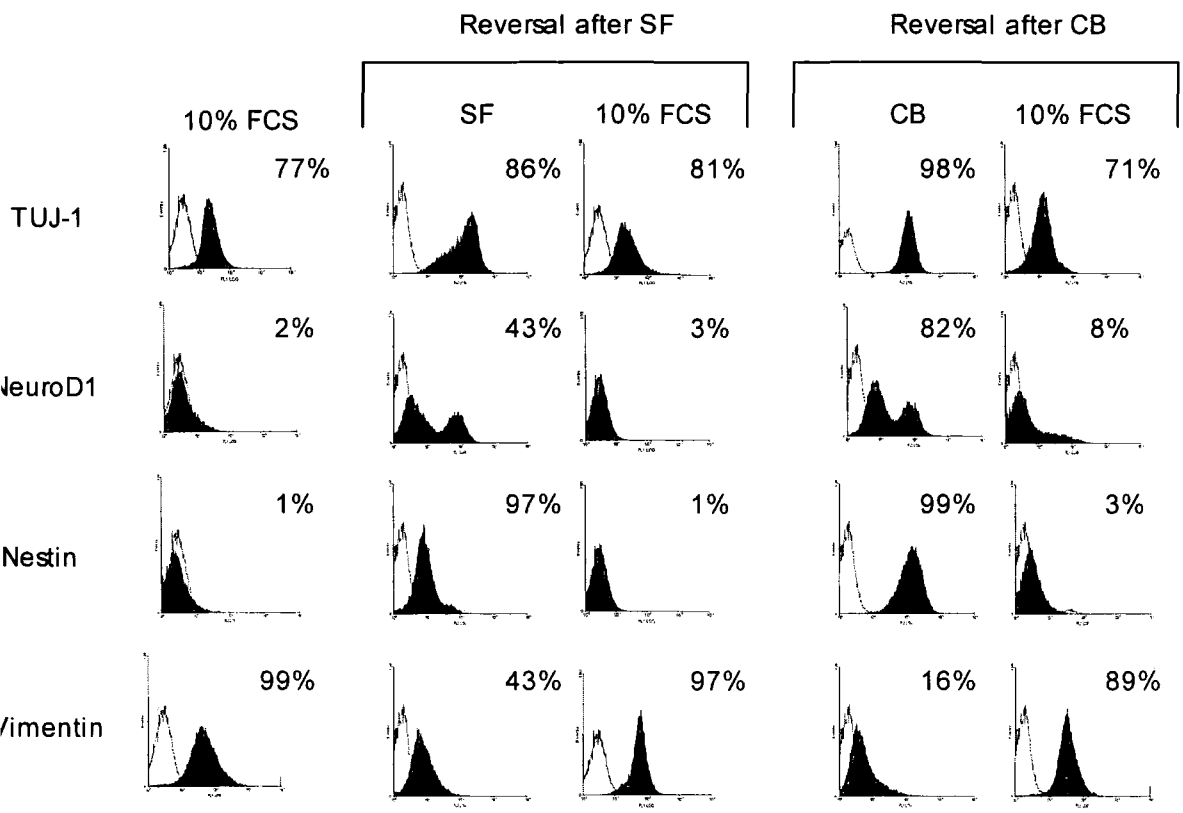


Figure 4.22: Effect of re-introduction of serum on the expression of neural or mesodermal marker proteins in cultures of rMSCs treated with CB or cells grown in serum free media. Cells were cultured for 5 hours in the absence of serum or the presence of CB (10μM) and then exposed to fresh growth medium supplemented with 10% FCS for a further 5 hours. Flow cytometric data from representative experiments are shown as histograms with fluorescence intensity on the x-axis (solid peaks) and cell count on the y-axis. Background fluorescence was excluded by the use of isotype matched control antibodies (open peaks). The number of positive cells for each antigen is indicated on the representative histogram. The cytometric data demonstrates that subsequent to growth in either serum free or CB treatment conditions, rMSCs can re-express neural and mesodermal markers just as they did prior to any treatment or growth condition when serum is re-introduced into the growth media.

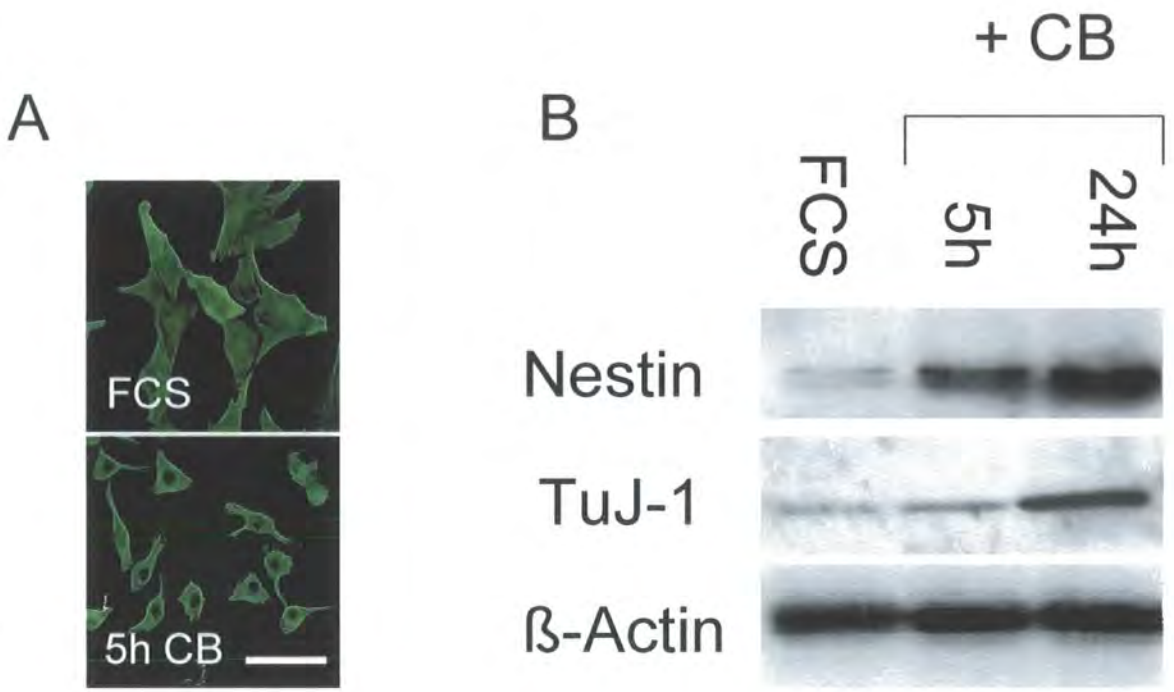


Figure 4.23: Effect of cytophasin-B (10μM) treatment on rat dermal fibroblasts. (**A**) Immunofluorescent staining of the Tubulin cytoskeleton in untreated fibroblasts (10% FCS) and cells exposed to CB (10μM) or vehicle for 5 hours. Fibroblasts underwent cytoskeletal collapse when exposed to CB in a similar fashion to rMSCs (*scale bar: 10μm*). (**B**) Western blot analysis revealed that rat dermal fibroblasts expressed almost undetectable levels of TuJ-1 and Nestin in serum containing cultures, however, these proteins were up regulated following CB treatment for 5-24 hours.

Table 4.1: Effect of broad spectrum PKC and MAPK inhibitors on the expression of nestin and NeuroD1 in MSCs following serum withdrawal and treatment with cytophasin B

PKC inhibitor (Staurosporine)

	NeuroD1	Nestin
Control	1.6 ± 0.8	1.3 ± 0.3
Control + Vehicle	2.3 ± 1.2	1.6 ± 0.9
SF + Vehicle	41.3 ± 2.6	48.0 ± 5.6
SF + Str	3.0 ± 0.5*	5.3 ± 1.4*
CB + Vehicle	68.6 ± 4.2	72.6 ± 8.5
CB + Str	1.7 ± 0.8*	2.6 ± 1.2*

PKC inhibitor (Chelerytrine)

	NeuroD1	Nestin
Control	1.0 ± 0.6	1.0 ± 0.5
Control + Vehicle	1.7 ± 0.6	1.6 ± 0.6
SF + Vehicle	45.0 ± 7.2	36.3 ± 6.9
SF + ChCl	2.3 ± 1.9	1.3 ± 0.8*
CB + Vehicle	48.6 ± 8.7	82.7 ± 3.1
CB + ChCl	3.3 ± 0.8*	1.3 ± 1.3*

MEK inhibitor (PD98059)

	NeuroD1	Nestin
Control	3.0 ± 1.2	1.3 ± 0.8
Control + Vehicle	2.3 ± 0.3	3.0 ± 0.9
SF + Vehicle	54.6 ± 4.9	49.0 ± 2.0
SF + PD 98059	59.3 ± 7.9	4.4 ± 5.7
CB + Vehicle	58.6 ± 6.6	90.6 ± 3.1
CB + PD 98059	42.6 ± 13.8	86.6 ± 4.0

P38 inhibitor (SB 202190)

	NeuroD1	Nestin
Control	1.7 ± 1.2	2.0 ± 1.2
Control + Vehicle	3.0 ± 0.5	3.3 ± 0.8
SF + Vehicle	50.3 ± 5.8	58.6 ± 10.4
SF + SB 202190	27.3 ± 2.7*	8.0 ± 2.3*
CB + Vehicle	51.6 ± 8/0	75.0 ± 4.0
CB + SB 202190	22.0 ± 5.0*	44.6 ± 5.2*

P38 inhibitor (SB 203580)

	NeuroD1	Nestin
Control	3.3 ± 0.8	2.3 ± 0.8
Control + Vehicle	5 ± 0.6	4.0 ± 1.1
SF + Vehicle	59.3 ± 10.7	67.6 ± 11.9
SF + 203580	27.6 ± 5.5*	21.0 ± 3.5*
CB + Vehicle	52.0 ± 3.6	74.3 ± 6.1
CB + 203580	22.0 ± 11.1*	26.6 ± 5.1*

JUNK inhibitor (SP 600125)

	NeuroD1	Nestin
Control	2.8 ± 0.4	4.7 ± 2.3
Control + Vehicle	4 ± 1.5	2.3 ± 1.2
SF + Vehicle	53.3 ± 9.02	64.0 ± 7.2
SF + SP600125	4.6 ± 0.8*	6.3 ± 4.1*
CB + Vehicle	60.3 ± 4.6	74.3 ± 5.7
CB + SP600125	27.3 ± 4.3*	12.7 ± 2.4*

Table 4.2: Expression of neural, mesodermal and endodermal cell markers in MSCs following CB induced actin disruption

Expression of neural proteins

	MSC Vehicle treated		MSC CB treated	
Protein	No. cells positive	MFI	No. cells positive	MFI
NeuN	41.2+2.3	2.23+0.4	86.7+4.1	9.09+0.8
GAP-43	72.7+3.3	18.5+1.2	92.3+6.8	24.6+2.7
Nestin	1.2+0.4	1.21+0.5	78.8+5.9	5.67+0.8
Syn	45+2.8	2.83+0.8	95+8.4	4.41+0.9
NF-68	43.5+1.5	3.03+0.6	22.3+1.2	1.52+0.3
NF-160	56.7+2.7	3.1+0.5	18.7+2.2	1.23+0.3
NF-200	33.4+5.6	2.8+0.4	21.6+1.7	1.11+0.3
GFAP	96.5+7.8	10.4+1.1	89.7+5.9	13.2+0.4
MAP-2	82.3+6.6	2.1+0.3	84.5+5.8	2.3+0.5

Expression of mesodermal and endodermal proteins

	MSC Vehicle treated		MSC CB treated	
Protein	No. cells positive	MFI	No. cells positive	MFI
Fibronectin	92.3+8.6	27.4+1.2	34.5+1.6	3.6+0.7
smooth muscle actin	91.2+7.8	22.9+2.1	93.4+7.8	21.3+2.1
Vimentin	97.8+5.6	4.88+1.5	28.9+3.3	2.1+1.7
cytokeratin 8	3.4+0.9	1.1+0.2	29.6+5.6	3.3+0.7
cytokeratin 18	2.5+1.1	1.2+0.1	41.3+1.7	2.7+1.2
alpha fetoprotein	71.4+8.8	3+0.3	64.6+8.4	4.7+0.2
Collagen II	2.9+1.1	1.2+0.5	31.2+3.6	2.8+0.3

CHAPTER 5

Mesenchymal Stem Cells Expressing Neural Antigens Instruct a Neurogenic Cell Fate Decision on Neural Stem Cells

5.1 Introduction

MSCs isolated from postnatal BM and expanded *ex vivo* have been shown to successfully engraft and survive in rodent brain following transplantation (Azizi et al., 1998; Kopen et al., 1999 and Deng et al., 2006). More importantly, infusion of whole BM (Chen et al., 2000; Li et al., 2001) or purified MSCs either directly into the CNS (Chen et al., 2001; Li et al., 2000; Li et al., 2001b) or indirectly into the systemic vasculature (Chen et al., 2001b; Li et al., 2002; Honma et al., 2006; Shen et al., 2006; Lu et al., 2006) has been associated with end-organ repair in several animal injury models including stroke (Chen et al., 2003; Li et al., 2001; Li et al., 2002), traumatic brain injury (Mahmood et al., 2001; Lu et al., 2001;) and spinal cord lesions (Chopp et al., 2000; Hofstetter et al., 2002; Neuhuber et al., 2005; Lu et al., 2005). Functional recovery and improved neurological outcome following an ischemic lesion in the CNS is a major therapeutic goal and recent reports that infusion of MSCs has a therapeutic benefit in animal models of injury are of major clinical importance.

However, despite the importance of these observations and extensive work by numerous investigators, the exact mechanisms by which MSCs promote functional recovery following injury remain elusive. Several publications have suggested that MSCs may contribute to the regeneration and repair of injured tissues by three mechanisms; by differentiating into appropriate cell phenotypes (trans-differentiation) (Mezey et al., 2000; Lu et al., 2006), by cell fusion with host cells (Ying et al., 2002; Terada et al., 2002) or by promoting endogenous restorative and regenerative mechanisms through the release of trophic factors and cytokines (Li et al., 2002; Chen et al., 2002; Chen et al., 2005; Kurozumi et al., 2005).

A number of studies support the concept that MSCs may have an intrinsic neurogenic potential and are thus able to differentiate into neural cell derivatives and replace lost or damaged host cells by a process of trans-differentiation. In support of this theory, MSCs expressing neural cell antigens such as GFAP, NeuN and Tuj-1 have been detected in both the intact (Kopen et al., 1999; Brazelton et al., 2000) and injured brain following MSC transplantation (Chen et al., 2000; Li et al., 2002). The

expression of these markers is confined to a small subpopulation and expression alone is insufficient to conclude neural differentiation. No evidence of a mature neural phenotype has been described for MSCs following transplantation into the host brain. In addition, previous reports of trans-differentiation may have been misinterpreted, as cells can acquire the characteristics of host cells through cell fusion and not differentiation (Mezey et al., 2004).

MSCs isolated from the adult BM can express neuronal and glial cell antigens under defined culture conditions (Sanchez Ramos et al., 2002; Hermann et al., 2004, 2006; Croft and Przyborski., 2004, 2006; Scintu et al., 2006) and following co-culture with neural stem/progenitor cells, independent of cell fusion events (Wislet-Gendebien et al., 2005; Rivera et al., 2006), MSCs retain their expression of these proteins following transplantation into the embryonic (Munoz et al., 2004) and adult rodent brain (Deng et al., 2006). MSCs maintained in culture have also been reported to spontaneously express neural proteins (Tondreau et al., 2004; Deng et al., 2006) and pluripotent stem cell markers (Lamoury et al., 2006; Pochampally et al., 2004). Presumptive neurons derived from MSCs have been shown to express a number of neurotransmitters and display electrophysiological activity consistent with a neuronal cell phenotype (Wislet-Gendebein et al., 2005; Tropel et al., 2006). These findings collectively support the notion that MSCs processes a limited neurogenic potential.

Reports of trans-differentiation of MSCs into neural cell phenotypes are controversial with numerous conflicting reports (Croft and Przyborski, 2006; Wagers et al., 2002; Bertani et al., 2005; Neuhuber et al., 2004; Castro et al., 2002). Although the contribution of trans-differentiation to repair of host tissue is uncertain, MSCs have been shown to engraft and migrate throughout the host CNS and express cell markers consistent with a neural cell fate in the absence of cell fusion (Mezey et al., 2003; Crain et al., 2005). The number of MSCs found to express neuronal and glial cell markers following transplantation are too few to fully account for the rapid improvements in neurological outcome (Chen et al., 2001; Li et al., 2002). Therefore tissue replacement either by trans-differentiation of MSCs or fusion with host cells is an unlikely mechanism by which MSCs promote restoration of function. The function

of neural protein expression in MSCs and the role of neural antigen positive MSCs at the injury site are unknown.

MSCs are thought to promote endogenous regeneration following brain injury by releasing numerous cytokines and trophic factors that activate restorative and regenerative processes in the host brain. MSCs produce numerous growth factors including; VEGF, NGF and BDNF in culture (Chen et al., 2005; Chen et al., 2002) and an up-regulated expression of NGF, VEGF, CNTF and FGF-2 have been reported in host tissue following transplantation (Munoz et al., 2005). The predominant effects of these factors are neuro-protection with a reduction in apoptopic cell death and trophic effects including neurogenesis, synaptogenesis and angiogenesis (For Review see Chopp and Li, 2002).

There is evidence to suggest that the transplantation of MSCs induces the proliferation and neurogenesis of endogenous neural stem/progenitor cells and promote the migration of newly formed neural cells to sites of tissue injury. It is now known that NSCs undergo neurogenesis in the adult brain and that newly formed cells can contribute to self-repair following injury although this is limited and unable to fully compensate for neuronal loss associated with injury or disease pathology. Many of the factors released by MSCs have also been found to regulate neurogenesis including BDNF, NGF, NT-3, FGF-2 and VEGF (Fiore et al., 2002; Scharfman et al., 2005; Schanzer et al., 2004; Palmer et al., 1995; Jin et al., 2003). Transplantation of MSCs in injury models is associated with an increase in the proliferation of neural progenitor cells in the subventricular zone (Chen et al., 2001; Chen et al., 2002). In addition, a recent study reported increased neurogenesis in the dentate gryus of the hippocampus following MSC transplantation into the intact brain, where the differentiated progeny of these cells were shown to migrate, engraft and survive within the adult brain (Munoz et al., 2005).

There is however little known about the interactions of MSCs with endogenous NSC/progenitor cells. Cell lineage commitment of NSCs is determined *in vitro* by trophic influences (Takahashi et al., 1999; Lim et al., 2000; Tanigaki et al., 2001; Hsieh et al., 2004) and because MSCs are known to release an array of growth factors

we hypothesized that in addition to many other effects on brain plasticity MSCs may influence the cell fate determination of resident neural progenitor cells by the release of soluble factors which influence differentiation.

In the present study we describe the co-cultivation of MSCs with multipotent neural progenitor cells isolated from day 14 rat embryos. We have developed an in vitro co-culture system in which NSC/progenitor cells and MSCs were cultured in the same environment separated by a porous membrane to specifically examine the soluble interactions of MSCs with NSC/progenitor cells isolated from the embryonic striatum. This provides a dynamic system in which intercellular signaling can operate between the two cell populations. To accurately model the effects of an MSC graft which in vivo is known to contain both neural antigen positive and neural antigen negative MSCs, we induced MSCs to express neural proteins in order to identify any differences in the interactions of these neural antigen positive cells with NSCs when compared to the effects of antigen negative MSCs.

The effects of MSC-derived soluble products from both induced and non-induced MSCs on the proliferation and cell lineage commitment of NSC/progenitor cells were examined. In addition, because reconstruction of neural circuitry will be vital for long- term functional recovery we also investigated the humeral effects of MSCs on the survival of neuronal progeny and the induction of neurite outgrowth and axonal development.

5.2 Materials and Methods

5.2.1 Cell Culture

Rat MSCs were isolated as previously described (Croft and Przyborski, 2006). Cells were cultured in complete culture medium (CCM; Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and 2μM L-glutamine (Sigma) and 1 x nonessential amino acids (Invitrogen)) in T-75cm^2 tissue culture flasks (Nunc). Cells were grown in CCM at 37°C and 5% CO^2 until >70% confluence at which stage cultures were passaged by enzymatic dissociation. Rat

MSCs were washed with phosphate buffered saline (PBS) and detached by incubation with 0.25% trypsin and 0.1% EDTA for 5-10 minutes at 37°C. CCM was added to inactivate the trypsin. The cells were centrifuged at 450 x g for 10 minutes, the medium was removed and cells were re-suspended in 1-10ml of CCM. The cells were counted in duplicate using a hemacytometer and then plated at a density of 10 cells / cm^2 for expansion. Passage 8 (approximately 25 population doublings) cells were used herein. The mutipotentcy of isolated rat MSCs was verified by their capacity to differentiate into mesodermal derivatives (bone and fat) in vitro and their cell surface expression of CD90, CD73, CD44 and absence of CD45 and CD11b (data not shown).

NSC/progenitor cells were isolated from the mesencephalon of day 14 rat embryos. Pregnant female Wistar rats at the specified gestational age of 14 days (E14) (the day of conception was confirmed by the presence of a vaginal plug, embryonic day 0) were killed by cervical dislocation and the uteri were aseptically removed and transferred to Petri dishes containing sterile Dulbecco's phosphate buffered saline (PBS) with 30% glucose and penicillin (20 units/mL), streptomycin (20 mg/mL). E14 striata were isolated and triturated in DMEM/F12 with a sterile Pasteur pipette. The cell suspension was filtered with a 70 μm-mesh and viable cells were estimated by typan blue exclusion. The cells were plated (1 x 106 cells/75-cm^2 uncoated tissue culture flask (Nunc)) in a chemically defined serum-free medium DMEM/F12 including 0.6% glucose, 2mM L-glutamine, 3mM sodium bicarbonate and 5mM HEPES buffer, supplemented with N-2 (a multi-component cell culture supplement), EGF (10ng/ml, Sigma) and FGF (10ng/ml, Sigma) and 2 μg/ml heparin. When the size of neurospheres reached approximately 50 cells, they were dissociated into a single cell suspension by titration and replated in fresh culture medium. Cultures were maintained in 37°C in a humidified incubator with 5% CO_2. Neurospheres with a maximum of 3 passages were used in this study. The phenotype of these cells was confirmed by the expression of the NSC/progenitor marker nestin (Figure 5.7) and capacity to differentiate into neural cell types (Figure 5.2).

Adult hippocampal progenitor cells (HCN/GFPH) from the subgranule cell layer of the dentate gyrus of F344 rats were clonally derived (Palmer et al., 1997) then

genetically modified via a retroviral based tetracycline-responsive vector containing a CMV promoter (Hoshimaru et al., 1996) to express the modified jellyfish (Aequorea Victoria) enhanced green florescent protein (eGFP) (Okada et al., 1999). HCN/GFPH cells were cultured on polyornithine/mouse Laminin-coated flasks in media consisting of DMEM/Ham's F12 containing 1mM l-glutamine and antibiotics supplemented with N2 medium and 20ng/ml bFGF. Cultures were maintained in 37°C in a humidified incubator with 5% CO_2. Adult GFPH cells were differentiated under control conditions as previously described (Palmer et al., 1997; Gage et al., 1998), because lammin promotes a neural cell fate decision during differentiation cells were plated on PLO coated tissue culture plastic as single cells at a density of 10,000 cells/cm^2. Cells were incubated for 12 days in DMEMF12 supplanted with N2 and 0.5% FCS. Lineage commitment was determined by immuno-staining for cell type specific markers.

The mouse neuroblastoma N2a cell line was originally obtained from the American Tissue Culture Collection. Cells were seeded in T-25 cm^2 tissue culture flasks (Gibco) plates at a density of 3 x 10^4 cells/cm^2 and grown in DMEM (Gibco) supplemented with 2 mM L-glutamine, penicillin (20 units/mL), streptomycin (20 mg/mL) and 10% FCS (Invitrogen). Cells were maintained in a humidified atmosphere containing 5% CO^2 at 37°C and grown to >70% confluence.

5.2.2 Differentiation of E14 derived rat neurospheres on different surface substrates

Culture substrate is known to have a profound effect of the cell fate determination and differentiation potential of NSC in culture including the promotion of a neurogenic cell fate (Song et al., 2002). Therefore because E14 derived neurospheres were a primary cell line derived in our laboratory it was necessary to optimize the differentiation protocols with a view to choosing a substrate that did not promote a neural cell fate. To access the differentiation potential, single cell suspensions were plated on either poly-L-ornithine (PLO) (250 μg/ml) or laminin (5 μg/ml) or fibronectin (25 μg/ml) coated tissue culture plastic at a density of 10,000 cells/cm^2. Cells were incubated for 7 days in N2 supplemented DMEMF12 with 0.5% FCS. Lineage commitment was determined by immuno-staining for cell type specific markers.

5.2.3 Induction of neural protein expression in MSCs

After passage 7 (25-30 population doublings, approx.) conversion of MSC into neurosphere-like structures was initiated as described previously (Hermann et al., 2004; 2006). Briefly, cells were dissociated with 0.05% trypsin/0.04% EDTA and plated on low attachment plastic tissue culture flasks (Nunc) at seeding density of 1-2x10^5 cells/cm^2 in DMEM:F12 supplemented with N2, 10 ng/ml EGF, 10 ng/ml FGF and heparin (sodium salt, 40ng/ml) and incubated at 5% CO_2, 92% N_2 and 3% O_2. After 7 days sphere formation could be observed. These spheres were propagated for an additional 2-3 passages by mechanical dissociation using a fire-polished pastuer pipette and replating of cells at a density of 1-2x10^5 cells/cm^2. The medium was changed once a week and growth factors added twice a week.

5.2.4 Co-culture and in vitro differentiation analysis

Differentiation of E14 derived neurospheres under control conditions was initiated by plating spheres on poly-L-ornithine coated tissue culture 12-well plates. Cells were transferred to serum free media (DMEMF12) devoid of growth factors but supplemented with N2 and heparin (10 units per ml). In co-culture assays, differentiation was initiated under identical conditions but in the presence of Millipore cell culture inserts in which MSCs were seeded at a density of 10,000 cells. In this system the MSCs share the same media environment as the NSC/progenitor cells and are separated only by a porous membrane through which diffusible factors can pass.
MSCs or MSC spheroids were seeded into 24mm diameter membrane cell culture inserts (Sigma) and put into six-well culture trays (Nunc). Co-culture of MSCs with adult GFPH cells was carried out under identical conditions to those described above for E14 derived neurospheres.

5.2.5 Bromodeoxyuridine (BrdU) and propidium iodide (PI) incorporation and analysis of proliferation and cell death.

Cell proliferation was accessed by addition of 10μM BrdU (S-phase marker) (Sigma) in the culture medium for a period of 24 hours 0, 1, 2 and 3 days after plating, immediately followed by fixation in 4% PFA and analysis of total cell numbers and BrdU incorporation. For dual staining of BrdU with Tuj-1, fixed cells were incubated with first anti-BrdU antibody (1:400 rat; Accurate) for 1 hour followed by anti-Tuj-1

antibody. Cells were then incubated with 2 N HCL for 30 minutes at 37°C and then washed with sodium perborate solution (50 mM, pH 8.5) and finally incubated with an anti-BrdU antibody for 1 hour at room temperature and then an FITC conjugated anti-rat secondary antibody for 1 hour at room temperature. Cells were washed once with HBSS followed by PBS, incubated for 3 minutes at room temperature for each wash. Cells were subsequently analysed by flow cytometry for quantification of the number of BrdU+. To access cell death samples of NSC/progenitor cells were obtained 0,1,2,3 or 4 days after plating under differentiation conditions. Cells were trypsinized, washed and processed for flow cytometry by forming a single cell suspension and maintained on ice. Cells were stained with 1 μg/ml propidium iodide (PI) (Sigma) just prior to analysis by flow cytometry.

5.2.6 Immunocytochemistry

PBS washed cells were fixed in 4% PFA (in PBS) for 1 hour at room temperature followed by immunocytochemical staining as previously described (Palmer et al., 1999). After fixation, cells were incubated with primary antibodies overnight at 4°C in blocking buffer (5% goat serum, 0.2% Triton X-100 in phosphate buffered saline (PBS)). Then, cells were incubated for 1 hour with secondary antibodies conjugated to fluorescein isothiocyanate or cyanin-3. Primary antibody concentrations used are as follows: rabbit anti-Tuj-1 ([Covance Research Products Inc., Berkley, CA] 1:500), GFAP (Sigma-Aldrich; 1:500), Nestin (Chemicon, 1:100), mouse anti-RIP (Chemicon, 1:100). Mouse anti-smooth muscle actin (Sigma, 1:200). Secondary antibodies were all purchased from Jackson ImmunoResearch laboratories and used at a concentration of 1:250 dilution. All cultures were counterstained with 1μg/ml Hoescht 33342 (Sigma-Aldrch) to visualize individual cells.

5.2.7 Evaluation and quantification of cells with different phenotypes

Labeled cells were cells visualized using an inverted fluorescent microscope (model E660 Nikon) and a CCD camera (Spot RT; diagnostic instruments) with individual filter sets for each channel. Color images were generated using Adobe Photoshop (Adobe systems, mountain view, CA) The mean percentage + standard error of the mean (S.E.M) of immunofluescent cells was determined by counting 15 high power (x20 magnification) visual fields (approx 25 cells/field; 375 cells/slide) systematically

across the slide, visualized under florescence. The total number of cells was determined from Hoescht 33342 positive cells. Data was gathered from 3 independently replicated experiments carried out under identical conditions.

5.2.8 Intracellular staining for flow cytometry

Suspensions of rMSCs (2 x 10^6 cells PBS washed cells) were pelleted in a 12 x 75 mm culture tube. The pellet was re-suspended in 0.875 ml of cold PBS. Then 0.125ml of cold 2% PFA was added and the suspension incubated at 4°C for 1 hour, centrifuged for 5 minutes at 250g, then the supernatant was removed. For permeabilisation cells were incubated with Triton X-100 (0.2% in PBS) at 37°C for 15 minutes. To terminate permeabilisation 1ml of buffer (1xPBS + 2% goat serum + sodium azide) and the suspension was spun for 5 minutes at 250g. The supernatant was removed and internal staining then proceeded as described for immunocytochemistry. Non-specific binding was blocked by incubation with 5% goat serum in PBS for 1 hour at room temperature. A primary isotype matched control was included in each experiment to exclude any background fluoresence. The cells used for this control analysis were fixed, permeabilised under identical conditions to experimental samples. For quantification of cells expressing a given marker, flow cytometry analysis was performed. The specificity of the assay was confirmed by the use of negative controls. Negative cells controls were HEK 233 cells for nestin, Tuj-1 and GFAP and primary rat astrocytes for fibronectin and smooth muscle actin.

5.2.9 Flow cytometry analysis

All samples were maintained on ice prior to analysis. Analysis was performed using a coulter EPICS XL flow cytometer. FITC and Cy-3 were identified by using a 530 and 585 band pass filter respectively, and quantification was completed using CellQuest Software (Becton Dickinson). Ten thousand events were acquired per sample with fluoresence measured on logarithmic scales. Forward and side light scatter gates were set to exclude dead debris and clumps of cells. To calculate the percentage of positive cells, linear gates were set at 0.1%, on samples stained only with secondary antibodies, and expression corresponding to a fluorescence signal exceeding this percentage was measured.

5.2.10 Measurement of neurite outgrowth

N2a cells or E14 progenitor cells were differentiated either alone or in the presence of induced or non-induced MSCs as described above. After 12 days cells were fixed in 4% PFA and processed for immunocytochemical staining with Tuj-1. Cells were viewed using an inverted fluorescent microscope and images acquired using digital camera. Images were taken of 10 non-overlapping visual fields (x10 magnification) for each culture condition and in 3 independent experiments in cells were cultured in parallel. The neurite lengths of every Tuj-1+ cell (10-15 cells/field approx) within each field of view was determined. Neurites exhibited by differentiating neuronal progeny from cultures of E14 neural progenitor cells or serum deprived N2a cells, that were immunopositive for TUJ1 were analysed using ImageJ 1.33 software, a public domain JAVA image processing program (NIH, USA). Pixel scale was set to microns according to image magnification. JPEG files obtained from light or fluorescent microscopy were opened in ImageJ and neurite lengths were measured by tracing along neurites with the freehand line tool then measuring length using the measurement tool.

5.2.11 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism Software version 4. Results were analysed for statistical significance using ANOVA and all error bars are expressed as standard error +/- mean. All data unless stated otherwise is expressed as Mean±SEM. Post hoc analysis was done using Bonferroni corrected planed comparison.

5.3 Results

5.3.1 Isolation of E14 derived NSC/progenitor cells and tri-lineage differentiation potential in vitro.

We first isolated of NSC/progenitor cells from day 14 embryos according to established procedures (Tropepe et al., 1999). There are no known antigens, which specifically define NSCs. Therefore the identity of these isolated cells was confirmed by morphology, formation of neurospheres structures and differentiation into the three principal lineages of the CNS system, i.e. neurons, glia and oligodendrocytes. The

concept of culturing CNS stem cells is a selective one in which most primary CNS differentiated cells are eliminated from culture soon after plating while undifferentiated cells enter a state of active proliferation in response to growth factors EGF and bFGF. Therefore 4 conditions must be satisfied for NSC to remain in propagate in culture and become the predominant cell type. Firstly cells should be cultured at low density (5 x 10^4 cells/cm^2), secondly cells must be cultured in serum free media, thirdly trophic support in form of growth factors should be present and finally the absence of a strong adhesive growth substrate. In initial early cultures most cells attach loosely to the surface of the tissue culture dish and most of them died within 2-3 days. There is at this stage some degree of background differentiation but a small fraction undifferentiated precursors become hypertrophic, round up and begin to proliferate, while remaining attached to the plate (Figure 5.1a). The progeny of the proliferating cells do not migrate away but instead selectively adhere to each other and form spherical clusters. These clusters because of their increasing mass eventually lift off the substrate and float in suspension as structures referred to as neurospheres (Figuure 5.1b,c,d). Cells can be grown as undifferentiated cells in monolayer cultures (Figure 5.1e) and on removal of growth factors will embark on differentiation (Figure 5.1f). It is important to note however, that not all the progeny found in a sphere are stem cells. Only a small fraction between 10-50% retains stem cell properties, the remaining cell undergoes spontaneous differentiation. Stem cell purification is by continual subculture. In the present study, Neurospheres were apparent by 7 days and could be serially passaged in culture (Figure 5.1).

To verify the identity of the stem cells the neurospheres were differentiated under established conditions. NSCs were plated on Laminin coated tissue culture plastic in media devoid of growth factors but supplemented with 0.5% FCS. Cells were plated either as single cells (Figure 5.2b,c,d,e,f) or as neurospheres (Figure 5.2a). After 12 days cells staining positive for Tuj-1 or GFAP or RIP were detected consistent with NSC having a trilineage potential. The number of cells expressing these markers progressively increased from day 3-6 (Figure 5.2e,f).

Culture substrate has been reported to promote differentiation towards specific cell lineages for example Laminin has been reported to promote neuronal differentiation

of adult hippocampal stem cells (Song et al., 2002; Palmer et al., 1997). E14 derived NSC/progenitors were plated on different substrates during differentiation to test the effect of substrate on the cell lineage commitment of these cells (Figure 5.3). Lineage commitment was determined by analyzing the expression of cell type specific markers using monoclonal antibodies: Tuj-1+ (neurons), oligodendrocytes (RIP+) and astrocytes (GFAP+) Tri-lineage differentiation was observed on all substrates tested including, Laminin, PLO and fibronectin. Differentiation on fibronectin and PLO both resulted in lineage commitment within the expected ratios with the majority of cells adopting an astrocytic cell fate (Tropepe et al., 1999). Laminin however, appeared to promote a neuronal cell fate because a higher number of neuronal cells (Tuj-1+) were generated when E14 neurospheres were plated on Laminin coated tissue culture plastic (29.3±3.2%) compared to cells plated on either PLO coated plastic (12.5±3.5%) or Fibronectin coated tissue culture plastic (7.7±3.6%) (Figure 5.3).

5.3.2 Induction of neural antigen expression in rat MSCs.

MSCs have been shown to express neural proteins in culture and following transplantation into the rodent brain (Tondreau et al., 2004; Deng et al., 2006). Although this expression can occur spontaneously in culture and is present at low levels in MSCs maintained under standard serum culture conditions, expression can be induced to increased levels through the culture of these cells under defined conditions (Hermann et al., 2006). Two populations of MSCs have been detected following transplantation into the intact and injured brain, those cells, which express markers of neural cell lineages, and those cells, which are negative for these markers. To investigate whether there are any differences in the interactions of these two populations with NSC/progenitor cells and their differentiated progeny we induced some MSCs to express neural proteins under defined culture conditions whilst others were maintained under standard culture conditions and retained a only a basal level of expression of these proteins (Figure 5.4).

We induced expression of neural proteins in MSCs according to previous reported methodology (Hermann et al., 2004; 2006). MSCs previously maintained under standard culture conditions and expanded until passage 8 were subsequently transferred to serum free media (DMEM F12) supplemented with N2, EGF (10ng/ml)

and FGF (10ng/ml) for 7 days in low attachment culture dishes. Under these conditions, small aggregations of cells were evident within 4-5 days of culture (Figure 5.4a). By 7 days these aggregations form free-floating cellular spheres (Figure 5.4b), which can be harvested and sub-cultured. Immuno-staining followed flow cytometry was used to quantify the expression of neural (Tuj-1, GFAP and nestin) and mesodermal (smooth muscle actin) proteins in induced and non-induced MSCs (Figure 5.4c). The number of positive cells and the mean fluorescence intensity (MFI) of staining were determined for each protein marker. Consistent with previous reports MSCs expanded ex vivo were found to express neural proteins (Tondreau et al., 2004). However, this expression was found to be confined to small subpopulation and of low mean florescence intensity (MFI) (Figure 5.4c; Nestin 0.7% MFI 1.03; Tuj-1 72% MFI 3.03; GFAP 38% MFI 2.41; Smooth Muscle Actin 98% MFI 6.78). In contrast following induction the number of cells positive for neural markers increased, as did the mean florescence intensity of this staining (Figure 5.4c; Nestin 99% MFI 6.02; Tuj-1 96% MFI 5.54; GFAP 98% MFI 6.78; Smooth Muscle Actin 23% 1.43). The expression of the mesodermal marker, smooth muscle actin was found to be down regulated in induced MSCs. The high numbers of cells expressing these markers suggests that there is considerable co-expression of these markers within these cells. Whether or not these cells represent a trans-differentiated phenotype is controversial and is currently under investigation by others and ourselves.

5.3.4 MSC soluble factors influence the cell fate determination of differentiating embryonic NSC/progenitor cells.

We first tested the multi-potency of NS/progenitor cells isolated from day 14 embryos under control conditions. These cells were propagated as neurosphere structures in non-adherent culture conditions according to established procedures (Tropepe et al., 1999). Under these conditions the cells retain their multi-potent stem cell characteristics. First, they give rise to all three principal neural cell types, as defined by cell type specific markers for neurons (Tuj-1+), Oligodendrocytes (RIP+) and astrocytes (GFAP+) in vitro (Figure 5.5 control). Second, undifferentiated cells are positive for nestin, an immature cell marker (97.8$\pm$2.2) (Figure 5.7) but negative for markers of differentiated cell types. Thirdly, undifferentiated cells proliferate in the presence of 10ng/ml EGF and 10ng/ml FGF. To initiate differentiation under control

conditions NSC/progenitor cells were plated on PLO coated tissue culture plastic followed by the withdrawal of growth factors from the medium and the introduction of 0.5% FCS to maintain cell viability upon plating. These conditions induced differentiation in the following proportions: 19.9±4.1 Tuj-1+ (neuronal), 41.4±2.0 GFAP+ (astrocyte) and 7±1 RIP+ (Oligodendrocyte) (Figure 5.5, 5.6) this was consistent with previous reports (Tropepe et al., 1999; Wislet-Gendebien et al., 2004).

We then analysed the effects of MSC derived soluble factors on the cell fate determination of NSC/progenitor cells using an in vitro co-culture system in comparison to progenitor cells cultured under control conditions in parallel cultures (Figure 5.5). NSC/progenitor cells were cultured either alone (control) or co-cultivated in the presence of MSCs (induced or non-induced) under standard differentiation conditions for a period 12 days. Cell phenotype specific markers (as described for control conditions) were used to monitor the lineage commitment of differentiated progeny (Figure 5.5). Quantification of lineage commitment was carried out by image analysis (Figure 5.6). Cells staining positive for GFAP and Tuj-1 (<1% approx) were discounted from the analysis.

In the presence of non-induced MSCs, 6.3±0.8% of NSC/progenitor cells differentiated into neurons compared to 19.9±4.1% of cells under control conditions. Statistical analysis of linage commitment revealed the number of Tuj-1 positive cells (as a percentage of the total cell population) was significantly reduced in co-cultures of NSC/progenitor cells and non-induced MSCs compared to differentiation under control conditions ($P<0.05$, one-way ANOVA with Bonferroni post hoc tests, Figure 5.6) consistent with a reduction in neuronal differentiation under these conditions. However, a significant increase in the percentage of cells NSC/progenitor cells expressing Tuj-1 was observed when cells were differentiated in the presence of induced MSCs (40.1±3.9%) compared to both control conditions (19.9±4.1%, $*P<0.05$ one-way ANOVA, bonferroni post hoc analysis, figure 5.6) and co-cultures of non-induced MSCs ($**P<0.01$). Therefore, differentiation in the presence of induced MSCs is associated with a statistically significant increase neuronal differentiation compared to control conditions and differentiation in the presence of non-induced MSC co-culture.

There was no significant difference in the percentage of GFAP+ cells between NSC/progenitor cells differentiated under control conditions (41.4±2.0%) and those differentiated in the presence of induced MSCs (33.8±2.1%, figure 3). However, co-culture of NSC/progenitor cells in the presence of non-induced MSCs was associated with a significant increase in the number of cells which differentiated into an astrocytic cell phenotype (GFAP+, 68.8±1.8) compared to control conditions (**P<0.01) and cells co-cultured with induced MSCs (**P<0.01, 1-way ANOVA, Bonferroni post hoc analysis, figure 5.6). Staining of cultures with RIP identified oligodrendrocyte differentiation. 5.5±0.7% of NSC/progenitor cells were RIP positive when cells were differentiated in the presence of non-induced MSCs compared with 5.1±0.6% in the presence of induced MSCs and 7±1.1% under control conditions. No significant difference in the number of RIP+ (oligodendrocyte) cells was observed between culture conditions (figure 5.6).

In summary, co-culture of NSC/progenitor cells with non-induced MSCs promoted a predominately astrocytic cell fate (GFAP+) in differentiating cells. In contrast, induced MSCs promoted neurogenic differentiation. This increase in neuronal differentiation occurs in the absence of a significant reduction in the number of RIP+ or GFAP+ cells compared to NSC/progenitor differentiated under control conditions. This indicates that the presence of induced MSCs promotes an increase in the total number of progenitor cells that differentiate compared to control conditions and that this additional differentiation is predominately neuronal.

5.3.5 Temporal expression of cell lineage specific markers in the differentiated progeny of E14 neuro-progenitors cultured in the presence of neural antigen positive MSCs.

The results demonstrate that soluble factors released by MSCs induce particular cell fate decisions in cultured NSC/progenitors. To investigate the potential the mechanism by which this is achieved we first examined the temporal expression of cell type specific markers at 0, 3, 6 and 12 days in differentiating cultures in either the presence of non-induced MSCs (MSC), induced MSCs (induced-MSC) or absence of co-culture (control) (Figure 5.7). The aim of these experiments was to determine

whether the increase in neurogenesis observed in the presence of induced MSCs was due to the selective survival of neuronal progeny from NSCs, the increased proliferation of progenitors, and/or the instructive differentiation of progenitor cells to the neuronal lineage. We first monitored the effects of co-culture on the expression of cell type specific markers in the progeny of NSCs over time (12 days). A significant time dependent decrease in the percentage of cells expressing nestin was observed in all conditions consistent with the differentiation of progenitor cells towards specific cell lineages. No significant difference was observed in the percentage of cells expressing nestin at any time point for any experimental condition.

Analysis of Tuj-1 expression revealed no significant differences in the percentages of Tuj-1 positive cells (neurons) between culture conditions 3 days post initiation of differentiation. By 6 days culture, the number of neurons had reached a maximum in control cultures and co-cultures of non-induced MSCs however; the number of neurons was significantly higher in co-cultures of induced MSCs compared to both control cultures (**$P<0.01$) and co-cultures of non-induced MSCs (**$P<0.01$, 1-way ANOVA and bonferroni post hoc analysis). Therefore the inductive neurogenic effect of induced MSCs was not apparent until 6 days co-culture and kinetics of Tuj-1 expression were comparable with control conditions. Under control conditions, however, the number of neurons at 12 days was significantly reduced compared to 6-day cultures (*$P<0.05$, 1-way ANOVA, Bonferroni post hoc analysis). A slight reduction in the percentage of TUJ-1 positive cells was observed in non-induced MSC co-cultures at day 12 compared to day 6, however this was not statistically significant. In contrast no reduction in neuron number was evident in co-cultures of NSC/progenitor cells with induced MSCs between 6 and 12 days. At day 12 the percentage of Tuj-1 positive cells in co-cultures of NSC/progenitor cells and induced MSCs was significantly higher (**$P<0.01$) than under control conditions or co-culture with non-induced MSCs. These results indicate that in addition to their inductive effects, the release of soluble factors by MSCs has a permissive effect on the long-term culture of differentiated neurons.

Analysis of GFAP expression was used as a marker of the number of astrocytic differentiation in the culture. Co-culture with induced MSCs did not significantly

affect the kinetics of GFAP expression with levels reaching a maximum by 6 days and remaining stable in long-term cultures (12 days). This observation was consistent with differentiation under control conditions and there was no significant difference in the level of expression of GFAP in control cultures compared to NSC/progenitor cells cultured in the presence of induced MSCs. Therefore soluble factors released by induced MSCs had no effect on astrocytic differentiation. In the presence of non-induced MSCs the number of astrocytes (GFAP+ cells) was significantly increased at day 6 (*$P<0.05$) and day 12 (***$P<0.001$, 2-way ANOVA, Bonferroni post hoc analysis), post differentiation when cultures were compared with control conditions and co-culture of NSC/progenitor cells with induced MSCs. The expression of GFAP in co-cultures of non-induced MSCs was similar to that observed in control conditions with the number of GFAP+ cells reaching a maximum by day 6 and remaining stable in long term cultures. There was an increase in the percentage of GFAP+ between day 6 and day 12-post differentiation, however this was not statistically significant.

Examining the expression of RIP accessed oligodendrocyte differentiation. The percentage of RIP positive cells increased from day 0 to day 12-post differentiation for all culture conditions. No significant difference in the percentage of RIP positive cells was observed following co-culture with either induced or non-induced MSCs.

5.3.6 MSCs provide instructive signals that regulate the cell fate determination of NSC/progenitor cells in culture

To determine whether the proliferation of progenitors might contribute to the observed increase in neurogenesis, 2.5 μM BrdU was added to parallel cultures for 12 hours at 0, 24, 48, 72 and 96 hours after plating, followed by fixation and quantification of total cell numbers and the percentage of BrdU+ cells. The number of BrdU+ cells was counted at each time point. Under control conditions the number of proliferating cells (BrdU+) progressively decreased over 4 days from 83.3 ± 1.3 at day 0 to 6.1 ± 2.0 at day 4 (Figure 5.8a). Similar kinetics of BrdU staining was observed for co-culture conditions. This pattern is consistent with the commitment of undifferentiated proliferating progenitor cells in differentiated cell phenotypes. No significant difference in the percentage of proliferating cells in each culture condition (control or co-culture with induced or non-induced MSCs) was observed. These

results indicate therefore that the neither the presence of neural antigen positive or neural antigen negative MSCs had any significant effect on the number of proliferating cells between 1 and 4 days.

Another possible mechanism by which the presence of MSCs may exert an influence on the percentage of cells of a particular cell lineage may be by selection through cell death. To examine this potential mechanism, the number of cells that incorporated the fluorescent exclusion dye propidium iodide (PI) was calculated. PI is excluded from healthy cells but enters dead cells following their loss of membrane integrity. It binds to exposed DNA and becomes highly fluorescent. This fluorescence was examined by flow cytometry, which determined the number of cells, which had incorporated PI and was therefore a measure of cell death. There was no significant difference in the numbers of PI positive cells between treatment groups at each time point over 4 days (Figure 5.8b). Therefore selection by cell death is unlikely to be the mechanism by which MSC derived soluble factors result in increased numbers of cells committed to particular cell lineages. A higher amount of cell death was observed on day 1 in all culture conditions as a result of the cell death associated with plating and induction of differentiation.

Cell death was investigated at intervals of 3, 6 and 12 days in differentiating NSC/progenitor cells cultured either alone (control) or in the presence of induced MSCs (MSC-induced) or non-induced MSCs (MSC). The number of the Tuj-1 positive cells that also stained positive for PI expressed as a percentage of the total Tuj-1+ cell population is shown in figure 5.8c for all culture conditions. This staining procedure allowed us to selectively examine cell death in neuronal cell progeny (Tuj-1+ cells). There was no significant difference in cell death in Tuj-1 positive cells at 3 or 6 day intervals as determined by flow cytometry following dual labeling of cells with Tuj-1 and PI. At 12 days, cell death in control cultures was significantly higher than that observed at 3 and 6-day intervals (*$P<0.05$, 2-way ANOVA, Bonferroni post hoc analysis) but in co-cultures of progenitor cells and induced or non-induced MSCs this increase in cell death was not observed. These results demonstrate that soluble factors released from MSCs promote neuronal cell survival in long-term cultures probably as a result of providing additional trophic support.

The induction of neurogenesis was evident from day 1 in both control cultures and co-cultures of induced MSCs with NSC/progenitor cells (Figure 5.8d). However, the significant increase in Tuj-1+ cells in co-cultures of induced MSCs and progenitor cells ($P<0.05$ compared to control cultures at 4-day time point, 2-way ANOVA, Bonferroni post hoc analysis) was not evident until 4 days, in which the numbers of Tuj-1+ cells were significantly increased compared to control cells. The observation that the increase in neurogenesis was evident in early (4 day) cultures and no evidence of increased cell death or proliferation of progenitors was found is consistent with MSCs soluble factors providing instructive signals rather than selective signals, which regulate the cell fate decisions of NSC/progenitor cells.

5.3.7 Soluble factors released by MSCs instruct an oligodendrocytic cell fate on adult NSCs isolated from the hippocampus

Following the observation that MSCs induced to express neural proteins can promote a neurogenic cell fate decision on differentiating embryonic NSC/progenitor cells, we next tested if the same effect could be observed in adult NSC/progenitor cells isolated from the hippocampus. Adult GFP+ hippocampal stem cells were differentiated under identical conditions to E14 derived neurospheres and lineage commitment was evaluated using cell type specific markers (Figure 5.9). Adult progenitor cells differentiated under control conditions adopted the following cell fates: $16.9\pm0.9\%$ neurons (Tuj-1+), $49.2\pm11.2\%$ astrocytes (GFAP+) and $2.9\pm0.8\%$ oligodendrocytes (RIP+) (Figure 5.9e). In the presence of induced or non-induced MSCs, neuronal commitment of differentiating adult hippocampal progenitor/stem cells was unaffected, with no significant difference in the number of Tuj-1+ generated during co-culture. Following co-cultivation with non-induced MSCs, the number of oligodendrocytes generated in differentiating stem cell cultures was increased to 26.6+6.1% significantly ($P<0.01$, 1-way ANOVA, Dunnetts post hoc analysis) higher than that observed in control conditions. The number of astrocytes (37.6 ± 5.1) and neurons (7.6 ± 2.8) has not significantly different from control conditions. This indicates that co-cultivation of adult stem/progenitor cells with non-induced MSCs promoted more progenitor cells to differentiate overall and the predominant phenotype was oligodendrocytic, however differentiation at the expense of other cell

fates was not observed. Therefore the additional differentiation was directed to an oligodendrocytes cell fate. Differentiation of adult stem/progenitor cells in the presence of induced MSCs resulted in a significant reduction in the number of cells differentiating into astrocytes and a trend towards an increase in oligodendrocytes.

5.3.8 Effect of MSC co-culture on the proliferation and viability of adult NSC/progenitor cells

Consistent with the results for E14 derived neurosphere differentiation, analysis of BrdU incorporation in the first 4 days revealed no significant difference in BrdU labeling between adult stem/progenitor cells differentiated in the presence or absence of non-induced or induced MSCs (Figure 5.10a). Also no significant difference was observed in cell death in cultures co-cultivated with MSCs or differentiated under control conditions (Figure 5.10b). Therefore effects of differentiation observed following co-cultivation such as increased commitment to the oligodendrocytes cell lineage is instructive and not selective.

5.3.9 Soluble factors released by MSCs promote the neurite outgrowth of differentiating neuronal progeny of NSC/progenitor cells

MSCs have been reported to promote axonal and neurite outgrowth of neuronal cell populations. As reconstruction of neural circuitry will be vital to promote recovery from CNS injury we investigated whether the soluble interactions of MSCs were extended to include effects on the differentiated progeny of progenitor cells. NSC/progenitor cells were first differentiated under control conditions to establish a baseline to compare the effects of MSC co-culture. Differentiated cultures were fixed and labeled with Tuj-1 at the specified time point in order to identify neuronal progeny.

We found that 12 days culture under differentiation conditions was sufficient to induce modest neurite outgrowth in Tuj-1+ cell progeny of progenitor cells (Figure 5.11). The average neurite length under control conditions was 22.06$\pm$2.73 μm and the average maximum neurite length with any one field of view was 212$\pm$68.64 μm. To determine if MSCs promote neuritogenesis in primary neurons described from NSC/progenitor cells we co-cultivated MSCs (induced and non-induced) in the

presence of NSC/progenitor from the onset differentiation. The average neurite length of neuronal progeny as defined by Tuj-1 was determined under each culture condition following image analysis of cells within 10 random fields of view in 3 independent experiments. The average neurite length of Tuj-1+ cells co-cultivated in the presence of non-induced MSCs was 38.63±4.2 μm and significantly increased compared to controls. Co-cultivation with induced MSCs also promoted a significant increase in neurite outgrowth compared to control it which the average neurite length of neuronal progeny was 57.48±5.7 μm. Therefore soluble factors released from MSCs promote neurite outgrowth of Tuj-1+ cell progeny and this effect was greater when cells were co-cultivated with induced MSCs. Both bipolar and multipolar neurons were observed under all culture conditions. Consistent with these observations the maximum neurite length (Mean±SEM) was significantly higher in so-cultures of progenitor cell and induced MSCs. Examples of neurite outgrowth under these co-cultures are illustrated in Figures 5.11 d-f which show both multipolar (d) and unipolar (e,f) neurons.

To test whether the induction of neurite outgrowth was the result of a direct action of MSC soluble factors or as a result of MSC induced activation of astrocytes, which subsequently release growth factors that promote neurite growth the effect of MSCs on the neurite outgrowth of differentiating N2a cells, was examined. N2a cells differentiate exclusively into neuronal progeny upon withdrawal of serum from the culture. Under these conditions modest levels of sprouting and neurite extension were observed by 12 days serum deprivation (Figure 5.12). Consistent with our previous observation with E14 neural progenitor cells, co-cultivation of differentiating N2a cells with MSCs significantly increased the average neurite length in both non-induced MSC co-cultures (22.34±1.94 μm) and induced MSC co-cultures (32.04±2.54 μm) compared to control (11.63±2.76 μm) conditions. Induction of neurite outgrowth was also significantly greater when N2a cells were co-cultivated with induced MSCs. The maximum neurite length in co-cultures of induced MSCs and N2a cells (650±70.50 μm) was significantly increased compared to control conditions (149.90±45.41 μm). Therefore a consistent pattern of the effects of MSC derived soluble factors on neurite outgrowth was observed in neuroblastoma cells.

5.4 Discussion

Infusion of MSCs is associated with improved functional recovery following CNS lesions in several animal models (For Review see Chopp and Li, 2002; Corti et al., 2003). The exact mechanism by which this therapeutic benefit is exerted is uncertain. MSCs have been shown to promote a number of activities within the adult CNS that improve neurological outcome following injury which include; angiogenesis (Hamano et al., 2000; Chen et al., 2001; Chen et al., 2003) neurogenesis (Li et al., 2002; Zhang et al., 2001), synaptogenesis, dendritic arborisation (Chopp et al., 1999; Zhang et al., 2006) and a selective reduction in apoptosis in ischemic boundary zone of a focal lesion (neuro-protection) (Chen et al., 2003). These effects on brain plasticity are mediated primarily by the release of cytokines and growth factors produced by MSCs, which activate endogenous restorative and possibly regenerative processes within the host brain (Li et al., 2002). Current work suggests that the therapeutic benefits of MSCs are not attributed to a single modification of brain plasticity or the effects of a single cytokine but the synergistic and cumulative action of several factors, which act to improve restoration of function.

Whilst the early effects of trans-planted MSCs are likely to result primarily from neuro-protective activities such as a reduction in cell death (Chen et al., 2003), the long-term restoration of function requires reconstruction of neural circuitry and replacement of lost or damaged neurons. It has also been proposed that MSCs have an intrinsic neurogenic potential and can differentiate to neural cell phenotypes both in vitro (Sanchez-Ramos et al., 2000; Deng et al., 2001; Kobas et al., 2002; Tondreau et al., 2004) and in vivo (Nakano et al., 2001; Hofstetter et al., 2002; Brazelton et al., 2000) and therefore may replace damaged CNS tissue by a process of trans-differentiation. These findings however, remain controversial and have been challenged by a number of recent publications offering alternative explanations for such unexpected cell fate changes including cell fusion (Alvarez-Dolado et al., 2003). Histological analysis in transplantation studies has revealed a small proportion of MSCs express neural markers (Chopp et al., 2000; Chen et al., 2002; Chen et al., 2003) but here is no evidence as yet, that these cells either fully differentiate into

neural cells or integrate into the host CNS. In addition, the proportion of cells which express these markers are too few to fully compensate for loss of function. The function of these cells is therefore poorly understood. Direct replacement of neural tissue by MSCs is therefore unlikely be the mechanism by which these exert their beneficial effects.

Recent experimental evidence from transplant studies has indicated an amplification of the endogenous neurogenic response to injury in MSC treated animals (Chen et al., 2002; Chen et al., 2001; Mahmood et al., 2005; Chen et al., 2004), suggesting that one therapeutic benefit of MSCs may be to promote the formation of new neurons in the adult brain. There is now consensus that neurogenesis occurs within discrete regions of the adult brain; including the subventricular zone lining the lateral ventricles, the subgranular zone in the dentate gyrus of the hippocampus and the olfactory bulb (Gage 2000; Gage et al., 1998). NSCs in these regions have the capacity to generate mature functional neurons (Song et al., 2002; Van Praag ey al., 2002). Following neurogenesis, the differentiated cell progeny can survive and contribute to tissue repair. Increased proliferation of progenitors in neurogenic regions has been observed following injury consistent with an increase in cell progeny expressing immature neuronal markers and an increase in the progenitor cell marker nestin at the periphery of the injury site (Zhang et al., 2001; Li et al., 2002). Endogenous NSC differentiate and proliferate into neurons at the foci of a lesion and stem cells distal to an ischemic lesion proliferate and migrate chemotactically to the infarct and differentiate into viable neurons (Nakatomi et al., 2002).

We used an in vitro co-culture system in which NSC/progenitor cells and MSCs were cultured in the same environment separated only by a Millipore porous membrane, (cell culture inserts) to specifically examine the soluble interactions of MSCs with NSC/progenitor cells isolated from the embryonic striatum. The advantages of this system are that live cells can be co-cultured and this allows intercellular signaling to operate between these two cell populations. This is crucial since in vivo these cells operate in such an environment in which they can respond to each other through the production of soluble factors. Therefore the effects of MSCs on NSCs must be the

result of the production of soluble factors by virtue of the system used to evaluate these effects.

In this paper we demonstrate that MSCs can stimulate the differentiation of multi-potent neural progenitors into specific cell fates by the release of soluble factors. These soluble factors provide instructive rather than selective regulation of cell fate commitment on NSC/progenitor cells as demonstrated by the fact that these factors had no effect on the proliferation of progenitors or cell death in short term cultures. More importantly we found that the nature of this signaling was distinctly different in MSCs induced to express high levels of neural proteins. Induced MSCs released factors that predominately directed a neurogenic cell fate decision on neural progenitor cells whereas non-induced MSCs instructed a predominately astrocytic cell fate decision. The conclusion from these observations is that MSCs provide instructive signals that regulate the cell lineage commitment of embryonic striatal NSC/progenitor cells, but that the nature of these signals is dependent on the developmental status of the MSCs.

Following the demonstration that co-cultivation of MSCs with embryonic NSC/progenitor cells increased neurogenesis of these cells we next examined the ability of MSC derived soluble factors to promote the neuronal differentiation of adult neural stem cells. A recent study showed that MSCs increased number of differentiating adult NSCs adopting an oligodendrocyte cell fate (Rivera et al., 2006). This observation is consistent with those findings of our studies. In the above study both MSCs and NSCs were maintained in 10% FCS even during differentiation, in contrast to our own experiments in which serum concentration was only 0.5% during differentiation. In the presence of induced MSCs a significant reduction in the number of astroicytes demonstrated was observed and the number of oligodendrocytes in differentiating cultures was increased, however in contrast to the observation of E14 derived NSCs no significant difference in neuronal commitment was observed. The reason why induced MSCs promote neurogenesis of E14 derived neurospheres and not adult NSC/progenitor cells is currently unknown. This effect may be specific to adult NSC isolated from the hippocampus as regional differences in differentiation potential of progenitor cells has been reported (Palmer et al., 1999; Kondo and Raff,

2000). Therefore to investigate this other stem cell populations from different brain regions need to be examined to determine their differentiation potential in the presence of induced and on-induced MSCs. It is also possible that these effects are the result of differences in the expression of different receptor types and densities and therefore the sensitivities of these cells to different neurotrophic factors.

We also observed that MSC derived soluble factors affect other aspects of neuronal plasticity and provide trophic support that maintains the long-term viability of NSC derived neuronal (Tuj-1+) progeny. We report for the first time that MSCs promote neurite outgrowth of the neuronal progeny (Tuj-1+) of neural progenitor cells. MSCs have previously been shown to promote axonal growth and neurite development in other neuronal cell populations both in vitro (Li et al., 2006; Tohill et al., 2004) and following injury in vivo (Hofstetter et al., 2002). The reduction in neuronal cell death observed in our long-term co-cultures is consistent with the reduction of cell death recently observed when neuroblastoma cell lines were induced to differentiate in the presence of MSCs (Crigler et al., 2006) and the increased survival of the differentiated progeny of endogenous progenitor cells in the intact brain (Munoz., 2005).

We are currently investigating the identity of the soluble factors released by MSCs that mediate their interactions with both progenitor cells and mature differentiated progeny. Identification of these factors may allow us to selectively direct the differentiation of NSC/progenitor into particular cell lineages. However, as described previously these effects are unlikely to be attributed to any single factor. MSCs have been shown to produce a variety of cytokines and growth factors the properties of which are influenced by the surrounding microenvironment (Chen et al., 2001c). MSCs have been shown to release VEGF, BDNF and NGF in response to conditioning with injured rat brain extracts (Chen et al., 2005). MSCs have been found to express bFGF, NGF, BDNF and their Trk A, Trk B receptor mRNAs (Garcia et al., 2004; Yaghoobi et al., 2006). A generalized up-regulation in the expression of these growth factors has been reported in the ipsilateral hemisphere of a cerebral lesion following transplantation of MSCs (Li et al., 2001).

Each of these growth factors has been shown to have independent effects on brain plasticity. Neurotrophic factors have a well-established role in survival, differentiation and function of CNS neurons. BDNF and NGF promote cell survival in the injured CNS tissue both in vivo and in vitro (Hefti, 1986; Kromer, 1987; Koliatsos et al., 1993; Bullock et al., 1999; Gage 2000). bFGF administered within hours of an ischemic insult reduces the infarcat size through a reduction in cell death in the penumbra region (Ay et al., 1999). VEGF can promote both angiogenesis and neural repair, including promotion of axonal outgrowth and nerve cell survival (Hamano et al., 2000; Jin et al., 2003; Papavassiliou et al., 1997; Zhang et al., 2000). Over expression of BDNF in MSCs results in axonal growth at sites of spinal cord injury (Lu et al., 2005). The effects of MSC co-culture is this study may be explained by the activities of such factors including increased cell survival and neurite outgrowth.

Several endogenous growth factors have been shown to increase within the brain in response to injury including bFGF (Hayashi et al., 1997; Gottlieb et al., 1999; Lin et al., 1997). bFGF has been shown to stimulate SVZ and hippocampal neurogenesis in vitro and in vivo (Palmer et al., 1999; Liu et al., 1994). MSCs enhance bFGF expression in the ischemic boundary zone and therefore may promote neurogenesis at the injury site by diffusible activity (Chen et al., 2003). Transplantation of MSCs into the ipsilateral hemisphere is associated also with increased expression of NGF and BDNF (Chen et al., 2002). BDNF promotes survival of neuronal progeny and NGF promotes regeneration of neural tissue (DeKosky et al., 1994; Dixon et al., 1997; Goss et al., 1998; Mocchetti and Wrathall, 1995). Therefore the up-regulation in the expression of these factors in CNS tissue following MSC transplantation may promote endogenous repair and regeneration.

Less is known however, as to how such soluble factors might affect cell fate decisions of resident neural progenitor cells. NSC/progenitor cells are known to respond to trophic influences from the microenvironment and neurogenesis in the adult brain is largely mediated by trophic influences (Song et al., 2002). bFGF is known to be important for the self renewal and maintenance of the stem cell pool (Zheng et al., 2004). CNTF is known to regulate neurogenesis in the adult mouse brain as well as EGF (Emsley and Hagg, 2003). NGF has been associated with regulation of neuron

number and BDNF is thought to play a key role in the maturation of neuronal progeny (Mattson et al., 2004; Louissaint et al., 2002). IGF-1 has recently been shown to induce an oligodendrocytic cell fate in vitro (Hsieh et al., 2004). Retinoic acid has also been found to be crucial in the early commitment of progenitor cells to the neuronal lineage (Takahashi et al., 1999).

The soluble factors released by MSCs may act directly on endogenous cell progeny or via the activation of astrocytes. In the present study we observed that astrocytes present in co-cultures displayed morphology consistent with activation (process bearing). Therefore it is possible that this activation may have played a role in the effects of MSCs. We were able to show that the effects of MSCs on neurite outgrowth were directly the result of MSC derived soluble factors as this effect was seen even in the absence of glial cells when MSCs were co-cultured with differentiating neuroblastoma cell lines (N2a cells). Although we cannot directly evaluate the role of MSC induced glial cell activation on progenitor cell fate, it is known that astroglia promote neurogenesis (Song et al., 2002). It is possible that MSC expressing neural antigens promote neurogenesiss from NSC/progenitor cells in part through the activation of glial cells.

The findings reported in this in vitro study draw many parallels with the effects of MSCs in recent transplant studies (Munoz et al., 2005; Chen et al., 2002). Although many of the effects of transplanted MSCs in vivo have been attributed to diffusible activity, in vivo data cannot be conclusive. A small number of investigations in vitro using either live cell co-culture or conditioned medium support a role of soluble factors released from MSCs affecting the behavior of NSC/progenitor cells and there progeny. We were able to demonstrate conclusively that these effects were mediated by soluble factors and not through the differentiation of MSCs or direct cell contact effects. We were able to show that MSCs provide instructive signals that regulate cell fate commitment of endogenous progenitor cells and that the nature of these signals is dependent on the developmental status of MSCs.

A small number of recent in vitro studies have investigated the possible interactions between MSCs and NSCs. Conditioned media prepared from adult MSCs cultured

under standard conditions has been shown to induce an oligodendrocytic cell fate on NSC/progenitor cells isolated from the adult hippocampus at the expense of astrocytic differentiation (Rivera et al., 2006). Conditioned media obtained from the culture of nestin positive cells has been shown to induce an astrocytic cell fate on NSC isolated from the embryonic striatum, an effect in part mediated by the release of BMP-4 (Wislet-Gendebien et al., 2004). In direct cell contact assays MSCs were shown to induce a neuronal cell fate in mesencephalic NSCs (Lou et al., 2003). The variation in these findings is likely to be attributable to a series of technical differences in the design of these studies and differences in experimental conditions. For example, the use of conditioned media is not always advantageous as some intercellular signaling may be mediated only by short-lived peptides, which are not stable in media. Co-culture of MSCs with conditioned media prepared from different CNS tissue regions induces a neural cell morphology and protein expression profile in MSCs consistent with a neuronal cell fate (Rivera et al., 2006b). The effect is greater in conditioned media prepared from neurogenic regions such as the hippocampus. This experimental data provides further evidence for the operation of two-way signaling between these populations. These reports suggest that the nature signaling mediated by diffusible factors between neural tissue and MSCs signaling may be regionally dependent. Therefore the interactions between the host brain and transplanted MSCs are likely to be complex and multidimensional and as of yet the exact nature of many of the factors, which mediate these interactions, is unknown.

The results indicate a role for neural antigen positive MSCs in promoting neurogenesis at the injury site. The findings suggest that the function of MSCs expressing neural markers in vivo following transplantation into the injured brain may be to provide trophic support and instructive signals that promote endogenous neurogenesis from resident NSC/progenitor cells and survival signals that maintain long term viability of neuronal progeny. In addition, the induction of extensive neurite outgrowth observed following co-culture with both populations of MSCs (neural antigen positive and neural antigen negative) will support the reconstruction of neural circuitry.

The inhibitory environment of surrounding neural tissue limits the neurogenic response to injury in the adult brain, trophic support by MSCs as demonstrated by the current study may provide growth permissive conditions for endogenous neurogenesis and axonal growth and development. Whilst the contribution that these events play to the overall restoration of function following injury remain uncertain, these activities will be undoubtedly be important aspects of MSC induced recovery from CNS injury. In conclusion we demonstrate that MSCs are capable of considerable interaction with NSC/progenitor cells and that the nature of these interactions is defined not only by the microenvironment but also the developmental status of MSCs themselves.

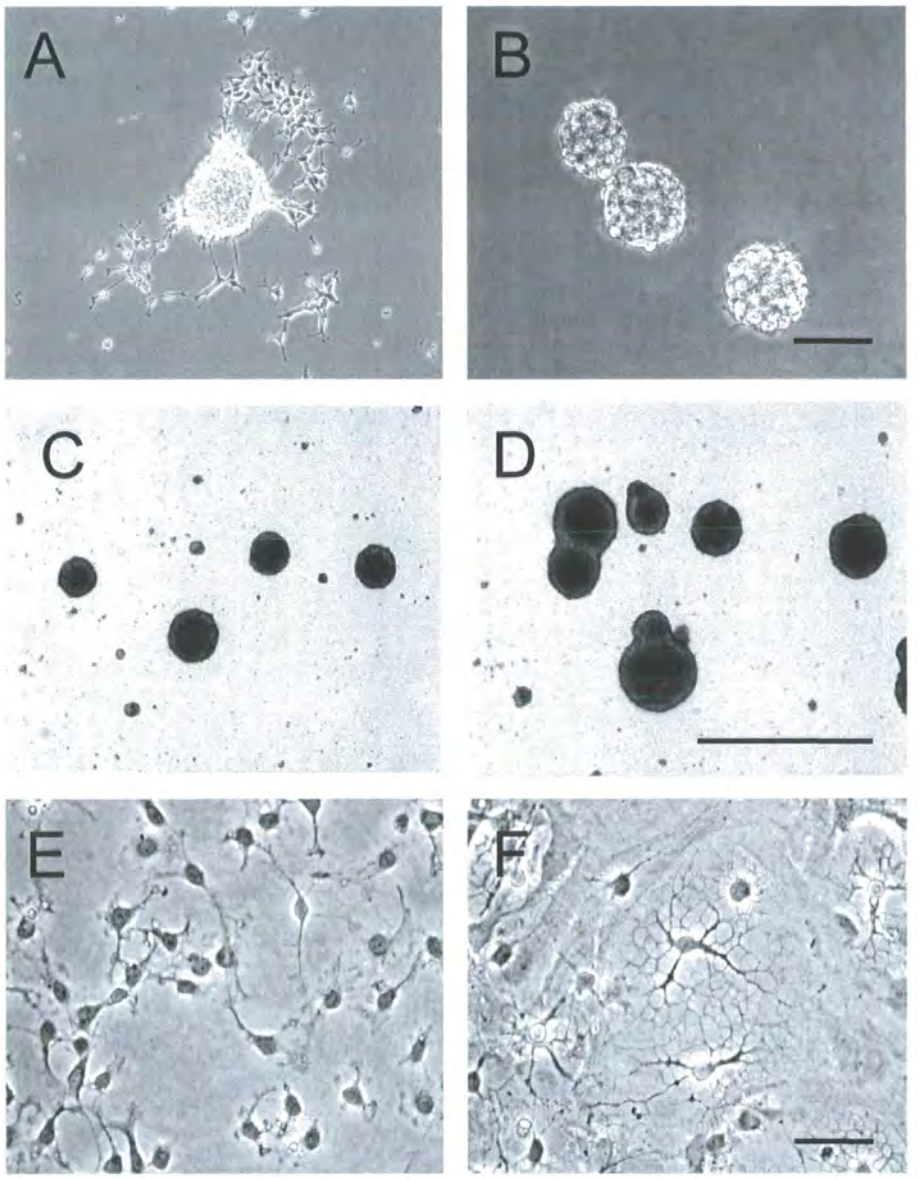


Figure 5.1: Isolation and characterisation of embryonic rat NSC/progenitor cells. NSC/progenitor cells were derived from the mesencephalon of day 14 rat embryos according to established procedures. Single phase bright cells proliferate and give rise to small spheres 7 days post isolation (**A**). Secondary neurospheres 7 days after sub-culturing were highly hypertrophic, with phase bright cells within the sphere (**B**). Secondary neurospheres were propagated in culture by serial passage, without loss of differentiation potential (**C, D**). NSC/progenitor cells were grown as a monolayer culture by seeding dissociated cells on fibronectin coated glass cover slips in the continual presence of growth factors (EGF + FGF) (**E**). Removal of growth factors for 12 days resulted in the differentiation of progenitor cells into mature neural cells (**F**). *Scale bars 50 μm.*

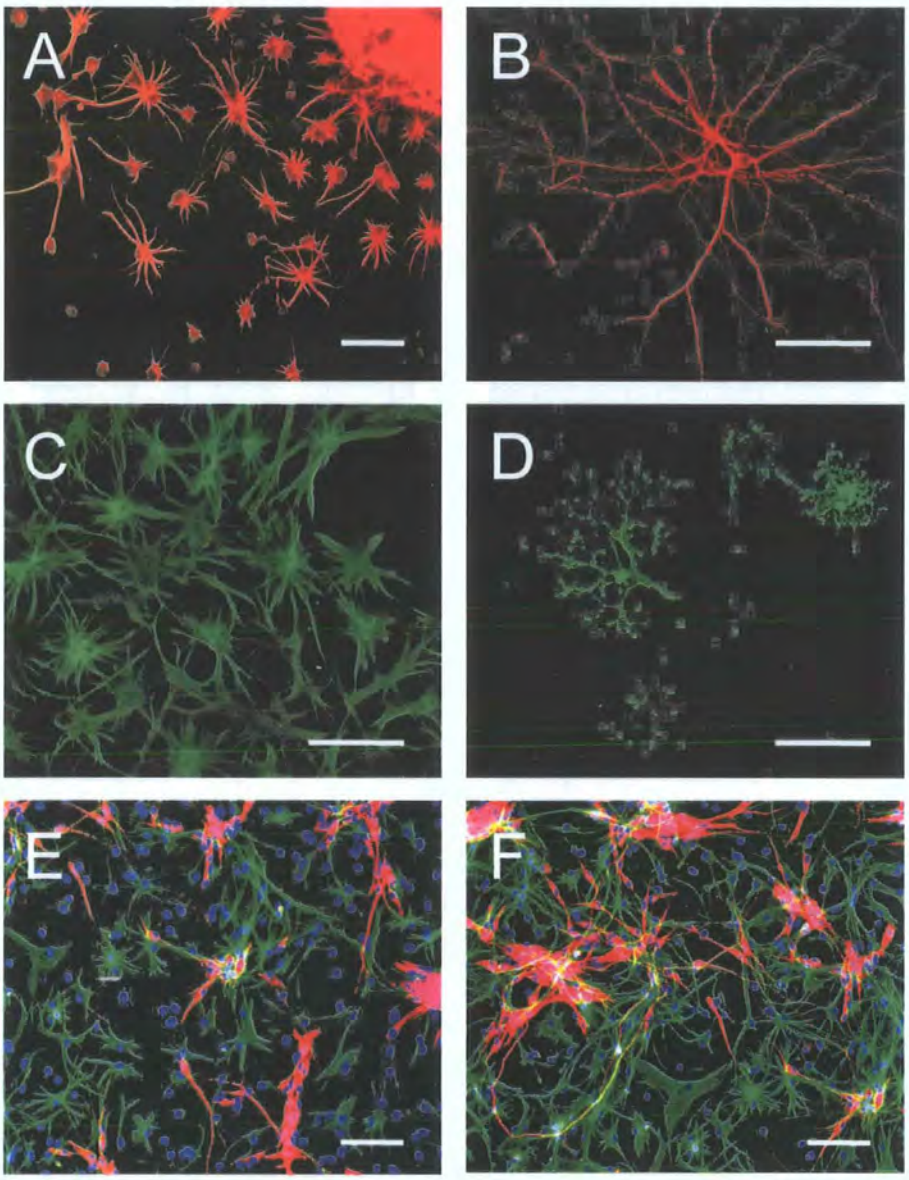


Figure 5.2: Multipotent differentiation of embryonic rat neurospheres. NSC/progenitor cells isolated from the embryonic rat mesencephalon displayed tri-lineage differentiation potential. Passage 2 neurospheres were differentiated by plating cells on adherent surfaces (Laminin coated tissue culture plastic) in the absence of growth factors. (**A**) 24 hours after plating, dissociated neurospheres expressed high levels (>98%) of the immature marker nestin as they migrate from the periphery of the sphere. After 12 days incubation in differentiation medium, cells were fixed in 4% PFA and stained for markers of neurons (Tuj-1) (**B**), astrocytes (GFAP) (**C**) and oligodendrocytes (RIP) (**D**). The number of cells expressing markers (GFAP + Tuj-1dual staining) of mature differentiated cells increased from day 3 (**E**) to day 6 of differentiation (**F**). *Scale bars 50 μm.*

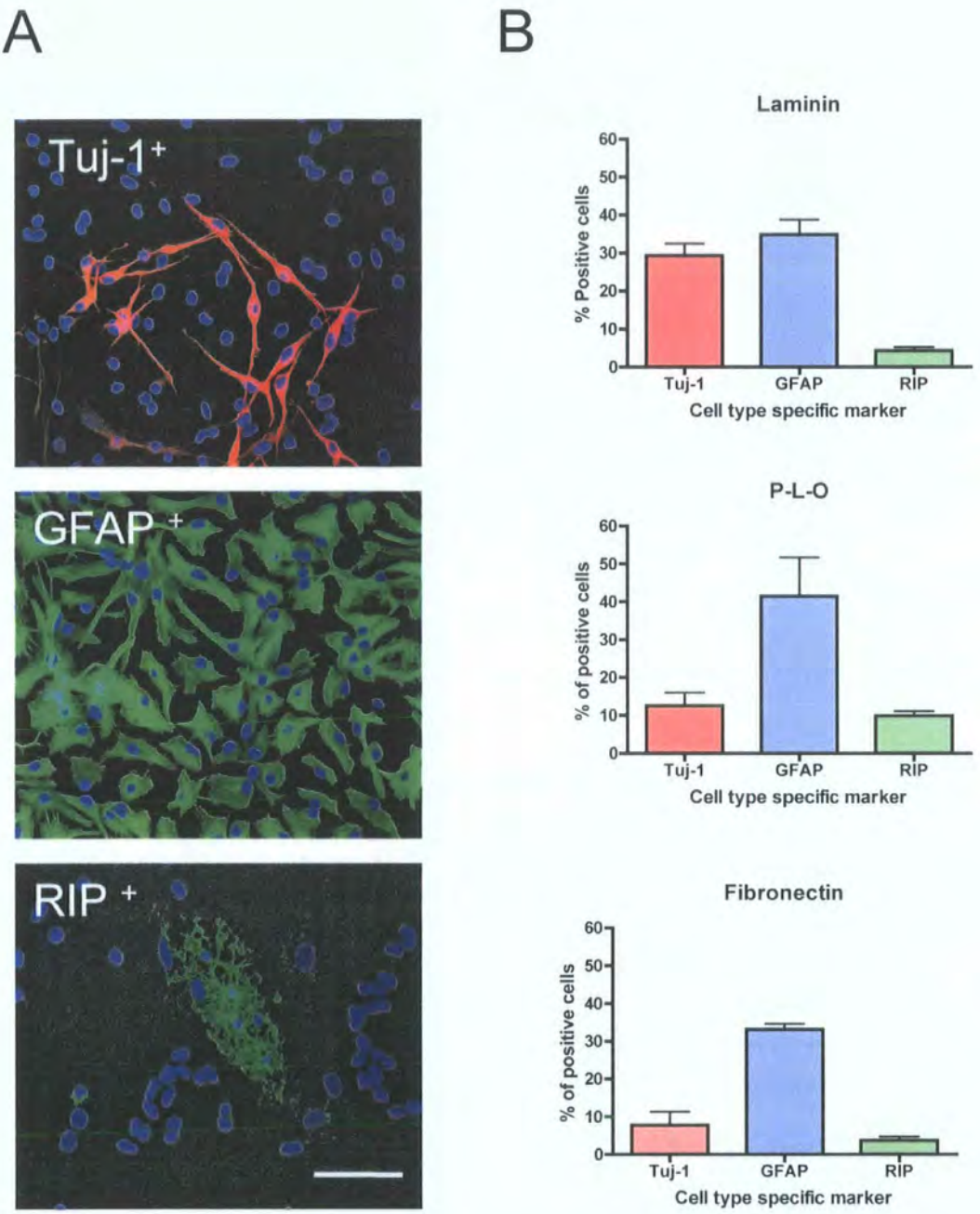


Figure 5.3: Effect of surface substrate on the differentiation of rat E14 derived NSC/progenitor cells. Embryonic NSC/progenitor cells were differentiated by plating cells on either Laminin, PLO or fibronectin coated tissue culture plastic dishes in N2 supplemented DMEM/F12 in the absence of growth factors and presence of 0.5% FCS. (**A**) Cells were stained for markers of neurons (Tuj-1, red), astrocytes (GFAP, green) and oligodendrocytes (RIP, green) and counterstained with Hoechst 33342 (blue). *Scale bars 50 μm.* (**B**) Quantification of cell types in differentiating conditions. All data are mean values±SEM acquired from the analysis of 15 non-overlapping fields of view and from 3 independent experiments in parallel cultures.

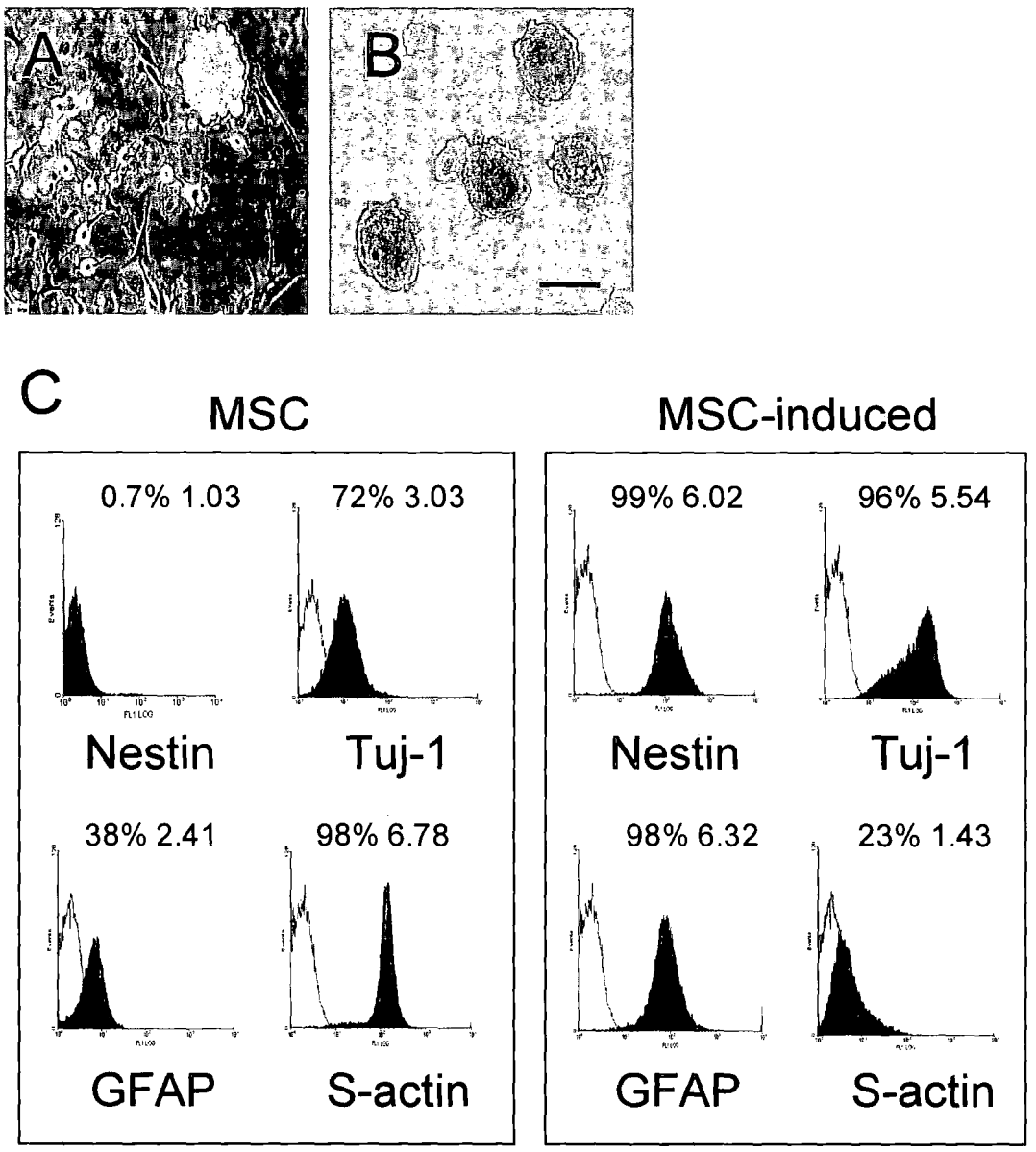


Figure 5.4: Generation of MSC cellular spheres positive for neural antigens following transfer to serum free media supplemented with EGF (10ng/ml) and FGF (10ng/ml). Photomicrographs show the presence of small aggregations of cells, which begin to develop on the bottom of the culture flask within 3-5 days (**A**). Free-floating spheres were evident within 7-10 days of culture (**B**). *Scale bars 50* μm. Flow cytometric analysis of neural and mesodermal cell markers (**C**). P8 MSCs were cultured under standard (**control**) or inductive (**induced**) cell culture conditions for 7 days. Cells were subsequently fixed, permeabilised and labelled with antibodies directed against intracellular neural (Tuj-1, GFAP and nestin) and mesodermal (smooth muscle actin, (S-actin)) antigens. FITC/Cy-3 conjugated secondary antibodies detected binding of the primary antibodies. The open peaks indicate IgG isotype control corresponding to the antibodies in which they were generated. The solid peaks indicate are counts of the cell population that is positive for the antibody indicated in each individual histogram. The number of positive cells is shown on the y-axis and the fluorescence intensity of staining on the x-axis.

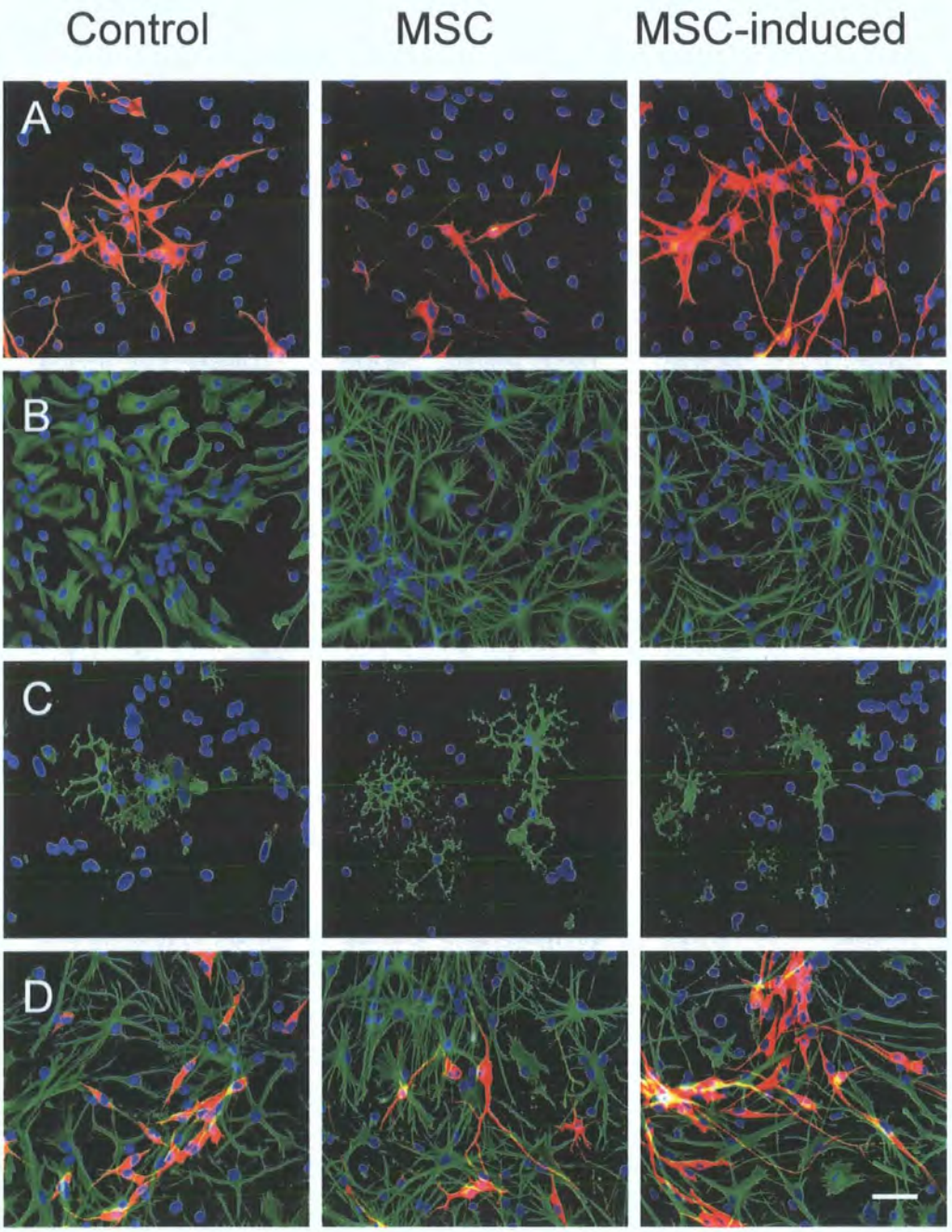


Figure 5.5: MSC soluble factors promote the differentiation of embryonic NSC/progenitor cells into distinct cell lineages. To induce multi-lineage differentiation under control conditions, progenitor cells were plated on poly-L-ornithine-coated dishes in the absence of growth factors and the presence of 0.5% FCS (control). For co-cultivation, differentiation was initiated under identical conditions but in the presence of induced or non-induced MSCs. After 12 days, cells were fixed and immuno-stained with monoclonal antibodies directed against Tuj-1 (**A**, neuron), GFAP (**B**, astrocyte) or RIP (**C**, oligodendrocyte). Some cultures were dual stained for GFAP (**D**, green) and Tuj-1 (**D**, red). All cells were counterstained with Hoechst 33342 (blue). *Scale bars: 50μm.*

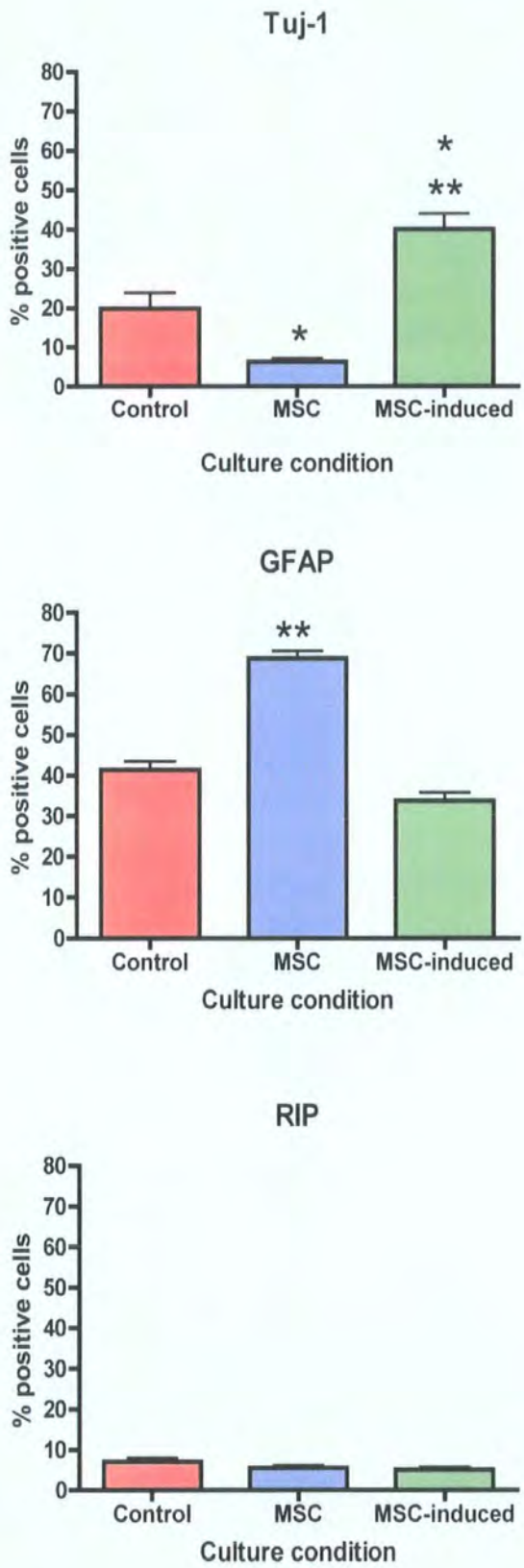


Figure 5.6: Quantification of cellular differentiation under co-culture conditions. The % of cells positive for each cell type marker (Tuj-1, GFAP and RIP) was determined for each culture condition (Mean±SEM; 10 fields of view) in 12-day cultures. All data shown are from at least three experiments in parallel cultures (Mean±SEM). Significant differences are indicated with an asterisk (*P<0.05, **P<0.01).

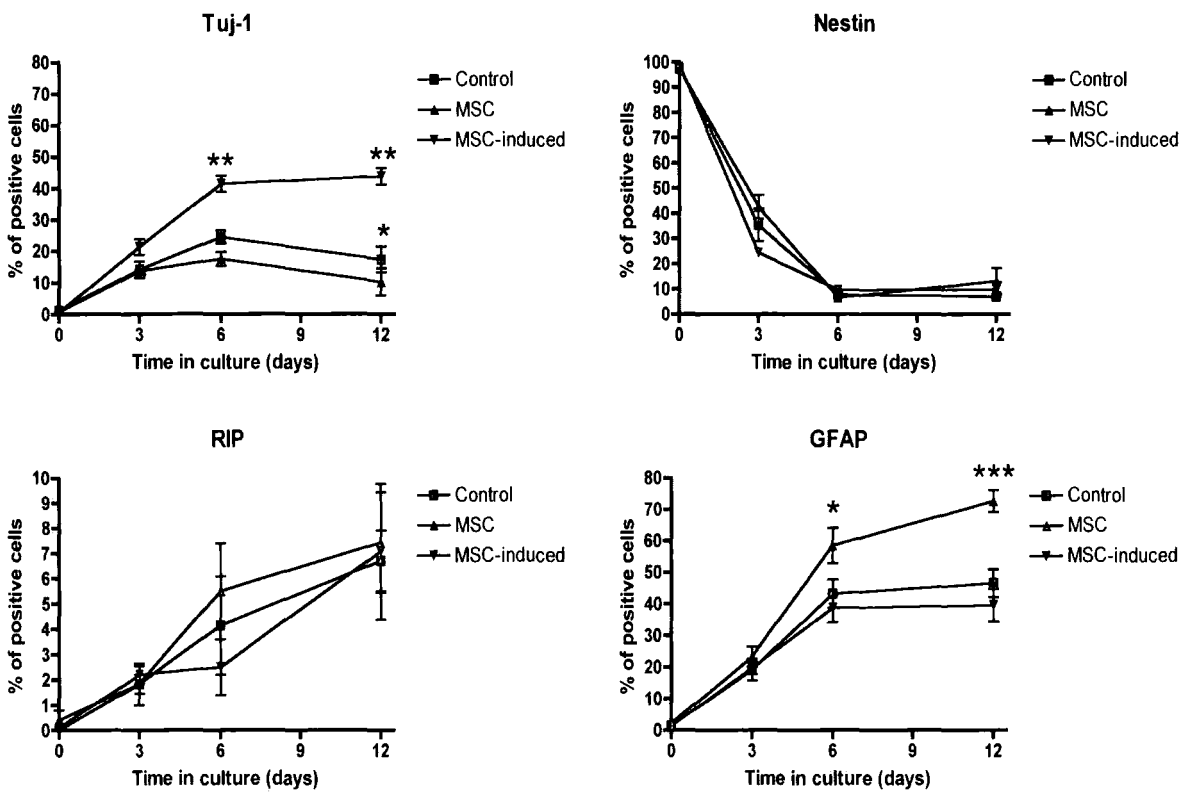


Figure 5.7: Quantification of the temporal expression of cell type specific markers during co-cultivation of NSC/progenitor cells with non-induced or induced MSCs for 12 days. Differentiation of NSC/progenitor cells from control and co-cultures (induced or non-induced MSCs) were subsequently fixed at 3, 6 and 12 days post differentiation and processed for immunocytochemistry. Graphs show the percentage of cells staining positive for each marker and at each time point analysed (mean $\pm$ SEM, 10 fields of view, experiment n=3). Significant differences are indicated with an asterisk (*P <0.05, **P <0.01, ***P<0.001).

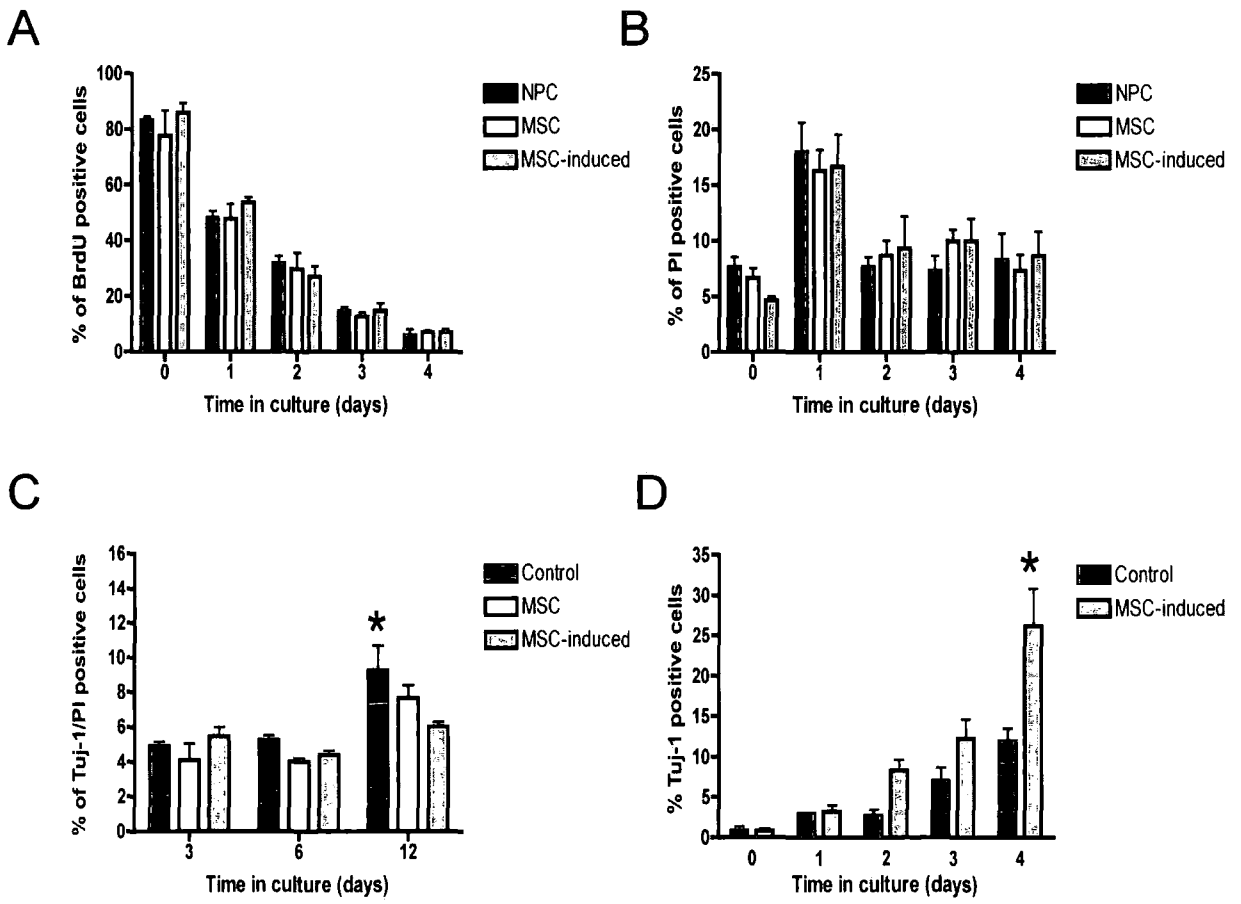


Figure 5.8: Effects of MSC co-culture on the survival, proliferation and neuronal commitment of striatal NSC/progenitor cells. (**A**) Proliferation of NSC/progenitor cells and their derivatives was accessed by BrdU incorporation. Parallel cultures were incubated with BrdU (2.5μM) on different time points for 24 hours, immediately followed by fixation and staining for BrdU. (**B**) Cell death in different culture conditions was accessed daily by live staining with PI (5μg/ml) and quantified by flow cytometry. All data are mean values±SEM from 3 independent experiments in parallel cultures. No significant differences in BrdU incorporation or cell death were found between culture conditions for each time point. (**C**) Double labelling of cells with PI and Tuj-1 at 3, 6 and 12 days post differentiation under control conditions (control) and in the presence of non-induced MSCs (MSC) or induced MSCs (induced-MSC). Quantification of staining was determined by flow cytometry. The % of TUJ-1+ cells staining positive for PI is indicated. (**D**) Percentage of Tuj-1 positive cells in 4-day cultures of differentiated progenitor cells (control) or co-cultures of progenitor cells and induced MSCs (induced-MSCs) as quantified by immuno-cytochemistry. Significant differences between control and co-culture groups are indicated with an asterisk (*$P<0.05$). All data are mean values±SEM from 3 independent experiments in parallel cultures.

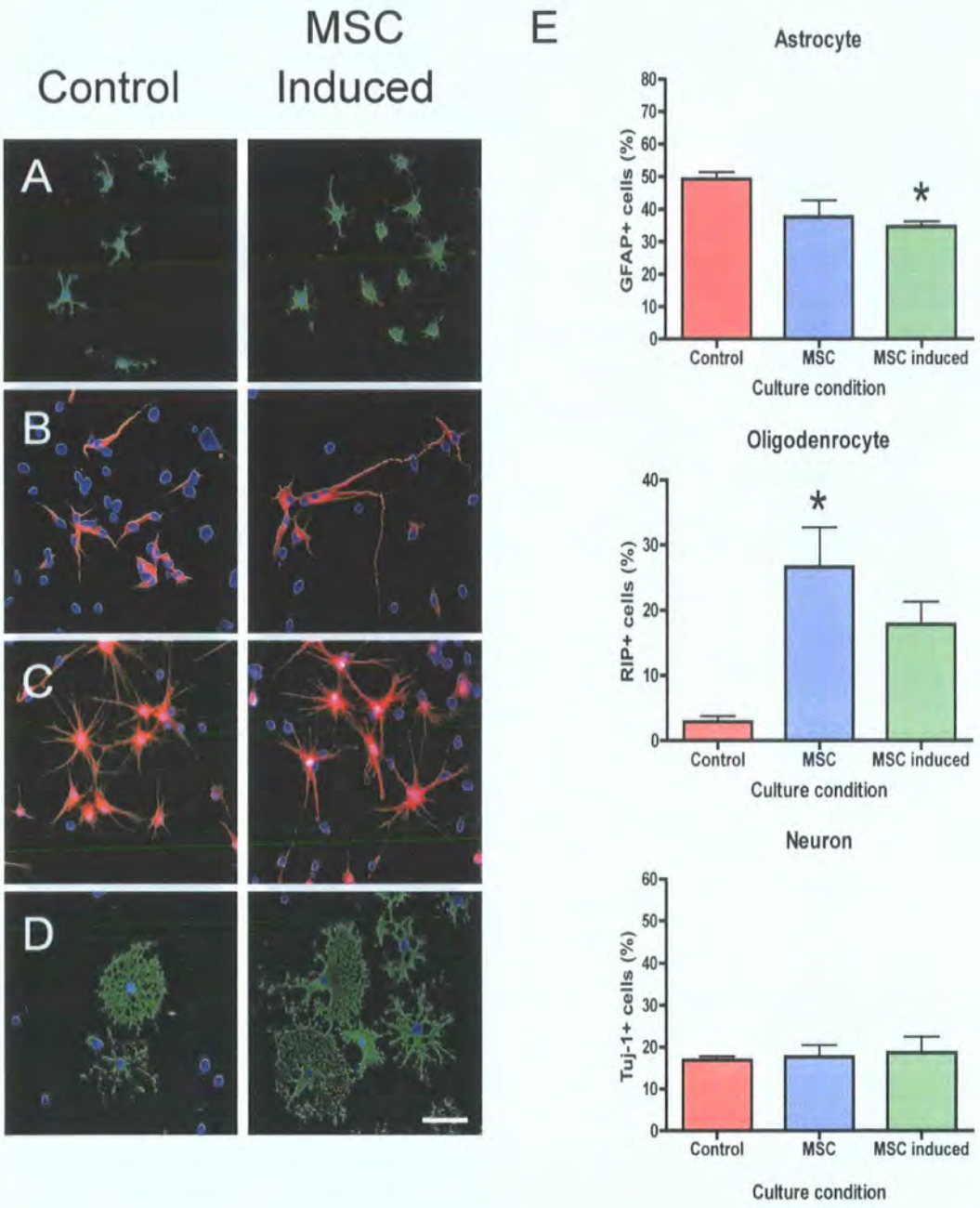
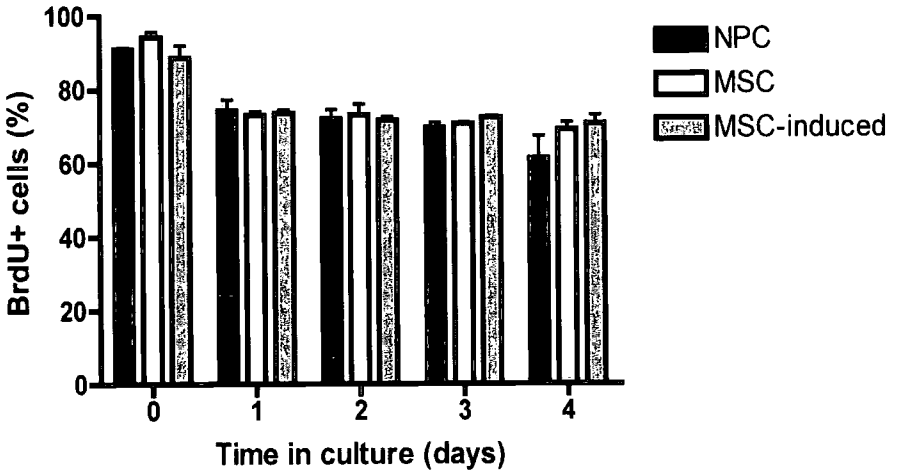


Figure 5.9: MSC soluble factors promote the differentiation of adult NSC/progenitor cells into distinct cell lineages. Undifferentiated adult NSC/progenitor cells (GFP+) were propagated in culture as mono-layers in the presence of bFGF (20ng/ml) on PLO coated tissue culture plastic. To induce multi-lineage differentiation under control conditions, progenitor cells were plated on poly-L-ornithine-coated dishes in the absence of growth factors and the presence of 0.5% FCS (**control, GFP A**). For co-cultivation, differentiation was initiated under identical conditions but in the presence of induced MSCs. After 12 days, cells were fixed and immuno-stained with monoclonal antibodies directed against Tuj-1 (**B**, neuron), GFAP (**C**, astrocyte) or RIP (**D**, oligodendrocyte). All cells were counterstained with Hoechst 33342 (blue). (**E**) Quantification of cellular differentiation under co-culture conditions. The number of cells positive (%) for each cell type marker was determined for each culture condition (mean±SEM; 10 fields of view) in 12 day cultures. All data shown are from at least three experiments in parallel cultures. Significant differences are indicated with an asterisk (*$P<0.05$) compared to control cells.

A



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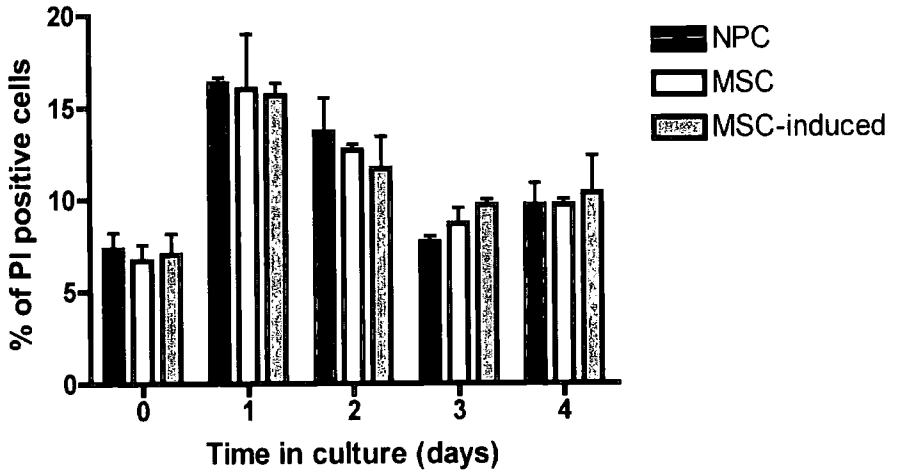

Figure 5.10: Effects of MSCs on the survival, proliferation and neuronal commitment of adult hippocampal NSC/progenitor cells. (**A**) Proliferation of progenitor cells was accessed by BrdU incorporation. Parallel cultures were incubated with BrdU (2.5μM) on different time points for 24 hours, immediately followed by fixation and staining for BrdU. (**B**) Cell death in different culture conditions was accessed daily by live staining with PI (5μ g/ml) and quantified by flow cytometry. All data are mean values±SEM from 3 independent experiments in parallel cultures. No significant differences in BrdU incorporation or cell death were found between culture conditions for each time point.

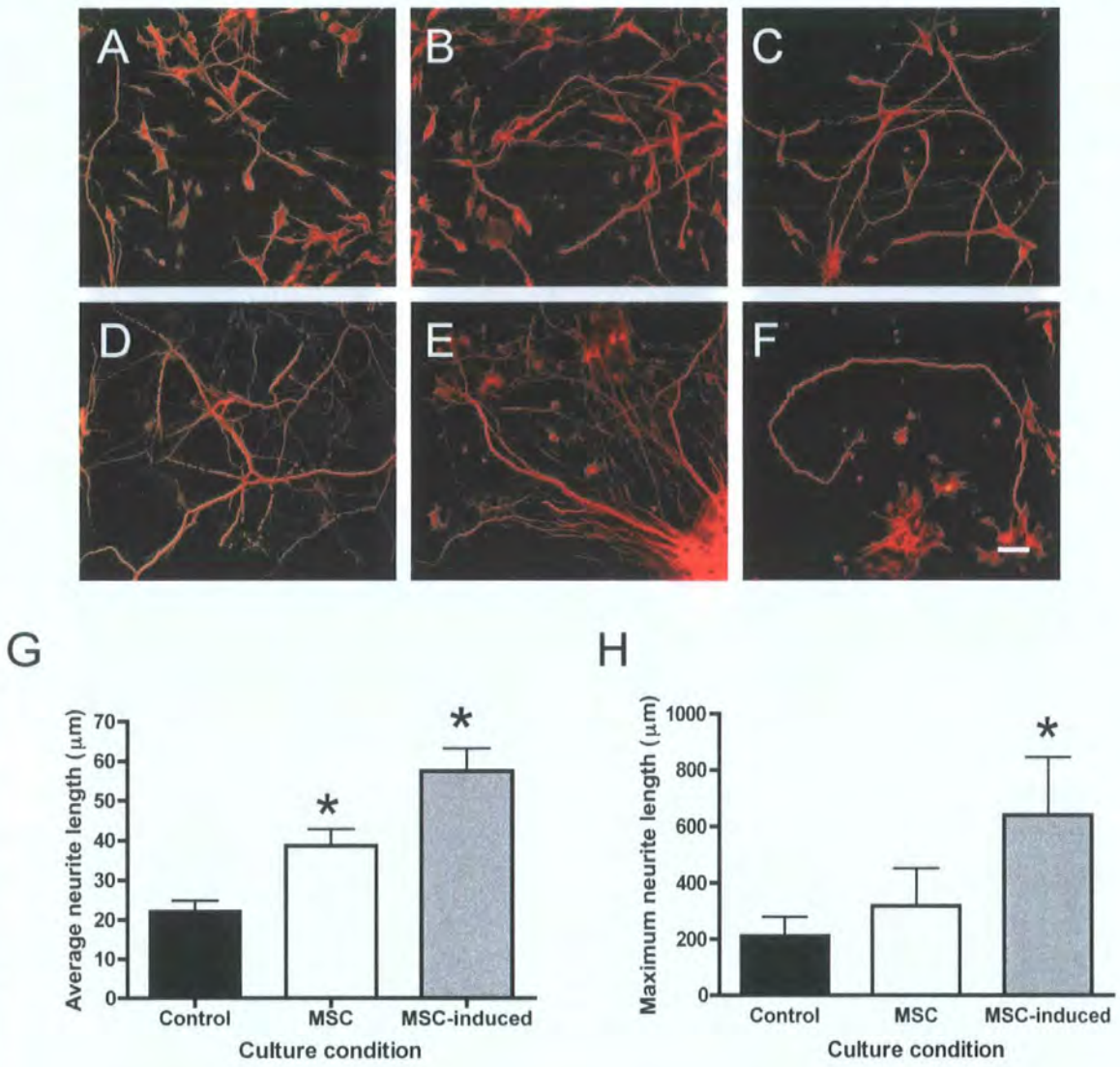

Figure 5.11: MSCs promote neuritogenesis of the Tuj-1 positive (neuronal) cell progeny of NSC/progenitor cells in 12-day cultures. Immunocytochemical staining (**A-F**) of 12 day differentiated cultures in the absence of MSCs (**A**), the presence of non-induced MSCs (**B**) and in the presence of induced MSCs (**C**). Further examples of extensive neurite outgrowth in co-cultures of induced MSCs with neural progenitor cells are illustrated in **D-E**. **G-H** Quantification of neurite outgrowth in control or co-culture conditions (induced or non-induced MSCs). After 12 days, cells were fixed and stained with Tuj-1 to identify neuronal progeny. Cells were then photographed and the average (**G**) and maximum (**H**) length of neurites was quantified from 10 randomly selected fields of view for each measurement. All data are mean values±SEM from 3 independent experiments in parallel cultures. Significant differences from the control group are indicated with an asterisk (*$P<0.05$).

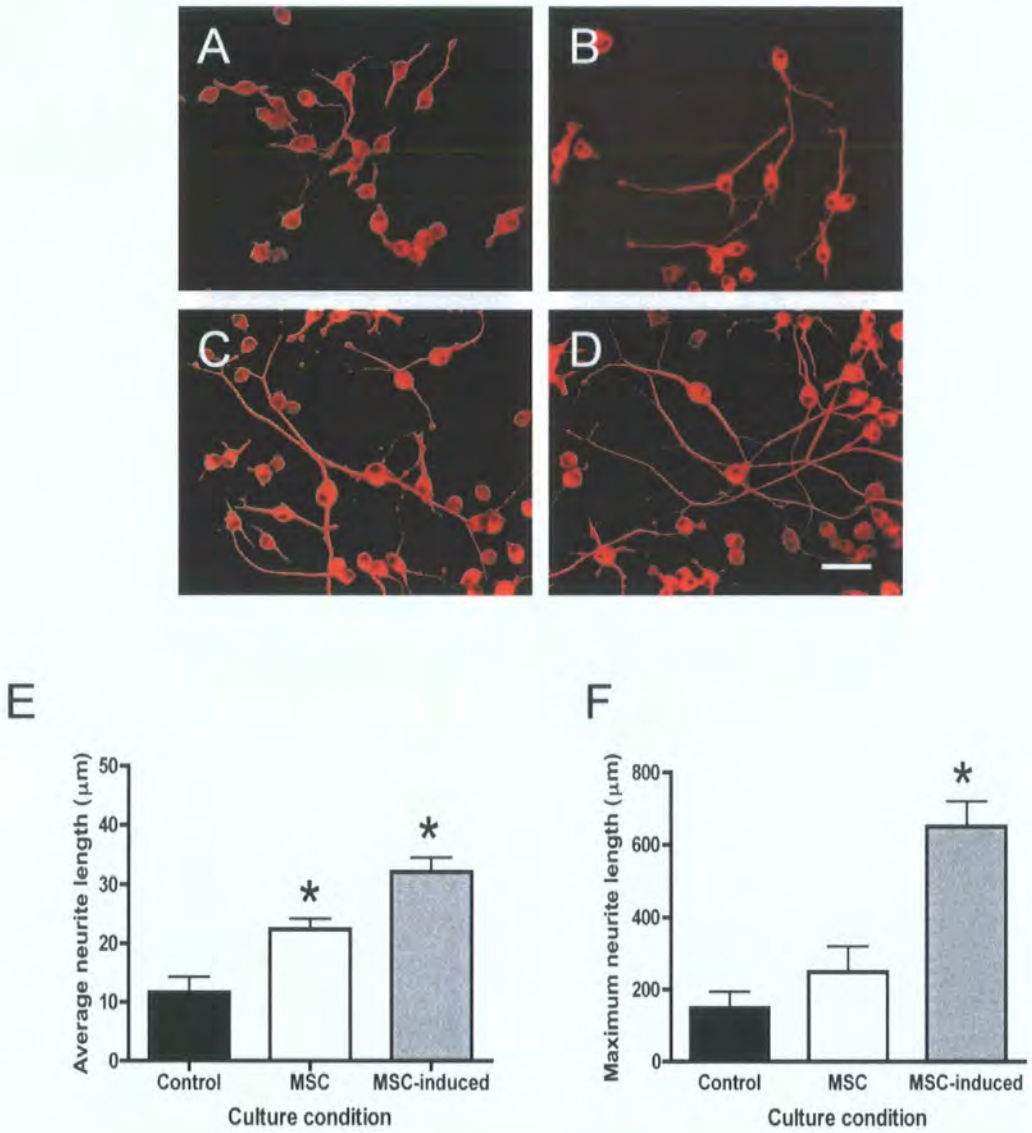

Figure 5.12 MSCs promote the neurite outgrowth of differentiating N2a cells. Undifferentiated N2a cells (**A**) were grown under standard culture conditions (DMEM+ 20% FCS) and used as a baseline for the determination of neurite outgrowth following differentiation. Control N2a were differentiated by the removal of serum for 12 days either alone (**B**) or co-cultivated with induced (**C**) or non-induced (**D**) MSCs. After 12 days, cells were fixed and stained with Tuj-1 to identify neurites. Cells were then photographed and the average (**E**) and maximum (**F**) length of neurites was quantified from 10 randomly selected fields of view for each measurement. All data are mean values±SEM from 3 independent experiments in parallel cultures. Significant differences from the control group are indicated with an asterisk (*$P<0.05$).

CHAPTER 6

General Discussion and Future Directions

6.1 Introduction

Most researchers will be increasingly aware of the surge in publications documenting the ability of adult stem cells to differentiate into cells and tissues which lie outside the organ in which they reside (Paul et al., 2002; Anderson et al., 2001; Blau et al., 2001; Tsai et al., 2002; Raff, 2003). In some cases, such differentiation can occur across germinal layers by a process referred to as 'transdifferentiation'. Results from these studies have implied that some adult stem cell populations may exist that retain a greater capacity for differentiation than previously appreciated. In some reports, certain adult stem cells have been described as possessing a developmental potential similar to stem cells derived from the early embryo (Jiang et al., 2002a). This work has been met with both interest and skepticism from the scientific community and has fueled the debate in the media as to whether such populations of adult stem cells could be used as an alternative to the more ethically controversial use of human embryonic stem cells (hES).

The potential use of stem cells for cell replacement therapy has been envisaged as a possible treatment for the damaged or diseased CNS for some time (review Corti et al., 2003). Whilst this is an attractive concept, it requires a readily available source of donor cells suitable for transplantation. The CNS does retain some capacity for self-renewal and the replacement of damaged cells although this regeneration is of limited capacity and occurs within distinct regions (Eriksson et al., 1998). Some of this regeneration is attributed to the presence of neural stem cells (NSCs), a multipotent population of cells that have the ability to generate in all three neural cell types (astrocytes, oligodendrocytes and neurons) (Gage, 2000). Such NSCs would in theory be the most valuable in terms of cell replacement therapy however, access to these cells from a healthy donor is practically impossible, therefore fetal and adult postmortem tissues remain as alternative sources.

hES cells derived from the human blastocyst have a unique capacity to differentiate into somatic cells of all three embryonic germ layers. This diverse potential for differentiation and their essentially unlimited proliferation in culture makes these cells an ideal starting population for the generation of purified populations of specific cell

types that could subsequently be used for cell replacement therapy. Several groups have described the differentiation of human ES cells into neuroectodermal derivatives *in vitro* (Carpenter et al., 1999), and others have reported the transplantation of these cells into the neonatal mouse brain as a testament of the ability of these cells to engraft into the CNS *in vivo* (Reubinoff et al., 2000). At present, only a subset of hES cells are able to differentiate into neural tissues following induction of differentiation (Carpenter et al., 2001). Subsequent isolation of these neural cells relies on selection and enrichment strategies involving the application of specific growth factors and mitogens to enhance the production of neural cells. Currently, this enrichment approach is unlikely to consistently produce the requisite amount of cellular material for cell transplantation therapies. In addition, the ethical debate surrounding research on hES cells continues and it is unlikely to be resolved in the near future.

The technical and ethical considerations of hES cell derived therapies have in part driven the search for adult stem cell populations that may be used as an alternative in the generation of neural cells for cell replacement therapies. Adult stem cells may offer a number of advantages to their embryonic counterparts. Adult stem cells with the capacity to form cells of unrelated organs could be isolated from non-diseased tissues of the body and used as autologous grafts for cell replacement. This would of course remove the problems of tissue matching and graft verses host disease encountered by the use of allogenic cell transplantation. Furthermore, there is no evidence that normal adult stem cells result in tumor formation following transplantation unlike the risks involved with grafted hES cells.

6.2 Trans-differentiation and the microenvironment (stem cell miche)

The test of trans-differentiation *in vitro* is based on the idea that the developmental potential of tissue specific stem and progenitor cells is dictated by their environment (the stem cell niche *in vivo*). Inhibition of the these signals by removing cells from their normal environment might allow cells to reprogram and differentiate into other cell lineages. MSCs have been shown to give rise to neurons (Woodbury et al., 2000; Deng et al., 2001; Hermann et al., 2004), glia (Wislet-Gendebein et al., 2005) *in vitro*, neurons and glia *in vivo* (Chen et al., 2001; Kopen et al., 1999; Eglitis and Mezey et al., 2000) and in some cases have been reported to have a multipotent capacity

consistent with that of NSC with the capacity to generate all three principal neural lineages *in vitro* (Suzuki et al., 2004). In co-culture studies MSCs have been reported to adopt neuronal and glial cell phenotypes when co-cultivated with primary hippocampal neurons (Riveria et al., 2006) and embryonic NSCs (Sanchez-Ramos et al., 2000). In most cases only morphological and immunological evidence of differentiation has been presented in these studies. A number of recent studies including the data presented in this thesis raise a number of important doubts as to the validity of using this approach in determining the trans-differentiation potential of MSCs (Lu et al., 2004; Neuhber et al., 2004; Bertani et al., 2005). In addition, cell fusion has been suggested as a possible alternative explanation for reports of trans-differentiation *in vivo* (Ying et al., 2002; Alvarez-Dolado et al., 2003). More recent developments in the post cell fusion era have been in understanding the molecular mechanism of possible trans-differentiation events *in vitro*.

At the heart of these issues are how to define cell identity and what the standards for the 'proof of principle' should be. In many respects the answers to these questions are tissue dependent. To establish a neuronal identity, one must first consider morphology. Neurons have a very characteristic morphology and are both variable and complex (Svendsen et al., 2001). Essentially in order to function electro-physiologically a neuron must produce a specialized growth cone, produce a single axonal fibre as it extends from the cell body and synapses with another neuron and extend a number of dendritic processes. This anatomical definition can be used to identify neurons (Svendsen et al., 2001), however such a definition has several disadvantages including misinterpretation of morphology, and other cells may mimic a neuronal morphology in appearance. One of the main obstacles to defining the identify of a cell is when we consider those cells on their way to becoming neuronal. Many of the early studies *in vitro* reported limited and immature differentiation towards the neural lineage (Sanchez-Ramos et al., 2000; Deng et al., 2001) and therefore under these culture conditions trans-differentiated cells may never reach the stage when they can be considered fully differentiated cells by a strictly anatomical definition, however these cells may still have the potential to become fully functional neurons under the appropriate conditions. Trans-differentiation of MSCs towards a neural cell fate, if it occurs is unlikely to involve two clearly defined stages of

differentiation i.e. mesodermal phenotype to neural phenotype, but is more likely to involve a progressive transition in which trans-differentiating cells retain some characteristics of both neuronal and mesodermal phenotypes. As a result one can envisage a stage in which cells may display an intermittent morphology. Recent work has shown that neural differentiation of MSCs is associated with a series of developmental processes, although these have not as yet been clearly defined (Wislet-Gendebein et al., 2005).

The studies presented in this thesis raise two major concerns when considering the phenotypic plasticity of MSCs in light of these findings. Most studies have and still do rely entirely on morphological and immunological characterization, as evidence of neuronal differentiation (Jin et al., 2003: Bossolasco et al., 2005; Long et al., 2005; 2005; For review see: Wislet-Gendebein et al., 2005; Lu et al., 2005; Song and Sanchez-Ramos; 2003; Chen et al., 2006). As stated above the problem is one of how do we define a neuron. Reh, 2002 when considering neural stem cell differentiation *in vitro* states that neuronal cells have to fulfill several criteria to be considered neuronal. They should be postmitotic, polarized with a single axon and have dendrites, able to conduct action potential and synapse with other neurons to form electrically active connections. Sevendsen et al., 2001 also suggested that these criteria should be meet when considering trans-differentiation events involving neuronal differentiation. So far no study has been able to fulfill all these criteria for putative neurons derived from MSCs.

6.3 Can MSCs form functional neurons?

A normal mature terminally differentiated CNS neuron is defined not only by its polarity and cytology and its specific protein expression in defined locations, but its excitability, and ability to fire action potentials and communicate with other neurons. Several recent studies have demonstrated the *in vitro* generation of mature functional neurons from embryonic stem cells (Miles et al., 2004; Okabe et al., 1996) and embryonic or adult NSCs (Morshead et al., 1994; Davis and Temple, 1994). Song et al., 2002 demonstrated that adult CNS stem cells isolated from the hippocampus could develop into electrically active neurons and form neural networks and functional synaptic transmission when co-cultured with primary neonatal hippocampal astrocytes

or neurons. Song et al., 2002 was able to clearly demonstrate the developmental processes, which occurred at the cellular level as these progenitor cells formed functional neurons. The progeny of adult NSC first established neuronal polarity with appropriate dendrite and axon formation, which was verified by immuno-labeling for GAP-43 (axon growth cone marker) which was detected in terminal dendrites and fine processes. MAP-2ab expression (dendrite specific marker) was found to be expressed in soma and thick processes. They were next able to demonstrate the presence and development of synapses. Synapse formation was demonstrated by immunolabelling for synapsin and confirmed by ultrastructural analysis. Whole-patch clamp analysis was then used to demonstrate that these synapses were functional and could conduct action potentials. The differentiated neurons were shown to conduct tetrodotoxin-sensitive action potentials after injection of depolarizing currents. Furthermore, spontaneous synaptic currents were detected in these neurons, demonstrating that they were able to receive inputs from surrounding neurons.

In comparison to the above study, no study at present has demonstrated the generation of functional neurons from MSCs to the standards described above. Most studies have used only morphological changes and expression of pan-neural markers as evidence of differentiation. In light of the findings presented in this thesis and that reported by other investigators, this criteria is clearly insufficient. Several previous reports have attempted to demonstrate electrophysiological properties of MSC derived neurons (Hofstetter et al., 2002; Padovan et al., 2003). Authors of these studies found no evidence of functional neurotransmitter receptors and cells lacked voltage gated Na+ and K+ channels. In contrast, Kohyama et al., 2001 using whole patch clamp analysis of MSC presumptive neurons demonstrated a resting membrane potential of -50mV by day 28. The authors claim that this resting membrane potential is consistent with primary neurons cultured as positive controls. However, other investigators have noted that this membrane potential is higher than normal functional neurons (Chen et al., 2006; Carleton et al., 2003). Hung et al., 2002 investigated the electrophysiological properties of size-sieved MSCs induced to differentiate in the presence of BME. An elevated [Ca^2+] occurred in MSC derived neurons in response to glutamate or high K+ buffer. However, this response was slow or delayed compared to a normal neuronal response. In addition, these studies failed to

demonstrate neuronal polarity and synapse formation, both critical parameters for demonstration of functional neurons. Following transfection with the BDNF gene MSCs are reported to differentiate into neural cells when treated with all-trans retinoic acid (RA) (Zhao et al., 2004). These MSCs derived neural cells expressed pan-neural markers such as O4, GFAP and NeuN. Electrophysiological analysis of these cells using patch clamp technique demonstrated that these cells had a resting membrane potential of approximately -65mV and processed voltage dependent K+/Ca2+ currents. A recent study by Wislet-Gendebien et al., 2005 recently demonstrated that adult rat, nestin positive MSCs were able to differentiate into excitable neuron-like cells following co-culture with cerebellar granule cells. These cells expressed Tuj-1, NeuN and MAP-2ab. In addition, the presumptive neurons formed from nestin positive MSCs were shown to be electrophysiologically active. The authors were able to document the maturation stages of this development according to criteria established by Carleton et al., 2003). After 4-6 days of co-culture, neural-like cells showed some response to neurotransmitters including: GABA, glycine, glutamate and serotonin. At this stage, no functional voltage gated sodium channels were detected and cells displayed only a low resting membrane potential. However in the 2nd week of co-culture, neurons started to display Na+ currents, which were reversibly inhibited by tetradoxinin demonstrating the presence of voltage gated Na+ channels. Cells were able to fire single action potentials and in more mature cultures the resting membrane potential approached more negative values.

Whilst the study by Wislet-Gendebein et al., 2005 provides the one of the most advanced reports of the electrophysiological characteristics of MSCs derived neurons, it still does not fulfill the criteria by which we would fully define a cell as neuron (Carleton et al., 2003). This study demonstrates that neuronal-like cells derived from nestin positive cells elicit electrical responses following application of several neurotransmitters and those responses can be inhibited by classical inotropic receptor blockers. However, the investigators have not yet demonstrated that these cells communicate via synapse formation. They report that they were unable to record any synaptic activity or repeated action potential firing. This is in contrast to neurons derived from adult stem cells, which display spontaneous firing and communication between cells. The authors did however, show a clear maturation and it is known that

in development *in vivo* synaptic activity and spontaneous firing are the last characteristics to be acquired (Carleton et al., 2003). It is also possible that terminal maturation requires additional signals, which may only be acquired *in vivo*.

It is my opinion that it may be premature to conclusively define a cell *in vitro* as a neuron without demonstrating its ability to communicate with other neurons through synapses and spontaneously fire action potentials. However, on a spectrum of maturation, spontaneous synaptic activity and spiking activity only occur during late maturation stages (Carleton et al., 2003). Therefore, it may be the conditions, which promote terminal maturation of MSC derived neurons cannot be easily recapitulated *in vitro,* even in co-culture studies. Therefore conclusive proof may require animals to be transplanted with MSCs and then electrophysiological activity measured by slice culture. Alternatively organotypic slice culture may be a useful alternative method to investigate the differentiation potential of MSCs by electrophysiology. However, it is also my opinion that it may be premature to dismiss the neurogenic potential of MSCs *in vitro* simply by quoting negative electrophysiological data. As described, if MSCs induced to differentiate into neuronal cells *in vitro* under defined culture conditions only achieve early stages of neuronal maturation then they may not be electro physiologically active however, this does not mean that such cells do not have a neurogenic potential. In fact, consistent with this concept is the recent success of *in vitro* studies utilizing a two stage approach in which MSCs are first manipulated *in vitro* under defined culture conditions and subsequently transplanted *in vivo* where cells appear to undergo further maturation (Deng et al., 2006). The question this raises is how do we define a neurogenic potential and the answer to this is probably to ultimately demonstrate functionality, however one must find the conditions in which to achieve this functional maturation.

An alterative but less satisfactory approach may be to analyze MSCs to see if they possess the machinery to terminally differentiate into neurons and this will require an understanding of the molecular mechanisms underlying the neural phenotypic plasticity of MSCs. These investigations have been approached in principally two ways, the first is an analysis of the gene expression profiles of MSCs (Woodbury et al., 2002) and the second is an analysis of the intrinsic and extrinsic factors that

govern cell fate decisions in MSCs (Chu et al., 2006; Jin et al., 2003; Bossolasco et al., 2005; Alexanian, 2005). Understanding the intrinsic and extrinsic factors that determine lineage commitment should allow a better understanding of proposed adult stem cell plasticity.

There have been very few studies that have analyzed the possible molecular mechanisms that result in neural trans-differentiation of MSCs. However, early studies did demonstrate that agents, which increase cyclic AMP, result in the formation of cells with a neuronal morphology expressing neuronal markers (Deng et al., 2001). Recently, Jori et al., 2005 analysed the biochemical pathways involved in neuronal commitment of MSCs. They analysed the effect of K252a an alkaloid compound, which is a general protein kinase C inhibitor that inhibits CaM Kinase II, protein kinase A (PKA) and protein kinase C (PKC) (Hashimoto et al., 1991; Twomey et al., 1991; Tapley et al., 1992). K252a treatment has been used to sustain differentiation of MSCs into neural derivatives in numerous studies (Woodbury et al., 2000; Deng et al., 2001; Black and Woodbury et al., 2001; Sanchez-Ramos et al., 2002; Woodbury et al., 2002). Therefore increasing intracellular cyclic AMP can induce neural cell differentiation of MSCs and the maturation process is further sustained by K252a treatment (Deng et al., 2001). However K252a treatment could not itself result in neural differentiation (Jori et al., 2004). Jori et al., 2004 determined that forskolin treatment and K252a treatment both resulted in elevated intracellular cyclic AMP that activated the classical protein kinase A pathway as opposed to the EPAC-RAP pathway (exchange protein, Rangarajan et al., 2003), which has been suggested to be involved in neural differentiation (Bos, 1998; de Rooij et al., 2000). Therefore cyclic AMP induction following forskolin treatment is recruited in the classical PKA-dependent pathway. However, neural induction in the presence of a PKA inhibitor did not result in differentiation suggesting that this pathway is critical. As described, K252a inhibits PKC, PKA and Ca MK II and the Trk A receptor (Ohmichi et al., 1992; Rovelli et al., 2002). However neural induction in the presence of selective inhibitors of PKC, PKA or Ca MH II did not inhibit neural differentiation but did induce significant cell death. Therefore it was concluded that the pro-differentiating effect of K252a was not the result of inhibition of these pathways, but it does rescue differentiating cells from cell death. The authors were able to

demonstrate that the neural induction effects of K252a were the result of inhibition of the calmodulin kinase, Ca MK II. This result is in contrast to several reports that demonstrate the induction of Ca MK II occurs with neuronal differentiation (Jensen et al., 1991; Donai et al., 2000; Bui et al., 2003). The investigators also found evidence that inhibition of the MEK-ERK pathway significantly reduced neural differentiation.

Several recent studies have reported that nestin expression is a required pre-requisite for neural differentiation in MSCs and other stem cell populations (Croft and Przyborski, 2004; Wislet-Gendebien et al., 2005). In culture, nestin expression by MSCs is known to be dependent on two conditions, firstly the absence of serum in the culture media and secondly a minimum passage number of 10 (25 population doublings) (Wislet-Gendebien et al., 2005). Nestin is predominately expressed in neural progenitor cells (Lendahl et al., 1990). Nestin in the embryo is expressed in migrating and proliferating cells whereas in adult tissues nestin expression is restricted to areas of regeneration (Wiese et al., 2004). Our understanding of the mechanisms that regulate nestin expression is poor.

The gold standard for proof of functionality in stem cell biology is the restoration of function following injury, through cell replacement. In the context of trans-differentiation this implies the restoration of function through replacement of damaged cells with the progeny of stem cells from a different dermal origin to the damaged cells. This criteria has not been definitively demonstrated for MSCs in relation to neuroectodermal differentiation. Infusion of purified populations of MSCs either systemically or intra-cerebrally has been shown to improve functional outcome in several animal models of CNS lesions (For review see: Chopp and Li 2002). In these transplantation studies MSC donor cells expressing neuronal and glial cell markers have been observed within the injured adult CNS following either direct or indirect infusion of MSCs or whole BM (Chen et al., 2001; Zhang et al., 2005; Li et al., 2002). It is not known however, whether such cells have differentiated into a neuronal phenotype and thus whether these findings indicate trans-differentiation events. The reason for this controversy is because of the markers used to define donor cell identity. Some investigators have interpreted the appearance of donor MSCs expressing neural proteins to represent differentiation towards a neural cell lineage

and thus MSCs trans-differentiate into neural phenotypes and improve functional neurological outcome through replacement of damaged endogenous cells (Lu et al., 2006). However transplant studies have revealed only a small fraction of MSCs express neural proteins and the numbers of these cells are insufficient to account for functional recovery and rapidity by which the therapeutic benefits of MSCs are observed (Chen et al., 2001; Li et al., 2002). In addition, the expression of these proteins by the small fraction of donor MSCs does not appear to correlate with the acquisition of a neural morphology. No significant branching, process formation or integration into host tissue architecture has been observed (Chen et al., 2001). In addition, recent studies have been able to offer alternate observations including cell fusion. Some investigators have found no evidence for trans-differentiation of BM derived stem cells in the absence of cell fusion (Alvarez-Dolado et al., 2003). It is important to note however, that this mechanism could represent a normal pathway towards differentiation and repair in damaged tissues (Blau 2002). The mechanism by which transplanted MSCs exert their therapeutic benefit is uncertain and the role of neural antigen positive donor MSCs is unknown.

Studies presented in this thesis may offer an alternate explanation for the appearance of donor derived MSCs expressing neural proteins. In addition, we were able to show with the current studies that MSCs can express a range of neuronal and glial cell markers without differentiating into neural cell phenotypes at least *in vitro*. Therefore our work casts further doubt as to whether MSCs are capable of generating neural cell derivatives *in vitro*.

6.4 Where does this leave neural differentiation of MSCs?

The observation that cell fusion could be responsible for the observed trans-differentiation events in the brains of adult mice following BM transplantation raises many doubts about the potential of BM stem cells to adopt neural cell fates (Rodic et al., 2004). However, cell fusion occurs at a very low frequency *in vivo* and cannot account for observations *in vitro* in which trans-differentiation occurs in cultures containing only one cell type (e.g. Hermann et al., 2004). In addition, recent work involving analysis of the brains of BM recipients has demonstrated that BM derived stem cells can contribute to host brain tissue in the absence of cell fusion events.

Cogle et al., 2004 analysed brain sections from three sex mismatched female BM transplant recipient patients. FISH and immunocytochemistry were performed to identify BM derived neural cells. In all three patients hippocampal cells containing Y chromosome positive cells were detected upto 6 years post transplant. These cells accounted for upto 1% of all neurons; cell fusion events could be ruled out since there was only one X chromosome detected. Crain et al., 2005 examined paraffin brain sections from female patients who had received BM transplants from male donors. They were able to detect Y-chromosome labeled cells in the neocortex, hippocampus, stratum and cerebellum. Both neurons and astrocytes were labeled, but most cells were of a glial phenotype. Cell fusion was eliminated as a possible explanation and therefore trans-differentiation of BM derived stem cells appeared to occur in these patients. However, these investigators report a very low number of trans-differentiation events, too low to adequately replace lost or damaged neurons following disease pathology or injury. These results are consistent with a earlier investigation by the same laboratory in which the brains of female recipients receiving BM transplants from male donors were analysed for Y chromosome positive cells. In this case Y chromosome positive cells were detected in several brain regions. However most of these cells were non-neuronal, but neuronal cells were detected in the hippocampus and cerebral cortex (Mezey et al., 2003).

Therefore these studies indicate that BM derived cells may contribute to adult brain regeneration by a process of neural trans-differentiation in the absence of fusion, but that the frequency of this differentiation is too low to have a significant impact on the regeneration of the adult CNS. Even following systemic transplantation of BM derived cells in animal models of CNS lesions the number of donor cells expressing neuronal and glial markers in animal models of injury is too low to account for the improvement in functional recovery following these transplants. Current work is focusing on determining under what conditions or circumstances, some form of BM transplantation might provide a adequate number of cells capable of replacing lost brain cells following injury or disease. Whilst these BM transplant studies demonstrate that stem cells present in BM can give rise to neural derivatives, it is not known what cell population undergoes this trans-differentiation under these conditions.

6.5 Therapy perspectives

At present, it is clear that under certain defined conditions MSCs are capable of differentiation into cells that resemble early neural cells and even possess these characteristics at a functional level in some cases (Hermann et al., 2004; Wislet-Gedebien et al., 2004; 2005). However, there remain doubts as to the validity of some of these observations and MSC derived presumptive neurons have not yet met all the criteria required for describing them as fully functional neurons. In addition, the contribution trans-differentiation of MSCs plays in the functional improvement observed in animals transplanted with MSCs is unknown since alternative mechanisms have been proposed; including as suggested by data presented in the present thesis the induction of neural differentiation of resident NSC/progenitor cells, the progeny of which could replace lost or damaged neurons. Therefore the mechanism of the therapeutic benefit of MSCs is unknown. They do however clearly contribute to functional recovery following CNS injury. The prospects of cell replacement therapies for specific disease processes are unknown. Parkinson's disease for example, involves the selective degeneration of dopaminergic neurons in s Substantia Nigra of the midbrain. For MSCs to be considered a source of cells for autograft therapy it will be necessary to promote them towards to dopaminergic cell fate. A number of investigators are working on developing these protocols but at present little is known about the neurotransmitter specification of these cells (Guo et al., 2005). This will of course involve an understanding of the environmental cues that regulate fate specification in MSCs in different micro-environments.

6.6 Concluding remarks

This work raises several key issues regarding the validity of *in vitro* observations and is particularly important to the field of transdifferentiation and the identity and behavior of cells *in vitro*. The excitement that surrounds the developmental potential of stem cells isolated from adult tissues is well justified, especially in its regard to neural differentiation, however, experimentation in this field must be closely controlled and the results carefully interpreted. The results presented here do not however, simply state that trans-differentiation of MSCs is not possible but, that they do emphasize that a knowledge of the molecular and cellular mechanisms underlying the regulation of neural protein expression and the acquisition of a neural morphology

are fundamental in establishing effective methods for induction of neural differentiation in MSCs. The criteria that an MSC derived presumptive neuron must meet is understandably high given the implications of these observations and the ultimate test for these cells is demonstration of differentiation into functional neurons that can integrate into the host CNS, synapse with neighboring neurons and spontaneously fire action potentials and replace lost or damaged neurons in the injured animal, and thus contribute to the restoration of function. It may just be matter of finding the right conditions to promote the terminal maturation of these cells, but alternatively these cells may be unable to attain such high levels of functional maturation, since it is not enough simply to express genes, cells must have the machinery to put all these proteins in place to allow them to attain the appropriate functions. Aside from the neural differentiation potential of MSCs, data presented in this thesis and by other investigators shows that these cells can act as reservoirs of soluble growth factors which are able to modify brain plasticity through their soluble interactions with resident NSCs and their associated progeny (Chopp and Li, 2002). These effects on brain plasticity undoubtedly contribute at least in part to the regeneration of the damaged brain. Understanding the chemical nature of these MSC derived signals may allow for the development of simple and effective treatments following CNS injury.

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