In Vitro and In Vivo neuronal differentiation capacity of human adult bone marrow-derived mesenchymal stem cells

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IN VITRO AND *IN VIVO* NEURONAL DIFFERENTIATION CAPACITY OF HUMAN ADULT BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Discovery of the ability of mesenchymal stem cells (MSCs) to differentiate into cells of non-mesodermal tissues, particularly neuronal cells, have raised the possibility of utilising MSCs in regenerative/reparative therapies for neurological disorders. However, a number of hurdles remain to be resolved. This thesis aims to address some of these issues by investigating the characteristics of bone marrow-derived human MSCs (hMSCs) during long-term culture, the potential of hMSCs to differentiate *in vitro* toward the neuronal lineage under the influence of cytokines, and the effects of intracerebral transplantation in the hemiparkinsonian rat model.

During expansion culture hMSCs were found to display the expected characteristics of MSC populations, and also constitutively expressed neural and pluripotency markers simultaneously with mesodermal markers. Analysis of hMSC long-term subcultivation revealed an optimal period for commencing neuronal differentiation (first 6-8 passages), and also showed the absence of spontaneous neural differentiation.

Application of neural-inducing cytokines and culture conditions resulted in the generation of an immature neuronal-like phenotype by hMSCs. Through live cell microscopy it was demonstrated for the first time that cytokine-based hMSC neuronal differentiation occurs through active and dynamic cellular processes involving outgrowth and motility of cellular extensions. In addition, single- and multiple-stage cytokine-based strategies for inducing dopaminergic neuronal-like cells from hMSCs were investigated. These studies revealed that FGF-2 and EGF exerted the greatest benefits for hMSC neuronal differentiation.

Undifferentiated and neuronal-primed hMSCs were transplanted intracerebrally into the striatum and substantia nigra of cyclosporine-treated hemiparkinsonian rats. Grafted hMSCs could be clearly identified at 1-day and 7-days post-transplantation; however, grafts were gradually lost over time, with complete absence by 21-days. Co-transplantation with olfactory ensheathing cells, neuronal-priming prior to grafting, and nigral as well as striatal grafting could not provide engraftment and differentiation advantages. Immunohistological analysis demonstrated the presence of innate inflammatory responses (microglia and astrocyte activation) at graft sites, fibronectin deposition by hMSCs, and lack of endogenous host neurogenesis.

The results of my PhD work indicate that cytokine-based culture methods are capable of differentiating hMSCs to an immature neuronal-like phenotype, and host-mediated innate inflammatory responses may be a key contributing factor for the failure of *in vivo* hMSC engraftment.

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ORIGINALITY STATEMENT

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ABSTRACT

Since the identification of mesenchymal stem cells (MSCs) in the bone marrow (BM) much interest has been generated in these cells by the development of methods to isolate, expand and differentiate MSCs. Discovery of the ability of MSCs to differentiate into cells of non-mesodermal tissues, particularly neuronal cells, has also raised the possibility of utilising MSCs in regenerative and reparative therapies for neurological disorders. However, a number of hurdles remain to be resolved. This thesis aims to address some of these issues by investigating the characteristics of BM-derived human MSCs (hMSCs) during long-term culture, the potential of hMSCs to differentiate *in vitro* toward the neuronal lineage under the influence of cytokines, and the effects of intracerebral transplantation of hMSC-derived cells in the 6-OHDA lesioned hemiparkinsonian rat model.

During expansion culture hMSCs were found to display characteristics of MSC populations, as assessed by morphological appearance, proliferative ability, surface antigen expression, gene and protein expression, and production of multi-lineage mesodermal derivatives. In addition, hMSCs constitutively expressed neural and pluripotency markers simultaneously with the expected mesodermal markers, indicating the importance of determining baseline expression levels prior to neural differentiation. Analysis of long-term serial passaging of hMSCs revealed maintenance of a stable *in vitro* phenotype over the initial 6-8 passages of expansion culture, with cells cultured beyond this period showing alterations in key phenotypic attributes, such as morphology, proliferative ability and gene and protein expression. However, extensive subcultivation of hMSCs did not result in spontaneous neural differentiation. These results allowed determination of the optimal window for hMSC expansion and commencement of neuronal differentiation for all subsequent experiments in this thesis.

Application of neural-inducing cytokines and culture conditions resulted in the generation of an immature neuronal-like phenotype by hMSCs, in terms of cellular morphology, gene expression and protein expression. Through live cell microscopy it was demonstrated for the first time that cytokine-based hMSC neuronal differentiation occurs through active and dynamic cellular processes involving outgrowth and motility of cellular extensions. Long-term subcultivation of hMSCs did not appear to prevent cells from acquiring immature neuronal-like characteristics, although use of hMSCs within the optimal window was associated with advantages, including higher proliferative ability and greater phenotypic stability of undifferentiated hMSCs and a lower proportion of cells unresponsive to differentiation conditions. Sequential application of cytokines chosen for their known involvement in midbrain dopaminergic neuronal differentiation *in vivo* was examined (MultiDA method) and compared to single-stage cytokine-based approaches (SingleDA and Single ND methods). The 3 different procedures investigated were observed to elicit differing effects on cellular morphology and gene expression; however, all methods investigated produced immature neuronal-like cells. Nevertheless, the first stage of the MultiDA method yielded the highest expression of early neuronal and early dopaminergic neuronal markers, and FGF-2 and EGF were found to exert the greatest benefits for hMSC neuronal differentiation.

Undifferentiated and neuronal-primed (first stage of the MultiDA method) hMSCs were transplanted intracerebrally into the striatum and substantia nigra of cyclosporine-treated hemiparkinsonian rats. Grafted hMSCs could be clearly identified at 1-day post-transplantation and were still detectable after 7-days; however, hMSCs were gradually lost over time, with complete absence by 21-days post-transplantation. Further differentiation of neuronal-primed hMSCs *in vivo* was not evident at either the

striatum or substantia nigra. In addition, transplantation of hMSCs did not elicit neurogenic effects on host endogenous neural stem cells or progenitors, as evidenced by lack of nestin expression. Co-transplantation of human olfactory ensheathing cells (OECs) with neuronal-primed hMSCs was unable to provide engraftment or differentiation advantages, and OECs also did not survive in the rat brain after transplantation. To my knowledge, it was shown for the first time that innate inflammatory responses (microglia and astrocytes) are activated following transplantation of hMSC-derived cells in the lesioned hemisphere of hemiparkinsonian rats. Continued fibronectin production by hMSCs was also found indicating retention of MSC characteristics, which are foreign to the host brain microenvironment. This suggests that further *in vitro* differentiation of hMSCs to the neuronal lineage may be required. The observed graft loss may therefore be the result of innate inflammatory responses, the xenogeneic nature of transplanted cells, the 'foreign' nature of hMSCs (which still expressed mesodermal/MSC traits), or the inability of the lesioned adult rat striatum and substantia nigra to support hMSC survival, engraftment and differentiation.

The results of my PhD work indicate that cytokine-based *in vitro* culture methods are capable of differentiating hMSCs to an immature neuronal-like phenotype, and that host-mediated innate inflammatory responses may be a key contributing factor for the failure of *in vivo* engraftment of hMSC-derived cells in a rodent model of Parkinson's disease. Further studies are required to gain a better understanding of this field and to improve the *in vitro* and *in vivo* procedures for directed neuronal differentiation and effective transplantation of hMSCs.

PUBLICATIONS, PRESENTATIONS AND AWARDS

Publications

Khoo, M.L.M., Shen, B., Tao, H. and Ma, D.D.F. (2008). Long-Term Serial Passage and Neuronal Differentiation Capability of Human Bone Marrow Mesenchymal Stem Cells. *Stem Cells and Development* **17**: 883-896.

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Presentations at Scientific Meetings

Khoo, M.L.M., Meedeniya, A.C.B., Tao, H., Pagendam-Turner, M., Nguyen, M., Mackay-Sim, A. and Ma, D.D.F. The Fate of Transplanted Neuronal-Primed Human Mesenchymal Stem Cells in the 6-OHDA Unilaterally Lesioned Parkinsonian Rat. *National Centre for Adult Stem Cell Research 'Adult Stem Cells: Biology and Clinical Applications' Conference, Brisbane, Australia, November 2008 (P01).*

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Khoo, M.L.M., Shen, B.J., Tao, H. and Ma, D.D.F. Stability of Characteristics (Stemness) of Human Bone Marrow Mesenchymal Stem Cells in Long Term Culture. *International Society for Stem Cell Research Annual Meeting, San Francisco, CA, USA, June 2005 (412).*

Shen, B.J., **Khoo, M.L.M.**, Tao, H. and Ma, D.D.F. Pluripotent and Differentiation Potential of Human Bone Marrow Derived Mesenchymal Stem Cells in Prolonged Expansion Culture. *Annual Meeting of the Australian Stem Cell Centre, Gold Coast, Australia, November 2005.*

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#	Including cytokines from Stage 1 (FGF-2 and EGF)
6-OHDA	6-Hydroxydopamine
AA	Ascorbic Acid
ACAN	Aggrecan
ADIPOQ	Adiponectin
AECs	Airway Epithelial Cells
α-FP	α-Fetoprotein
ALB	Albumin
APCs	Antigen-Presenting Cells
BDNF	Brain-Derived Neurotrophic Factor
BGLAP	Osteocalcin
BHA	Butylated Hydroxyanisole
BM	Bone Marrow
BME	β-mercaptoethanol
BMP	Bone Morphogenetic Protein
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Mono-Phosphate
CFU	Colony-Forming Unit
CFU-F	Colony-Forming Unit – Fibroblast
CK	Cytokeratin
CNPase	2',3'-Cyclic nucleotide 3'-phosphohydrolase
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
COL1A1	Type I Collagen
COL2A1	Type II Collagen
c-RET	RET Receptor Tyrosine Kinase
CSF	Colony-Stimulating Factor
CSPG4	Chondroitin Sulfate Proteoglycan 4
CTL	Cytotoxic T Lymphocyte
d	day
DA	Dopamine
DAPI	4'6-diamidino-2-phenylindole
DAT	Dopamine Transporter
dbcAMP	Dibutyryl Cyclic Adenosine Mono-Phosphate
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DMEM-LG	Dulbecco's Modified Eagle's Medium – Low Glucose
DMSO	Dimethylsulfoxide
dPBS	Dulbecco's Phosphate Buffered Saline
E	Embryonic Day
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EN	Engrailed
ESC	Embryonic Stem Cell
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum

FGF	Fibroblast Growth Factor
FOXA2	Forkhead Box A2 (also known as HNF-3β)
Fzd	Frizzled
GABA	γ-Aminobutyric Acid
GALC	Galactosylceramidase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDNF	Glial-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GFRA1	GDNF Family Receptor Alpha 1
GM-CSF	Granulocyte-Macrophage-CSF
GVHD	Graft-Versus-Host Disease
HGF	Hepatocyte Growth Factor
HBSS	Hank's Balanced Salt Solution
HLA	Human Leukocyte Antigen
hMSC	Human Mesenchymal Stem Cell
HNA	Human Nuclear Antigen
HNF	Hepatocyte Nuclear Factor
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HSC	Haematopoietic Stem Cell
Iba1	Ionised Calcium Binding Adaptor Molecule 1
IBMX	Isobutyl Methyl Xanthine
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iPS	Induced Pluripotent Stem
ITS	Insulin Transferrin Selenium
i.v.	Intravenous
JAK	Janus Kinase
LDL	Low-Density Lipoprotein
L-DOPA	3,4,-Dihydroxyphenylalanine
LMX	LIM homeobox transcription factor
LPL	Lipoprotein Lipase
MAP	Microtubule-Associated Protein
MAPC	Multipotent Adult Progenitor Cell
MAPK	Mitogen-Activated Protein Kinase
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
min	Minutes
MNC	Mononuclear Cell
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MSC	Mesenchymal Stem Cell
MultiDA	Multiple-Stage Dopaminergic Neuronal Differentiation
NANOG	Nanog Homeobox
NES	Nestin
NeuN	Neuronal Nuclear Antigen
NEUROD1	Neurogenic Differentiation 1
NEUROG	Neurogenin
NF-M	Neurofilament protein subunit of medium molecular weight

NGF	Nerve Growth Factor
NICD	Notch Intracellular Domain
NK	Natural Killer
NR4A2	Nuclear Receptor Subfamily 4, Group A, Member 2 (also known as NURR1)
NSC	Neural Stem Cell
NSE	Neuron-Specific Enolase
OECs	Olfactory Ensheathing Cells
OSM	Oncostatin M
OTX	Orthodenticle Homeobox
P	Passage
PAX	Paired Box Gene
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PDGF	Platelet-Derived Growth Factor
PEG	Polyethylene Glycol
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PI	Propidium Iodide
PITX3	Paired-Like Homeodomain Transcription Factor-3
PKA	Protein Kinase A
POU5F1	POU Class 5 Homeobox 1 (also known as OCT3/4)
PTC	Patched
RA	Retinoic Acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
s	Seconds
S	Stage
SC	Stem Cell
SHH	Sonic Hedgehog
SingleDA	Single-Stage Dopaminergic Neuronal Differentiation
SingleND	Single-Stage Neuronal Differentiation
SMO	Smoothened
SOX2	Sex Determining Region Y-box 2
SSEA	Stage-Specific Embryonic Antigen
STAT	Signal Transducer, and Activator of Transcription
TGF	Transforming Growth Factor
TH	Tyrosine Hydroxylase
T_H	T Helper
TNF- α	Tumour Necrosis Factor α
TPA	4β-12-O-tetradecanoylphorbol 13-acetate
VMAT2	Vesicular Monoamine Transporter 2
VPA	Valproic Acid
VTA	Ventral Tegmental Area
WNT	Wingless-Type MMTV Integration Site Family

CHAPTER 1

LITERATURE REVIEW

1.1 Bone Marrow-Derived Mesenchymal Stem Cells

1.1.1 Adult Bone Marrow-Derived Stem Cells

Stem cells (SCs) are regarded as possessing a unique position in the scheme of cell biology, due to the defining properties of self-renewal, extensive proliferation, differentiated progeny generation, and broad developmental potential (Anderson *et al.*, 2001; Blau *et al.*, 2001). Traditionally, a hierarchical system based on developmental potential has been employed for the classification of vertebrate SCs (Figure 1.1). Embryonic SCs (ESCs) derived from the inner cell mass of the blastocyst possess the greatest developmental potential with the ability to generate all differentiated cell types in the body, and are termed pluripotent. Whereas, tissue-committed SCs are restricted to producing cell types found within that tissue, and are termed multipotent. A range of terminologies have been used in describing committed SCs, but for the purposes of this thesis tissue-committed SCs will be described using the tissue of origin as a prefix, and progenitor cells will refer to the more committed progeny of the tissue-committed SCs. Interest in this area of cell biology has been fuelled by discoveries of SC populations residing in multiple adult tissues and organs, including the bone marrow (BM), epidermis, gastrointestinal epithelium and nervous system. The first adult tissue described to contain a population of SCs was the BM (Cole *et al.*, 1955; Lorenz *et al.*, 1951; Nowell *et al.*, 1956; Till *et al.*, 1961).

In the early 1950's, several independent studies suggested the presence of a factor within BM that, upon transplantation, was capable of preventing death in laboratory animals after otherwise lethal whole body irradiation (Cole *et al.*, 1955; Lorenz *et al.*, 1951; Nowell *et al.*, 1956). This culminated in the Nobel prize-winning discovery, by Till and McCulloch, of BM-derived cells that were capable of

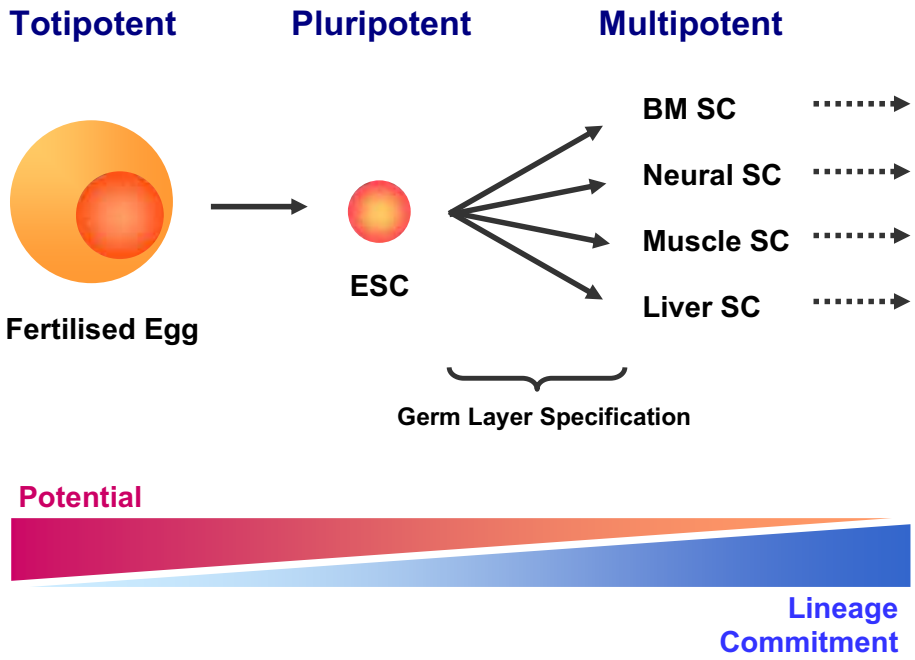

Figure 1.1 Stem cell developmental potential.

Stem cells (SCs) are typically classified according to developmental potential, with pluripotent embryonic SCs (ESCs) possessing the greatest capacity for differentiation. Multipotent tissue-committed SCs are restricted to producing cell types of the tissue from which the SCs are derived. A long-held belief of the process of SC commitment is that it proceeds unidirectionally from left to right, with decreasing potential and increasing lineage commitment. More recently, the extent of lineage commitment of tissue SCs has been challenged by findings of transdifferentiation of cells from one lineage to a different lineage under certain circumstances, giving rise to the concept of adult SC plasticity. BM, bone marrow.

giving rise to colonies of rapidly proliferating and differentiating haematopoietic tissue in the spleen (Till *et al.*, 1961). These populations of "colony-forming units (CFUs)" are today known to be haematopoietic SCs (HSCs), which have the ability to reconstitute cells of the entire haematopoietic spectrum. To this day, of all the SCs identified in adult tissues, HSCs are perhaps the most well characterised both *in vitro* and *in vivo*, and much knowledge has been attained of the hierarchical lineage progression from HSC to mature progeny. This is due in part to the relative ease of assaying HSCs *in vivo*, due to the ability of prospectively isolated HSCs to circulate and home to permissive niches, the continuous and rapid turnover of HSCs and HSC-derived progeny, and the systemic distribution of haematopoiesis in the body (Bianco *et al.*, 2008; Ma, 1990). BM is now known to consist of a heterogeneous population of cells, including two types of SCs, HSCs as well as mesenchymal stem cells (MSCs), also known as multipotent mesenchymal stromal cells.

1.1.2 The Mesenchymal Stem Cell Identity

Evidence for the existence of a cell population within the BM that produced non-haematopoietic progeny emerged in the mid-1960's with the pioneering work of Friedenstein and colleagues (Friedenstein *et al.*, 1970; Friedenstein *et al.*, 1968; Friedenstein *et al.*, 1966). These studies demonstrated the occurrence of *in vivo* osteogenesis in transplants of BM (Friedenstein *et al.*, 1968; Friedenstein *et al.*, 1966), as well as *in vitro* clonal fibroblast colony formation in monolayer culture (also known as CFU – fibroblastic (CFU-F) formation) (Friedenstein *et al.*, 1970). This provided the framework for subsequent studies, particularly by the development of a rudimentary technique to isolate the MSC fraction from BM by adherence to cultureware and removal of non-adherent haemopoietic cells through a complete medium change at 24-48 hours post-plating (Friedenstein *et al.*, 1970; Friedenstein *et al.*, 1974b). These

findings were extended by a number of independent investigators, who employed the isolation and culture techniques of Friedenstein, *et al.*, and together these studies demonstrated that MSCs were multipotential and differentiated into cells of the mesodermal lineage, including osteoblasts (Beresford *et al.*, 1992; Cheng *et al.*, 1994; Friedenstein *et al.*, 1987; Howlett *et al.*, 1986; Rickard *et al.*, 1994), chondroblasts (Friedenstein *et al.*, 1987; Johnstone *et al.*, 1998; Mackay *et al.*, 1998), adipocytes (Beresford *et al.*, 1992; Lanotte *et al.*, 1982) and myoblasts (Wakitani *et al.*, 1995). The marrow stromal SC concept was proposed in 1988 by Owen and Friedenstein (Owen *et al.*, 1988), whereas the currently popular term "MSC" was later coined by Caplan (Caplan, 1991). Collectively, these studies have paved the way for the development of the field of MSC research as we know it today.

Since these initial reports, scientific and clinical research interest in MSCs has increased exponentially. It is now generally recognised that MSCs possess the *in vitro* characteristics of SCs with the ability to proliferate, symmetrically divide, and produce multi-lineage mesodermal derivatives, making MSCs an attractive candidate for use in potential cellular therapies. In addition, MSCs exhibit further promising qualities for regenerative medicine, including relatively easy isolation from small aspirates of BM, as well as relatively easy expansion in culture with low tumorigenicity and teratoma formation. Furthermore, MSCs have been reported to display immunosuppressive properties that are advantageous for allogeneic transplantation (see Section 1.1.5), and in ideal settings, autologous transplantation is also possible. Subpopulations of MSCs have also been reported to be capable of differentiation to non-mesodermal lineages (see Section 1.2.2, 1.2.3, and 1.3), indicating the potential application of MSCs in a wider range of diseases.

The results of decades of research are beginning to elucidate the factors affecting the complex regulation of MSC differentiation and proliferation pathways. However, despite the intense interest and research activity surrounding MSCs, there remain large gaps in our knowledge and understanding of these cells. Currently, distinctive phenotypic markers of MSCs are still yet to be identified, and MSCs have primarily been characterised *in vitro*, with *in vivo* validation of SC qualities yet to be shown. In addition, laboratories investigating MSCs have employed different methods of isolation, expansion and characterisation of these cells (see Section 1.1.3). MSC-like cells have also been isolated from a variety of tissue sources, including adipose tissue, skeletal muscle and umbilical cord, leading to the suggestion that cells with MSC-like characteristics reside in virtually all postnatal organs and tissues. Cumulatively, this has led to difficulties in comparing and contrasting study outcomes, and has added to the controversy and confusion in the field.

Consequently, criteria for defining MSCs has increasingly been sought, and the International Society for Cellular Therapy has proposed minimal criteria for defining human MSCs (hMSCs) in an effort to promote more uniform characterisation of MSCs and facilitate data exchange amongst laboratories (Dominici *et al.*, 2006). This proposal was based on the best available data at the time, and recognised that the emergence of new data would require revisions of the criteria. Three criteria were proposed to define hMSCs: 1) MSCs must be adherent to plastic when cultured in standard conditions; 2) MSC populations must express specific surface antigens CD105, CD73 and CD90 (at ≥ 95% purity), but lack expression of haematopoietic surface antigens (≤ 2% positive), such as pan-leukocyte marker CD45, primitive haematopoietic progenitor and endothelial marker CD34, monocyte and macrophage markers CD14 and CD11b, B lymphocyte markers CD79α and CD19, and human leukocyte antigen (HLA)-DR

molecules; and 3) MSCs must exhibit multipotent differentiation potential by differentiating to osteoblasts, adipocytes and chondroblasts under standard *in vitro* tissue culture-differentiating conditions with confirmation of differentiation using histochemical and immunohistochemical staining (Dominici *et al.*, 2006). However, this proposal only addresses the *in vitro* characteristics of MSCs. The reason for this is a limitation common across most fields of adult SC research, that of primary examination of adult SCs only in *in vitro* systems, with little known of the function or existence of these cells *in vivo* (HSCs excepted). In the case of MSCs, while it is accepted that *in vitro* CFU-F potential and multipotent mesodermal differentiation may indicate MSC activity, it has thus proven far more challenging for MSCs, than for HSCs, to demonstrate *in vivo* long-term survival with self-renewal capacity and tissue repopulation with multi-lineage differentiation (Horwitz *et al.*, 2005). Therefore, whilst it is common practice to refer to MSCs as a type of SC, and while it is also generally accepted that a bona fide MSC exists, it must be acknowledged that the *in vivo* evidence for this remains to be demonstrated.

The issue of terminology remains controversial, and further suggestions have recently been made, with Bianco and colleagues (Bianco *et al.*, 2008) suggesting that the term "skeletal SC" be used until it has been definitively proven that these cells can produce tissues other than skeletal cell types. Bianco, *et al*. (Bianco *et al.*, 2008) also suggest that the term CFU-F be used to describe a cell that is assayed as clonogenic in culture, with a prefix to denote the tissue of origin, e.g. BM-CFU-F for CFU-F derived from BM.

1.1.3 Isolation and Expansion of Mesenchymal Stem Cells

The identification of MSCs has been limited as the biologic property which most uniquely identifies MSCs, that of multi-lineage mesodermal differentiation (Pittenger *et*

al., 1999; Prockop, 1997), does not permit prospective isolation. The plastic-adherence method of isolation, described by Friedenstein, *et al.* (Friedenstein *et al.*, 1970) more than 35 years ago, is still commonly used for MSC isolation today. Additionally, in this time little progress has been made in further optimising MSC culture conditions, with the majority of studies depending on the addition of foetal bovine serum (FBS) for MSC expansion. The reported frequency of MSC colony formation from the mononuclear cell (MNC) fraction of BM is approximately 1 in 10^5 to 1 in 10^6 (Bruder *et al.*, 1997; Reyes *et al.*, 2001), highlighting the need for increased homogeneity in isolated cells and the necessity of efficient expansion methods to generate sufficient cells for use in downstream applications.

Since the 1990s, efforts have been made by independent laboratories to improve the purity of isolated cells and to achieve clonal MSC populations. The persistence of haematopoietic cells in MSC cultures (Cheng *et al.*, 1994; Friedenstein *et al.*, 1970) prompted some investigators to implement antibody-mediated negative selection procedures. Cells expressing CD14, CD31 and CD11a have been removed from adherence isolated BM stromal cells via magnetic Dynabeads and a magnetic particle concentrator (Rickard *et al.*, 1996). CD45-positive cells and glycophorin A-positive cells have been depleted from the MNC fraction by means of immunomagnetic microbeads and MACS separation after Ficoll-Paque density gradient centrifugation (Reyes *et al.*, 2001; Tondreau *et al.*, 2004a). Most recently, haematopoietic cells have also been removed using tetrameric antibody complexes, which link the target cells to erythrocytes for removal during Ficoll-Paque centrifugation (Tondreau *et al.*, 2004a).

Alternatively, antibody-mediated positive selection procedures for prospective MSC isolation have also been investigated; however, identification of the most appropriate markers for this purpose remains an issue. Markers that have been examined

for positive selection include: STRO-1, which has been utilised after MNC isolation by Ficoll density gradient centrifugation (Gronthos *et al.*, 1994; Simmons *et al.*, 1991); low affinity nerve growth factor receptor, also known as p75 neurotrophin receptor or CD271 (Quirici *et al.*, 2002); CD49a, the α1-integrin subunit of VLA-1 (very-late antigen 1) integrin, which is a receptor for both collagen and laminin (Deschaseaux *et al.*, 2000; Deschaseaux *et al.*, 2003); neural ganglioside GD2 (Martinez *et al.*, 2007); stage-specific embryonic antigen (SSEA)-4, an early embryonic glycolipid antigen (Gang *et al.*, 2007); and melanoma-associated cell adhesion molecule, MCAM/CD146, which was applied following $CD45^+$ cell depletion (Sacchetti *et al.*, 2007). Nonetheless, despite intense research, no consensus has been reached on a definitive BM MSC marker for prospective isolation and *in situ* identification. However, the recent finding of CD146-expressing subendothelial cells within BM stroma, may provide a promising lead for future studies, as the CD146 marker identified self-renewing osteoprogenitors that were capable of establishing the haematopoietic microenvironment at heterotopic sites *in vivo* (Sacchetti *et al.*, 2007).

Attempts have been made to improve MSC culture conditions with the intent of generating more pluripotent cells or to meet standards for clinical use. However, these newer methods have yet to be implemented in mainstream use. Furthermore, the use of different tissue sources and isolation and culture techniques by different laboratories has resulted in the isolation of subpopulations of MSCs with different characteristics. This has added further complexity to the field, and highlights the need for independent examination of the baseline characteristics of MSC populations employed in different studies.

1.1.4 Characterisation and Function of Mesenchymal Stem Cells

Although the discovery of MSCs occurred several decades ago, the properties and function of these cells are still being elucidated today. The multi-lineage mesodermal differentiation capacity of MSCs has been known for some time, however, research is currently underway to determine the mechanisms underlying these processes and methods for directed differentiation towards functional mature cell types. The search for a definitive marker of MSCs is also ongoing, and much progress has been made in characterising the MSC surface marker phenotype. The migratory capacity, cytokine secretion and unique immunological features of MSCs are also under further investigation. Furthermore, research aimed at defining the microenvironmental niche and the role of MSCs *in vivo* continues to be examined.

1.1.4.1 Initial Observations

Initially, the adherent cells isolated by Friedenstein, *et al.* were described as a heterogeneous population, which contained a number of isolated large fibroblast-like cells by the third day post-explantation (Friedenstein *et al.*, 1970; Friedenstein *et al.*, 1976). Haematoxylin staining of these cultures revealed the fibroblast-like cells to display a pale cytoplasm and ovoid nuclei containing several distinct nucleoli. After 5-6 days, discrete foci of 4-8 fibroblasts were observed in the cultures, and the cells multiplied rapidly to form large discrete colonies of 10^2-10^3 fibroblasts by the 10^{th} day, with colonies fusing together on the 15^{th} day. Friedenstein, *et al.* also demonstrated the osteogenic ability of these colony-forming fibroblasts after transplantation in diffusion chambers *in vivo* (Friedenstein *et al.*, 1970), and importantly, showed retention of the capacity for osteogenesis and chondrogenesis after 20-30 cell doublings *in vitro* (Friedenstein *et al.*, 1987).

1.1.4.2 Role of MSCs in Supporting Haematopoiesis In Vivo

The essential role of BM stroma in supporting *in vivo* haematopoiesis was established in early experiments, which demonstrated successful formation of the haematopoietic microenvironment at ectopic sites following transplantation of BM fragments or BM stromal cells (Friedenstein *et al.*, 1974a; Friedenstein *et al.*, 1968; Maniatis *et al.*, 1971; Tavassoli *et al.*, 1968). Tavassoli and Crosby showed that transplantation of large autologous marrow fragments resulted in initial necrosis and phagocytosis of haematopoietic elements, leaving behind a network of mesenchymal cells (Tavassoli *et al.*, 1968). These cells proliferated and acquired osteoblastic morphology with development of a lattice of trabecular bone throughout the implant by day 6, and later also formed the lining layer of sinusoids. After establishment of the sinusoidal microcirculation, haematopoietic elements appeared around the sinusoids in the interstices of the bone, which was gradually resorbed, leaving only a shell of bone encapsulating the new haematopoietic tissue by the 5^{th} week (Tavassoli *et al.*, 1968).

Semi-syngeneic BM transplantation experiments revealed that by 12-14 months the structures formed were chimaeric, with the haematopoietic tissue of recipient origin, whilst the osteogenic tissue was donor-derived (Friedenstein *et al.*, 1968). And subsequently, through irradiation of BM prior to transplantation, Maniatis, *et al*. found that the osteogenic precursor cells were associated with the transplanted BM stroma, and that donor-derived haematopoietic cells were not necessary for regeneration of marrow implants (Maniatis *et al.*, 1971). However, the precise identity and anatomical location of the cells within the BM fragments that established the heterotopic haematopoietic microenvironment remained unknown.

Recent evidence has emerged describing CD146 as a potential marker of these cells (Sacchetti *et al.*, 2007). CD146 was found to label subendothelial (adventitial)

reticular cells in sinusoidal walls, which projected reticular processes that associated with haematopoietic cells. Furthermore, subcutaneous transplantation of human $CD146^+$ CFU-Fs in immunocompromised mice revealed a defined developmental sequence of bone formation followed by the appearance of a sinusoidal system, and finally colonisation of the ossicle with murine haematopoietic tissue by 8 weeks (Sacchetti *et al.*, 2007).

1.1.4.3 Role of MSCs In Supporting Haematopoiesis In Vitro

Early studies on HSCs also identified that BM-derived adherent cells provide an important microenvironment in *in vitro* culture systems for the long-term maintenance, proliferation and differentiation of HSCs. Dexter and colleagues found that the introduction of a BM-derived adherent cell layer significantly improved *in vitro* murine HSC culture, and allowed granulopoiesis and HSC production to be maintained for 2-3 months, whereas previous systems were only successful for several days to a few weeks (Dexter *et al.*, 1977). In addition, cultures established without the adherent cell layer showed rapid decline in the numbers of cells within 1-2 weeks, as well as disappearance of HSCs and granulocyte precursors within 2-3 weeks (Dexter *et al.*, 1977).

Gartner and Kaplan adapted this liquid medium and BM feeder layer culture system to the *in vitro* culture of human HSCs (Gartner *et al.*, 1980). They found the production of granulocytic-macrophage progenitor cells to be dependent on the BM-derived adherent layer of cells, and the continuation of haematopoiesis for at least 20 weeks. Furthermore, active haematopoiesis was observed to occur in “cobblestone”-like areas, which contained myeloid and monocytoid cells. The non-adherent cells were very closely associated with the stromal layer, and monocytoid cells were seen scattered

throughout the stromal layer in islands of haematopoiesis and in large tight clusters within the stromal layer.

Later studies showed that haematopoiesis-supporting stromal cells secrete biologically functional cytokines (including colony-stimulating factor (CSF)-1, granulocyte-CSF, granulocyte-macrophage-CSF (GM-CSF), interleukin (IL)-6 and c-kit ligand), which are involved in the development of haematopoietic cells, in particular those of the myeloid lineage (Eaves *et al.*, 1991; Kittler *et al.*, 1992). In addition, the adherent stromal cells synthesise abundant extracellular matrix, consisting of basal lamina and interstitial collagen types, including collagen types I, III, IV and V, as well as fibronectin and proteoglycans, in particular chondroitin sulfate-containing proteoglycans (Clark *et al.*, 1995).

1.1.5 Immunomodulatory Properties of MSCs

Further clinical interest in MSCs has been fuelled by an emerging body of data indicating that MSCs possess immunomodulatory properties. MSCs had been long thought of as having an immune privileged status, but are now recognised to possess the dual ability to both suppress and/or activate immune responses, depending on the stimulus to which the cells have been exposed (Locatelli *et al.*, 2007; Stagg, 2007). Initially, investigations centred around the immunosuppressive nature of MSCs in relation to T lymphocytes (Di Nicola *et al.*, 2002; Krampera *et al.*, 2003; Le Blanc *et al.*, 2003). However, increasing evidence has revealed that MSCs may exert effects on a broad range of immune cells, including antigen-presenting cells (APCs) (Aggarwal *et al.*, 2005; Beyth *et al.*, 2005; Jiang *et al.*, 2005; Nauta *et al.*, 2006a; Zhang *et al.*, 2004), natural killer (NK) cells (Aggarwal *et al.*, 2005; Krampera *et al.*, 2006; Maccario *et al.*, 2005; Rasmusson *et al.*, 2003; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2006) and

B cells (Augello *et al.*, 2005; Corcione *et al.*, 2006; Glennie *et al.*, 2005; Krampera *et al.*, 2006).

1.1.5.1 Effects of MSCs on T cells

In vitro studies with human, baboon and murine MSCs have demonstrated that MSCs are capable of inhibiting the proliferation of T cells induced by allogeneic cells or mitogens such as phytohemagglutinin and concanavalin A (Bartholomew *et al.*, 2002; Di Nicola *et al.*, 2002; Le Blanc *et al.*, 2003; Tse *et al.*, 2003), as well as the activation of T cells by CD3 and CD28 antibodies (Krampera *et al.*, 2003; Tse *et al.*, 2003), in a dose-dependent manner. Furthermore, MSCs have been reported to escape recognition by cytotoxic T lymphocytes (CTLs), inhibit the formation of CTLs, induce formation of regulatory $CD8^+$ T cells, decrease the secretion of interferon (IFN)-γ by T helper (T_H) 1 cells and increase IL-4 secretion by T_H2 cells, favour the differentiation of $CD4^+CD25^+$ regulatory T cells and decrease the alloantigen-specific cytotoxic capacity of CTLs or NK cells (Aggarwal *et al.*, 2005; Djouad *et al.*, 2003; Maccario *et al.*, 2005; Rasmusson *et al.*, 2003).

The suppressive effect of MSCs on T cell proliferation was found to be independent of the major histocompatibility complex (MHC) (Krampera *et al.*, 2003; Le Blanc *et al.*, 2003), with the majority of studies showing involvement of soluble factors secreted by MSCs, such as hepatocyte growth factor (HGF), transforming growth factor (TGF)-β1, prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO; which depletes tryptophan, an essential factor for lymphocyte proliferation), nitric oxide and IL-10 (Aggarwal *et al.*, 2005; Di Nicola *et al.*, 2002; Djouad *et al.*, 2003; Meisel *et al.*, 2004; Rasmusson *et al.*, 2003; Rasmusson *et al.*, 2005; Sato *et al.*, 2007; Tse *et al.*, 2003). However, due to conflicting data, there remains no consensus on which soluble factors are essential for suppression. In addition, a role for direct cell-to-cell contact

mechanisms in MSC-mediated suppression has also been described (Augello *et al.*, 2005; Krampera *et al.*, 2003).

Another possible mechanism of MSC-mediated immune suppression is induction of T cell anergy. MSCs express MHC class I molecules which may activate T cells, but lack surface expression of costimulatory molecules, such as CD80 and CD86, resulting in MSCs being unable to provide a secondary signal and thereby leaving T cells anergic (Chamberlain *et al.*, 2007; Nauta *et al.*, 2007; Stagg, 2007). MSCs have been demonstrated to induce division arrest anergy in T cells, in which T cell proliferation was inhibited (arrested at G_1 phase) and IFN-γ production was decreased (Glennie *et al.*, 2005). However, this condition differed to classical anergy, which can be reversed by exogenous IL-2, as T cell proliferation could not be restored upon addition of exogenous IL-2.

Overall, these studies have been interpreted as indicative of an immunosuppressive role of MSCs, however, it has been acknowledged that the current evidence only shows targeting of T cell proliferation *in vitro* rather than T cell effector function, and therefore may suggest a general non-specific anti-proliferative effect of MSCs (Nauta *et al.*, 2007; Ramasamy *et al.*, 2007; Ramasamy *et al.*, 2008).

1.1.5.2 Effects of MSCs on Professional Antigen-Presenting Cells

The differentiation, maturation and function of the initiators of the immune response, the professional APCs e.g. dendritic cells (DCs), are also disrupted by MSCs. Several studies have shown that the presence of MSCs significantly inhibited differentiation of both monocytes and $CD34^+$ progenitors into DCs, and prevented the increase of CD1a, CD40, CD80, CD86 and HLA-DR expression. During maturation, MSCs prevented up-regulation of CD40 and CD86, while mature DCs treated with MSCs also showed reduced CD83 expression, suggesting skewing toward an immature

status. Furthermore, exposure to MSCs during differentiation and maturation resulted in cells with decreased capacity for inducing T cell proliferation, showing that DC phenotype and function are affected by MSCs (Jiang *et al.*, 2005; Nauta *et al.*, 2006a; Zhang *et al.*, 2004). It has also been reported that hMSCs induce APCs toward a regulatory phenotype, and that the cells produced possess T cell-suppressive properties (Beyth *et al.*, 2005). Additionally, alterations in DC cytokine production have been demonstrated, with hMSCs found to cause decreased secretion of pro-inflammatory cytokines tumour necrosis factor-α (TNF-α) and IL-12, and increased production of anti-inflammatory cytokine IL-10 (Aggarwal *et al.*, 2005; Beyth *et al.*, 2005; Jiang *et al.*, 2005; Zhang *et al.*, 2004). Together these results indicate that MSCs are capable of suppressing DC differentiation and cause the formation of immature DCs that display a suppressor or inhibitory phenotype (Nauta *et al.*, 2007).

1.1.5.3 Effects of MSCs on B cells

MSCs have also been shown to exert immunosuppressive activities on B cells. Corcione, *et al.* (Corcione *et al.*, 2006) demonstrated that hMSCs inhibited *in vitro* human B cell proliferation by arresting cells in the G_0/G_1 cell cycle phase, prevented differentiation to antibody-secreting cells as seen by impaired immunoglobulin (Ig)M, IgG and IgA production, and interfered with chemotaxis through down-regulation of CXCR4, CXCR5 and CCR7 expression. However, no effect on B cell costimulatory molecule expression and cytokine production was observed, while paracrine secretion of soluble factors was implicated in the mechanism of B cell suppression. Murine studies have also observed similar findings of inhibition of B cell proliferation following stimulation with anti-CD40 monoclonal antibody and IL-4 or pokeweed mitogen, with soluble factors playing a role in suppression (Augello *et al.*, 2005; Glennie *et al.*, 2005). Additionally, co-culturing of B cells from a murine model of

systemic lupus erythematosus with allogeneic MSCs resulted in inhibition of B cell proliferation, activation and IgG secretion (Deng *et al.*, 2005). However, contradictory data has been reported, which found that B cell proliferation could only be inhibited by MSCs in the presence of exogenous IFN-γ (Krampera *et al.*, 2006). Nonetheless, these studies suggest that MSCs are capable of modulating B cell functions at multiple levels.

1.1.5.4 Effects of MSCs on Natural Killer Cells

Several independent groups have shown that MSCs also inhibit NK cell proliferation in response to IL-2, IL-15 and alloantigens (Krampera *et al.*, 2006; Maccario *et al.*, 2005; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2006). However, proliferation of activated NK cells was only partially suppressed by MSCs (Spaggiari *et al.*, 2006). Additionally, co-culture with MSCs was found to suppress the effector functions of NK cells. Secretion of cytokines, including IFN-γ, IL-10 and TNF-α, were reduced after culturing in the presence of MSCs (Aggarwal *et al.*, 2005; Krampera *et al.*, 2006; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2008), and NK cell cytotoxic activity was also impaired (Maccario *et al.*, 2005; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2008).

Spaggiari, *et al.* (Spaggiari *et al.*, 2008) showed that the suppression of cytotoxic activity was related to the down-regulation of surface expression of activating NK receptors NKp30 and NKG2D, which are involved in NK cell-activation and target cell killing, and absence of NKp44 activating receptor. However, other studies have found that cytolytic activity is not inhibited in freshly isolated NK cells (Rasmusson *et al.*, 2003), or was restricted to HLA-class I-expressing targets (Sotiropoulou *et al.*, 2006).

Initially, MSCs were thought to escape recognition by NK cells, since it was reported that MSCs were not lysed by killer cell immunoglobulin-like receptor ligand

mismatched alloreactive NK cells (Rasmusson *et al.*, 2003). However, recent reports have shown that hMSCs are highly susceptible to lysis by activated NK cells, and that this is mediated by activating NK receptors NKp30, NKG2D and DNAM-1 and the expression of the corresponding ligands by MSCs (Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2006). Interestingly, exposure of hMSCs to IFN-γ caused protection from NK cell-mediated lysis due to the up-regulation of surface HLA-class I expression (Spaggiari *et al.*, 2006).

The mechanisms of MSC-mediated NK cell suppression are still under investigation, but recent reports have found involvement of soluble factors IDO, PGE2 and TGF-β (Krampera *et al.*, 2006; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2008) as well as cell-to-cell contact (Sotiropoulou *et al.*, 2006), suggesting the existence of different mechanisms.

1.1.5.5 MSC Immunomodulation in In Vivo Studies

The immunosuppressive effects and immunogenicity of MSCs have primarily been demonstrated in *in vitro* studies, and only a few studies have investigated these properties in *in vivo* or clinical settings. However, the limited data available at present provides little evidence that donor MSCs are able to engraft after infusion or transplantation (Fibbe *et al.*, 2007; Le Blanc *et al.*, 2005).

In one of the earliest *in vivo* studies, Bartholomew and colleagues (Bartholomew *et al.*, 2002) showed that intravenous administration of allogeneic, MHC-mismatched MSCs prolonged the survival of allogeneic skin grafts in baboons to 11 days, in comparison with 7 days in control animals. Engraftment in multiple tissues and site-specific differentiation of hMSCs has also been reported after intrauterine transplantation in foetuses of sheep, even after development of immunocompetency (Airey *et al.*, 2004; Liechty *et al.*, 2000).

MSCs have also been found to exert therapeutic effects on severe graft-versus-host disease (GVHD), which was first reported in a landmark case study showing that repeated infusion of MSCs from the patient's HLA-haploidentical mother with cyclosporine treatment, completely reversed grade IV acute GVHD of the gut and liver (Le Blanc *et al.*, 2004). Additionally, systemic infusion of adipose-derived MSCs has been found to control lethal GVHD in mice, with greater efficiency if MSCs were administered early after HSC transplantation, and possible requirement of repeated doses (Yanez *et al.*, 2006). However, another murine study failed to show any effect of MSC transplantation on the incidence and severity of GVHD, although this may be due to administration of a single MSC dose alone (Nauta *et al.*, 2007; Sudres *et al.*, 2006). Horwitz, *et al*. have also administered allogeneic MSCs to children with osteogenesis imperfecta via intravenous (i.v.) infusion and demonstrated potential engraftment, at least for short-term periods of 4-6 weeks (Horwitz *et al.*, 2002).

Contrary to these findings, MSCs have been found to be immunogenic in certain transplant scenarios, indicating that MSCs are not intrinsically immune privileged. Xenogeneic transplantation of hMSCs into myocardium of immunocompetent rats resulted in an intense cellular immune response (primarily macrophage infiltration) and graft rejection (Grinnemo *et al.*, 2004). In contrast, myocardial engraftment was seen after transplantation into RNU athymic or tacrolimus-immunosuppressed rats. Furthermore, significant rat lymphocyte proliferation was observed when hMSCs were co-cultured with lymphocytes of rats previously exposed to hMSCs, indicating the presence of a sensitisation reaction. Another study examining the effect of MSCs on allogeneic BM transplantation in sublethally irradiated mice found that addition of syngeneic host MSCs significantly enhanced long-term engraftment, whereas infusion of donor-derived MSCs not only failed to prevent rejection, but increased rejection

(Nauta *et al.*, 2006b). This study further showed that MSCs were capable of inducing a memory T cell response after injection into immunocompetent hosts. Together with a previous study showing rejection of allogeneic MSCs after transplantation in MHC-mismatched mice (Eliopoulos *et al.*, 2005), these findings indicate that MSCs can be immunogenic under appropriate circumstances.

1.1.5.6 Controversy Related to MSC Immunomodulation

The precise mechanisms underlying the immunomodulatory properties of MSCs remain largely unresolved, despite being of increasing importance. The currently available data is controversial, and the majority of reports including a clinical study performed by our group (Ma *et al.*, 1987), have not demonstrated long-term engraftment of donor MSCs. Le Blanc and Ringden (Le Blanc *et al.*, 2005) have commented that the diversity in findings could be attributed to the use of different techniques for obtaining MSCs, different stimuli, culture conditions, doses and kinetics, and different lymphocyte populations. Furthermore, after *in vivo* transplantation it has been difficult to detect or recover MSCs from the BM of recipients, which could be due to a number of reasons, including homing of MSCs to other sites for mediating immune suppression, non-specific lodging in capillary beds of other tissues (particularly pulmonary tissue), the possibility that MSCs exert effects by production of growth factors and die after completion of this role, or perhaps difficulty in detecting MSCs within BM aspirates due to the endosteal location of these cells (Le Blanc *et al.*, 2005; Nauta *et al.*, 2007).

1.1.6 Classical Multi-Lineage Mesodermal Differentiation Capacity of MSCs

Consistent with the germ layer origin of these cells, it was demonstrated that under specific induction conditions, MSCs were capable of differentiation into the mesodermal cell types typically found in bone, cartilage and adipose tissue. The ability of MSCs to generate cell types of multiple mesodermal lineages has now been well-

established. Techniques for the *in vitro* differentiation of MSCs towards osteocytes, adipocytes and chondrocytes are widely used and highly reproducible amongst different laboratories.

Osteocytic differentiation is typically achieved through culturing a confluent layer of MSCs under the influence of dexamethasone, β-glycerol phosphate and ascorbate for 1-3 weeks (Pittenger *et al.*, 1999). This results in the formation of aggregates or nodules that stain with Alizarin Red S and Von Kossa techniques for detecting mineral deposition. In addition, increases in alkaline phosphatase activity and calcium accumulation are observed.

Adipogenesis of MSCs can be accomplished by treatment with isobutyl methyl xanthine (IBMX), dexamethasone, insulin and indomethacin (Pittenger *et al.*, 1999). Differentiation towards adipocytes is marked by expression of peroxisome proliferation-activated receptor γ2, lipoprotein lipase and fatty acid-binding protein aP2, and accumulation of intracellular lipid-rich vacuoles, which coalesce with continued culture to eventually fill the cell. These lipid-rich vacuoles can be stained through histochemical procedures using Oil Red O.

Differentiation of MSCs into chondrocytes *in vitro* is induced through gentle pelleting of cells into a micromass by centrifugation and culturing with TGF-β in the absence of serum (Johnstone *et al.*, 1998; Mackay *et al.*, 1998; Pittenger *et al.*, 1999). Histological analysis of the cell pellets post-differentiation revealed strong staining with toluidine blue and Safranin O indicating production of a glycosaminoglycan- and proteoglycan-rich extracellular matrix. Additionally, the extensive extracellular matrix was found to be abundant in type II collagen (COL2A1), type X collagen, aggrecan (ACAN), link protein, biglycan, decorin, chondroitin-4-sulfate and keratan sulfate, which are typical of cartilaginous tissues (Johnstone *et al.*, 1998; Kopen *et al.*, 1999;

Mackay *et al.*, 1998; Pittenger *et al.*, 1999; Yoo *et al.*, 1998). Chondrogenic differentiation was further marked by the formation of large cells embedded within lacunae, which morphologically resembled hypertrophic chondrocytes.

Further research is currently being conducted to extend these findings and to uncover the mechanisms involved during differentiation. In particular, differentiation procedures for obtaining specific subtypes of mesodermal cells are being examined for transplantation into specific tissues. These procedures are also being optimised, in terms of isolation and culturing techniques as well as removal of xenogeneic elements, in preparation for potential clinical use. Recent reports from our laboratory have also demonstrated that supplementation with bone morphogenetic protein (BMP)-2 enhances TGF-β3-mediated *in vitro* chondrocytic differentiation of BM-derived hMSCs (Shen *et al.*, 2009b), whereas BMP-13 was found to inhibit *in vitro* osteogenic differentiation of hMSCs (Shen *et al.*, 2009a).

Since the discovery that MSCs were capable of differentiation into multiple mesodermal lineages, numerous studies have now shown MSCs to also be capable of differentiation into non-mesodermal cell types under certain circumstances. The observation of MSC plasticity or multi-lineage differentiation has generated great interest in the biology of these cells, and will be the focus of the following section.

1.2 Lineage Conversion Potential of BM-Derived MSCs

The traditional model of embryonic development centres upon a strict hierarchy of stem cell subsets that display progressive and orderly differentiation in a unidirectional manner, finally culminating in a terminally differentiated cell type (Anderson *et al.*, 2001; Blau *et al.*, 2001; Eisenberg *et al.*, 2003; Goodell, 2003; Lemischka, 2002a; Morrison, 2001; Orkin *et al.*, 2002; Wagers *et al.*, 2004). Central to this view is the early specification of cells during development into the three embryonic

germ layers in the course of gastrulation. The segregation of cells into each germ layer was thought to be an irreversible event, and subsequent tissue regeneration and cell replacement were believed to occur through tissue-resident SCs only capable of generating mature cell types of that tissue. However, these established paradigms have been challenged in the past decade with reports demonstrating the surprising ability of adult SCs to cross lineage boundaries, bringing about a remarkable evolution in the field of SC biology (Anderson *et al.*, 2001; Blau *et al.*, 2001; Eisenberg *et al.*, 2003; Goodell, 2003; Lemischka, 2002a; Morrison, 2001; Orkin *et al.*, 2002; Phinney *et al.*, 2007; Prockop, 2007; Wagers *et al.*, 2004).

In particular, an increasing number of reports, from our laboratory and others, have shown MSCs to be capable of differentiation into a wide range of non-mesodermal cell types both *in vitro* and *in vivo*, including ectodermal neuronal and glial cells (Chen *et al.*, 2001; Hermann *et al.*, 2004; Jiang *et al.*, 2003; Jiang *et al.*, 2002; Kopen *et al.*, 1999; Munoz-Elias *et al.*, 2004; Sanchez-Ramos *et al.*, 2000; Tao *et al.*, 2005; Woodbury *et al.*, 2000; Zhao *et al.*, 2002), as well as endodermal hepatic cells (Jiang *et al.*, 2002; Schwartz *et al.*, 2002; Wang *et al.*, 2004). These findings have challenged the extent of lineage commitment of MSCs, and together with similar findings in other adult SC populations, have suggested the potential of adult SCs to be capable of transdifferentiation, which has consequently given rise to the concept of adult SC plasticity (Wagers *et al.*, 2004). Wagers and Weissman (Wagers *et al.*, 2004) have described transdifferentiation as “the conversion of a cell of one tissue lineage into a cell of an entirely distinct lineage, with concomitant loss of the tissue-specific markers and function of the original cell type, and acquisition of markers and function of the transdifferentiated cell type”. Whereas for SC plasticity, Blau and colleagues (Blau *et al.*, 2001) have proposed that the fate of SCs in adult tissues may be amenable to

change, rather than restricted to a specific lineage, depending on the type of microenvironmental cues present.

The observations of MSC plasticity or multi-lineage differentiation, while generating great interest in the potential therapeutic value of these cells, challenges the long-held dogma of irreversible tissue specification during development, and remains a highly controversial area. It is generally accepted that more rigorous and consistent scientific evidence is necessary to verify these claims before the basic tenets of developmental biology are discarded, in line with the ideology that extraordinary claims require extraordinary proof (Anderson *et al.*, 2001; Eisenberg *et al.*, 2003). It has also been proposed that higher standards of evidence should be established for demonstrating adult SC transdifferentiation (Anderson *et al.*, 2001). The following sections will discuss the recent advances in our understanding of MSC plasticity, as well as alternative arguments that have been put forward to explain transdifferentiation events and plasticity-related improvements observed in animal disease models.

1.2.1 Evidence for a Pluridifferentiated Stromal Progenitor Cell

The controversy surrounding the MSC plasticity debate has been further fuelled by recent findings that MSCs not only acquire non-mesodermal marker expression following exposure to differentiation conditions, but also express genes and proteins of heterologous lineages prior to differentiation, leading to the suggestion that MSCs possess a 'multidifferentiated' or 'pluridifferentiated' phenotype (Blondheim *et al.*, 2006; Deng *et al.*, 2006; Deng *et al.*, 2001; Lamoury *et al.*, 2006; Minguell *et al.*, 2005; Phinney *et al.*, 2006; Sanchez-Ramos *et al.*, 2000; Seshi *et al.*, 2003; Suon *et al.*, 2004; Tao *et al.*, 2005; Tondreau *et al.*, 2004b; Tremain *et al.*, 2001; Vogel *et al.*, 2003; Wislet-Gendebien *et al.*, 2003; Woodbury *et al.*, 2002; Woodbury *et al.*, 2000). Whilst, only one study has detected endodermal gene expression in undifferentiated MSCs

(Woodbury *et al.*, 2002), numerous reports have demonstrated that rodent and human MSCs express ectodermal markers before differentiation, in particular those of the neural lineage (Blondheim *et al.*, 2006; Deng *et al.*, 2006; Deng *et al.*, 2001; Lamoury *et al.*, 2006; Minguell *et al.*, 2005; Phinney *et al.*, 2006; Sanchez-Ramos *et al.*, 2000; Seshi *et al.*, 2003; Tao *et al.*, 2005; Tondreau *et al.*, 2004b; Tremain *et al.*, 2001; Vogel *et al.*, 2003; Wislet-Gendebien *et al.*, 2003; Woodbury *et al.*, 2002; Woodbury *et al.*, 2000). In addition, rat and murine MSCs have recently been found to express germ-line specific and pluripotency markers, including protamine 2, POU5F1 (POU class 5 homeobox 1; also known as OCT3/4) and zinc finger protein 42 homolog (also known as REX1) (Jiang *et al.*, 2002; Lamoury *et al.*, 2006; Woodbury *et al.*, 2002).

The significance of these findings is three-fold: firstly, it reveals the necessity of ascertaining the basal levels of lineage marker expression by undifferentiated MSCs, and also uncovers possible repercussions for previous reports claiming transdifferentiation without examining expression levels prior to commencement of differentiation; secondly, these findings provide additional support for the propensity of MSCs to undergo neural differentiation, by demonstrating the ease with which neural properties are spontaneously expressed by MSCs; and thirdly, the constitutive expression of both mesodermal and ectodermal phenotypes suggests that MSCs have a 'multidifferentiated' (Woodbury *et al.*, 2002) or 'pluridifferentiated' (Seshi *et al.*, 2003) phenotype.

The detection and level of neural marker expression by MSCs appears to vary from laboratory to laboratory. This confounding variability possibly arises from the use of different isolation and culturing techniques, which may have led to the isolation of different subpopulations of cells, and which is partially due to the lack of a defined marker/s for the identification of MSCs. Previously, our laboratory has shown that

undifferentiated hMSCs are capable of expressing a wide range of neural genes and proteins, including NF-M (neurofilament protein subunit of medium molecular weight), nestin (NES), β tubulin III, microtubule-associated protein (MAP)-2, neuron-specific enolase (NSE), myelin basic protein (MBP) and myelin-associated enzyme 2',3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase) (Tao *et al.*, 2005). These markers were generally expressed at low levels and, with the exception of MBP and CNPase, are typically markers of a primitive neural phenotype or early neural development. However, astrocyte and neuroglial precursor marker glial fibrillary acidic protein (GFAP), axonal marker Tau, and markers of mature neurons, including neurotransmitters tyrosine hydroxylase (TH), serotonin, glutamic acid decarboxylase, and γ-aminobutyric acid (GABA), were unable to be detected (Tao *et al.*, 2005).

In contrast, an early study by Woodbury, *et al.* initially reported lack of NF-M and Tau protein expression, with only low levels of NSE protein in undifferentiated rat MSCs (Woodbury *et al.*, 2000). In a later report, they then showed that rat MSCs also expressed neuroectodermal genes *GFAP*, *NEUROD1* (neurogenic differentiation 1), aldolase C, amyloid precursor protein, N-methyl D-aspartate glutamate binding subunit, and syntaxin 13, but lacked expression of mature neurotransmission markers synaptophysin and choline acetyltransferase(Woodbury *et al.*, 2002). Other early studies detected low levels of NES, neuronal nuclear antigen (NeuN) and GFAP protein expression in hMSCs prior to neural induction (Sanchez-Ramos *et al.*, 2000), or expression of vimentin, NSE, MAP-1B and β tubulin III in the absence of NF-M, MAP-2, Tau, S-100, GFAP and MBP (Deng *et al.*, 2001).

In recent times, a number of studies have further examined this apparent neural predisposition of MSCs (Blondheim *et al.*, 2006; Deng *et al.*, 2006; Minguell *et al.*, 2005; Tondreau *et al.*, 2004b). Tondreau, *et al.* observed constitutive expression of

immature neuronal markers NES and β tubulin III in the majority of hMSCs (greater than 80%; both gene and protein expression detected), with the presence of mature neuronal and glial proteins, MAP-2, TH and GFAP, at lesser degrees after 4-5 passages of culture (Tondreau *et al.*, 2004b). Suon and colleagues also found low level expression of β tubulin III, neurofilament, NSE and GFAP mRNA and protein in hMSCs (Suon *et al.*, 2004). Neural lineage markers, NES, NEUROD1, NeuN and β tubulin III were also detected in certain subpopulations of hMSCs by Minguell, *et al.* (Minguell *et al.*, 2005). Deng and colleagues described spontaneous neural protein expression by murine MSCs, with nearly 100% of murine MSCs positive for intermediate filament protein NES (Deng *et al.*, 2006). Additionally, mouse MSCs were shown to express β tubulin III (12%), NF-M (13.2%) and S100B (calcium binding protein; 15%), however, were negative for the polysialylated-neural cell adhesion molecule, GFAP and vimentin (Deng *et al.*, 2006). Whereas, Blondheim, *et al.* (Blondheim *et al.*, 2006) reported hMSC expression of 12 neural genes (including neurofilament protein subunit of heavy molecular weight, *NF-M*, *NES*, *NSE* and *NeuN*), 8 genes related to the neuro-dopaminergic system (including *TH*, dopamine transporter (DAT), aromatic L-amino acid decarboxylase, engrailed-1 (*EN1*), *NR4A2* (nuclear receptor subfamily 4, group A, member 2; also known as *Nurr1*) and paired-like homeodomain transcription factor-3 (*PITX3*)), and 11 transcription factors with neural significance (including neural zinc fingure 3, paired box gene (*PAX*) 3 and *PAX6*). In addition, NES, NSE, NeuN, TH and CNPase proteins were detected in hMSCs by Western blot analysis (Blondheim *et al.*, 2006). Overall, the commonly reported neural markers in undifferentiated MSCs include NES, NSE, β tubulin III, NF-M, NeuN and GFAP.

1.2.2 Evidence for Non-Mesodermal Differentiation of MSCs *In Vitro*

Of the known adult SC populations, BM-derived MSCs are one of the more extensively studied in terms of differentiation potential, as it is an easily accessible and expandable population with demonstrated multi-lineage differentiation potential. However, variability in the differentiation capacity of MSCs exists between laboratories, which may at least be partially accounted for by discrepancies in the isolation and culture expansion procedures of different laboratories. The majority of studies also require expansion of MSCs in culture, however, the expansion methods are often inconsistent and the effect of *in vitro* culture (sometimes long-term) on MSC plasticity has not been well-documented (e.g. possible introduction of epigenetic and genetic modifications, impact of cell density and confluence, cell senescence). Additionally, the conditions used to drive differentiation are not well-defined. Currently, the mechanisms involved in differentiation and development *in vivo* remain largely undetermined, adding to the difficulties of this field. In general, the differentiation methods employed involve the manipulation of culture microenvironments, mainly through addition of cytokines, signalling molecules, conditioned medium and/or chemicals, and alterations to the growth substrate.

The potential of MSCs to generate non-mesodermal cell types, such as neural, hepatic and epithelial cells, has been investigated in view of the possibility of utilising these cells in the treatment of degenerative diseases of these organs. The *in vitro* neurogenic potential of MSCs will be discussed in greater depth in Section 1.3. Generation of endodermal hepatocyte-like cells from MSCs *in vitro* has been achieved through culturing MSCs on Matrigel and supplementing media with fibroblast growth factor (FGF)-4 and HGF (Jiang *et al.*, 2002; Schwartz *et al.*, 2002), serum starvation of MSCs followed by HGF addition (Wang *et al.*, 2004), supplementation with HGF,

FGF-2 and nicotinamide, followed by a second step of oncostatin M (OSM), dexamethasone and insulin transferrin selenium (ITS) mix (Lee *et al.*, 2004), addition of FGF-4 and OSM (Shi *et al.*, 2008; Shi *et al.*, 2005), co-culture with damaged liver tissue (Luk *et al.*, 2005), pellet culture with HGF and FGF-2 supplementation followed by OSM (Ong *et al.*, 2006), sequential addition of FGF-4, HGF, and HGF + ITS + dexamethasone (Snykers *et al.*, 2006), hepatocyte-conditioned medium with type I collagen (COL1A1) culture surface coating (Chen *et al.*, 2007c), and epigenetic modification through valproic acid (VPA) treatment followed by sequential addition of FGF-4, then HGF, and HGF + OSM + ITS + dexamethasone (Chen *et al.*, 2008). Cells obtained through these procedures were demonstrated to display epithelioid morphology, with some cells becoming binucleated, and expressed hepatocyte markers including hepatocyte nuclear factor (HNF)-3β, transcription factor GATA-4, cytokeratin (CK) 19, transthyretin, α-fetoprotein (α-FP), CK18, HNF-4, HNF-1α, albumin (ALB), glucose-6-phosphatase, and tyrosine-aminotransferase (Chen *et al.*, 2007c; Chen *et al.*, 2008; Jiang *et al.*, 2002; Lee *et al.*, 2004; Luk *et al.*, 2005; Ong *et al.*, 2006; Schwartz *et al.*, 2002; Shi *et al.*, 2008; Shi *et al.*, 2005; Snykers *et al.*, 2006; Wang *et al.*, 2004). Functional characteristics of hepatocytes were also acquired by MSCs post-differentiation. Schwartz and colleagues (Schwartz *et al.*, 2002) showed hepatocyte-like MSCs were capable of secreting urea and ALB, taking up low-density lipoprotein (LDL), expressing functional hepatocyte-specific cytochrome p450 and storing glycogen. Lee, *et al.* (Lee *et al.*, 2004) also demonstrated the properties of LDL uptake, cytochrome p450 enzyme presence, glycogen storage, urea production, as well as the expression of bile canaliculi-specific antigen in differentiated MSCs. Whilst other studies showed production of ALB and urea secretion (Shi *et al.*, 2008), together with

glycogen accumulation (Chen *et al.*, 2007c; Ong *et al.*, 2006; Shi *et al.*, 2005), and inducible cytochrome p450-dependent activity (Snykers *et al.*, 2006).

MSCs have also been reported to differentiate *in vitro* into epithelial-like cells following co-culture with heat-shocked small airway epithelial cells (AECs) or in an air-liquid interface co-culture system with AECs (Spees *et al.*, 2003; Wang *et al.*, 2005). The co-cultured MSCs gained epithelium-like characteristics, including a broad, flattened cytoplasm with an elevated perinuclear region, or an epithelial-like cuboidal or columnar morphology, expression of epithelial-specific markers such as keratin 17, 18 and 19, as well as CD24 (epithelial mucin-like glycoprotein), CC26 (marker of clara, serous and goblet cells in the lung) and occludin (tight junction protein), and a gene expression profile that closely resembled that of small AECs (Spees *et al.*, 2003; Wang *et al.*, 2005). Furthermore, the morphologically differentiated MSCs were found to form adherens junctions with neighbouring small AECs (Spees *et al.*, 2003). Surprisingly, while direct differentiation of some MSCs into epithelial-like cells was observed after incorporation of MSCs into the epithelial monolayer, Spees, *et al.* also detected fusion of MSCs with adjacent epithelial cells (Spees *et al.*, 2003). The frequency of this cell fusion was higher than previously reported, and in some cases nuclear fusion also occurred.

1.2.3 *In Vivo* Transplantation Studies Supporting MSC Plasticity

The prospect of using MSCs in autologous transplantation therapies provides the advantages of overcoming the ethical issues and constraints associated with the acquisition of other types of donor tissue and ESCs, and circumvents the need for anti-rejection drugs and the possibility of transplant rejection. Additionally, transplantation of MSCs is not associated with teratoma formation, unlike ESC transplantation. These factors, and the abilities to expand MSCs relatively easily in culture, as well as to

differentiate MSCs into many cell types *in vitro*, clearly make these cells attractive for potential cell-based patient-specific therapies. The fate of MSCs after transplantation *in vivo* has been investigated widely, with much interest garnered through reports of MSC-mediated disease improvement in experimental animal models. However, despite recent progress in our understanding, much remains to be determined concerning the *in vivo* behaviour and function of MSCs.

1.2.3.1 MSC Transplantation at Early Stages of Development

Transplantation studies have found MSCs to be capable of *in vivo* differentiation across germinal boundaries, in a manner that appears dependent on cues provided by the microenvironment of different organs. This cross-lineage potential was observed after transplantation of hMSCs intraperitoneally in foetal sheep early in gestation (Liechty *et al.*, 2000). Engraftment was observed in multiple tissues, where hMSCs underwent site-specific differentiation to form chondrocytes, adipocytes, myocytes, cardiomyocytes, BM stroma and thymic stroma. Furthermore, hMSCs persisted in tissue for 13 months post-transplantation, and long-term engraftment was observed even with transplantation after the development of immunocompetence. These results suggest that hMSCs are capable of migration, integration and survival in xenogeneic host tissue, and may possess unique immunologic characteristics.

Transplantation of a subset of MSCs, known as multipotent adult progenitor cells (MAPCs), into early blastocysts has resulted in the generation of chimaeric mice (Jiang *et al.*, 2002). Murine MAPCs were observed to contribute to many somatic tissues, including brain, retina, lung, myocardium, skeletal muscle, liver, intestine, kidney, spleen, BM, blood and skin. Furthermore, injection of a single MAPC could generate balanced chimaeras, and chimaerism was not only detected in mouse embryos, but also in 6-20 week old mice. This study additionally showed evidence of robust,

early and persistent engraftment of MAPCs after i.v. infusion into non-irradiated and minimally irradiated post-natal non-obese diabetic/severe combined immunodeficient mice. MAPC engraftment and differentiation were seen in haematopoietic tissues (blood, BM and spleen) and lung, liver, and intestinal epithelium of all recipient animals.

Plasticity of MSCs *in vivo* has also been demonstrated in a chick embryo model, which showed engraftment and partial differentiation of MSCs in the absence of cell fusion (Pochampally *et al.*, 2004a). Four days after infusion of rat MSCs into 1.5-2 day old chick embryos, cells were found to migrate into multiple host tissues, and histological analysis revealed the presence of MSCs in developing organs such as the heart, liver, brain, and spinal cord. Additionally, the number of MSCs increased 1.5-33 fold in one-third of surviving embryos, indicating engraftment and expansion of donor cells. MSCs were most commonly detected in the heart, perhaps due to infusion in the area just above the dorsal aorta, and some cells expressed markers of cardiomyocytes, including cardiotin and α-heavy-chain myosin, suggesting partial differentiation of MSCs.

1.2.3.2 MSC Transplantation into Neural Tissues

The ability of MSCs to contribute to particular organ systems has also been the focus of intense investigation. Studies have examined the potential of MSCs to integrate and differentiate after transplantation into normal tissue, as well as developing and injured post-natal tissue. Numerous reports have now described the remarkable efficacy of MSCs in ameliorating damage caused by injury and disease in a wide range of organs and tissues. The mechanisms underlying the observed improvements are yet to be clearly defined; however, transdifferentiation and replacement of damaged cells,

secretion of trophic factors, interaction with endogenous host cells, alteration of immune responses, or a combination of these, have been proposed to be involved.

The majority of initial reports demonstrating neural engraftment and restoration by MSCs examined direct intracerebral transplantation of cells into neural regions, including the corpus striatum of normal albino rats (Azizi *et al.*, 1998), the lateral ventricles of neonatal mice (Kopen *et al.*, 1999), the ventricles of embryonic rats *in utero* (Munoz-Elias *et al.*, 2004), and the ischemic boundary zones in rat models of cerebral ischemia (Chen *et al.*, 2001; Zhao *et al.*, 2002). Injection of MSCs into uninjured animal models resulted in engraftment, migration along known neural SC (NSC) migratory pathways, incorporation into successive layers of the brain, and phenotypic expression of markers in a manner suggesting the involvement of region-specific signals within the different neural microenvironments to produce characteristics of radial glia, subventricular zone progenitors, migratory cells, parenchymal neurons, and glia (Azizi *et al.*, 1998; Kopen *et al.*, 1999; Munoz-Elias *et al.*, 2004). In addition, Azizi, *et al.* (Azizi *et al.*, 1998) found an approximate MSC engraftment rate of 20%, loss of COL1A1 and fibronectin production by MSCs post-transplantation, and lack of an inflammatory response or rejection of grafted cells. MSCs also exhibited long-term survival at 1 and 2 postnatal months when infused in rat ventricles *in utero* at embryonic day 15.5, and were detected in distant locations throughout the brain, including the olfactory bulbs, rostral migratory stream, frontal, parietal and occipital cortices, hippocampus, dentate gyrus, and the telencephalic ventricular and subventricular zones (Munoz-Elias *et al.*, 2004). In experimental models of stroke, intracerebral transplantation of MSCs was found to result in significant recovery of motor and somatosensory deficits (Chen *et al.*, 2001; Li *et al.*, 2000; Zhao *et al.*, 2002). In these studies MSCs were observed to survive and migrate along white matter tracts, as well as

differentiate to express neural phenotypic proteins, including astrocytic marker GFAP, oligodendrocytic marker GALC (galactosylceramidase), and neuronal markers NeuN, MAP-2, β tubulin III , NSE, and neurofilament proteins, when grafted into the ischemic brain microenvironment. However, while behavioural improvement was detected, the grafted cells did not re-establish a normal tissue cytoarchitecture, with Zhao and colleagues noting that grafted cells displayed a spherical morphology with few processes. From these observations the authors suggested that cell replacement and integration into host circuitry may not be responsible for functional recovery, and this could instead be mediated by trophic factor secretion by MSCs, or by host cells in response to interaction with MSCs (Chen *et al.*, 2001; Zhao *et al.*, 2002). Li, *et al.* (Li *et al.*, 2001a) also demonstrated functional improvement following administration of MSCs via an intracarotid arterial route in a rat middle cerebral artery occlusion model of cerebral ischemia. MSCs were observed throughout the ischemic region in middle cerebral artery territory and in multiple areas of the ipsilateral hemisphere, however, more than 90% of MSCs were localised in the ischemic core and boundary zone. In addition, approximately 10% of MSCs in the ipsilateral hemisphere were observed to express GFAP, and 1% expressed MAP-2, suggesting possible differentiation *in vivo* towards astrocytes and neurons respectively. Furthermore, the results of this study suggested that intra-arterial administration of MSCs was superior to using an intracerebral route, and transplantation of MSCs did not appear to evoke an inflammatory response. However, the mechanisms underlying the observed neurological recovery remained undefined, although the authors commented that it was unlikely to be due to the apparent adoption of a neural phenotype by MSCs, and could be related to trophic factors produced by MSCs or interaction of MSCs with the host brain (Li *et al.*, 2001a). Intracerebral and i.v. transplantation of MSCs into rodent models of traumatic

brain injury have also yielded successful results with observations of neurological improvement in the recipient animals (Lu *et al.*, 2001a; Mahmood *et al.*, 2003; Mahmood *et al.*, 2002; Mahmood *et al.*, 2001). Promising outcomes have also been obtained in animal models of Parkinson's disease (PD) following transplantation of MSCs, and these reports will be discussed in greater detail in Section 1.4.4. Collectively these studies highlight MSCs as a promising therapeutic modality for degeneration, ischemia and injury of the nervous system, and are indicative of the ability of MSCs to respond to specific signals in different microenvironments *in vivo*.

1.2.3.3 MSC Transplantation into Hepatic Tissues

The possible contribution of BM cells to liver cell types was initially observed in studies investigating cross-gender BM or liver transplantation in rodent models with or without hepatic injury (Petersen *et al.*, 1999; Theise *et al.*, 2000a), as well as a retrospective study examining human archival autopsy and biopsy liver specimens from recipients of therapeutic BM transplantations or orthotopic liver transplantations (Theise *et al.*, 2000b). However, transdifferentiation of MSCs toward hepatocyte-like cells *in vivo* remains controversial. Several studies have demonstrated engraftment of MSCs, induction of a hepatocyte-like phenotype and partial restoration of function after transplantation in the uninjured and injured liver of rodents (Aurich *et al.*, 2007; Chen *et al.*, 2007c; Chen *et al.*, 2008; Luk *et al.*, 2005; Oyagi *et al.*, 2006; Sato *et al.*, 2005). Whilst Fang and colleagues (Fang *et al.*, 2004) reported functional improvement in rodent liver fibrosis models, but could only show a low frequency of donor cell engraftment and transdifferentiation, with alleviation of injury attributed to endogenous hepatic regeneration elicited through interaction between MSCs and host cells. In contrast, the results of other studies suggested that MSCs recruited to chronically injured liver may instead display pro-fibrogenic myofibroblast-like properties and

contribute to liver fibrosis, with limited hepatocellular differentiation (di Bonzo *et al.*, 2008; Russo *et al.*, 2006). Nonetheless, marked differences exist between these studies, including the transplantation of uninduced MSCs or differentiated hepatocyte-like MSCs, the use of different routes or time points of administration, as well as different harvest time points, with some of these factors being touted as crucial to the success of the study in question.

1.2.3.4 MSC Transplantation into Epithelial Tissues

Reports have also documented the differentiation of MSCs *in vivo* toward a variety of epithelial cell types, including those of the lung (Kotton *et al.*, 2001; Ortiz *et al.*, 2003; Rojas *et al.*, 2005), retina (Arnhold *et al.*, 2007; Arnhold *et al.*, 2006; Kicic *et al.*, 2003), skin (Fu *et al.*, 2006a; Nakagawa *et al.*, 2005), and kidney (Herrera *et al.*, 2004; Morigi *et al.*, 2004). In the bleomycin-induced rodent pulmonary fibrosis model, transplanted MSCs were found to engraft in recipient lung parenchyma, localise to areas of injury, and differentiate into cells resembling type I pneumocytes (Kotton *et al.*, 2001) and type II pneumocytes (Ortiz *et al.*, 2003), as well as other lung cell types such as fibroblasts and endothelial cells (Rojas *et al.*, 2005). The rescue effects of MSCs were also detected in rodent models of retinal degeneration, with donor cells expressing markers of photoreceptor cells, adopting morphology similar to retinal pigment epithelial cells, integrating into the host retinal pigment epithelial cell layer and neuroretina layers, and establishing tight junction contacts with adjacent host cells (Arnhold *et al.*, 2007; Arnhold *et al.*, 2006; Kicic *et al.*, 2003). Animal grafting experiments with MSCs also revealed enhanced wound healing properties and possible generation of *de novo* intact skin, with transdifferentiation into epithelium (Nakagawa *et al.*, 2005), and formation of epidermal cells and sebaceous duct cells (Fu *et al.*, 2006a). Contribution of MSCs to renal repair was observed in murine models of acute renal

failure, in which i.v. transplantation of MSCs resulted in engraftment in the damaged kidney, differentiation into tubular epithelial cells, and restoration of morphological and functional alterations (Herrera *et al.*, 2004; Morigi *et al.*, 2004). Nonetheless, the majority of these studies recognised that the observed protective effects of MSCs may involve mechanisms other than transdifferentiation and replacement of lost cells, such as roles of MSCs in the suppression of inflammation, reduction of collagen deposition and production of reparative growth factors. Additionally, most were unable to rule out the possibility of cell fusion. Further controversies surround the reports describing MSC epithelial differentiation, with several studies arguing that MSCs show little evidence of differentiation into cells of the tubular epithelium after ischemic renal injury (Duffield *et al.*, 2005), or into corneal epithelial cells when grafted to chemically-damaged corneal surfaces (Ma *et al.*, 2006), or into photoreceptor cells in a rat model of retinal degeneration (Inoue *et al.*, 2007).

Taken together, these studies suggest that MSCs may have extraordinary potential for contributing to and restoring the structure and function of many different organ systems. However, the contradictory findings across the different studies, and the fact that the underlying mechanisms still remain largely undetermined, all point towards the need to consider alternative explanations to the observations of MSC plasticity and transdifferentiation. These possible alternatives and potential caveats will be examined in the following section.

1.2.4 Alternative Explanations for MSC Plasticity

Together with the numerous reports published demonstrating evidence for plasticity and transdifferentiation of MSCs, also come equally numerous studies containing contradictory findings that question these concepts. Several alternative explanations and mechanisms have been proposed to account for MSC plasticity,

including the presence of multiple distinct SC or progenitor cells within the heterogeneous BM compartment, cell-cell fusion leading to the transfer of markers between unrelated cell types, acquisition of broader developmental potential through *in vitro* culturing techniques, difficulties in tracking transplanted SCs and the progeny of these cells, as well as difficulties in ascertaining cellular identity through examination of only a few or several markers (Anderson *et al.*, 2001; Goodell, 2003; Morrison, 2001; Orkin *et al.*, 2002; Wagers *et al.*, 2004; Wulf *et al.*, 2001). Furthermore, observations of functional improvement in animal models of disease and injury credited to MSC transdifferentiation and cellular replacement, could instead be due to other mechanisms, such as the production of soluble factors, modulation of immune responses, transfer of mitochondria, and interaction with, or stimulation of endogenous host responses (Phinney *et al.*, 2007; Prockop, 2007).

A significant caveat of all studies involving MSCs, whether *in vitro* or *in vivo*, is the heterogeneous nature of MSC populations. Furthermore, the BM origin of MSCs is known to possess pre-existing heterogeneity. The complexity of these cellular populations allows for alternative interpretations of the proposed transdifferentiation events, since multiple SC types could be present and therefore account for the generation of multiple cellular lineages (Orkin *et al.*, 2002). This has been shown in studies investigating the production of haematopoietic cells from muscle, which demonstrated that itinerant HSCs residing within muscle were responsible for all muscle-associated haematopoietic activity, negating earlier studies supporting the plasticity of muscle-derived cells (Issarachai *et al.*, 2002; Kawada *et al.*, 2001; McKinney-Freeman *et al.*, 2002). A recent study has also reported the presence of neural crest-derived SCs within a variety of adult mouse tissues, including the BM (Nagoshi *et al.*, 2008). These findings suggest that the observed neural differentiation

potential of BM cells may actually reflect the potential of the neural crest-derived SCs that reside within BM. Further to this, Pierret and colleagues have put forward the hypothesis that adult SCs are all derived from the neural crest, which would then explain the reported plasticity seen in adult SC subsets (Pierret *et al.*, 2006). Phinney and Prockop (Phinney *et al.*, 2007) have also commented that the heterogeneity of MSCs and the complexity of the BM organ system may contribute to the therapeutic efficacy associated with these cell populations, but on the contrary, makes assessment of the transdifferentiation potential more difficult. Additionally, these authors proposed that evolutionary mechanisms have conferred plasticity to adult mesenchyme, in view of the fact that during development there are multiple transitions from epithelium to mesenchyme, and vice versa (Phinney *et al.*, 2007).

Reports of somatic SC plasticity often describe these events as infrequent or rare, leading to the possibility that alternative low-probability or aberrant mechanisms are responsible (Anderson *et al.*, 2001; Lemischka, 2002a), one of which is cell fusion (Alvarez-Dolado *et al.*, 2003; Terada *et al.*, 2002; Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003; Weimann *et al.*, 2003; Ying *et al.*, 2002). Moreover, certain cell types, including myocytes and hepatocytes, have a natural propensity for cell fusion. Initially, cell fusion events were demonstrated in co-culture experiments with ESCs and BM cells or central nervous system (CNS) cells (Terada *et al.*, 2002; Ying *et al.*, 2002). The BM and CNS cells were found to undergo spontaneous fusion with the ESCs, as well as adoption of an ESC phenotype. Shortly after these studies, additional findings were published documenting fusion of MSCs or BM cells with hepatocytes, cardiomyocytes and Purkinje neurons post-transplantation to form multinucleated cells (Alvarez-Dolado *et al.*, 2003; Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003; Weimann *et al.*, 2003). Furthermore Alvarez-Dolado, *et al*. (Alvarez-Dolado *et al.*, 2003) found no evidence of

MSC transdifferentiation or contribution to non-haematopoietic cell types without cell fusion *in vivo*. Importantly cell fusion does not appear to explain all instances of transdifferentiation. Transplanted BM-derived cells have been found to contribute *in vivo* to buccal epithelial cells of the cheek, insulin-producing pancreatic endocrine cells, and cardiomyocytes, in the absence of cell fusion (Ianus *et al.*, 2003; Pochampally *et al.*, 2004a; Tran *et al.*, 2003). Nonetheless a study investigating *in vitro* co-culture of MSCs with heat-shocked lung epithelial cells did observe the presence of fusion events; however, these phenomena could only account for a minority portion of the total transdifferentiation events (Spees *et al.*, 2003).

Technique-related factors have also been proposed to account for the discrepancies between studies examining SC plasticity and transdifferentiation. One such factor is the impact of *in vitro* culturing on developmental potential (Anderson *et al.*, 2001; Lemischka, 2002b; Morrison, 2001; Wagers *et al.*, 2004). The concern here arises from the possibility of cells accruing changes during extensive *in vitro* culture (such as reported for extensively cultured NSCs (Morshead *et al.*, 2002)) or after exposure to mitogens in culture, raising the question of whether the plasticity exhibited is a normal property possessed *in vivo*, or if cells are endowed with broader potential by the culture conditions employed. If this is indeed the case, then a greater concern emerges regarding the possibility that the manipulated cells could exhibit unpredictable behaviour if used therapeutically (Anderson *et al.*, 2001).

Another potential source of experimental variation lies in the difficulties with detecting donor cells post-transplantation and identifying the differentiation status of these cells (Goodell, 2003; Wulf *et al.*, 2001). Commonly used techniques for labelling or identifying donor cells are all subject to artifact and misinterpretation, for example, the high technical difficulty of *in situ* hybridisation techniques used for detecting the Y-

chromosome of male donor cells in female recipients, and limitation in the *lacZ* transgene reporter system due to the presence of endogenous β-galactosidase activity in lysosomes of mammalian cells, such as macrophages (Goodell, 2003; Wulf *et al.*, 2001). Labelling with green fluorescent protein (GFP) can also yield false positive results as a consequence of endogenous autofluorescence, which can be visualised at the same wavelengths used for detecting GFP fluorescence (Goodell, 2003; Wulf *et al.*, 2001). Furthermore, tissue resident macrophages of the host animal may phagocytose protein products from donor cells that could be detectable histochemically, again leading to erroneous results (Lemischka, 2002a). On the contrary, false negative results can also occur due to loss or reduction of transgene fluorescence. In addition, demonstration of donor cell differentiation is typically achieved through examining markers indicative of the tissue type, however, in most situations this does not allow definitive identification since most commonly used markers are expressed in a variety of tissues (Goodell, 2003; Wulf *et al.*, 2001).

Mechanisms for MSC-related *in vivo* functional improvement, other than transdifferentiation, have already been alluded to in Section 1.2.3. A number of studies have suggested that the alleviation of injury and disease in animal models following MSC transplantation could be attributed to the secretion of trophic factors, which could enhance repair by stimulating the regeneration of damaged cells or endogenous tissue SCs or progenitor cells (Chen *et al.*, 2001; Fang *et al.*, 2004; Inoue *et al.*, 2007; Li *et al.*, 2001a; Lu *et al.*, 2001a; Mahmood *et al.*, 2003; Mahmood *et al.*, 2002; Mahmood *et al.*, 2001; Munoz *et al.*, 2005; Phinney *et al.*, 2007; Prockop, 2007; Zhao *et al.*, 2002). MSCs have also been shown to promote functional recovery in a rat spinal cord injury model, by forming bundles that bridged the epicentre of the injury and that guided regeneration of host axons through the spinal cord lesion (Hofstetter *et al.*, 2002). These

lesions were typically filled with debris and macrophages, therefore, the MSCs re-established a degree of cellular organisation within the injury zone, and may have promoted nerve-fibre outgrowth by providing a physical growth-permissive surface (Hofstetter *et al.*, 2002). In ischemic renal injury, resident epithelial cells were found to restore kidney epithelial integrity rather than the transplanted MSCs, however, MSCs may have played an indirect role by contributing to the repopulation of the injured vascular endothelium (Duffield *et al.*, 2005). Modulation of immune responses by MSCs, discussed in Section 1.1.5, may also play a role in functional improvement *in vivo*. Support for this is seen in a recent study by Ohtaki and colleagues, which found improvement in neurological function and decreased neuronal death after transplantation of MSCs in a mouse model of transient forebrain ischemia (Ohtaki *et al.*, 2008). The beneficial effects observed were attributed to the ability of MSCs to modulate inflammatory and immune responses through alternative activation of microglia and/or macrophages and subsequent establishment of a T_H2 cell immune bias, together with the up-regulation of factors that decrease apoptosis (Ohtaki *et al.*, 2008). Another possible mode of tissue repair by MSCs could involve the transfer of intact mitochondria or mitochondrial DNA, as MSCs have been reported to rescue aerobic respiration in mammalian cells lacking functional mitochondria through this action, without evidence of cell fusion (Spees *et al.*, 2006).

Given the wide variance in the prevalence of transdifferentiation, the rarity of such occurrences, and the continued controversy surrounding this topic, a call has been made for a higher standard of evidence to verify claims of plasticity (Anderson *et al.*, 2001; Blau *et al.*, 2001; Morrison, 2001; Wagers *et al.*, 2004). Stringent criteria have been proposed for the demonstration of a bona fide somatic SC transdifferentiation event, including the necessity for prospective isolation of SC populations and clonal

analysis, minimal manipulation for assessment of intrinisic developmental potential, evidence of morphologically indistinguishable integration into the host tissue structure, demonstration of function, exclusion of cell fusion, production of robust and sustained regeneration of target tissues, detailed examination of co-localised protein signals by laser scanning confocal or deconvolution microscopy, and independent replication of results in different laboratories and in different experimental models (Anderson *et al.*, 2001; Blau *et al.*, 2001; Morrison, 2001; Wagers *et al.*, 2004). However, there is recognition of the validity of at least some of the plasticity findings, although the frequency at which transdifferentiation occurs may only be exceedingly rare (Eisenberg *et al.*, 2003; Goodell, 2003). And, there is general agreement that given the potential importance of this phenomenon, efforts must be undertaken to further understand these properties of SCs.

1.3 Neural Differentiation of BM-Derived MSCs *In Vitro*

Upon the realisation of the MSC transdifferentiation phenomenon, an immense effort across the field was channeled into determining whether differentiation toward specific cell types could be achieved and controlled in culture. Additionally, endeavours were made to understand the mechanisms that govern such cell fate decisions. An aim of many of these studies was the derivation of neural cells from MSCs. A wide variety of methods for neural differentiation of MSCs have been investigated, with some studies yielding promising results, including the induction of neural gene and protein expression and, in some cases, action potential generation. However, like many areas in the field of SC research, much remains to be determined.

The generation of neuroectodermal-like derivatives from MSCs *in vitro* is typically achieved through manipulation of the culture microenvironment. In general, at the commencement of neural differentiation, the MSC expansion medium is altered or

substituted with culture media, supplements and coating substrates characteristically used in neural cell culture. Depending on whether a specific neural cell type is desired, a range of different supplements and substrates have been employed. Surface coating substrates that have been utilised in MSC neural differentiation include fibronectin (Jiang *et al.*, 2003; Jiang *et al.*, 2002; Kim *et al.*, 2002a; Tao *et al.*, 2005), poly-D-lysine (Jin *et al.*, 2003; Kondo *et al.*, 2005), poly-L-lysine (Guo *et al.*, 2005; Hermann *et al.*, 2004; Long *et al.*, 2005), poly-L-lysine/laminin (Joannides *et al.*, 2003), poly-ornithine (Wislet-Gendebien *et al.*, 2003) and poly-L-ornithine/laminin (Suzuki *et al.*, 2004).

The main approaches taken for MSC neural induction primarily involve the use of chemicals, growth factors and/or signalling molecules, conditioned media, or co-culture systems. On a lesser scale, some studies have employed a genetic engineering approach, whilst others have combined various aspects of the different approaches. The different methods that have been employed are further discussed in the following sections.

1.3.1 Induction of Neuronal Differentiation of MSCs

1.3.1.1 Chemical Induction

Rapid *in vitro* transformation of human and rat MSCs into neuronal-like cells using a simple chemical treatment protocol was first reported by Woodbury and colleagues (Woodbury *et al.*, 2000). This study proposed that treatment with pre-induction media, containing Dulbecco's Modified Eagle's Medium (DMEM), 20% FBS, 1 mM β-mercaptoethanol (BME) and FGF-2 for 24 hours, followed by neuronal induction media composed of DMEM, 2% dimethylsulfoxide (DMSO), and 200 μM butylated hydroxyanisole (BHA) produced cells exhibiting neuronal morphology and protein expression (NSE, NF-M and Tau) in a very short period (changes observed as early as 60 min to 3 hours) (Woodbury *et al.*, 2000). This procedure was also used by a

separate group in a neurosphere formation step (Suzuki *et al.*, 2004), and was later optimised further through the addition of 10 μM forskolin, N-2 supplement, 2 mM VPA, 5 nM K252A, and 10 mM KCl (Munoz-Elias *et al.*, 2003). The method incorporating these additional factors resulted in greater than 70% morphologic conversion and expression of Tau, NeuN, NSE, and TUC-4 within 24 hours in the absence of mitosis (Munoz-Elias *et al.*, 2003).

Induction of early neural progenitors from hMSCs has also been achieved through the use of conditions that increase intracellular cyclic adenosine mono-phosphate (cAMP; 0.5 mM IBMX / 1 mM dibutyryl cAMP (dbcAMP) in DMEM/20% FBS) (Deng *et al.*, 2001). Treatment with IBMX/dbcAMP for 6 days resulted in conversion of 20% of MSCs to neuronal-like morphology and increased expression of NSE and vimentin protein (Deng *et al.*, 2001). A study comparing the Woodbury and Deng methods found a higher percentage of neuron-like cells generated from the Woodbury protocol, however, this method also yielded a much higher degree of cell death at 53% of total cells, compared to the less than 5% cell death seen in the Deng protocol (Rismanchi *et al.*, 2003). Alternative techniques that have been reported for hMSC neural differentiation include the application of 10^{-3} M BME +/- 5 x 10^{-7} M all-*trans*-retinoic acid (RA) to attain NES, NSE, NeuN and β tubulin III protein expression within 5 hours (Hung *et al.*, 2002), and treatment with 3 μM 5-bromo-2-deoxyuridine (BrdU) for 3 weeks to increase hMSC multi-lineage differentiation potential (Qu *et al.*, 2004).

Recent findings have raised doubts over the phenomenon observed during chemical induction and question whether these changes occur through true differentiation processes or through alternative cellular events. The factors underlying these concerns include the seemingly unrealistic time frame of the observed changes,

which occurred rapidly over several hours, as well as the lack of neurite growth cone formation with development of neuronal morphology, and the high degree of cell death accompanying the induction process. Recent independent studies using chemical neuronal induction techniques, in particular the DMSO/BHA and IBMX/dbcAMP methods, were unable to reproduce the results of the original studies (Bertani *et al.*, 2005; Choi *et al.*, 2006; Deng *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004; Suon *et al.*, 2004; Tao *et al.*, 2005). Furthermore, the morphological changes and increases in certain neuronal markers elicited by chemical exposure were demonstrated to be due to cellular toxicity, cell shrinkage and disruption of the actin cytoskeleton in response to environmental stress, rather than complex regulated cellular differentiation processes (Bertani *et al.*, 2005; Lu *et al.*, 2004; Neuhuber *et al.*, 2004; Suon *et al.*, 2004). Observations of similar cellular responses by a range of cell types, including primary fibroblasts, HEK293 cells and NIH3T3 cells (Bertani *et al.*, 2005; Deng *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004), and the lack of *de novo* neural-specific protein translation (Bertani *et al.*, 2005; Choi *et al.*, 2006), further substantiated these results.

1.3.1.2 Cytokine and Signalling Molecule Induction

The use of different combinations of cytokines and signalling molecules, such as RA, have been utilised for MSC neural differentiation in attempts to mimic the physiological environment thought to drive *in vivo* neural development. Additionally, clues garnered from the culture requirements of NSCs have also been implemented. Sanchez-Ramos and colleagues (Sanchez-Ramos *et al.*, 2000) conducted the first study examining cytokine and signalling molecule induction of MSCs toward neural-like cells. This work demonstrated that human and mouse MSCs cultured with 10 ng/mL epidermal growth factor (EGF) or 0.5 μM RA and 10 ng/mL brain-derived neurotrophic factor (BDNF) could be induced toward a neuronal phenotype (Sanchez-Ramos *et al.*,

2000). These factors caused large flat fibronectin-positive MSCs to transform into smaller ovoid or spindle-shaped cells, which expressed neural markers and decreased expression of fibronectin (Sanchez-Ramos *et al.*, 2000). Shortly afterwards, Kim, *et al.* (Kim *et al.*, 2002a) showed that combined stimulation of hMSCs with FGF-2, RA and fibronectin substrate yielded a significant increase in expression of neuronal lineage marker NF-M from <1.0% to 40%. This combination proved to be the most effective in generating NF-M expression, over the other growth factors (RA alone, nerve growth factor (NGF) and EGF) and culture subtrates (laminin, gelatin, collagen and poly-ornithine) examined. However, the neurofilament protein-expressing cells obtained were considered to be immature neuronal lineage progenitors, since action potential-like responses could not be observed in whole cell patch-clamp studies (Kim *et al.*, 2002a).

Experiments conducted in our laboratory with hMSCs have utilised FGF-2, EGF, platelet-derived growth factor (PDGF) and fibronectin surface coating to produce a stable neuronal-like phenotype that is maintained for up to 3 months *in vitro* (Tao *et al.*, 2005). Changes in cell morphology were observed at 2 weeks post-cytokine induction, with the majority (80-95%) of cells displaying retraction of the cytoplasm towards the nucleus, formation of refractile cell bodies and development of long branching processes (Tao *et al.*, 2005). Additionally, increased expression of neuronal markers, including NF-M, NSE, β tubulin III, MAP-2 and Tau, were detected post-induction, and neuronal-like cells stained positively for neurotransmitters or associated proteins, such as GABA, TH and serotonin, suggesting generation of GABAergic, dopaminergic and serotonergic neuron-like cells *in vitro* (Tao *et al.*, 2005). Significantly, this work also showed in parallel experiments using sister cultures of MSCs that growth factors were capable of inducing a neuronal-like phenotype in MSCs,

while chemical agents elicited changes that were not associated with physiological development and also caused cell death after 48 hours (Tao *et al.*, 2005).

A different combination that has been investigated in mouse MSCs by Jin and colleagues, uses EGF, FGF-2, RA and NGF, for production of cells with neuronal-like processes and neuronal marker expression (NeuN, MAP-2, Tau, and synaptic function-associated proteins, including synaptophysin, GABA, α1A and α1B calcium channel subunits and NR2A glutamate receptor subunits), albeit in the absence of appropriate intracellular localisation (Jin *et al.*, 2003). Whereas, the MAPC subset of the MSC population of rodents have been stimulated sequentially with FGF-2, followed by FGF-8, and finally BDNF, resulting in a more mature neural phenotype than stimulation with FGF-2 alone (Jiang *et al.*, 2002). MSCs stained positively with markers for neurons (neurofilament-200; 68%), astrocytes (GFAP; 15%) and oligodendrocytes (GALC; 12%) when cultured with FGF-2 alone, but with the sequential cytokine induction approach, mature neuronal markers for dopamine (DA)-containing neurons (30% dopa decarboxylase and TH positive), serotonin-containing neurons (20% serotonin positive) and GABA-containing neurons (50% GABA positive), as well as MAP-2 and Tau could be detected (Jiang *et al.*, 2002). In a later study, sonic hedgehog (SHH) was added to the second step (FGF-8) of the neuronal differentiation process, and murine MAPC-derived neuron-like cells were demonstrated to be capable of acquiring functional voltage-gated sodium channels; however, this could only be achieved after co-culture with astrocytes (Jiang *et al.*, 2003).

Interestingly, several studies have also reported the formation of neurosphere-like aggregates from rat and human MSCs, that are morphologically similar to those produced by NSCs (Hermann *et al.*, 2004; Suon *et al.*, 2006; Suzuki *et al.*, 2004; Wislet-Gendebien *et al.*, 2003). Suzuki, *et al.* induced neurosphere formation in rat

MSCs using FGF-2 alone, and neurosphere differentiation by plating spheres onto poly-L-ornithine/laminin coated surfaces with media containing FGF-2 for the first 1-2 days, followed by BDNF for up to 7 days (Suzuki *et al.*, 2004). Whereas, Hermann and colleagues observed neurosphere-forming capacity with the addition of FGF-2 and EGF to hMSC cultures, and also induced terminal neural differentiation by plating onto poly-L-lysine-coated surfaces and treating with RA and BDNF for neuronal differentiation, or RA and PDGF for glial differentiation (Hermann *et al.*, 2004). Furthermore after neuronal differentiation neurosphere-derived cells were found to be capable of DA production and potassium-dependent release. However, neither mature MAP-2ab-positive neurons nor TH-positive/DA producing cells were obtained through this protocol, and cells continued to express pluripotency marker *POU5F1* (Hermann *et al.*, 2004). Sphere formation by hMSCs was initiated in a similar manner by Suon, *et al.*, through suspension culture and supplementation with EGF and FGF-2 (Suon *et al.*, 2006). However, for further differentiation neurosphere-like aggregates were plated onto poly-ornithine/laminin-coated surfaces in serum-free medium containing IBMX, forskolin and TPA (4β-12-O-tetradecanoylphorbol 13-acetate) for 3 hours, followed by dbcAMP for 7 days, producing neuronal-like cells with GABAergic-like and dopaminergic-like traits (Suon *et al.*, 2006). While NES expression was found by another study to be necessary for neurosphere formation by rat MSCs (Wislet-Gendebien *et al.*, 2003).

1.3.1.3 Conditioned Media or Co-Culture Systems

Co-culturing of MSCs with a variety of neural cell types or treatment with neural cell-conditioned media has also been performed in attempts to achieve a more mature neuronal phenotype. The study by Sanchez-Ramos and colleagues (Sanchez-Ramos *et al.*, 2000) that reported the earliest cytokine- and signalling molecule-based

neural induction of MSCs, also demonstrated that co-culturing murine MSCs with primary foetal murine midbrain cells provided enhanced differentiation in comparison with culturing in induction medium alone. After co-culturing, the number of MSC-derived cells expressing NeuN and GFAP increased by at least two-fold, supporting the hypothesis that cell-to-cell contact plays an important role in MSC differentiation, in addition to signalling with cytokines and trophic factors (Sanchez-Ramos *et al.*, 2000).

Astrocytes have been utilised by Joannides, *et al.* (Joannides *et al.*, 2003) and Jiang, *et al.* (Jiang *et al.*, 2003) in co-culture and conditioned media experiments, since hippocampus-derived astrocytes have been shown to have a role in instructing neuronal fate specification of NSCs and promoting neuronal maturation and functional synapse formation (Song *et al.*, 2002a; Song *et al.*, 2002b). Application of hippocampus astrocyte-conditioned medium to hMSCs achieved neurofilament and β tubulin III-expressing cells, which displayed greater process elaboration in comparison to DMSO-treated cells, and also fewer GFAP-positive astrocyte-like cells (Joannides *et al.*, 2003). Meanwhile, Jiang, *et al.* did not observe significant enhancement of morphologic maturation following the addition of astrocyte-conditioned medium as a final differentiation step after sequential cytokine induction of mouse MAPCs (Jiang *et al.*, 2003). However, co-culturing with foetal brain astrocytes resulted in prolonged survival, and cells acquired a much more mature neuronal morphology with a more elaborate array of axons. Continued expression of dopaminergic, serotonergic and GABA-ergic markers was also seen. In addition, electrophysiological properties typical of neurons were only observed following co-culturing with astrocytes, and not in MAPCs exposed to astrocyte-conditioned medium or cytokine induction alone (Jiang *et al.*, 2003).

NSCs have also been employed in co-culture systems for rodent MSC neural differentiation (Alexanian, 2005; Wislet-Gendebien *et al.*, 2003). In a study by Wislet-Gendebien, *et al.*, NES-positive rat MSCs were co-incubated with mouse NSCs for 48 hours, resulting in aggregation of cells and sphere formation, followed by plating of cells on poly-ornithine substrate (Wislet-Gendebien *et al.*, 2003). Under these conditions, NES-positive rat MSCs were able to express astrocytic markers GFAP and GLAST (glial high affinity glutamate transporter), but lacked expression of neuronal markers, NeuN, NSE, β tubulin III and MAP-2b, and oligodendroglial markers, O4 and A2B5 (Wislet-Gendebien *et al.*, 2003). In a later study, Wislet-Gendebien, *et al.* further showed that rat MSCs co-cultured with murine cerebellar granule neurons express neuronal markers, including Tuj1 and NeuN, and display electrophysiological properties of immature neurons, such as generation of single-action potentials and response to several neurotransmitters (Wislet-Gendebien *et al.*, 2005). Whereas, Alexanian, *et al.* co-cultured mouse MSCs with proliferating or fixed mouse NSCs, and generated NSC-like cells with increased expression of NES and SOX2 (sex determining region Y-box 2); and after further differentiation, β tubulin III and GFAP expression could also be detected, along with neuronal and glial morphologies (Alexanian, 2005).

Kondo and colleagues have also cultured murine MSCs in the presence of an organ of Corti explant, which is the target tissue for developing cochlear ganglion neurons, with neural induction medium consisting of FGF-2, forskolin, IBMX, BDNF and BME (Kondo *et al.*, 2005). Additionally, mouse MSCs were also cultured in conditioned medium from mouse embryonic day (E)10 hindbrain/somite/otocyst and neural induction medium (Kondo *et al.*, 2005). Incubation of MSCs with neural induction medium and conditioned medium resulted in strong up-regulation of sensory neuron marker and POU-domain transcription factor Brn3a, as well as basic helix-loop-

helix genes *NEUROD1* and neurogenin (*NEUROG*) 1, which are essential for cranial sensory neurogenesis (Kondo *et al.*, 2005). When co-cultured with an E18 organ of Corti tissue, MSCs survived and propagated vigorously, and on exposure to neural induction reagents, ceased proliferation and gained neuronal-like morphology with extension of processes toward sensory hair cells in the explants, accompanied by expression of defined sensory neuron markers, such as Brn3a, glutamate receptor 4, and calretinin (Kondo *et al.*, 2005). The microenvironment created by tissue explant cultures was utilised by another study examining murine MSC differentiation in an organotypic hippocampal slice co-culture model (Abouelfetouh *et al.*, 2004). In this system, differentiation of mouse MSCs to neuron-like cells occurred within the hippocampal slice boundaries starting from day 3 of co-culture, whereas formation of network-like connections were observed at approximately day 14 (Abouelfetouh *et al.*, 2004). Axon-like processes were also observed to penetrate into the hippocampal tissue, however migration of mouse MSCs was not observed (Abouelfetouh *et al.*, 2004). Furthermore, it was shown that addition of RA to the culture system increased the number of differentiated cells (both neuron-like and non-neuron-like) and 9.6% of these cells were positive for NeuN, while no staining could be detected for astrocytic marker GFAP, or microglial marker Iba1 (ionised calcium binding adaptor molecule 1) (Abouelfetouh *et al.*, 2004).

1.3.1.4 Genetic Engineering

A genetic engineering approach has also been employed to attain MSC-derived neural-like cells through the over-expression of specific genes known to be important for neural development and function. Genes that have been over-expressed in MSCs for generation of neural cells include: Noggin (Kohyama *et al.*, 2001), the Notch

intracellular domain (*NICD*) (Dezawa *et al.*, 2004), and the *BDNF* gene (Zhao *et al.*, 2004).

Transfection of MSCs with the Noggin gene caused cells to aggregate and detach from culture surfaces, and form non-adherent neurosphere-like clusters (Kohyama *et al.*, 2001). Plating of the cluster-forming cells onto poly-L-ornithine/fibronectin-coated surfaces and growth in medium containing a cytokine cocktail, elicited differentiation into neuronal-like cells, with greater than 50% of cells expressing MAP-2, and evidence of calcium influx in response to depolarising stimuli (Kohyama *et al.*, 2001). Dezawa and colleagues (Dezawa *et al.*, 2004) employed *NICD* transfection of MSCs together with application of FGF-2, ciliary neurotrophic factor (CNTF), forskolin and in some cases glial-derived neurotrophic factor (GDNF) or BDNF/NGF, which resulted in generation of neuronal-like cells capable of electrophysiological activity, as well as function in an animal disease model. Whilst, combination of *BDNF* gene transfection with RA induction was found to produce cells with significantly increased expression of neural markers, such as NES, NeuN, O4 and GFAP, and the ability to generate voltage dependent K^+/Ca^{2+} currents using the patch clamp technique (Zhao *et al.*, 2004).

Studies have also genetically engineered MSCs with the *TH* gene (Lu *et al.*, 2005), as well as the two genes required for 3,4,-dihydroxyphenylalanine (L-DOPA) synthesis (Schwarz *et al.*, 2001), to assess the ability of MSCs to act as gene delivery vehicles for gene therapy of PD. Upon transplantation of *TH*-engineered MSCs into a rat model of PD, behavioural improvement was observed (assessed by apomorphine-induced rotational behavior), TH expression was detected around the graft sites and the levels of DA in the lesioned striatum were elevated in comparison with levels in control *lacZ*-MSC treated rats (Lu *et al.*, 2005). Whereas, Schwarz, *et al.* introduced human TH

type I (rate limiting enzyme in DA biosynthesis) and rat GTP cyclohydrolase I (enzyme providing tetrahydropterin cofactor for TH) into rat MSCs and found synthesis and secretion of L-DOPA together with 1000-fold expansion over 3-4 weeks (Schwarz *et al.*, 2001).

1.3.1.5 Combined Approach

A number of studies have also combined elements from the different induction methods. Variations that have been investigated include: cytokine induction and addition of factors that increase intracellular cAMP concentrations (Barzilay *et al.*, 2008; Kan *et al.*, 2007a; Kondo *et al.*, 2005; Long *et al.*, 2005; Suon *et al.*, 2006; Tondreau *et al.*, 2008); cytokine induction with factors to increase intracellular cAMP and use of conditioned medium (Guo *et al.*, 2005; Kondo *et al.*, 2005); genetic manipulation and cytokine induction (Dezawa *et al.*, 2004; Kohyama *et al.*, 2001); and co-culture systems with RA or cytokine induction (Abouelfetouh *et al.*, 2004; Jiang *et al.*, 2003).

1.3.2 Evidence of Neuronal Differentiation of MSCs

The results of the above-mentioned studies together with those of *in vivo* MSC transplantation studies holds great promise for the eventual use of MSCs in cellular therapies for neurodegenerative diseases. However, recent inconsistent results and findings of MSC fusion with mature cell types have revealed the necessity for further detailed analyses in order to gain a greater understanding of the true nature of MSC multi-lineage differentiation events. Moreover, a number of factors complicate the interpretation of published results, as the methods employed for neural differentiation and assessment of produced cells are often fragmented and inconsistent (Phinney *et al.*, 2007). Additionally, many markers used for classifying neural cell types are not entirely

neural-specific, and *in vitro* expansion of MSCs in culture may introduce artifacts (Phinney *et al.*, 2007).

Currently, the majority of studies have relied on morphological, immunological and gene expression changes as evidence of neural plasticity of MSCs. However, several criteria have been proposed to define whether a functional neuron has indeed been generated (Reh, 2002; Svendsen *et al.*, 2001). According to Reh (Reh, 2002), the proposed neuronal cell should be (1) post-mitotic, (2) polarised, with a single axon and multiple dendrites, (3) capable of firing voltage-gated action potentials, and (4) able to communicate with other neurons through synapses, requiring both neurotransmitter release and neurotransmitter receptors. Svendsen, *et al.* also believe that in defining a new neuron, not only should the correct anatomy of a neuron be displayed, but the cell should display appropriate developmental maturation, that is, the anatomical features of a maturing neuron should be matched with the expression of neuronal-specific markers (Svendsen *et al.*, 2001). However, the authors point out that since the specificity of the markers relies heavily on the quality of the antibody, markers should only be considered as indicative of neuronal identity, but cannot confirm neuronal identity on their own (Svendsen *et al.*, 2001). Therefore, the ultimate tests for identification of neuronal cells are assessment of the ability to connect and form synapses with other neurons and to subsequently affect the behavioural function of an animal; these are typically examined through electrophysiological techniques and transplantation into animal disease models with demonstration of graft function through behavioural testing (Svendsen *et al.*, 2001). To date, none of the studies examining MSC-derived neuronal-like cells have shown fulfillment of all these criteria.

The expression of neural genes and proteins by undifferentiated MSCs prior to application of differentiation stimuli (discussed in Section 1.2.1), emphasises the need

for examination of basal expression levels in MSCs, and for caution in interpretation of results. Therefore, in neuronal differentiation of MSCs it would be necessary to demonstrate significant up-regulation of an already expressed neuronal marker/s, and/or *de novo* expression of neuronal-specific marker/s. Additionally, if genes and proteins from multiple lineages are constitutively expressed in MSCs, it would be expected that concomitant down-regulation of non-neuronal markers should be observed. Expression of NES by MSCs has frequently been reported in the literature as evidence of a NSC-like or neural progenitor-like phenotype, since NES was originally described as a marker of NSCs (Lendahl *et al.*, 1990). However, NES expression has now been documented in a variety of cells and tissues, including developing and regenerating muscle (Kachinsky *et al.*, 1994; Sejersen *et al.*, 1993; Vaittinen *et al.*, 2001), newly formed endothelial cells (Klein *et al.*, 2003; Mokry *et al.*, 1998a; Mokry *et al.*, 1998b), epithelial cells of the developing lens (Mokry *et al.*, 1998b), and activated hepatic stellate cells (Niki *et al.*, 1999). In addition, non-neural cells such as chondrocytes, myoepithelial cells and certain fibroblast populations have been reported to express GFAP, neurofilament and/or NSE proteins (Egerbacher *et al.*, 1995; Hainfellner *et al.*, 2001). Phinney and Prockop (Phinney *et al.*, 2007) have also commented that while many reports have shown expression of a wide range of neural proteins in MSC-derived neuron-like cells, only an unbalanced repertoire of neuronal markers has been described, with cells often lacking the functional properties of bona fide neurons. Furthermore, limitations with the current evidence of electrophysiological activity also exist, as those studies which have shown acquisition of neuronal functional properties have not verified the presence of all electrical characteristics of neurons, or are unable to show these attributes (Phinney *et al.*, 2007).

Recently, time-lapse microscopy has been employed for examining the process of neural differentiation from rat and human MSCs using the chemical induction method and serum withdrawal (Bertani *et al.*, 2005; Croft *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004). Using this technique it was shown that the acquired morphological changes after application of BME, DMSO/BHA or serum withdrawal were due to cellular shrinkage rather than new neurite outgrowth, and that there was no motility or further elaboration of the processes remaining after cytoplasmic retraction (Bertani *et al.*, 2005; Croft *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004). Furthermore, these morphological changes could be reproduced in normal primary fibroblasts, and could be mimicked by drugs that elicit cytoskeletal collapse and disruption of focal adhesion contacts (Bertani *et al.*, 2005; Croft *et al.*, 2006; Neuhuber *et al.*, 2004), or by various cellular stressors such as detergents, high-molarity sodium chloride, and extremes of pH (Lu *et al.*, 2004). With respect to the use of morphological analysis as evidence of MSC neural differentiation, it appears that time-lapse microscopy is a useful technique for ascertaining the validity of morphological changes, and to ensure that any putative axons/dendrites were formed through outgrowth rather than cellular shrinkage. Therefore, this technique will be employed in my PhD studies to confirm the morphological observations during cytokine-based MSC neural differentiation.

Another challenging aspect in characterising MSC neuronal differentiation, involves the identification of MSC-derived neurons after grafting. Much of the difficulty with this is in distinguishing between host and graft cells. Currently, for grafting cells of the same species, identification of donor cells is mainly achieved through the use of genetic modification to label cells (e.g. with GFP or β-galactosidase), transplanting male donor cells to allow for Y chromosome detection, pre-labelling cells

with fluorescent dyes, or using BrdU or tritiated thymidine to label dividing cells before transplantation (Svendsen *et al.*, 2001). In addition, if hMSCs are to be grafted into an animal model, then it is possible to stain for detection of human specific markers such as human nuclear antigen (HNA). Using the above cell identification techniques together with neural-specific markers, therefore, allows identification of grafted cells and an indication of whether neuronal differentiation events have occurred. However, caution should be taken in analysing tissue-sections using this method, as false double-positive cells may be detected with standard fluorescence microscopic analysis (i.e. single-labelled cells juxtaposed onto one another). Instead, detailed analysis of tissue sections using high-powered analytical equipment, such as confocal microscopes, would be useful to reduce the likelihood of detecting false double-positive cells (Svendsen *et al.*, 2001). The possibility of cell fusion must also be considered, particularly with *in vivo* transplantation experiments or *in vitro* co-culture systems, in which the MSCs are in direct contact with other cell types. The reported frequency of cell fusion events is typically low, however, it has been detected in lethally irradiated animals receiving BM grafts (Alvarez-Dolado *et al.*, 2003; Weimann *et al.*, 2003), and in mouse BM cells co-cultured with mouse ESCs (Terada *et al.*, 2002). Nevertheless, cell fusion cannot account for all MSC neural differentiation events, as *in vitro* studies have shown acquisition of neural phenotypes by MSCs that were not in contact with neural cells.

While much remains to be determined, continued investigations conducted in a meticulous and systematic manner in areas such as MSC prospective isolation, efficient clonal selection, optimal expansion and *in vitro* differentiation, will undoubtedly provide a great deal of knowledge and understanding for the proper development of therapeutic approaches involving MSCs. Advancements have already been made with an increasing number of studies reporting evidence for the acquisition of

electrophysiological properties by MSCs post-neuronal differentiation *in vitro* (Cho *et al.*, 2005; Greco *et al.*, 2007b; Kan *et al.*, 2007a; Trzaska *et al.*, 2007). Functionality has been shown in these studies by demonstrating neurotransmitter synthesis and packaging (Cho *et al.*, 2005; Trzaska *et al.*, 2007), neurotransmitter release in response to depolarising stimuli (Greco *et al.*, 2007b; Kan *et al.*, 2007a), presence of spontaneous post-synaptic currents (Cho *et al.*, 2005; Greco *et al.*, 2007b), constitutive DA release (Trzaska *et al.*, 2007), and up-regulation of voltage-gated potassium (Trzaska *et al.*, 2007) and sodium channels (Greco *et al.*, 2007b).

1.3.3 Possible Molecular Control of MSC Neuronal Differentiation

In recent years, increasing efforts have been directed towards identifying the molecular mechanisms underlying MSC plasticity. To date, the underlying mechanisms remain elusive, and the great variety of differentiation methods reported adds further complications. The critical transitions of MSCs to neuronal-like cells are controlled by signalling pathways and subject to subsequent transcriptional regulation (Blondheim *et al.*, 2006). Jori and colleagues have recently investigated the biochemical pathways involved in neuron-like commitment and maturation of rat MSCs via induction with forskolin (which increases cAMP), DMSO, BHA, VPA and KCl (Jori *et al.*, 2005). The findings of this study suggests that: (1) increases in cAMP induced by forskolin treatment, activates the classical protein kinase A (PKA) pathway, however not through the exchange protein directly activated by cAMP, a guanine nucleotide exchange factor for the small GTPase Rap1 and Rap2; (2) MEK–ERK signalling could contribute to neural commitment and differentiation; (3) CaM KII activity seems dispensable for neuron differentiation, but its inhibition could contribute to rescuing differentiating cells from death (Jori *et al.*, 2005). Additionally, this study found that these *in vitro* differentiation agents are required for the early steps of neural differentiation, but are

unable to further sustain this process and therefore are not useful for long-term *in vitro* survival of neurons (Jori *et al.*, 2005). Chu and colleagues also showed involvement of cAMP-PKA activation of cAMP response element binding protein for inducing neurite outgrowth and reducing astrocyte and oligodendrocyte differentiation in a neural induction protocol utilising IBMX or forskolin (Chu *et al.*, 2006).

Dezawa, *et al.* utilised a genetic engineering approach (transfection with *NICD* followed by application of cytokines) to obtain neuronal-like cells from rat MSCs and hMSCs, that were capable of action potential generation and behavioural improvement in a 6-hydroxydopamine (6-OHDA)-lesioned rat model of PD (Dezawa *et al.*, 2004). Analysis of the effect of *NICD* transfection showed that down-regulation of signal transducer, and activator of transcription (*STAT*) 1 and *STAT3* were tightly associated with *NICD*-mediated neuronal induction (Dezawa *et al.*, 2004). Additionally, treatment with Janus kinase (JAK)/STAT inhibitor alone, instead of *NICD*-transfection, was able to elicit neuron-like cells though to a lesser extent, emphasising the importance of STAT inhibition in this neuronal induction procedure (Dezawa *et al.*, 2004). It was also suggested that the mitogen-activated protein kinase (MAPK)-signalling pathway may be involved, since FGF-2, CNTF and forskolin (applied after *NICD*-transfection) activate the MAPK cascade, which may then activate the transcription of neuronal genes (Dezawa *et al.*, 2004).

Studies performed by Wislet-Gendebien and colleagues have led them to propose that NES expression by rat MSCs is a prerequisite for acquiring the capacity to progress toward the neural lineage (Wislet-Gendebien *et al.*, 2003). It was found that only NES-positive rat MSCs were capable of: (1) sphere formation when cultured in conditions typically used for NSC culture, and (2) differentiation into GFAP-positive cells when co-cultured with NSCs (Wislet-Gendebien *et al.*, 2003). In addition, they

have also recently shown that NES-positive rat MSCs over-expressed transcription factors SOX2, SOX10 and PAX6, WNT (wingless-type MMTV integration site family) receptors frizzled (Fzd)1, Fzd2 and Fzd5, and neuregulin receptors ErbB2 and ErbB4, in comparison to NES-negative rat MSCs (Wislet-Gendebien *et al.*, 2005).

As further investigations are being conducted into the molecular mechanisms underlying the neural induction of MSCs, a greater understanding of this complex process will be obtained. It is anticipated, however, that the regulation of MSC neural differentiation involves complex interactions between a range of signalling pathways, transcription factor cascades and microRNAs. Although some of the key studies that pioneered this area (Deng *et al.*, 2001; Woodbury *et al.*, 2000), have now been found to describe culture artifacts rather than true neuronal differentiation, promising results have recently been obtained, which demonstrate the acquisition of electrophysiological properties characteristic of neurons, as well as synthesis of neurotransmitters. While mature neuronal cell types have yet to be generated, the progress and knowledge achieved since the first reports of MSC neuronal differentiation continue to fuel and encourage this field of research.

1.4 Parkinson's Disease and Cell-Based Therapies

The development of methods to induce neuronal differentiation of hMSCs has opened the possibility of applying these cells in regenerative or reparative therapies of the CNS. The loss of neurons characterises many neurodegenerative diseases, such as PD, Alzheimer's disease, and Huntington's disease, and injuries such as stroke, traumatic brain injury, and spinal cord injury. Furthermore, the CNS is known to have a poor capacity for generating new neurons and oligodendrocytes after injury or degeneration, with the adult and aged CNS, in particular, possessing a prevailing non-neurogenic and growth-inhibitory milieu that negatively influences these processes

(Horner *et al.*, 2002; Ourednik *et al.*, 2004). For these reasons, therapies endeavouring to replace the damaged or missing neurons and restore some degree of function have been the focus of much attention. Positive outcomes, including neurological recovery, have been observed following transplantation of MSCs into animal models of CNS disorders (some of which are discussed in Section 1.2.3.2), revealing MSCs to be a promising therapeutic modality for the treatment of CNS diseases and injuries.

1.4.1 Overview of Parkinson's Disease

PD is a chronic neurodegenerative disorder characterised by the progressive loss of nigrostriatal neurons that synthesise the neurotransmitter DA (Cotran *et al.*, 1999). The cell bodies of affected dopaminergic neurons reside within the substantia nigra pars compacta, while the nerve terminals project rostrally to innervate the striatum (also known as the caudate putamen; see Figure 1.2). Consequently, neuronal degeneration in PD is associated with a reduction in striatal DA content, with the severity of symptoms being proportional to the DA deficiency (Cotran *et al.*, 1999).

1.4.1.1 Neuropathological Features of Parkinson's Disease

A number of neuropathological features define PD, including the specific loss of pigmented catecholaminergic neurons of the substantia nigra (A9 group of dopaminergic neurons), the presence of Lewy bodies in remaining nigral neurons, and the normal appearance of the striatum (Gelb *et al.*, 1999; Klockgether, 2004; Schulz *et al.*, 2004; Teismann *et al.*, 2004). The classic macroscopic finding of substantia nigra depigmentation or pallor results from the depletion of neurons which normally contain conspicuous amounts of neuromelanin (Schulz *et al.*, 2004). Lewy bodies were first described by Lewy in 1912, and are single or multiple, eosinophilic, round to elongated inclusions in the cytoplasm of affected neurons, which are composed of numerous proteins, including misfolded synaptic protein α-synuclein, parkin, ubiquitin,

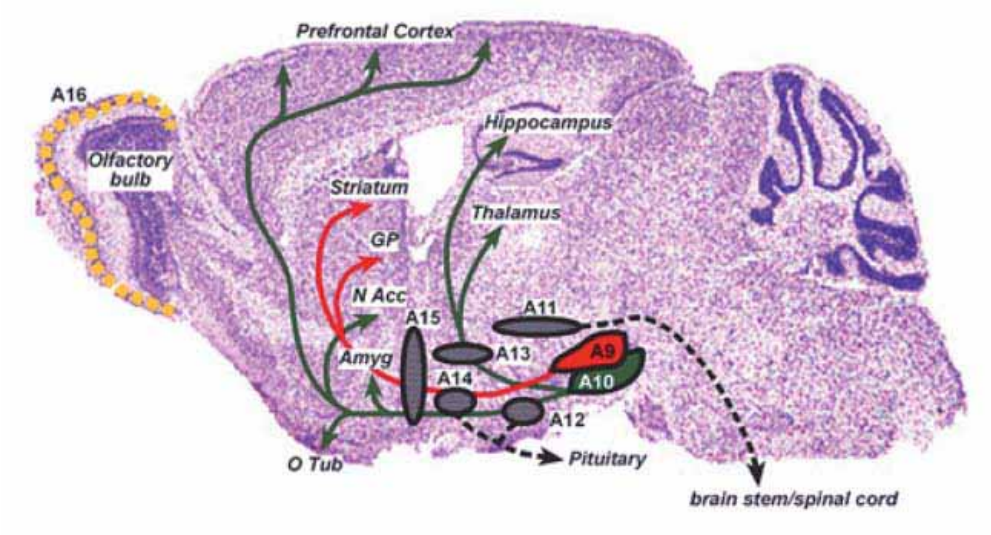

Figure 1.2 Position and axonal projection of dopaminergic cell groups within the adult rodent brain (sagittal view).

The cell bodies of mesencephalic dopaminergic neurons reside in the substantia nigra pars compacta (A9 group; red), ventral tegmental area (A10 group; green), and in the retrorubral field (A8 group). Different dopaminergic cell groups possess characteristic projection areas, with neurons of the A9 group innervating the dorsolateral striatum (caudate nucleus and putamen) and globus pallidus (GP), forming the nigrostriatal pathway, while A10 and A8 neurons project to the ventral striatum (nucleus accumbens (N Acc), amygdala (Amyg) and olfactory tubercle (O Tub)) as part of the mesolimbic system, and to the prefrontal cortex (mesocortical system). Additional connections from the A10 group are also established with the thalamic and hippocampal regions. Approximate locations are shown for the diencephalic groups (A11-15) of the hypothalamus/ventral thalamus (grey) and the associated projections into the brain stem/spinal cord and anterior pituitary (dashed lines). Periglomerular interneurons within the olfactory bulb form the telencephalic A16 group (yellow dashed line). The A8 and A17 (retina amacrine interneurons) groups have not been depicted. Figure from (Prakash *et al.*, 2006).

synphilin and neurofilaments (Cotran *et al.*, 1999; Dunnett *et al.*, 1999; Schulz *et al.*, 2004). Often, these protein aggregates have a dense hyaline core surrounded by a clear halo, and ultrastructurally, are composed of fine filaments densely packed together at the core, but loose at the rim (Cotran *et al.*, 1999; Schulz *et al.*, 2004). Additionally, Lewy neurites can be observed, which occur when proteinaceous aggregates form in dendrites producing a local swelling (Schulz *et al.*, 2004). Interestingly, in PD the mesolimbic dopaminergic neurons, which reside in the ventral tegmental area (VTA) adjacent to the substantia nigra pars compacta, are much less affected (Schulz *et al.*, 2004). Further neurodegeneration also exists in PD beyond that observed in dopaminergic neurons, with degeneration and Lewy body formation evident in noradrenergic (locus coeruleus), serotonergic (raphe), and cholinergic (dorsal motor nucleus of vagus, nucleus basalis of Meynert) systems, and in the cerebral cortex (especially the cingulate gyrus and parahippocampal gyrus), olfactory bulb and autonomic nervous system (Cotran *et al.*, 1999; Gelb *et al.*, 1999; Schulz *et al.*, 2004). However, degeneration of dopaminergic neurons of the substantia nigra pars compacta remains the earliest and most consistent neuropathologic feature of PD.

1.4.1.2 Clinical Features of Parkinson's Disease

The clinical features of PD were initially described almost two centuries ago by James Parkinson in 1817 (Dunnett *et al.*, 1999; Langston, 1998; Orr *et al.*, 2002). The cardinal symptoms of PD include bradykinesia (slow movement), muscular rigidity and resting tremor, although postural instability, impaired gait, depression, dementia and other motor and non-motor symptoms may also be involved (Dunnett *et al.*, 1999; Gelb *et al.*, 1999; Klockgether, 2004). Of the cardinal symptoms, bradykinesia primarily contributes to the disability experienced by PD patients, whilst tremor is the most conspicuous feature and stigmatises patients as PD sufferers (Klockgether, 2004).

Degeneration of dopaminergic neurons is well underway by the time symptoms become apparent, with loss of approximately 50% of nigral dopaminergic neurons and depletion of 70-80% of DA in the striatum (Bernheimer *et al.*, 1973; Dunnett *et al.*, 1999; Fearnley *et al.*, 1991). Estimates from the rates of substantia nigra cell loss suggest that disease onset in PD patients commences approximately 5 years prior to the onset of symptoms (Fearnley *et al.*, 1991).

Although the symptoms and pathology of PD have been long identified, the mechanisms underlying the dysfunction and progressive loss of dopaminergic neurons is still poorly defined. Idiopathic PD is usually sporadic, with major risk factors of aging and environmental exposure, but evidence of a genetic component has also been demonstrated with a 2-14 fold increase in relative risk found in close relatives of PD patients, and some concordance between identical twins who develop PD at less than 50 years of age, or when examining subclinical dysfunction of the nigrostriatal dopaminergic system by positron emission tomography (PET) imaging (Brooks, 1998; Dunnett *et al.*, 1999; Gasser, 1998; Olanow *et al.*, 1999; Veldman *et al.*, 1998). Nonetheless, investigation of an environmental cause was the focus of much research, with epidemiological studies showing geographical variation in incidence that appeared to be associated with living in a rural environment, well-water consumption, and exposure to pesticides, herbicides, farming and industrial chemicals (Dick, 2006; Langston, 1998; Olanow *et al.*, 1999; Veldman *et al.*, 1998), and the finding of a PD syndrome resulting from a single exposure to a toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston *et al.*, 1983). Surprisingly, an inverse relationship has also been found between cigarette smoking and the risk for PD, which has been considered to provide support for an environmental cause of PD (Langston, 1998).

1.4.1.3 Aetiology and Pathogenesis of Parkinson's Disease

Advances in understanding the pathogenesis of this disease have recently been achieved through findings of causal genetic, molecular and cellular tissue changes. Mechanisms involving protein aggregation, dysfunction of the ubiquitin-proteasome pathway, mitochondrial dysfunction, oxidative stress, activation of the c-Jun N-terminal kinase system, and inflammation have all been implicated in PD pathogenesis, and may also further interact and amplify one another (Dunnett *et al.*, 1999; Schulz, 2008; Schulz *et al.*, 2004). A growing number of disease-related genes have been identified to cause hereditary forms of PD with autosomal dominant or autosomal recessive inheritance (see Table 1.1). While most single gene mutations are only responsible for a small number of patients with PD, these discoveries have provided important insights into the possible disease mechanisms underlying PD. One possible pathway involves disturbances in protein quality control, which could result from point mutations or gene duplications and triplications in the α-synuclein gene, or mutations in the Parkin or ubiquitin-C-terminal hydrolase-L1 genes that would cause dysfunction of the ubiquitin-proteasome protein degradation system, therefore, leading to accumulation of aggregated α-synuclein (Schulz, 2008). Another potential mechanism includes altered mitochondrial function and oxidative stress, suggested by mutations in the *PINK1* and *DJ-1* genes, respectively, as well as polymorphisms in the *Htra-2/Omi* gene (Schulz, 2008). Further to this, dopaminergic neurons are known to be particularly susceptible to oxidative injury since DA production and use depends on oxidative mechanisms (Jenner *et al.*, 1998). Aberrant kinase activities may also underlie PD pathogenesis with observations of c-Jun N-terminal kinase activation in animal studies, and mutations in *LRRK2* resulting in increased kinase activity that may cause neuronal toxicity (Schulz, 2008). Cell-cell interactions and immune regulation have also been identified as being

Table 1.1 Genetics of Parkinson's Disease

Locus	Chromosomal Localization	Gene product	Inheritance	Lewy body pathology	Specific clinical symptoms
PARK 1	4q21	α-Synuclein	AD	Yes	Dementia
PARK 2	6q25.2-27	Parkin	AR	No	Early onset, L-Dopa-induced dyskinesias, improvement during sleep, foot dystonia
PARK 3	2p13	?	AD	Yes	Dementia
PARK 4	4q21	α-Synuclein	AD	Yes	Dementia, postural tremor
PARK 5	4p14	UCH-L1	AD	No report	Not described
PARK 6	1p35-36	PINK-1	AR	No report	Early onset, tremor dominant
PARK 7	1p36	DJ-1	AR	No report	Early onset, dystonia, psychiatric alterations
PARK 8	12cen	LRRK2/Dardarin	AD	Yes/but also Tau pathology	Tremor, late onset
PARK 9	1p36	ATP13A2	AR		Kufor-Rakeb syndrome, very early onset
PARK 10	1p32	?	AD (?)	No report	Late onset
PARK 11	2q34	?	AD (?)	No report	Late onset
	5q23	Synphilin-1	Susceptibility	No report	Late onset
PARK 13	2p13	HtrA2/Omi	Susceptibility	No report	Late onset

AD, autosomal dominant

AR, autosomal recessive

(Table from (Schulz, 2008))

significant in PD pathogenesis (Orr *et al.*, 2002). Nigral dopaminergic neurons are selectively vulnerable to inflammatory attack due to the presence of higher numbers of inflammatory microglia in the substantia nigra (Kim *et al.*, 2000; Lawson *et al.*, 1990), and evidence of a role for inflammation in PD is supported by findings that substantial microglial activation has been directly associated with dopaminergic neuronal death in PD (Orr *et al.*, 2002).

1.4.1.4 Treatments for Parkinson's Disease

The current treatment of PD involves alleviation of symptoms through pharmacologic replacement of DA with L-DOPA (the immediate precursor of dopamine; also referred to as Levodopa (International Nonproprietary Name)) (Cotran *et al.*, 1999; Davie, 2008; Dunnett *et al.*, 1999). Additionally, a peripheral DOPA-decarboxylase inhibitor is co-administered to control disabling side effects, and together these drugs effectively alleviate akinesia and rigidity in early- and middle-stage disease (Dunnett *et al.*, 1999). This combination therapy has been the gold standard for symptomatic treatment of PD for four decades (Davie, 2008). Other drugs have also been developed such as dopamine receptor agonists, slow-release L-DOPA formulations, inhibitors of degrading enzymes catechol-O-methyltransferase and monoamine oxidase B, dopamine transport blockers, anti-cholinergic drugs, and anti-glutamatergic drugs (Davie, 2008; Dunnett *et al.*, 1999; Klockgether, 2004). However, these medications do little to prevent or reverse disease progression, and efficacy declines several years after disease onset (Cotran *et al.*, 1999; Dunnett *et al.*, 1999). Furthermore, after long-term levodopa therapy patients frequently develop severe side effects, particularly in the form of dyskinesias (Davie, 2008; Dunnett *et al.*, 1999).

The use of neurosurgery as a therapy for PD has been revisited after originally being implemented over 50 years ago (Davie, 2008; Dunnett *et al.*, 1999). At that time,

surgical intervention was not very reliable, and could only address tremor symptoms. Presently, neurosurgery has shown promise with the advent of modern imaging techniques, electrophysiological monitoring during surgery, improved understanding of basal ganglia circuits and disturbances caused by PD that have allowed identification of critical target sites, as well as the introduction of chronic deep-brain stimulation for functional and reversible inhibition of target areas (Davie, 2008; Dunnett *et al.*, 1999; Klockgether, 2004). Now, rather than focusing on primary symptom relief, surgical intervention has been applied for controlling levodopa-induced dyskinesias, therefore extending pharmacotherapy to advanced patients (Dunnett *et al.*, 1999). New approaches are needed for pharmacological therapies for PD, and while drug trials concentrate primarily on motor symptoms and complications, studies have found that quality of life is, instead, determined by other factors, including depression and dementia (Klockgether, 2004). Another approach under investigation as a potential treatment for PD is the transplantation of DA-producing cells, which will be further discussed in Section 1.4.2.

1.4.1.5 Experimental Animal Models of Parkinson's Disease

A number of experimental models of PD are available, which have proven to be valuable tools in evaluating clinical approaches for symptomatic treatments. This has allowed the development of effective DA replacement therapies that are now routinely used in clinical settings, and is one of the major reasons for the advancement of PD therapies in comparison with other neurodegenerative diseases, which still lack effective symptomatic treatments (Jenner, 2008). Nevertheless, whilst many approaches have been taken for producing animal models of PD, including use of a range of toxins, transgenic expression of mutant proteins involved in familial PD, and deletion of genes affecting dopaminergic neuronal development (knock-out models), there are still no

accepted experimental models that closely resemble the progression and pathogenesis of PD as observed in humans (Jenner, 2008). Additionally, while the existing models have demonstrated utility in identifying symptomatic treatments, these models have been much less successful in discovering neuroprotective therapies (Jenner, 2008). For this purpose, new models may be required which more accurately portray PD pathogenesis as it affects humans (Jenner, 2008).

Two of the most extensively studied models of PD are the 6-OHDA and the MPTP toxin-based models (Jenner, 2008; Schwarting *et al.*, 1996; Teismann *et al.*, 2004). Once it was found that 6-OHDA exhibited toxicity towards catecholaminergic neurons (dopamine and noradrenaline), it was applied in the depletion of these neurotransmitters in the forebrain and opened the way for toxin-based models of PD (Jenner, 2008; Teismann *et al.*, 2004; Ungerstedt, 1968; Ungerstedt *et al.*, 1970). Destruction of catecholaminergic neurons is believed to occur through the selective uptake of 6-OHDA by these neurons, and autooxidation of 6-OHDA following intracellular metabolism, resulting in the simultaneous formation of several cytotoxic products (Sachs *et al.*, 1975). The unilateral 6-OHDA–lesioned rat has become the universal standard model for assessing potential PD pharmacotherapies (Teismann *et al.*, 2004), and it is this model that will be used for the present study. Typically, the neurotoxin is stereotaxically injected into the medial forebrain bundle or substantia nigra of one hemisphere, causing unilateral degeneration of the nigrostriatal pathway and depletion of striatal DA (Jenner, 2008; Teismann *et al.*, 2004). Unilateral lesioning results in a quantifiable functional asymmetry that is readily measurable by inducing rotational behaviour in rats with administration of direct or indirect DA agonists, apomorphine and amphetamine, respectively (Schwarting *et al.*, 1996; Teismann *et al.*, 2004; Ungerstedt *et al.*, 1970). Furthermore, several other deficits exist in this model

that correspond with the degree of nigrostriatal loss, including abnormalities in sensorimotor performance, posture and paw usage (Henderson *et al.*, 2003; Schwarting *et al.*, 1996).

Although, the 6-OHDA model is useful for testing symptomatic treatments, there exist several discrepancies between this experimental model and PD in humans. Firstly, it only reproduces a single component of the cell death process rather than the complex cascade of events that occurs in PD; secondly, there is little evidence of Lewy body formation, despite this being a characteristic pathological hallmark of PD; thirdly, the pattern of pathology and cell loss in different neural regions is not reproduced; and fourthly, 6-OHDA administration leads to acute death of dopaminergic neurons in the substantia nigra, rather than the progressive degeneration usually observed in PD (Gerlach *et al.*, 1996; Jenner, 2008; Teismann *et al.*, 2004). Other drawbacks of this model include the need for stereotactic application of 6-OHDA to appropriate regions of the brain, and that using rotational behaviour as a measure of functional improvement is not relevant to the pathophysiology of PD symptoms (Gerlach *et al.*, 1996).

The discovery that MPTP causes an irreversible and severe parkinsonian syndrome in humans that is almost indistinguishable from PD provided perhaps the greatest advance in PD experimental models, and also led to the first effective non-human primate model of PD (Jenner, 2008). Generally, the MPTP model is produced with systemic administration of MPTP, and a murine model is widely utilised, but, this is not as robust and reproducible a model of PD as sometimes reported (Jenner, 2008). The mechanism of action of MPTP involves entry of the neurotoxin into the CNS, followed by metabolism into the active form (1-methyl-4-phenylpyridinium) by astrocytes, and entry into dopaminergic neurons via the dopamine transporter, where it inhibits complex I of the mitochondrial electron transport chain (Du *et al.*, 2001; Orr *et*

al., 2002). Important roles for glial activation and the accompanying up-regulation of inducible nitric oxide synthase in MPTP neurotoxicity have also been discovered (Du *et al.*, 2001; Orr *et al.*, 2002). Although, like most toxin-based PD models, the MPTP model is also divergent from PD in humans, for the same reasons that the 6-OHDA model differs from PD in humans (Gerlach *et al.*, 1996; Jenner, 2008). Additional drawbacks exist with the MPTP model, such as the lack of permanency of motor deficits, and tremor symptoms are not often observed (Gerlach *et al.*, 1996).

1.4.2 Cellular Replacement Therapies for Parkinson's Disease

The primary aim of cell-based treatments for PD is to restore neuronal function by transplanting cells with DA-producing capacity into the striatum in an attempt to replace the lost nigrostriatal neurons. Ideally, the implanted cells should also reconstitute neural networks and provide physiologically-regulated feedback-controlled DA release to prevent graft-related side effects (Isacson *et al.*, 2008). Cell transplantation therapy is believed to hold great potential for PD, due to the localised nature of this lesion, however, at present research in this area is still largely exploratory (Isacson *et al.*, 2008). In addition, several issues concerning cell transplantation exist that have limited research progress, including scientific, technical and ethical difficulties (Klockgether, 2004). Nonetheless, encouraging results have been reported in both animal models and human clinical trials, suggesting that further understanding of appropriate cell populations and target sites for transplantation, as well as improved surgical and technical approaches, could lead to the development of much needed neuroprotective or neurorestorative interventions for PD.

1.4.2.1 Foetal Neural Tissue Transplantation in PD

A variety of cellular sources have been investigated for the potential to act as a renewable supply of transplantable dopaminergic neurons for PD, including foetal brain

tissue, embryonic and adult SC populations, and xenogeneic porcine foetal nigral tissue. In preclinical studies involving neurotoxin-induced parkinsonian rodents and non-human primates, transplantation of embryonic or foetal dopaminergic neuron-rich mesencephalic tissue resulted in the amelioration of at least some aspects of lesion-induced motor deficits (Bjorklund *et al.*, 1979; Bjorklund *et al.*, 1981; Dunnett *et al.*, 1981; Perlow *et al.*, 1979; Redmond *et al.*, 1986; Wuerthele *et al.*, 1981). Additionally, these studies revealed that grafted cells survived, produced DA, formed effective dopaminergic terminals and synapses, and re-innervated the striatum. Furthermore, graft survival and functional improvements were also reported in xenogeneic situations, with transplantation of human foetal mesencephalic tissue from 6.5-9 week old or first trimester donors into the striatum of Parkinsonian rats (Brundin *et al.*, 1986; Brundin *et al.*, 1988; Stromberg *et al.*, 1986).

These promising findings led to the initiation of clinical trials in the late 1980s, which employed transplantation of foetal ventral mesencephalic tissue from aborted human foetuses into patients with PD (Hitchcock *et al.*, 1988; Lindvall *et al.*, 1989; Lindvall *et al.*, 1988). The majority of the early studies were open-label, uncontrolled trials, which reported a remarkable ability of transplanted human foetal dopaminergic cells to survive and provide clinically meaningful improvement that in some cases could replace pharmacological therapies (Brundin *et al.*, 2000; Freed *et al.*, 1992; Freeman *et al.*, 1995; Hagell *et al.*, 1999; Hauser *et al.*, 1999; Kordower *et al.*, 1995; Lindvall *et al.*, 1990; Lindvall *et al.*, 1994; Peschanski *et al.*, 1994; Wenning *et al.*, 1997). However, subsequent double-blind, placebo-controlled trials were unable to replicate these findings, and failed to show evidence of significant clinical benefit, with a few patients also developing dyskinesias or abnormal movements (Freed *et al.*, 2001; Isacson *et al.*, 2008; Olanow *et al.*, 2003; Svendsen, 2008).

Recent clinical trial results have proven more optimistic, with most human dopaminergic neuron-containing transplants found to display functional activity for at least a decade (Kordower *et al.*, 2008; Mendez *et al.*, 2008; Piccini *et al.*, 1999). Moreover, Mendez and colleagues detected the presence of graft survival in the absence of pathology for at least 14 years after transplantation (Mendez *et al.*, 2008). Notably, other reports found that in a few long-term cases (11 years post-transplant), a minor proportion of surviving dopaminergic neurons showed signs of pathological changes associated with PD, including protein aggregation and fibrillar changes suggestive of Lewy body formation (Kordower *et al.*, 2008; Li *et al.*, 2008). The mechanism underlying this observed pathology remains unknown, and reflects the many biological and technical challenges that lie ahead in developing a cell-based therapy for PD.

1.4.2.2 Neural Stem Cell Transplantation in PD

One of the limitations of using foetal neural tissue for cellular therapies is the rarity of developing dopaminergic neurons appropriate for transplantation, which are only present in 6-8 week old embryos (Sayles *et al.*, 2004; Svendsen, 2008). This, together with the low post-operative survival of these cells, necessitates the sourcing of mesencephalic tissue from multiple aborted foetuses, as well as transplantation within hours of tissue harvesting (Sayles *et al.*, 2004). To address this problem, research efforts since the early 1990s have been directed towards investigating the potential of different SC populations to act as an alternative dopaminergic cell source (Isacson *et al.*, 2008; Svendsen, 2008).

At first, *in vitro* culture expanded human foetal neural precursor cells were employed by Svendsen and colleagues in the 6-OHDA unilaterally lesioned Parkinsonian rat model (Ostenfeld *et al.*, 2000; Svendsen *et al.*, 1997; Svendsen *et al.*, 1996). These studies revealed that upon transplantation into the lesioned adult striatum,

expanded neural precursors were capable of survival, migration and differentiation towards glia and neurons, including TH-immunoreactive neurons. Although, in contrast with the large self-contained tissue masses formed by primary foetal neural tissue grafts, the expanded neural precursors formed only thin grafts containing small numbers of surviving cells, and did not migrate extensively into host tissues. Occasional TH-positive cells were detected in low numbers, with the observed expression perhaps being a transient event. In most cases, the small number of these cells was unable to elicit any functional effects, except in 2 animals that displayed partial reversal of lesion-induced rotation deficits. While the generation of some TH-positive neurons in these studies provided encouragement, it was clear that improvements were necessary for increased graft survival and dopaminergic differentiation.

Studer, *et al*. (Studer *et al.*, 1998) addressed these issues through *in vitro* culture expansion and pre-differentiation of neural precursors toward dopaminergic neurons prior to implantation. Using this procedure the yield of dopaminergic neurons was increased and partial alleviation of behavioural deficits in hemiparkinsonian rats could be achieved. However, *in vitro* expansion was only performed for a short period (6-8 days) and the degree of expansion was small (Sayles *et al.*, 2004). Subsequent studies have continued to examine these issues, and have tested the use of NSCs from different neural regions, different expansion and pre-differentiation techniques, *ex vivo* genetic modification of cells, and administration of trophic factors together with cell transplantation, with varying degrees of success. Common to the majority of these studies is the limited access to autologous NSCs from patients, due to the location of NSCs within the brain. Interestingly, a possible autologous cell source for PD cellular therapy has recently been described by my co-supervisor's laboratory (Murrell *et al.*,

2008), in a study showing functional improvement in the 6-OHDA rat model using SCs from rat and human olfactory mucosa.

1.4.2.3 Embryonic Stem Cell Transplantation in PD

Another potential source of cells that have been investigated are ESCs, which also provide opportunities for the development of large quantities of therapeutically useful cells. The first demonstration of TH-positive neuronal cell derivation from ESCs was reported by Lee, *et al*. (Lee *et al.*, 2000). This study implemented a differentiation protocol that was comprised of multiple induction stages, including embryoid body generation, selection of CNS SCs with a defined medium, proliferation of these cells using mitogen FGF-2, together with SHH and FGF-8, and differentiation and maturation following removal of FGF-2 and supplementation with ascorbic acid (AA). Cells obtained through this procedure were found to express CNS- and midbrain-specific genes in a pattern suggestive of progressive restriction to a mesencephalic and metencephalic CNS SC fate. Furthermore, the murine ESC-derived neurons were shown to produce DA, respond to neurotransmitters, and exhibit spontaneous synaptic activity. By the end of the final stage, this system yielded >30% TH-positive neurons, giving strength to the possibility of using ESCs as a reliable donor cell source for dopaminergic neuron transplantation in PD (Lee *et al.*, 2000).

This prospect was further extended by studies investigating the *in vivo* transplantation potential of ESCs in the 6-OHDA hemiparkinsonian rodent model. Transplantation of low numbers of undifferentiated murine ESCs into the rat striatum resulted in spontaneous generation of dopaminergic neurons capable of gradual and sustained restoration of motor asymmetry from 5-9 weeks post-transplantation (Bjorklund *et al.*, 2002). In addition, PET and functional magnetic resonance imaging scans also confirmed that the ESC-derived dopaminergic neurons had become

integrated within the host brain circuitry and mediated functional improvement. Nevertheless, 20% of rats receiving grafts developed teratomas within 9 weeks at the site of transplantation(Bjorklund *et al.*, 2002), indicating the need for caution and removal of proliferating and non-neuronal cells prior to application of this technique in the clinic.

However, another study reported the absence of teratoma formation and proliferative marker Ki-67, when pre-differentiated *NR4A2*-transfected ESCs were grafted into the striatum of 6-OHDA lesioned rats (Kim *et al.*, 2002b). Graft analysis revealed the presence of TH-positive cells that possessed complex cellular morphologies, with the immunoreactive cell bodies restricted to the graft region, whereas TH-positive cell processes extended into the parenchyma of the host striatum up to 2 mm away from the graft site. Additionally, these cells were found to release DA, form functional synaptic connections and modulate spontaneous and pharmacologically-induced behaviours, strongly supporting the capability of ESC-derived neurons to survive and function after intrastriatal grafting (Kim *et al.*, 2002b). Following these findings, other studies have continued to improve these procedures by examining different methods of pre-differentiation (cytokines, inductive factors, genetic modification, co-culture systems, and combinations of these), selection and enrichment of dopaminergic neuron populations, and combining trophic factor administration with transplantation.

1.4.2.4 Patient-Specific Cellular Transplantation in PD

Recent advances in the understanding of pluripotent SC biology have extended this work to include the possibility of patient-specific dopaminergic neuron replacement therapies. Studer and colleagues (Tabar *et al.*, 2008) utilised therapeutic cloning strategies to generate nuclear transfer ESCs, using donor fibroblasts obtained from

individual 6-OHDA lesioned hemiparkinsonian mice. The genetically matched ESCs were then differentiated into midbrain dopaminergic neurons and transplanted back into the original donor mouse. Significantly, this study found enhanced graft survival and amelioration of parkinsonian behaviour with autologous grafts, in comparison with allogenic grafts, which appeared to be related to decreased graft immunogenicity (Tabar *et al.*, 2008).

In another study by Jaenisch and colleagues (Wernig *et al.*, 2008), the need for nuclear transfer techniques was circumvented through the use of induced pluripotent stem (iPS) cells (Takahashi *et al.*, 2006; Yamanaka, 2007). Murine iPS cells were successfully differentiated into dopaminergic neurons of midbrain character through application of regional patterning factors (SHH and FGF-8) to FGF-2 responsive, iPS cell-derived neural precursor cells. Intrastriatal transplantation of these cells into the 6-OHDA unilaterally lesioned rat model of PD resulted in marked improvement in rotational behaviour at 4 weeks post-grafting. Attempts were also made to minimise the risk of teratoma formation by employing fluorescence-activated cell sorting (FACS) to remove contaminating pluripotent cells (SSEA1-positive fraction) from the cell suspension prior to transplantation (Wernig *et al.*, 2008).

A number of alternative non-neural adult SC sources have also been examined for potential in dopaminergic neuron replacement therapy for PD, in particular BM-derived MSCs, which will be discussed in detail in Section 1.4.4.

1.4.2.5 Challenges in Cellular Replacement Therapies for PD

Many important biological, technical, and surgical hurdles and challenges need to be addressed before cellular replacement therapy for PD patients can be implemented in the clinic. Currently, detailed knowledge is still required concerning critical issues, such as: 1) the appropriate neuronal (and perhaps also glial) cell type for transplantation,

including the cellular and biochemical characteristics necessary in the donor cell population; 2) the correct anatomical location for cell administration; 3) suitable selection criteria for identifying patients most likely to respond to cellular replacement therapy, which may be influenced by the stage of the disease and responsiveness to levodopa; 4) effective parameters for cell preparation and delivery to obtain optimal graft survival, including the optimal volume, dosage and format of cells; 5) mechanisms for limiting host immunological responses to donor cells; and 6) connectivity variables for functional reconstitution of neurocircuitry (Isacson *et al.*, 2008). Another crucial challenge for clinical application is the optimisation of graft function, whilst preventing graft-related side effects, since midbrain dopaminergic neurons are highly specialised cell types that must be capable of physiologically appropriate DA release and establishment of specific short- and long-distance connections within a complex cellular network (Isacson *et al.*, 2008). Additionally, investigation of cellular transplantation therapies must take into account the rate of cellular maturation when progenitor cell populations are used, since this is donor dependent and currently available data indicates that long-term clinical evaluation periods may be necessary (Isacson *et al.*, 2008). Production of clinical grade, individual, patient-specific cell lines also presents a considerable logistical challenge (Svendsen, 2008). And, it must also be remembered that the pathological changes present in PD are not restricted to dopaminergic neurons alone, with degeneration occurring in other neural regions (Svendsen, 2008). However, while gaps exist in our current knowledge, renewed hope is provided through the major research efforts directed towards understanding and developing novel cellular therapies for PD.

1.4.3 Lessons from Midbrain Dopaminergic Neuron Development *In Vivo*

In order to achieve efficient directed differentiation of MSCs into the neural lineage, it is surmised that the *in vitro* differentiation procedures utilised should mimic neural development as it occurs *in vivo*. Consequently, for the generation of midbrain dopaminergic neuronal cell types from SC populations, it is expected that the growth factors and signalling molecules important for directing the development of these cell types will be of significant relevance. Additionally, when SC populations are subjected to these differentiation inducing signals, it is also anticipated that changes in gene and protein expression would occur in a manner that reflects the patterns observed *in vivo* in developing dopaminergic neurons. Although the exact mechanisms of these processes are yet to be fully elucidated, current knowledge indicates the involvement of several critical signalling molecules and transcription factor cascades in complex cell extrinsic and intrinsic mechanisms that govern midbrain dopaminergic neuron specification and differentiation (Abeliovich *et al.*, 2007; Prakash *et al.*, 2006; Smidt *et al.*, 2007).

Cell fate specification in the vertebrate nervous system is believed to occur in accordance with a grid of Cartesian co-ordinates established by gradients of signalling molecules secreted along the dorso-ventral and rostro-caudal (anterior-posterior) axes of the neural tube (Maxwell *et al.*, 2005; Ye *et al.*, 1998). Neural progenitors consequently assume distinct cell fates according to the positional identity conferred by the grid location. Additionally, refinement of cell fate is achieved through multiple local signalling centres at the rostro-caudal level, which specify the identity and stereotypic locations of mature neuronal cell types within the major brain subdivisions.

After restriction of neuronal fate by these extrinsic cues, further differentiation into mature post-mitotic neurons is directed by intrinsic signals, often transcription factors (Maxwell *et al.*, 2005; Ye *et al.*, 1998). The development of midbrain

dopaminergic neurons can be broadly divided into 4 stages, the first of which involves migration of neuroblasts from the ventricular zone to the central and lateral ventral mesencephalon (Abeliovich *et al.*, 2007; Isacson *et al.*, 2008). The second stage is comprised of specification of progenitors to a dopaminergic neuronal precursor cell fate, followed by the third step, in which dopaminergic precursors exit the cell cycle and commence expressing markers indicative of an early midbrain dopaminergic neuron phenotype. Lastly, functional maturation of early dopaminergic neurons takes place, which is marked by expression of mature midbrain dopaminergic neuronal markers, axonal pathfinding and establishment of specific connectivity (Abeliovich *et al.*, 2007).

1.4.3.1 Extrinsic Cues Involved in Midbrain Dopaminergic Neuron Specification

The specification of the permissive region for midbrain dopaminergic neuron generation is a fundamental event that is initiated by the positioning of 2 key signalling centres, the floorplate, which is present at the ventral midline of the neural tube, and the isthmic organiser (also known as the isthmus) at the midbrain-hindbrain boundary (Abeliovich *et al.*, 2007; Prakash *et al.*, 2006). Cells of the floorplate secrete SHH, which influences cell fate along the dorso-ventral axis, whereas FGF-8 is secreted by the isthmus and exerts patterning activity along the rostro-caudal axis. Midbrain dopaminergic neurons develop at sites of SHH and FGF-8 intersection (see Figure 1.3), and Ye and colleagues (Ye *et al.*, 1998) have shown that induction with these 2 extrinsic factors is necessary and sufficient for dopaminergic neuron induction in multiple locations in the neural tube. Other extrinsic factors that have been demonstrated to be significant in specifying the site of dopaminergic neuron development include TGF-β, Nodal, WNT signalling (WNT1 and WNT5A), and RA (Abeliovich *et al.*, 2007; Prakash *et al.*, 2006; Smidt *et al.*, 2007).

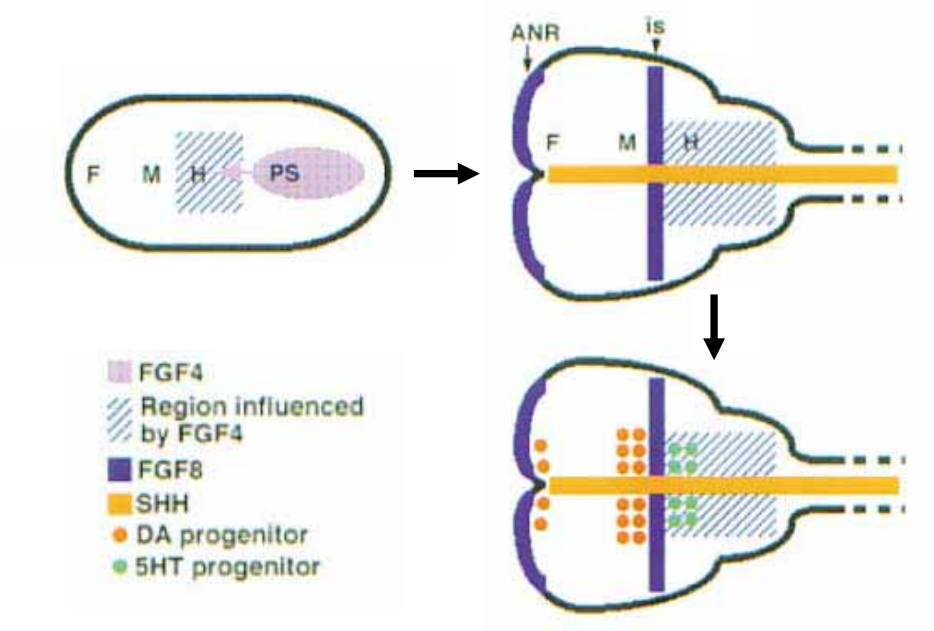

Figure 1.3 A model illustrating the mechanisms controlling the positions and cell fates of DA neurons during early development.

The specification of the region for midbrain DA neuron generation is controlled by 2 signalling centres, SHH expression in the floor plate, and FGF-8 expression along the isthmus. Initially, FGF-4 expression in the primitive streak influences the hindbrain region and prepatterns this area to block DA neuron formation. Following this SHH is expressed along the floor plate, and FGF8 is expressed at the isthmus and anterior neural ridge. Areas influenced by both SHH and FGF-8 signals produce DA neurons, while the influence of SHH and FGF-8 on the FGF-4-prepatterned hindbrain produces serotonin neurons. Anterior is to the left. F, forebrain; M, midbrain; H, hindbrain; is, isthmus; PS, primitive streak; ANR, anterior neural ridge; DA, dopaminergic; 5HT, serotonergic. Top panel, presomitic embryo; middle and bottom panel, late somitogenic embryo. Figure from (Ye *et al.*, 1998).

The migration of young midbrain dopaminergic neurons has been associated with a small number of molecules (Smidt *et al.*, 2007). However, little is known of the specific roles of these molecules in migration and some of the reports have presented conflicting findings. The signalling molecules that have been implicated in early dopaminergic neuronal migration include axon guidance cue netrin 1 and its receptor DCC (deleted in colorectal cancer), reelin, proteoglycan phosphacan 6B4 together with L1 cell adhesion molecule, neural cell adhesion molecule and polysialic acid expression, and PAX6 (Smidt *et al.*, 2007).

Several secreted factors have been found to be implicated in midbrain dopaminergic neuronal differentiation and functional maturation (Abeliovich *et al.*, 2007; Smidt *et al.*, 2007). These include RA, which appears to be locally synthesised during all steps of development, docosahexanoic acid as a ligand for retinoid X receptor, WNT family members, FGF family members, and TGF signalling (TGF-α and TGF-β), which appears to act in conjunction with SHH and FGF-8, as well as possibly inducing neurotrophic support. Currently, it is not known whether other extrinsic factors that have a role in earlier developmental stages, such as SHH and FGF-8, play an additional role in post-mitotic development (Abeliovich *et al.*, 2007; Smidt *et al.*, 2007).

Many factors have been identified as having a role in midbrain dopaminergic neuron maintenance and neurotrophic support (Smidt *et al.*, 2007). Proteins with known neurotrophic effects include GDNF, BDNF, TGF-α, TGF-β, neurotrophin 3, -4 and -5, TrkB and TrkC receptors for neurotrophins, neurturin, neublastin/artemin, persephin, growth/differentiation factor 5, neuregulin 1, BMPs, heparin-binding EGF, and FGF-2. Of these factors, GDNF has shown the most promise in increasing midbrain dopaminergic neuronal survival. A role for GDNF signalling in long-term maintenance was also suggested in a recent *in vivo* study (Kramer *et al.*, 2007), which examined mice

with regionally selective ablation of the RET receptor tyrosine kinase (c-RET; receptor for GDNF). This work showed progressive, adult-onset loss of dopaminergic neurons specifically in the substantia nigra with c-RET ablation. Additionally, crosstalk between GDNF signalling and TGF-β signalling has also been described (Smidt *et al.*, 2007).

1.4.3.2 Cell Intrinsic Signals Directing Dopaminergic Neuron Differentiation

In conjunction with the cell extrinsic cues that instruct midbrain dopaminergic neuronal development, also exist cell intrinsic programs that act in concert with extrinsic factors to progressively restrict cell fate choices (Abeliovich *et al.*, 2007)(see Figure 1.4). Recent advances through *in vivo* gene ablation studies have elucidated networks of transcriptional factors with sequential and parallel action that are implicated in the induction of specific phenotypic characteristics of midbrain dopaminergic neurons (Isacson *et al.*, 2008; Smidt *et al.*, 2007). None of the identified transcription factors are sufficient for individually instructing the midbrain dopaminergic neuronal phenotype, providing further support for a network model of transcription factor involvement (Abeliovich *et al.*, 2007).

The permissive region formed early in development by organising centres is defined by a specific pattern of gene expression (Smidt *et al.*, 2007). The expression domains of two transcriptional repressors orthodenticle homeobox (OTX) 2, at the presumptive fore- and midbrain, and gastrulation brain homeobox 2, at the presumptive hindbrain and spinal cord, establish the midbrain-hindbrain boundary (Abeliovich *et al.*, 2007; Maxwell *et al.*, 2005; Prakash *et al.*, 2006; Smidt *et al.*, 2007). Subsequent transcriptional regulation through LIM homeobox transcription factor (LMX) 1A, msh homeobox 1, and NEUROG2 has been found to play an important role in specifying the midbrain dopaminergic neuronal phenotype (Abeliovich *et al.*, 2007; Andersson *et al.*, 2006; Smidt *et al.*, 2007). Studies have also found that the development of the final

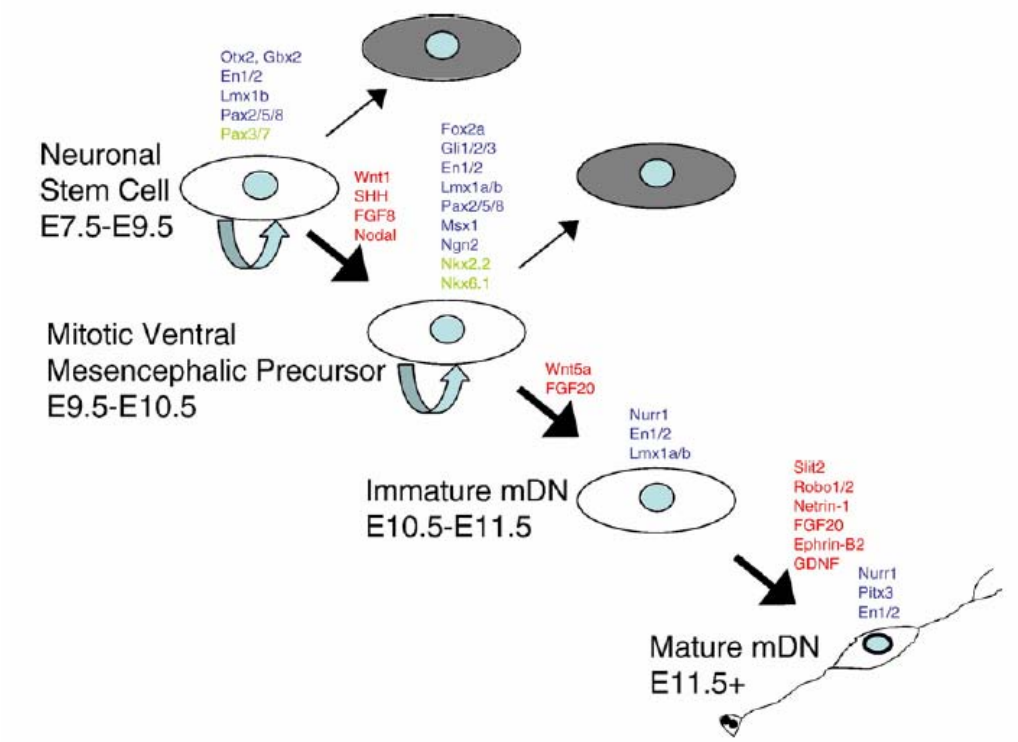

Figure 1.4 Intrinsic and extrinsic factors affecting the specification and development of midbrain dopaminergic neurons.

The developmental program of midbrain dopaminergic neurons consists of cell intrinsic programs that act in concert with cell extrinsic cues to progressively restrict cell fate choices. Initially, neuronal stem cells are patterned toward a ventral cell fate, and away from a dorsal fate (grey). Subsequently, mitotic ventral mesencephalic precursors are specified toward a dopaminergic fate, and away from alternative fates including the serotonergic cell fate (grey). Networks of transcription regulatory factors with sequential and parallel action have been implicated in these processes. Transcription factors that promote a dopaminergic neuronal fate are shown in blue, whilst inhibitory transcription factors are depicted in green. Additionally, secreted factors known to be involved in the different stages of the dopaminergic developmental program are shown in red. E, embryonic day; mDN, midbrain dopaminergic neurons. Figure from (Abeliovich *et al.*, 2007).

adult dopaminergic phenotype is dependent on several critical transcription factors, including EN1, EN2, LMX1B, NR4A2 and PITX3 (Abeliovich *et al.*, 2007; Maxwell *et al.*, 2005; Prakash *et al.*, 2006; Smidt *et al.*, 2007). In particular, NR4A2 has been proven to be necessary for the proper differentiation and survival of dopaminergic neurons at later developmental stages, but does not appear to be essential for the specification and initial birth of midbrain dopmainergic precursors. The majority of neurotransmitter-related genes and some trophic signalling pathways are also regulated by NR4A2, including several proteins required in DA synthesis and regulation, such as TH, vesicular monoamine transporter 2 (VMAT2), DAT and c-RET. Additionally, PITX3 is specifically expressed in midbrain dopaminergic neurons of the CNS, and appears to be critical for the proper terminal differentiation or maturation, and/or early maintenance of the subset of dopaminergic neurons that form the substantia nigra. Furthermore, the *TH* gene may be directly regulated by PITX3, and PITX3-deficient Aphakia mice exhibit specific loss of TH-expressing cells in the substantia nigra, whereas the VTA dopaminergic neurons were less affected (Abeliovich *et al.*, 2007; Maxwell *et al.*, 2005; Prakash *et al.*, 2006; Smidt *et al.*, 2007).

In recent years, many of the molecular processes underlying midbrain dopaminergic neuronal development, maintenance and function have been elucidated (Smidt *et al.*, 2007). These advances in our understanding provide many opportunities for the development of strategies to induce efficient generation of midbrain dopaminergic neurons from SC populations. Since successful cell replacement therapies are believed to depend upon the knowledge of how to make appropriate dopaminergic neurons, it is hoped that further unravelling of these mechanisms and continued research efforts will eventually lead to the development of functional cell-replacement therapies for PD. The present study aims to employ extrinsic factors, which are known to be

important in midbrain dopaminergic neuron specification and differentiation, in *in vitro* culture systems for the dopaminergic neuronal differentiation of hMSCs. Furthermore, cell intrinsic factors will be examined by investigating the gene expression changes elicited in hMSCs during *in vitro* dopaminergic neuronal differentiation.

1.4.4 Dopaminergic Neuronal Differentiation of MSCs *In Vitro* and Transplantation into Animal Models of Parkinson's Disease

A number of investigations have been performed to determine the potential of MSCs to differentiate into functional DA-producing neuronal-like cells, and to examine the capability of MSC grafts to re-innervate the striatum and ameliorate behavioural deficits in animal models of PD. To date, these studies have exhibited varying degrees of success, ranging from the generation of TH-positive cells, to observations of electrophysiological activity and DA secretion, to significant improvements in pharmacologically-induced rotational behaviour in hemiparkinsonian rodent models. Although, amongst the different studies a diverse array of methodologies have been employed and variations exist in the cell types obtained, which together have caused difficulty in the evaluation and comparison of experimental outcomes. Nevertheless, the promising results that have been reported provide much encouragement for the prospect of developing a MSC-based cellular therapy for PD.

Similar methods have been investigated for dopaminergic neuronal differentiation of MSCs as have been utilised in general MSC neuronal differentiation (discussed in Section 1.3.1), but with the inclusion of factors more specific to midbrain dopaminergic neuron development (see Section 1.4.3). At the time that the neuronal differentiation work in the present study was commenced, there were 6 reports in the literature describing dopaminergic neuronal differentiation of MSCs from rodents (Dezawa *et al.*, 2004; Guo *et al.*, 2005; Jiang *et al.*, 2003) and humans (Dezawa *et al.*,

2004; Fu *et al.*, 2006b; Hermann *et al.*, 2004; Suon *et al.*, 2006). The majority of these studies employed the addition of extrinsic factors to cell cultures, such as cytokines and small molecules; however, the effects of gene transfection, conditioned medium and co-culturing, as well as combinations of these methods were also investigated. These studies are summarised in Table 1.2.

The initial report by Jiang and colleagues (Jiang *et al.*, 2003) described a multi-stage differentiation strategy capable of generating midbrain neuronal-like cells from the MAPC subset of murine MSCs. The first stage of differentiation consisted of supplementation with FGF-2 for 7 days, followed by a combination of FGF-8 and SHH for 7 days, then BDNF for 7 days, and finally 5-12 days of co-culture with foetal brain astrocytes. The resulting cells exhibited phenotypical and electrophysiological characteristics similar to midbrain neurons, with approximately 25% efficiency of dopaminergic neuron generation. Also, co-culturing with astrocytes for 7-12 days resulted in between 80% and 100% of cells acquiring functional voltage-gated sodium channels. In addition, activation of transcription factors known to be involved in neuroectodermal development, such as *SOX1*, *OTX2*, *OTX1*, *PAX2*, *PAX5* and *NR4A2*, were detected in a similar developmental pattern as described for NSC and ESC dopaminergic differentiation (Jiang *et al.*, 2003). However, a number of caveats are associated with this approach, including lack of specificity for dopaminergic neuronal production, since serotonergic (approximately 25%) and GABA-ergic (approximately 50%) neurons were also produced, and as a consequence of this, requirement for selection of desired dopaminergic cells. Additionally, astrocyte-conditioned medium or cytokines alone were unable to provide appropriate differentiation signals. Therefore, astrocyte co-culture is necessary for prolonged survival and maturation toward electrophysiologically active cells, which impedes clinical translation of this method.

Table 1.2 *In Vitro* Dopaminergic Neuronal Differentiation of MSCs

Differentiation Method	Cell Type	Results (In vitro/In vivo)	References
Multi-stage method: 1) FGF-2 (7d) 2) FGF-8, SHH (7d) 3) BDNF (7d) 4) Astrocyte co-culture (5-12d)	Murine MAPC	25% efficiency of dopaminergic neuron generation (IF); electrophysiological characteristics similar to midbrain neurons	Jiang, *et al*. (2003)
NICD transfection, FGF-2+CNTF+ FBS +forskolin (5d), GDNF or BDNF+NGF (7-11d)	Human and rat MSCs	41% TH-positive (IF); electrophysiological properties; DA release after depolarisation; functional improvement in hemiparkinsonian rats	Dezawa, *et al*. (2004)
Neurosphere formation with EGF+FGF-2 (2-10 weeks), BDNF (10-14d)	Human MSCs	11% TH-positive (IF); DA release after depolarisation; loss of osteogenic differentiation capacity	Hermann, *et al*. (2004)
Neurosphere formation with IBMX+forskolin+ TPA (3hrs), dbcAMP (7d)	Human MSCs	15% of β tubulin III-positive cells also TH-positive (IF); lack of graft survival and differentiation in PD model	Suon, *et al*. (2006)
IBMX (2d), GDNF+ IL-1β+mesencephalic glial-conditioned medium+flash-frozen mesencephalic cell fragments (7-15d)	Rat MSCs	35% TH-positive (IF)	Guo, *et al*. (2005)
Expansion (10% FBS+ DMEM; 3d), neuron-conditioned medium alone (6-9d), SHH+ FGF-8+neuron-conditioned medium (3-12d)	Human MSCs from Wharton's jelly of umbilical cord	12.7% TH-positive (IF); DA secretion; partial correction of amphetamine-evoked rotational behaviour	Fu, *et al*. (2006)

d, days
IF, immunofluorescence staining

Furthermore, this study was conducted using murine cells, and whether the same results can be obtained with human cells must be confirmed.

The next key study demonstrating production of dopaminergic neuronal-like cells from MSCs was reported by Dezawa and colleagues (Dezawa *et al.*, 2004), who employed transfection of the *NICD* gene and administration of trophic factors FGF-2 and CNTF with forskolin, followed by addition of GDNF. Importantly, it was shown that this approach could generate TH-positive cells with 41% efficiency, which was at the time the highest reported rate, and the cells obtained also displayed *in vitro* and *in vivo* functional properties of neurons, with little glial differentiation. Delayed rectifier potassium currents were recorded after treatment with FGF-2, CNTF and forskolin, whereas voltage-gated fast sodium currents and action potentials were only obtained with further administration of BDNF and NGF. Treatment with GDNF elevated the expression of transcription factors *NR4A2*, *LMX1B*, *EN1* and *PITX3*, and also resulted in *in vitro* DA release in response to high K^+ depolarising stimuli. Further evidence for the dopaminergic neuronal differentiation of MSCs was demonstrated through observations of significant improvements in hemiparkinsonian rats, in terms of apomorphine-induced rotational behaviour and non-pharmacological assessments, such as the step adjustment and paw-reaching tests, and also through examining DA production by the graft in cultured brain slices. Additionally, the authors confirmed that similar results could also be obtained when using rat or human MSCs (Dezawa *et al.*, 2004). However, this study was not without limitations. The use of gene transfection may be problematic for application in the clinic, although the authors commented that this could be avoided through induction with a JAK/STAT inhibitor (Dezawa *et al.*, 2004). Also, while this study employed the use of neural trophic factors, those typical of midbrain dopaminergic neuronal specification, SHH and FGF-8, were not included,

suggesting that the observed differentiation may be divergent from that which occurs typically during normal development.

Application of extrinsic factors alone have also resulted in the induction of MSCs toward a dopaminergic neuronal phenotype (Guo *et al.*, 2005; Hermann *et al.*, 2004; Suon *et al.*, 2006). Hermann and colleagues (Hermann *et al.*, 2004) devised a protocol for producing a NSC-like population from hMSCs, through the formation of neurosphere-like structures in low-attachment culture flasks in the presence of serum-free medium containing EGF and FGF-2. The majority of the neuroprogenitor-like cells were found to have upregulated expression of NES protein to high levels, while mesodermal protein fibronectin was downregulated. Quantitative PCR analysis showed decreases in *SOX1*, *POU5F1*, and neurotrophic tyrosine kinase receptor type 1, whereas neuroectodermal transcripts *OTX1*, *NEUROD1*, *NEUROG2*, musashi and *NES* were acquired with neurosphere formation. Further differentiation of MSC-derived neuroprogenitors was achieved using BDNF for neuronal differentiation (42% of resulting cells were positive for β tubulin III), or PDGF for glial differentiation (45% GFAP-positive, and 27% GALC-positive). Interestingly, with this general neuronal differentiation procedure, 11% of cells were also shown to express TH, and were capable of releasing DA in response to membrane depolarisation *in vitro*. In addition, osteogenic differentiation capacity was lost after induction into neuroprogenitor-like cells (Hermann *et al.*, 2004). However, the authors were unable to examine the electrophysiological function of neuronal-like cells due to technical constraints, and further assessment of function through *in vivo* transplantation is also required. This system does not appear capable of generating mature neuronal cells, although the authors suggest that immature cells are more suitable for transplantation, since fully differentiated mature neural cells poorly survive detachment and subsequent

transplantation (Hermann *et al.*, 2004). Additionally, MSC dopaminergic neuronal differentiation occurred at a low rate of 11%, although even this level of efficiency may be somewhat surprising given the lack of specific dopaminergic inductive cues in the culture media.

Neural progenitor-like sphere formation by hMSCs was also utilised by Suon, *et al*. (Suon *et al.*, 2006) prior to dopaminergic neuronal differentiation by plating spheres on a poly-ornithine/laminin-coated surface in serum-free medium containing IBMX, forskolin and TPA for 3 hours, followed by dbcAMP for 7 days. Down-regulation of mesenchymal genes was observed after differentiation, with concomitant up-regulation of neural-associated genes. Additionally, 15% of β tubulin III-positive cells also expressed TH. Surprisingly, all of the cells were found to express GABA, including those found to be TH-positive. Intrastriatal transplantation of MSC-derived spheres in 6-OHDA unilaterally lesioned rats resulted in short-term graft survival, however, no further differentiation into dopaminergic neuronal cells could be observed, and instead only GABA-positive cells were detected. The lack of survival of hMSCs transplanted in the rat PD model, despite administration of cyclosporine A, reveals a potential limitation in this method of assessment of hMSC *in vivo* function.

Yet another differentiation approach was taken by Guo, *et al*. (Guo *et al.*, 2005), which yielded a rate of 35% TH-positive cells from rat MSCs, after exposure to IBMX for 2 days, followed by application of GDNF, IL-1β, mesencephalic glial cell-conditioned medium, and flash-frozen mesencephalic cellular fragments for 7-15 days. However, examination of DA secretion and excitability of the resulting cells was not performed, and assessment of this system using hMSCs remains to be determined. In addition, treatment with glial-conditioned medium and membrane fragments is unsuitable for use in clinical applications.

Fu and colleagues (Fu *et al.*, 2006b) isolated MSCs from Wharton's jelly of human umbilical cord and employed a multi-stage differentiation method consisting of expansion in 10% FBS-DMEM for stage 1, neuronal differentiation through incubation with neuron-conditioned medium in stage 2, and induction of a dopaminergic neuronal phenotype with application of neuron-conditioned medium, SHH and FGF-8. TH-positive neurons were generated with approximately 12.7% efficiency, and DA secretion into the culture medium was detectable. Furthermore, functional effects were observed *in vivo* following transplantation into the striatum of hemiparkinsonian rats, as seen by partial correction of amphetamine-evoked rotational behaviour (Fu *et al.*, 2006b). The primary caveat of this work was the low efficiency of dopaminergic neuronal differentiation, which may have contributed to the incomplete behavioural recovery. In addition, the electrophysiological characteristics of the resulting cell population were not examined, and it should be noted that umbilical cord-derived MSCs have been reported to possess different properties than adult BM-derived MSCs (Kern *et al.*, 2006).

The above studies that have examined the *in vivo* functional potential of MSC-derived dopaminergic neuron-like cells have found behavioural improvement with transplantation of these cells over that of control undifferentiated MSCs. However, other studies have reported survival, migration, generation of TH-positive cells and functional improvement with transplantation of undifferentiated MSCs (Hellmann *et al.*, 2006; Li *et al.*, 2001b). Li and colleagues (Li *et al.*, 2001b) performed the initial study investigating intrastriatal MSC transplantation in a murine MPTP-induced bilateral model of PD. Behavioural recovery was analysed using the rotarod test, with results showing that mice with a MSC graft exhibited prolonged duration on the rotarod at day 35, when compared with control sham grafted mice. Additionally, MSCs were

found to survive within graft sites for at least 4 weeks post-transplantation, and a small proportion of scattered MSCs were TH-immunoreactive. However, the authors hypothesised that the therapeutic benefits most likely originated from MSC secretion of growth factors capable of promoting survival and plasticity within the damaged brain, since improvement was observed rapidly and few MSCs were present in the target tissue (Li *et al.*, 2001b). Hellmann, *et al.* (Hellmann *et al.*, 2006) also transplanted undifferentiated MSCs into parkinsonian rodents (6-OHDA unilaterally lesioned model), but for the purposes of assessing MSC survival, migration and differentiation. It was found that MSCs exhibited higher survival in the 6-OHDA lesioned hemisphere, in comparison with the unlesioned side. In addition, MSC transplantation into the striatum contralateral to the lesion resulted in migration of MSCs through the corpus callosum into the striatum, thalamic nuclei and substantia nigra of the 6-OHDA lesioned hemisphere (Hellmann *et al.*, 2006). These studies suggest that the therapeutic benefits observed following MSC transplantation in rodent PD models may be conferred through mechanisms other than cellular replacement and DA production.

A variety of distinct approaches have been implemented in the quest for MSC dopaminergic neuronal differentiation and application in potential PD therapies. Nonetheless, the reported findings possess a number of unifying elements, although these mainly lie in the realm of newly raised questions and issues that remain to be resolved. In the midst of these pertinent issues, are questions regarding the optimal developmental point at which to transplant differentiating cells, the relevance of animal models and studies to the situation in humans, the little-defined potential immunological responses to undifferentiated and differentiated MSC grafts, and the suitability for translation into the clinic. While much progress has been achieved and promising results have been obtained, the generation of mature functional midbrain dopaminergic neurons

from MSCs remains elusive. However, the current evidence of phenotypic conversion and acquisition of functional characteristics *in vitro* and *in vivo*, support the feasibility of this approach and continue to render research efforts in this field as worthwhile pursuits.

1.5 Aims of the Current Study

The overall hypothesis of the current study is that human adult BM-derived MSCs can be induced to differentiate toward the neuronal lineage and in particular into midbrain dopaminergic neuronal-like cells. The project aims to investigate this hypothesis through firstly establishing cultures of hMSCs and characterising these cell populations in the undifferentiated state. The functional capacity of hMSCs to differentiate *in vitro* towards the neural lineage and the expected mesodermal lineages would then be examined, and the resulting cells characterised. Subsequently, the function of MSCs *in vivo* will be explored through transplantation into a rodent model of PD, in order to determine the anatomical and functional effects of MSC transplantation into neural regions. Within these general aims, there are three specific aspects that are to be investigated, and the following outlines these in greater depth.

Human BM-derived MSCs have previously been demonstrated to be capable of transdifferentiation, but have also been shown to have a finite expansion potential. Therefore, it is hypothesised that hMSCs display a pluridifferentiated phenotype, and that long-term *in vitro* culture alters the hMSC phenotype. The first section of this project aims to test these hypotheses through the characterisation of undifferentiated human BM-derived MSCs, in terms of expression of markers of pluripotency and the three germ lineages, and to determine the baseline expression profile of these cells prior to commencing differentiation.

Our laboratory has previously shown that hMSCs are able to adopt a neuronal-like phenotype upon cytokine exposure. In view of this, human BM-derived MSCs are hypothesised to be capable of responding to sequential stimuli with cytokines that have been reported to be physiologically important in midbrain dopaminergic neuron development. This project aims to differentiate hMSCs into dopaminergic neuronal-like cells through the manipulation of *in vitro* culture conditions. In addition, this work aims to examine the morphological changes associated with cytokine-induced neuronal differentiation, to determine whether the observed changes result from culture artifacts due to environmental stress during the differentiation procedure. Live cell imaging techniques will be employed to record changes in cellular morphology for these purposes. Human BM-derived MSCs undergoing neuronal differentiation will also be characterised in terms of gene and protein expression, to determine whether a neuronal-like phenotype is acquired, together with a concomitant loss of mesodermal lineage and pluripotency markers. Furthermore, the effect of long-term serial passaging on the neuronal differentiation capability of hMSCs will also be investigated, as this has previously been reported to affect the mesodermal differentiation ability of MSCs.

Finally, it is also hypothesised that human BM-derived MSCs are capable of differentiation to dopaminergic neuronal-like cells *in vivo* when transplanted directly into the brains of 6-OHDA unilaterally-lesioned Parkinsonian rats, and also that partial-differentiation of hMSCs prior to transplantation enhances engraftment. This will be investigated through analysis of the survival, anatomical integration and functional effects of the hMSC graft. The specific aims of this final section are: a) To transplant hMSCs (undifferentiated and partially differentiated) into the hemiparkinsonian rat model, and investigate the survival, differentiation and migration of these cells in the brain; b) To determine whether the substantia nigra or the striatum is the optimal

transplantation site for hMSCs; c) To evaluate any effects of hMSCs on host cells, for example, neurotrophic effects, or immunomodulatory effects; and d) To discover if there exists an advantage or synergistic effect of co-transplanting hMSCs with olfactory ensheathing cells (OECs) into the hemiparkinsonian rat model. A significant part of this *in vivo* study was conducted at the National Centre for Adult Stem Cell Research (Griffith University, Brisbane, QLD), under the supervision of Prof. Alan Mackay-Sim.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell lines

SK-N-SH, HepG2, U-87 MG and MG-63 cell lines from the American Type Culture Collection (ATCC) Global Bioresource Center (Manassas, VA, USA). HUH7 cell line was a gift from Prof. Bernard Tuch (Prince of Wales Hospital, Sydney).

2.1.2 Cell Isolation and Culture Reagents

The tetrameric antibody mixture RosetteSep™ Bone Marrow Progenitor Cell Pre-Enrichment Cocktail was obtained from StemCell Technologies (Vancouver, Canada) for depleting lineage-committed cells in BM expressing CD3, CD11b, CD14, CD16, CD19, CD56, CD66b and Glycophorin A/CD235a.

DMEM – Low Glucose (DMEM-LG) containing L-glutamine and sodium pyruvate, DMEM/nutrient mixture F12, DMEM/nutrient mixture F12 (without HEPES buffer), Neurobasal medium, DMEM/HAM F12, Dulbecco's Phosphate Buffered Saline (dPBS), 25 U/mL penicillin/25 μg/mL streptomycin, L-glutamine, sodium pyruvate, FBS (Lot No. 1236374), N-2 supplement, B-27 serum-free supplement (50x) liquid, and fibronectin (human plasma) were purchased from Gibco/Invitrogen (Carlsbad, California, USA). MCDB-201 medium, Hank's Balanced Salt Solution (HBSS), ITS liquid media supplement (100x), linoleic acid-albumin, AA 2-phosphate, dexamethasone, β-glycerophosphate, proline, bovine serum albumin (BSA), IBMX, insulin, indomethacin, poly-L-ornithine, and laminin (human placenta) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse laminin was obtained from BD Biosciences (Bedford, MA, USA). All cultureware (sterile, tissue culture-treated plastic flasks and chamber slides) was purchased from Falcon/Becton Dickinson Labware (Franklin Lakes, NJ, USA), except for chamber slides used in live cell imaging, which

were from Lab-Tek, Nunc (IL, USA). Glass coverslips were sourced from Mediglass/Lomb Scientific (NSW, Australia).

The following growth factors/cytokines were purchased from R&D Systems (Minneapolis, MN, USA): recombinant human EGF, recombinant human FGF basic (FGF-2), recombinant mouse FGF-8b, recombinant human GDNF, human PDGF, recombinant human SHH amino terminal peptide, and recombinant human TGF-β3. Recombinant human FGF-8 was also purchased from PeproTech, Inc. (NJ, USA). All cytokines were reconstituted in 0.2% BSA in dPBS and stored at -80°C until use.

2.1.3 Enzymes and enzyme buffers

Platinum® SYBR® Green qPCR SuperMix-UDG and SuperScript™ III First-Strand Synthesis System for RT-PCR (reverse transcription-polymerase chain reaction) were obtained from Invitrogen. 0.5% trypsin-ethylenediamine tetraacetic acid (EDTA) was purchased from Gibco/Invitrogen.

2.1.4 Oligonucleotides

Sense and anti-sense primers for real-time RT-PCR were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and synthesised by Sigma-Aldrich.

2.1.5 Antibodies

Antibodies used in flow cytometry were CD29, CD73, CD45, CD14, CD34, CD166, HLA Class I and HLA Class II from BD Biosciences Pharmingen (San Jose, CA, USA), CD44 from Beckman Coulter (Fullerton, CA, USA) and CD105 from Chemicon (Millipore, NSW, Australia).

Primary antibodies used in immunofluorescent staining were mainly sourced from Chemicon (Millipore), including mouse monoclonal antibodies POU5F1, NES,

SSEA-1, SSEA-4, TRA-1-60, A2B5, NeuN, and human nuclear antigen (HNA; clone 235-1; MAB1281), and rabbit polyclonal antibody Forkhead Box A2 (FOXA2; also known as HNF3β). Other mouse monoclonal antibodies were obtained from BD Pharmingen (CD44), Sigma-Aldrich (MAP-2 and GFAP-Cy3) and Immunostar Incorporated (WI, USA; TH). Rabbit polyclonal primary antibodies were also sourced from Sigma-Aldrich (Fibronectin), Dako Cytomation (Glostrup, Denmark; GFAP), Covance (CA, USA; β tubulin III), and Wako Pure Chemical Industries Ltd (Osaka, Japan; Iba-1). Secondary antibodies used were Alexa Fluor 488 or 594 highly cross adsorbed goat anti-rabbit or anti-mouse antibodies from Molecular Probes (Invitrogen).

2.1.6 Equipment

For flow cytometric analysis, cells were acquired and analysed on a FACSCalibur flow cytometer (BD) with Cell-Quest software.

The NanoDrop 1000 spectrophotometer used to quantify total RNA was obtained from Thermo Scientific. A Rotor-Gene RG3000 machine (Corbett Research, NSW, Australia) was used for performing real-time RT-PCR reactions. The Gel Doc-1000 photographic system (Bio-Rad Laboratories, NSW, Australia) was used for agarose gel electrophoresis. Statistical analysis was performed using GraphPad Prism from GraphPad Software Incorporated (La Jolla, CA, USA) and Microsoft Office Excel.

Routine morphological observations of cultured cells were recorded using an inverted light microscope and digital camera from Olympus (Tokyo, Japan). Live cell imaging was performed with an inverted microscope equipped with CO_2- and temperature-controlled stage from Carl Zeiss (Germany). Zeiss inverted fluorescent microscope, Axiocam digital camera and Axiovision software used for imaging hMSCs cultured on slides and coverslips were also from Carl Zeiss.

The stereotaxic frame and microinjector unit used for intracerebral transplantation were obtained from Kopf Instruments (USA). The syringe and needle used for cell injection were from Hamilton Company (Reno, NV, USA).

Confocal imaging was performed using a Leica laser scanning confocal microscope (DM IRE2 TCS SP2 AOBS) from Leica Microsystems (Wetzlar, Germany). Images were prepared as figures using Adobe Photoshop (Adobe Systems Incorporated, CA, USA). Three-dimensional rendering of graft sites was prepared using Imaris software from Bitplane (Zurich, Switzerland). Images for rendering were captured using a Zeiss Axio Imager Z1 with ApoTome and Axiovision software (Carl Zeiss).

Amphetamine-induced rotational behaviour was measured using the Rotometer Activity System from San Diego Instruments (San Diego, CA, USA).

2.1.7 General materials and chemicals

All general reagents used in molecular biology were of molecular biology grade. TRIzol reagent was obtained from Invitrogen. RNeasy® Micro kit and Mini kit for total RNA isolation and RNase-free DNase I were obtained from Qiagen (Basel, Switzerland). Human brain total RNA was obtained from Stratagene (Texas, USA). Human ESC cDNA was kindly provided by Dr Justin Lees (Prince of Wales Hospital, NSW, Australia). Chloroform was obtained from Sigma-Aldrich. Agarose was obtained from Promega (Madison, USA). GeneRuler Ultra Low Range 10-300 bp DNA ladder was purchased from Fermentas (Ontario, Canada).

Sodium heparin tubes were obtained from Becton Dickinson. Ficoll-Paque™ Plus solution was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Trypan blue solution (0.4%) was purchased from Sigma-Aldrich.

Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Biosciences. Trisodium citrate was obtained from Sigma-Aldrich.

Paraformaldehyde (PFA) powder and Triton X-100 solution were obtained from Sigma-Aldrich. Normal goat serum was obtained from Chemicon. DAPI (4',6-diamino-2-phenylindole) was obtained from Molecular Probes (Invitrogen). Glycerol was sourced from Bacto Laboratories (NSW, Australia).

Cyclosporine A was obtained from Sandimmune, Sandoz Pharmaceutical (East Hanover, NJ, USA). Isofluorane gas was from Affane, Bomac (NSW, Australia). Lethabarb-Sodium Pentabarbitone was from Virbac Animal Health (TX, USA). Polyethylene glycol (PEG) and D-amphetamine were obtained from Sigma-Aldrich. D-amphetamine was used in compliance with the regulations for Schedule 4 Controlled Substances and with approval from the Chief Pharmacist of QLD and NSW.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Isolation of Human Bone Marrow-Derived Mesenchymal Stem Cells

Whole BM (1-2 mL) was collected into sodium heparin tubes from the posterior iliac crest of haematologically normal donors following written informed consent as approved by the Human Research Ethics Committee of our Institute (St Vincent's Hospital Sydney and The University of New South Wales). hMSCs were isolated using antibody-mediated negative selection and density gradient centrifugation, followed by plastic adherence. For negative selection, BM specimens were incubated for 20 minutes (min) with a tetrameric antibody mixture (RosetteSep Bone Marrow Progenitor Cell Pre-Enrichment Cocktail) for depleting lineage-committed cells expressing CD3, CD11b, CD14, CD16, CD19, CD56, CD66b and Glycophorin A/CD235a. Subsequently, MNCs were separated on a Ficoll-Paque density gradient (30 min at 400 *g*). Enriched cells were re-suspended in expansion medium (see next section) and

seeded in culture flasks at a density of less than 8 x 10^4 cells/cm^2. hMSCs were isolated from 12 BM specimens using this procedure.

2.2.1.2 Expansion of Human Bone Marrow-Derived Mesenchymal Stem Cells

hMSCs were expanded in medium (adapted from (Pittenger *et al.*, 1999), (Reyes *et al.*, 2001), (Gronthos *et al.*, 2003)) consisting of 60% DMEM-LG, 40% MCDB-201 medium, 1x ITS, 1x linoleic acid-albumin, 10^{-9} M dexamethasone, 10^{-4} M AA 2-phosphate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated FBS. FBS was heat-inactivated by incubating room temperature FBS in a water bath at 56°C for 30 min. This medium was chosen as it was demonstrated to expand BM MSCs with multi-lineage differentiation potential (Jiang *et al.*, 2003; Jiang *et al.*, 2002; Reyes *et al.*, 2001). Cultures were maintained at 37°C, 5% CO_2, and 20% O_2. Non-adherent cells were removed 3 days after initial plating, and medium replaced every 3-4 days. When cultures reached 80-90% confluency, hMSCs were harvested by incubating cultures with 0.05% trypsin/0.53 mM EDTA for 5 min at 37°C, followed by immediate inactivation of trypsin with serum-containing media. Exposure to trypsin was minimised and cells were thoroughly washed and re-plated as Passage (P) 1 in expansion medium at a density of 1.7-2.0 x 10^3 cells/cm^2. Cells were subcultured weekly, and population doubling and cell viability (assessed by trypan blue dye exclusion) were recorded. The formula used for calculating the number of population doublings was: $X \times 2^N = Y$ (where X is the number of cells initially seeded, N is the number of population doublings, and Y is the final cell number obtained after culturing. Cumulative doubling was calculated by adding together the calculated population doublings. For cryopreservation, cells were stored in cryovials with 10% DMSO, 20% FBS and 70% HBSS and cooled slowly to -80°C in an isopropanol tank, prior to storage in liquid nitrogen.

2.2.1.3 Induction of Multi-lineage Mesodermal Differentiation

Osteogenic, chondrogenic and adipogenic differentiation were induced in P4-6 hMSCs to confirm the MSC identity of isolated cells. All multi-lineage mesodermal differentiation experiments were performed by Dr. Bojiang Shen (St Vincent's Hospital Sydney, NSW Australia), according to published protocols, with some modifications (Abdallah *et al.*, 2005; Khoo *et al.*, 2008; Schutze *et al.*, 2005). Briefly, for osteogenic differentiation, confluent cultures were exposed to DMEM-LG, 100 nM dexamethasone, 50 µg/mL AA 2-phosphate and 10 mM β-glycerophosphate for 2 weeks. For chondrogenic differentiation, 1 x 10^6 hMSCs were placed in a 15 mL polypropylene tube and centrifuged to form 3-D pellet cultures. Pellets were cultured for 3 weeks with DMEM-LG, 100 nM dexamethasone, 50 µg/mL AA 2-phosphate, 100 µg/mL sodium pyruvate, 40 µg/mL proline, 1% ITS, 5.35 µg/mL linoleic acid, 1.25 mg/mL BSA, and 10 ng/mL TGF-β3. For adipogenic differentiation, confluent cultures were exposed to DMEM-LG, 10% FBS, 1 µM dexamethasone, 0.5 mM IBMX, 10 µg/mL insulin and 100 µM indomethacin for 2 weeks. Standard histochemical staining procedures were performed on differentiated and undifferentiated hMSCs (cultured in parallel) to confirm mesodermal differentiation. Osteogenic differentiation was confirmed by Alkaline Phosphatase staining, chondrogenic differentiation by Alcian Blue staining, and adipogenic differentiation by Oil Red O staining. For osteogenic and chondrogenic differentiation, multiple fields were examined to determine the area of extracellular matrix deposition as a percentage of the total area of confluent cultures (as described (Digirolamo *et al.*, 1999)). Adipogenic differentiation was assessed by performing cell counts of multiple fields to determine the percentage of positively stained cells.

2.2.1.4 Induction of Neuronal Lineage Differentiation – Single-Stage Cytokine-Based Method (SingleND)

Neuronal differentiation of early (P4-5) and late (P11-12) stage hMSC cultures was induced using a cytokine-based single-stage neuronal differentiation (SingleND) method as reported previously (Tao *et al.*, 2005), with medium containing DMEM/nutrient mixture F12, 1x N-2 supplement, 100 U/mL penicillin, and 100 µg/mL streptomycin, supplemented with 10 ng/mL FGF-2, 10 ng/mL EGF and 1 ng/mL PDGF. hMSCs were plated in neuronal differentiation medium (0.11 mL/cm^2) at 6.7 x 10^3 cells/cm^2 (i.e. for a T75 flask, cells were seeded at 5.0 x 10^5 cells in 8 mL medium). Cultureware was pre-coated with 10 µg/mL Fibronectin to facilitate cell adhesion in serum-free neuronal differentiation conditions. Cultures were incubated at 37°C, 5% CO_2, and 20% O_2 with daily replenishment of growth factors and medium changed every 2 days. Cells were subcultured weekly with 0.05% trypsin/0.53 mM EDTA in the same way as for subculturing hMSCs (see Section 2.2.1.2), however, cells were only exposed to trypsin/EDTA for 2 min at 37°C, due to serum-free conditions. Proliferation and cell viability were assessed by trypan blue dye exclusion. In these experiments, neuronal differentiation conditions were applied for a maximum of 3 weeks, although cells have been shown to survive in these conditions for up to 3 months (Tao *et al.*, 2005). Neuronal-induced cells were harvested for characterisation at 1, 2 and 3 weeks of differentiation. The SingleND method was used for the neuronal differentiation of 4 hMSC cultures obtained from different donors.

2.2.1.5 Induction of Dopaminergic Neuronal Differentiation – Multiple-Stage Cytokine-Based Method (MultiDA)

A multiple-stage protocol was designed with the purpose of using cytokines reported to be important in dopaminergic neuronal development to direct hMSCs more

specifically toward a dopaminergic neuronal fate. Cytokine-based multiple-stage dopaminergic neuronal differentiation (MultiDA) was induced in exponential growth phase hMSC cultures (P5-6) from 3 individuals after the usual MSC subculturing procedure. Cells were seeded into cultureware pre-coated with 2 µg/cm^2 Poly-L-ornithine (overnight incubation at 37°C) and 1 µg/cm^2 Mouse Laminin (>3 hour incubation at 37°C) at 6.0 x 10^3 cells/cm^2 in 0.11 mL/cm^2 differentiation medium (i.e. for a T75 flask, cells were seeded at 4.5 x 10^5 cells in 8 mL medium). The medium used in all 3 stages of dopaminergic neuronal differentiation contained DMEM/nutrient mixture F12 (without HEPES buffer), 1x N-2 supplement, 100 U/mL penicillin, and 100 µg/mL streptomycin. The aim of Stage 1 of the MultiDA method was to prime hMSCs toward a neural fate. Stage 1 medium was supplemented with 10 ng/mL FGF-2 and 10 ng/mL EGF. The purpose of Stage 2 was to initiate midbrain specification of hMSCs, and Stage 2 medium was supplemented with 100 ng/mL SHH, 10 ng/mL FGF-8 (human) and 200 µM AA 2-phosphate. Stage 3 of the MultiDA method aimed to induce differentiation and maturation of hMSCs toward a dopaminergic neuronal phenotype. Stage 3 medium was supplemented with 50 ng/mL GDNF and 200 µM AA 2-phosphate. Each stage was applied for 1 week. Additionally, different combinations of the stages were also examined over the 3 week differentiation period. Cultures were incubated at 37°C, 5% CO_2, and 20% O_2 with daily replenishment of growth factors and medium changed every 2 days. Cells were subcultured at the end of each stage (weekly) with 0.05% trypsin/0.53 mM EDTA in the same way as for subculturing neuronal-induced hMSCs (Section 2.2.1.4). Cell counts and viability analysis were performed using trypan blue dye exclusion. Dopaminergic neuronal-induced hMSCs were harvested for characterisation at the end of each stage of differentiation. The MultiDA method was used for differentiation of 3 hMSC cultures obtained from different donors.

2.2.1.6 Induction of Dopaminergic Neuronal Differentiation Via Published Protocol – Single-Stage Cytokine-Based Method (SingleDA)

Differentiation was induced in exponential growth phase hMSCs (P5-6) from 3 individual donors, using a cytokine-based single-stage dopaminergic neuronal differentiation (SingleDA) protocol that had recently been published (Trzaska *et al.*, 2007). Briefly, hMSCs were seeded into 6 well plates at a density of 1 x 10^5 cells per plate, with MSC expansion medium. After 1 day, the expansion medium was replaced with dopaminergic neuronal differentiation medium, containing Neurobasal medium, 0.25x B-27 supplement, 250 ng/mL SHH, 100 ng/mL FGF-8 (mouse), and 50 ng/mL FGF-2. Cells were cultured at 37°C, 5% CO_2, and 20% O_2, and media was not replaced during the induction period. Dopaminergic neuronal-induced hMSCs were harvested for characterisation after 6 days and 12 days of differentiation, and cell proliferation and viability were assessed by trypan blue dye exclusion. The SingleDA method was used for differentiation of 3 hMSC cultures obtained from different donors.

Table 2.1 summarises the different neuronal differentiation methods employed in the present study.

2.2.1.7 Cell Lines

The cell lines SK-N-SH, HUH7, U-87 MG, HepG2, and MG-63 were used as positive controls and were cultured according to the recommended protocols.

2.2.2 Flow Cytometric Analysis

2.2.2.1 Detection of Apoptosis

For detection of apoptosis, hMSCs (4 cultures) were harvested at P2, P5, P8 and P11 by trypsinisation, washed with 10% FBS in HBSS, and resuspended in cold dPBS. hMSCs were stained with Annexin V-FITC (early apoptosis) and Propidium Iodide (PI;

Table 2.1 Summary of the Neuronal Differentiation Procedures

Procedure	Stage	Harvest*	Cytokines	Surface Coating	Medium
SingleND	1	D7, 14, 21	10 ng/mL FGF-2 + 10 ng/mL EGF + 1 ng/mL PDGF	Fibronectin	DMEM/F12 + N-2
MultiDA	1	D7	10 ng/mL FGF-2 + 10 ng/mL EGF	Poly-L-ornithine + Laminin	DMEM/F12 w/o HEPES + N-2 AA (Stage 2 & 3)
	2^	D7	100 ng/mL SHH + 10 ng/mL FGF-8		
	3~	D7	50 ng/mL GDNF		
SingleDA	1	D6, 12	250 ng/mL SHH + 100 ng/mL FGF-8 + 50 ng/mL FGF-2	None - hMSCs allowed to adhere prior to induction	Neurobasal + B-27

D, day

*cells were harvested following application of the indicated cytokines for the indicated number of days

^Stage 2 applied following induction with Stage 1 conditions

~Stage 3 applied following induction with Stage 2 conditions

late apoptosis) using Annexin V-FITC Apoptosis Detection Kit I, and following the manufacturer's recommendations. Cells (>10,000) were acquired and analysed on a FACSCalibur flow cytometer with Cell-Quest software.

2.2.2.2 Characterisation of Surface Antigen Expression

For characterisation of surface antigen expression, hMSCs were harvested at P4 and P8 by trypsinisation, washed with 10% FBS in HBSS, and resuspended in cold dPBS as reported previously (Tao *et al.*, 2005). hMSCs were incubated with normal human AB plasma (1:10) at 4°C for 30 min, then washed with FACS buffer (dPBS, 13.6 mM tri-sodium citrate and 1% BSA), and labeled with FITC-, PE- or PerCP-conjugated antibody in the dark at 4ºC for 30 min. Antibodies used were CD29, CD44, CD73, CD105, CD166, CD14, CD34, CD45, HLA Class I and HLA Class II. Cells were washed, resuspended in FACS buffer and >10,000 cells were acquired and analysed on a FACSCalibur flow cytometer with Cell-Quest software. This experiment was performed twice ($n = 2$). Similar findings were observed in additional experiments performed by Jean Hsu (St Vincent's Hospital Sydney, NSW Australia).

2.2.2.3 Statistical Analysis

For the apoptosis detection assay, data are presented as mean ± SEM. Statistical analysis was performed using the Wilcoxon Signed Ranks Test to determine the equality of the means between paired time points P2–P5, P2–P8 and P2–P11. The significance level was set at 0.05.

2.2.3 RNA Extraction

Total RNA was extracted from undifferentiated hMSC cultures at P2, P5, P8 and P11, and hMSC cultures pre- and post-differentiation using TRIzol reagent and RNeasy Micro kit. Cells were harvested by trypsinisation, as described previously, and

resuspended in cold dPBS, followed by centrifugation and removal of all the supernatant, including the last drop using a pipette. Cold TRIzol reagent was added to the cell pellet, at a volume of 500 µl TRIzol for every 1 x 10^6 cells, and the solution was resuspended by pipetting. Samples were stored at -80°C or immediately utilised for RNA extraction. For RNA extraction, samples were incubated at room temperature for 2-3 min, before addition of 20% volume of chloroform and mixture by shaking. Samples were centrifuged at 13,000 rpm for 15 min at 4°C. The top supernatant layer was immediately removed and an equal volume of room temperature 70% ethanol was added, mixed by pipetting, and immediately transferred to an RNeasy spin column. The manufacturer's recommendations were followed for the remainder of this procedure. To exclude genomic DNA contamination, samples were also treated with RNase-free DNase I, in accordance with the manufacturer's protocol.

Total RNA was also isolated from cell lines SK-N-SH, U87, HUH7, HepG2, and MG-63, for use in primer optimisation and as positive controls in RT-PCR, since these cell types are known to express the genes of interest. Other positive control samples included RNA obtained from human brain, chondrocytes and ESCs.

The total RNA extracted from samples was quantified using a Nanodrop spectrophotometer, and samples were aliquoted into 1-2 µg aliquots and stored at -80°C for use in cDNA synthesis reactions.

2.2.4 Real-Time RT-PCR Analysis

In order to examine the expression of markers of hMSCs, pluripotency, and the three germ lineages, as well as genes indicative of neural and dopaminergic neuronal development, real-time RT-PCR was performed. Table 2.2 lists the genes that were examined for mRNA expression by real-time RT-PCR, as well as the corresponding forward and reverse primers used.

Table 2.2 Primer Pairs for Real-Time PCR

Gene	GenBank Accession Number	Sequence (5'-3') Sense	Antisense	Product Size (bp)	Positive Control
Pluripotency Markers					
POU5F1	NM_002701	ctcaccctgggggttctatt	agcttcctccacccacttct	129	hESCs
NANOG	NM_024865	aactggccgaagaatagcaa	catccctggtggtaggaaga	86	hESCs
Ectodermal Lineage Markers					
NES	NM_006617	tccaagacttccctcagctt	tcaggactgggagcaaagat	145	SK-N-SH
MAP-2	NM_002374	agaccaccattgacgactcc	tctccgagcttccttttcag	134	SK-N-SH
CSPG4	NM_001897	gaaggaggacggacctcaa	gatccatctcggaggcatta	143	U-87 MG
GFAP	NM_002055	atcgagatcgccacctacag	caccacgatgttcctcttga	150	Brain
ENO2	NM_001975	aggccagatcaagactggtg	caagcagaggaatcacagca	148	Brain
Endodermal Lineage Markers					
FOXA2	NM_021784	cgctctccttcaacgactgt	tagcagccgttctcgaacat	115	HUH7
ALB	BC039235	acattcaccttccatgcaga	aaagctgcgaaatcatccat	146	HepG2
Mesodermal Lineage Markers					
CD44	NM_000610	aagacacattccaccccagt	ggttgtgtttgctccacctt	98	hMSCs
COL1A1	NM_000088	gagagcatgaccgatggatt	atgtaggccacgctgttctt	149	MG-63
BGLAP	NM_199173	ggcgctacctgtatcaatgg	tcagccaactcgtcacagtc	106	MG-63
COL2A1	NM_033150	gtgacaaaggagaggctgga	acctctagggccagaaggac	146	Chondrocyte
ACAN	NM_013227	tcaacaacaatgcccaagac	aaagttgtcaggctggttgg	128	Chondrocyte
ADIPOQ	NM_004797	cctggtgagaagggtgagaa	ctcctttcctgccttggatt	124	
LPL	NM_000237	gtggccgagagtgagaacat	gaaggagtaggtcttatttgtggaa	66	
Dopaminergic Neuronal Development Transcription Factors					
NR4A2	NM_006186	gctgttgggatggtcaaaga	ctgtgggctcttcggtttc	87	Brain
PITX3	NM_005029	gctaccccgacatgagcac	gcgaagctgcctttgcat	148	Brain
EN2	NM_001427	gcgtgggtctactgtacgc	tgtcctctttgttcgggttc	91	Brain
Dopaminergic Neuronal Markers					
TH	NM_000360	cagccctaccaagaccagac	gtacgggtcgaacttcacg	129	Brain
VMAT2	NM_003054	ctttttgcccctctctgctt	gggcagttgtgatccatga	83	Brain
DAT	NM_001044	tcgagagaaactggcctacg	aggatgacttcctggggtct	138	Brain
Ubiquitous Marker					
HPRT1	NM_000194	gaccagtcaacaggggacat	cctgaccaaggaaagcaaag	132	
GAPDH	NM_002046	aatcccatcaccatcttcca	tggactccacgacgtactca	82	

hESCs, human embryonic stem cells
hMSCs, human mesenchymal stem cells

2.2.4.1 Primer Design and Optimisation

A three-step procedure was utilised for the design and optimisation of primers used in this study:

1) Identification of coding sequences from NCBI (Entrez Nucleotide) – http://www.ncbi.nlm.nih.gov/.

2) Design of sense and anti-sense primers using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with subsequent NCBI BLAST (blastn) performed for designed primers to check potential homology with other human genes. In addition, to exclude genomic DNA contamination, primers were designed to be intron-spanning.

3) Optimisation by real-time RT-PCR. Primer optimisation was carried out using cDNA from the positive control for the respective genes.

2.2.4.2 cDNA Synthesis

Total RNA (1 μg) was reverse transcribed into cDNA using the Superscript™ III First Strand Synthesis Kit, with 25 ng random hexamer and 1.25 μM Oligo$(dT)_{20}$ primers, according to manufacturer's recommendations. Following cDNA synthesis, samples were treated with RNase H for 20 min at 37°C. The cDNA was stored at -80°C and diluted 1:20 for use in real-time RT-PCR.

2.2.4.3 Real-Time RT-PCR Procedure

Real-time RT-PCR was performed to examine gene expression using the Platinum SYBR Green qPCR SuperMix-UDG on a Rotor-Gene RG3000 machine. The PCR reaction contained 9 μL of diluted (1:20) cDNA, 1x Platinum SYBR Green qPCR SuperMix-UDG, 0.25 μM of each primer (or 0.125 μM of each primer for *PITX3*), and RNase-free water to make a final volume of 20 μL. All reactions were performed in

duplicate within the same PCR plate. The thermal profile for all reactions was: 2 min at 50°C, 2 min at 95°C, 40 cycles of 30 seconds (s) at 95°C, 30 s at 60°C and 30 s at 73°C. Melt curve analysis was also performed with ramp from 72°C to 95°C, rising by 1°C in each step (the first step being 45 s and each step afterwards 5 s).

Quantitation analysis was performed using the Rotor-Gene software with Dynamic Tube and Slope Correct options selected, threshold set at 0.007, and elimination of cycles before 7. For experiments with control untreated and treated samples the ratio of the target gene expression in experimental/control ('fold change in target gene'/'fold change in reference gene') was determined using the ΔΔCt method (Livak *et al.*, 2001). For other experiments target gene expression was expressed as a ratio relative to the reference gene. Melt curves were also assessed for the presence of possible non-specific products and primer dimer.

The reference genes used were *HPRT1* (hypoxanthine phosphoribosyltransferase 1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Water amplification controls were included in each reaction to control for contaminating cDNA and genomic DNA.

2.2.4.4 Electrophoresis of PCR Products

Following primer optimisation the size of PCR products were confirmed using gel electrophoresis. PCR products were visualised following separation in a 3% (w/v) agarose gel prepared in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) with 1 µg/mL ethidium bromide. PCR product was mixed with 1/6th volume of 6x DNA loading dye (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol in Baxter water). GeneRuler Ultra Low Range 10-300 bp DNA ladder was loaded in parallel with samples to approximate the size of PCR products.

Electrophoresis was conducted in 1x TAE buffer at 100 volts. Gel images were captured digitally by transillumination using the Gel Doc-1000 photographic system.

2.2.4.5 Statistical Analysis

Gene expression data were prepared as figures using computer package GraphPad Prism. Data obtained were expressed as mean fold change in mRNA expression + SEM relative to control undifferentiated hMSCs. The student's *t*-test was performed to determine significant differences using Microsoft Excel, with statistical significance set at $p < 0.05$.

2.2.5 In Vitro Morphological Analysis

2.2.5.1 Routine Morphological Recordings

For morphological analysis hMSCs cultured in expansion and differentiation conditions were examined on a daily basis and observations were recorded. Phase-contrast images were routinely captured prior to subculturing or harvesting using an Olympus upright light microscope and digital camera. Cells undergoing neuronal differentiation procedures were imaged more frequently at least every 2 days.

2.2.5.2 Live Cell Imaging

To further examine the morphological changes induced in hMSCs by neuronal differentiation, cells were differentiated for 3 weeks using the SingleND method, trypsinised, and cultured in chamber slides for examination by live cell imaging. Images were captured immediately after re-adherence of cells (commencing at 30 min post-trypsinisation) using a Zeiss inverted microscope equipped with a CO_2- and temperature-controlled stage. Images were recorded at 5 min intervals for periods longer than 18 hours.

2.2.6 Immunofluorescence Microscopy of *In Vitro* Cultured Cells

Human MSCs cultured on chamber slides (BD Falcon) or glass coverslips were fixed with 4% PFA (warmed to 37°C) at room temperature for 20 min. For intracellular antigens, cells were permeabilised using 100% DMSO for 10 min, and then washed 3x with 0.1% Triton X-100 in phosphate buffered saline (PBS; used for all washes unless otherwise stated; each wash was 5 min long for all washes). Slides/coverslips were blocked with normal goat serum (10%) in PBS for 1 hour, followed by incubation with primary antibodies at room temperature for 1 hour. Primary antibodies included: mouse monoclonal antibodies (all IgG unless specified) against POU5F1 (1:50), NES (1:200), SSEA-1 (1:50; IgM), SSEA-4 (1:50), TRA-1-60 (1:50; IgM), CD44 (1:100), MAP-2 (1:100), A2B5 (1:200; IgM), NeuN (1:200) and TH (1:1000); and rabbit polyclonal antibodies against Fibronectin (1:400), FOXA2 (1:100), GFAP (1:200) and β tubulin III (1:2000). Slides/coverslips were washed 3x and then incubated with Alexa Fluor 488 or 594 highly cross adsorbed goat anti-rabbit or anti-mouse secondary antibodies at 1:400 dilution for 1 hour. Negative controls were performed in all cases, consisting of secondary antibody application in the absence of primary antibody. Slides/coverslips were washed 3x, after which nuclei were counterstained with 300 nM DAPI in PBS for 10-15 min, followed by a final wash with PBS (1 min; 3x) and mounting in 100% glycerol. Cells were examined and imaged using a Zeiss inverted fluorescent microscope with Axiocam digital camera and Axiovision software. For undifferentiated hMSCs, immunofluorescence analysis was performed on cultures from 4 different donors. For all neuronal differentiation procedures, immunofluorescence analysis was performed on 3 hMSC cultures, with control undifferentiated hMSCs from the same cultures examined in parallel.

2.2.7 Cell Preparation and Stereotactic Intracerebral Transplantation

The *in vivo* experiments in my thesis were performed at the National Centre for Adult Stem Cell Research, Griffith University (Brisbane, QLD) in the laboratory of my co-supervisor Prof. Alan Mackay-Sim. Additional supervision was also provided by Dr. Adrian Meedeniya, and technical assistance was received from Maria Nguyen, Mary Pagendam-Turner, Brenton Cavanagh, Joseph Kan and Nathalie Romond. Preparation of undifferentiated and neuronal-primed (partially differentiated) hMSCs and OECs for transplantation, as well as immunohistological analysis of graft sites was performed by me, primarily at St Vincent's Hospital Sydney, and also at Griffith University.

2.2.7.1 Animal Welfare

Animal studies were performed under approval from the Animal Ethics Committee of Griffith University (Brisbane, QLD), and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animal subjects were housed in pairs with a 12 hour light/dark cycle and with food and water *ad libidum*.

2.2.7.2 Experimental Design

Part I of this study employed the use of 6-OHDA unilaterally-lesioned adult female Sprague-Dawley rats (Taconic Farms, NY, USA), weighing 250-350 g at the start of the experiment, for all surgeries. This source of animals was selected as successful published studies had also used these animals for the same purposes (Roy *et al.*, 2006; Sanchez-Pernaute *et al.*, 2001). Animal subjects received single intrastriatal grafts of undifferentiated hMSCs. Amphetamine-induced rotational behaviour was assessed once prior to transplantation and was intended to be assessed every 4 weeks after transplantation. Graft phenotype was intended to be examined at 3 months post-transplantation, however due to the early experimental endpoint immunohistological

analysis of graft sites was performed at a little over 2 months post-transplantation. Table 2.3 outlines the experimental plan for Part I.

Part II was performed to address issues raised in Part I. For Part II, an alternative source, sex and strain of animal subjects was utilised. Adult male 6-OHDA unilaterally-lesioned Wistar Ob rats weighing 250-350 g were obtained from a source within Australia, the Integrative Neuroscience Facility (Howard Florey Institute, VIC, Australia). Transplantation into the striatum, as well as the substantia nigra was performed, and hMSCs underwent cytokine-based neuronal-priming prior to transplantation (Stage 1 of the MultiDA method; see Section 2.2.1.5). The experiment was conducted over a shorter period of 21 days, and co-transplantation with OECs was also performed. Table 2.4 describes the experimental plan for Part II.

2.2.7.3 Preparation of hMSCs for Transplantation

Both undifferentiated and neuronal-primed hMSCs were prepared at St Vincent's Hospital Sydney and transported to Griffith University for further culturing and transplantation. Undifferentiated hMSCs were expanded in culture as described in Section 2.2.1.2, until the middle of P6 when cells were transported to Griffith University. For transportation, T25 flasks containing adherent hMSCs were filled to the neck with DMEM-LG medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Flasks were then sealed and stored upright in an esky during transportation (approximately 6 hours). Cells were returned to expansion culture conditions upon arrival at Griffith University.

Neuronal-primed hMSCs were obtained following exposure of undifferentiated P5 hMSCs to Stage 1 of the MultiDA method (Section 2.2.1.5) for 1 week and then harvested by trypsin/EDTA (described in Section 2.2.1.4) for transportation. Cells were resuspended in complete MultiDA – Stage 1 medium at approximately 50,000 cells

Table 2.3 Experimental Plan for Part I

Time Point	Undifferentiated hMSCs	Sham
3 months	8	8

NB: Transplantation into striatum only

Table 2.4 Experimental Plan for Part II

Time Point	PhMSCs	PhMSCs + OECs	Sham
Day 1	3	3	3
Day 7	3	3	3
Day 21	3	3	3

NB: Transplantation into both striatum and substantia nigra

PhMSCs, Neuronal-Primed hMSCs

per mL and transferred into T25 culture flasks, which were sealed and stored upright in an esky during transportation. Cells were returned to Stage 1 differentiation conditions upon arrival at Griffith University.

Prior to transplantation, cells were harvested by trypsinisation as described in Sections 2.2.1.2 and 2.2.1.4, and washed twice with HBSS. Undifferentiated hMSCs were resuspended at a concentration of 30,000 cells per µL in HBSS, and 3 µL of the cell suspension were injected into the lesioned striatum (total of 90,000 cells per rat). Neuronal-primed hMSCs were resuspended at 50,000 cells per µL in HBSS, and 2 µL of the cell suspension were injected into the striatum and substantia nigra of the lesioned hemisphere (total of 100,000 cells per site).

2.2.7.4 Preparation of OECs for Transplantation

OECs were prepared from frozen stocks available at the National Centre for Adult Stem Cell Research, generated with the previously described protocol (Bianco *et al.*, 2004). OECs were maintained in DMEM/HAM F12 medium containing 10% foetal calf serum, and penicillin/streptomycin (100 U/mL and 100 µg/mL respectively), and passaged at 80% confluency. For transplantation, OECs were harvested by trypsinisation, using the same procedure as for trypsinising hMSCs, and washed twice with HBSS. Subsequently, OECs were resuspended at 25,000 cells per µL in HBSS together with the neuronal-primed hMSCs (total of 50,000 OECs per site).

2.2.7.5 Stereotactic Intracerebral Transplantation in Rat PD Model

Surgical procedures were performed by Dr. Adrian Meedeniya and Mary Pagendam-Turner. Briefly, rats were anaesthetised using isofluorane gas in an induction chamber, and anaesthesia was maintained during surgery via a nose cone. Rats were placed in a small animal stereotaxic frame with microinjector unit, and cell suspensions were injected into the lesioned hemisphere of each subject according to the following

co-ordinates: undifferentiated hMSCs into the striatum at AP: 1.2, L: -2.5, V: 5.0, and neuronal-primed hMSCs and OECs into the striatum at the same co-ordinates as above, as well as the substantia nigra at AP: -5.2, L: -3.2, V: -7.4. Cells were injected with a 27-gauge injection needle connected to a Hamilton syringe. The needle was inserted at the injection co-ordinates and a cavity was created for the injection through overshooting by 200 μm. After waiting 2 min the cell suspension was injected at 1 μL per min. Following infusion, the needle was left in place for 2 min and then slowly withdrawn. Sham-operated animals were treated in the same manner, but HBSS was infused alone without cells. Transplant groups and harvest time points are shown in Table 2.3 and 2.4. All subjects were immunosuppressed with daily injections of cyclosporine A (10 mg/kg; subcutaneous) from 3 days prior to grafting until harvest.

2.2.7.6 Tissue Processing

Tissue processing procedures were performed by Maria Nguyen and Joseph Kan. Subjects were anaesthetised with Lethabarb-Sodium Pentabarbitone (1.5 mL per animal) and perfused transcardially with PBS followed by Zambonie's Fixative. Brains were harvested and immersed in Zambonie's Fixative overnight under vacuum, then rinsed twice in PBS for 30 min, and placed in PBS-azide (0.1%). Specimens were dehydrated through graded alcohols (50% ethanol for 30 min, 70% ethanol for 30 min, and finally 100% ethanol for 60 min), permeabilised with DMSO for 60 min or until sunken (under vacuum), rinsed in 100% ethanol for 5 min, and placed in fresh 100% ethanol for a further 60 min. Brains were embedded in PEG by placing in PEG 400 overnight at room temperature under vacuum, PEG 1000 at 48ºC under vacuum until sunk (approximately 60 min), PEG 1450/1000 mix at a ratio of 4:1 at 48ºC under vacuum until sunk (approximately 60 min), and embedded in PEG 1450/1000 mix at a ratio of 4:1. Sectioning was performed at room temperature on a rotary

microtome to obtain 30 μm thick sections, which were placed 10 per well in PBS-azide (0.1%) and stored at 4ºC.

2.2.8 Immunohistological Analysis

Characterisation of graft sites was performed on free-floating brain sections in 24 well plates using the same procedure as described in Section 2.2.6, with some modifications. These modifications included: incubation and washing steps were all performed with agitation on a shaker; washes were all performed for 10 min; permeabilisation with 100% DMSO for 20 min; overnight incubation with primary antibodies at 4ºC; and incubation with secondary antibodies for 3 hours. Additional primary antibodies used were rabbit anti-Iba-1 (1:2000), mouse anti-GFAP-Cy3 (1:400), and mouse anti-Human Nuclear Antigen (HNA; 1:100). Furthermore, the permeabilisation step for HNA staining was performed with 0.1% Triton X-100 in PBS, rather than 100% DMSO, for 20 min.

Confocal imaging was performed using a Leica laser scanning confocal microscope (DM IRE2 TCS SP2 AOBS). Individual colour channels were captured separately and merged in Adobe Photoshop. Co-localisation of HNA staining with DAPI-positive nuclei was confirmed through analysis of z-series confocal reconstructions and corresponding orthogonal planes under 40x and 100x. Contralateral brain regions served as internal controls to confirm primary antibody specificity. Images presented are 'maximum projection images' from z-stacks, with 10 sections captured over the thickness of the brain section (30-45 μm). Three-dimensional rendering of graft sites in Part I was prepared by Brenton Cavanagh using Bitplane Imaris software from images captured using a Zeiss Axio Imager Z1 with ApoTome and Axiovision software.

2.2.9 Amphetamine-Induced Rotation Testing

Behavioural testing was conducted by Maria Nguyen. Briefly, rotational behaviour was measured prior to transplantation in Part I and II to confirm the lesioning of nigrostriatal dopaminergic neurons, and was intended to be assessed every 4 weeks post-transplantation of undifferentiated hMSCs in Part I. Rats were placed in a Rotometer and allowed to habituate prior to injection of D-amphetamine (4 mg/kg, intraperitoneal). Net rotation over a period of 90 min was recorded, commencing from 15 min post-injection. All behavioural testing was conducted in a closed room with a controlled environment and no exposure to loud or sudden noises or lighting changes.

CHAPTER 3

CHARACTERISATION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AND THE EFFECT OF LONG-TERM SERIAL PASSAGE

3.1 Introduction

Since the identification of MSCs in the BM by Friedenstein and colleagues (Friedenstein *et al.*, 1970; Friedenstein *et al.*, 1968; Friedenstein *et al.*, 1966), increasing interest in these cells has been stimulated by the potential use of MSCs in regenerative and reparative therapies. The characteristic feature that most uniquely distinguishes MSCs is the ability to differentiate into multiple mesodermal cell types, typically osteocytes, adipocytes and chondrocytes (Pittenger *et al.*, 1999; Prockop, 1997). However, this property does not allow prospective MSC isolation, and consequently, the identification of MSCs has been restricted.

Due to the low frequency of MSCs in BM (Bruder *et al.*, 1997; Reyes *et al.*, 2001), it is necessary to expand MSCs extensively *in vitro* to acquire sufficient cells for use in research and clinical trials, and potentially for therapeutic applications. Various expansion methods exist, which are often inconsistent, and the effects of long-term serial passage are not well defined among the different protocols. Several reports on the extensive subcultivation of BM-derived MSCs have described changes in morphology, proliferation, telomere length and mesodermal differentiation capacity (Baxter *et al.*, 2004; Bruder *et al.*, 1997; Digirolamo *et al.*, 1999; Mets *et al.*, 1981). In particular, it was observed that while MSCs retained osteogenic differentiation potential through 10-15 passages, adipogenic potential was lost (Digirolamo *et al.*, 1999). In addition, spontaneous osteogenic differentiation with deposition of mineral was observed in some cultures (Digirolamo *et al.*, 1999). These findings suggest that the use of MSCs at different passages may contribute to discrepancies observed between studies.

In vitro and *in vivo* studies have shown that certain BM-derived MSC populations are capable of multi-lineage differentiation. Under select culture conditions, MSCs have been reported to differentiate into ectodermal derivatives including neuronal

and glial cells (Hermann *et al.*, 2004; Jiang *et al.*, 2003; Jiang *et al.*, 2002; Sanchez-Ramos *et al.*, 2000; Tao *et al.*, 2005; Woodbury *et al.*, 2000), as well as endodermal derivatives, such as hepatic cells (Jiang *et al.*, 2002; Lee *et al.*, 2004; Schwartz *et al.*, 2002; Shi *et al.*, 2005; Wang *et al.*, 2004). Transplantation of MSCs into post-natal animals has yielded engraftment and differentiation of donor-derived cells into lung, liver and intestinal epithelium, whereas introduction into early blastocysts has resulted in contribution to most somatic tissues (Jiang *et al.*, 2002). Furthermore, expression of genes indicative of the three germ lineages have been detected in undifferentiated MSCs (Minguell *et al.*, 2005; Seshi *et al.*, 2003; Tondreau *et al.*, 2004b; Woodbury *et al.*, 2002), providing further evidence for a 'multidifferentiated' (Woodbury *et al.*, 2002) or 'pluridifferentiated' (Seshi *et al.*, 2003) MSC state.

Recognition of the potential for BM-derived MSCs to act as a renewable cell source for cellular therapies has led to investigations aimed at defining the optimal conditions required for differentiation towards particular lineages. However, it is not known whether BM-derived hMSCs simultaneously express pluripotency and lineage-specific genes and proteins, or whether long-term *in vitro* expansion culture affects the 'pluridifferentiated' nature of hMSCs.

This study aimed to address these issues by investigating the effects of long-term serial passage on the characteristics of BM-derived hMSC populations by concurrently assessing growth kinetics, apoptosis, expression of cell surface antigens, and gene and protein expression. Since hMSCs have been reported to possess multi-lineage differentiation potential, the expression of markers of pluripotency and the three germ lineages will be examined to determine whether the expression profile is suggestive of a 'pluridifferentiated' phenotype, and also whether there exists an optimal period to initiate differentiation towards particular lineages. Furthermore, these studies would

allow the establishment of a baseline expression profile of undifferentiated hMSCs prior to the commencement of differentiation.

3.2 Results

3.2.1 MSC Morphology and Growth Kinetics

Primary colonies of hMSCs at P0 contained small raised cells with a fibroblast-like appearance (Figure 3.1A). With continued growth *in vitro*, cells gradually gained a larger and more flattened morphology, but maintained a consistent appearance from P1 up to P6-8 (approximately 8-10 weeks; Figures 3.1B and C). Beyond this period (up to 19 weeks), the cells were large, flat, and difficult to harvest by trypsinisation. In addition, cultures contained fewer dividing cells and greater amounts of cell debris (Figure 3.1D).

The proliferative capability of hMSC populations was examined by calculating the number of population doublings at each subculture. A linear correlation between cumulative doubling and passage number was observed up to approximately P6-8 (8-10 weeks; Figure 3.2A), and in one case up to P10 (14 weeks; $R^2 = 0.9857$), indicating the presence of symmetric cell division. Beyond this period, a reduction in the proliferation rate was observed, which corresponded with the observed morphological changes, and suggested deviation toward asymmetric division.

Viability of hMSC populations at each subculture was consistently above 85% (Figure 3.2B). The Pearson correlation coefficient for viability versus passage number was 0.16 with a *p*-value of 0.31 (two-tailed test), indicating that cell viability was not affected by time in culture. This was confirmed using an apoptosis assay in which hMSC cultures were found to contain an average of 70-85% viable cells (Figure 3.2C) with no statistical difference observed across all time points (Wilcoxon Signed Ranks Test for paired data).

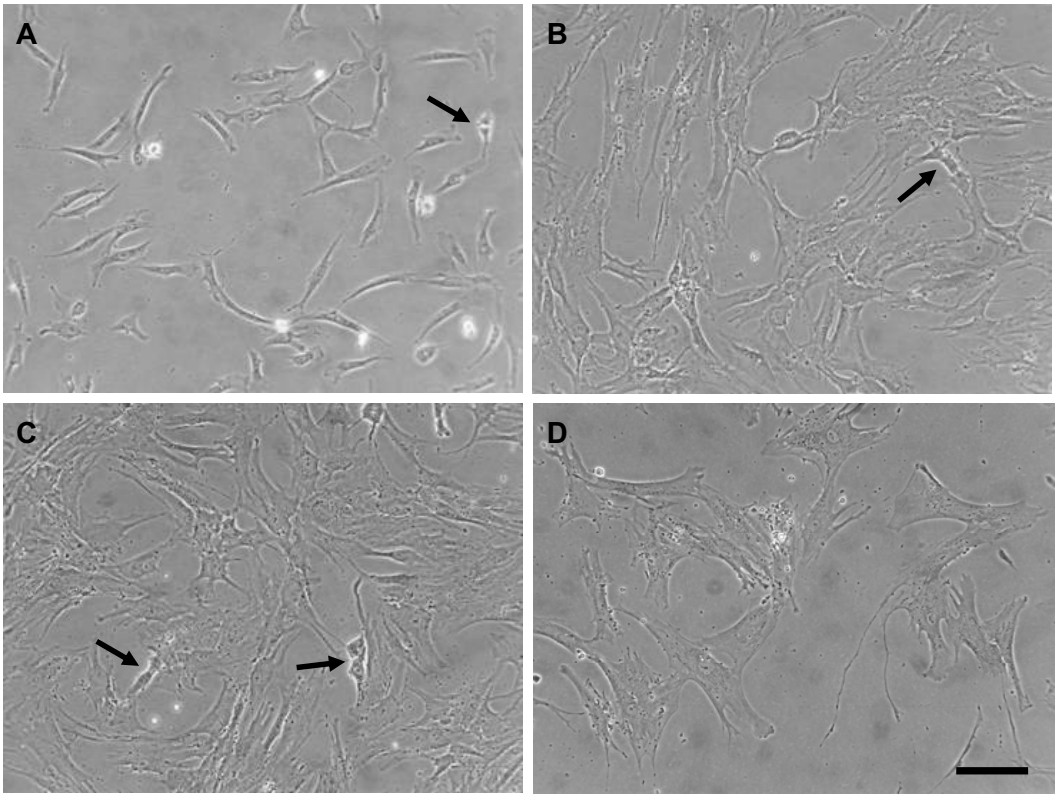

Figure 3.1 Morphology of hMSCs at early and late culture stages.
Representative phase-contrast micrographs showing morphology of hMSC cultures at (**A**) P0 (Day 14), (**B**) P5 (Day 64), (**C**) P8 (Day 85), and (**D**) P15 (Day 134). Cells gradually gain a larger and more flattened morphology with growth *in vitro*. A fairly consistent appearance is maintained from P1 to P8. Arrows indicate dividing cells. Scale bar: 100 μm.

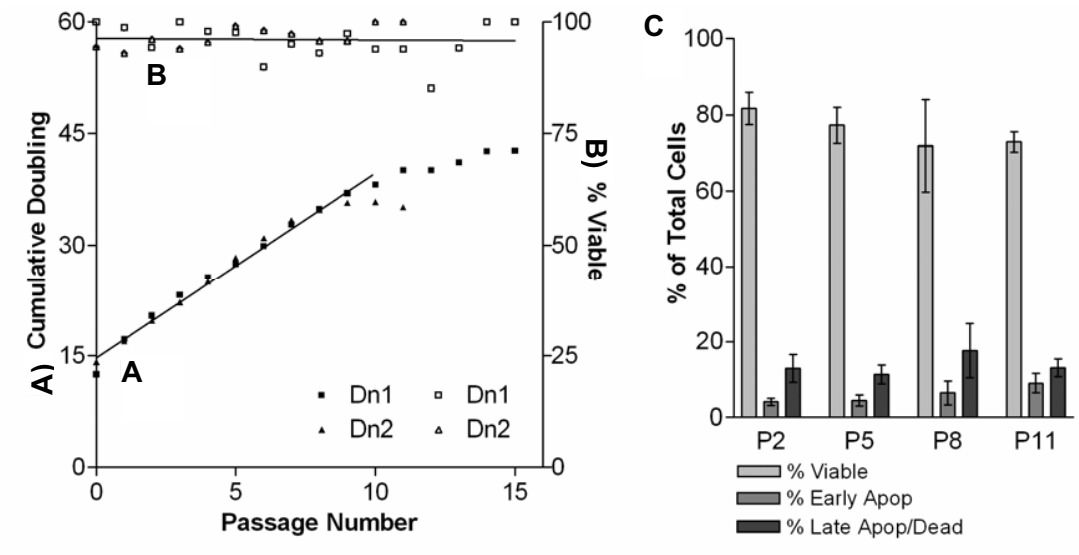

Figure 3.2 Proliferation and viability of hMSCs at early and late stages of culture.

(**A**) Cumulative population doubling of hMSCs from 2 representative donors. Cumulative doubling is linearly correlated to days in culture (Dn1: $R^2 = 0.9857$), indicating the presence of symmetric division. Each data point represents the number of doublings obtained at the end of the indicated passage. (**B**) Cell viability by trypan blue dye exclusion performed at each subculture. Viability of hMSC populations is consistently >85%, and is not correlated to days in culture ($R = 0.159$). (**C**) Detection of apoptosis by Annexin V-FITC and PI staining with flow cytometric analysis (mean ± SEM; $n = 4$ donors). P, passage; Dn, donor.

3.2.2 Immunophenotype of Human MSCs by Flow Cytometry

Undifferentiated hMSCs in expansion cultures were examined at P4 and P8 for the expression of cell surface antigens commonly used in the characterisation of hMSC populations. Cells expressed the expected pattern of surface antigens for human MSCs (CD44, CD73, CD105, CD166, CD29 and HLA Class I), and lacked expression of haematopoietic markers CD45, CD14 and CD34, as well as HLA Class II (Figure 3.3). The pattern of surface antigen expression was maintained consistently in cultures up to P8.

3.2.3 Multi-Lineage Mesodermal Differentiation

The MSC identity of cells isolated in this study was confirmed with the ability to differentiate into chondrocytic, adipocytic, and osteocytic mesodermal lineages. Chondrocytic differentiation was evident in micromass pellet cultures after 3 weeks in differentiation conditions, with proteoglycan deposition detected in >70% of the total area by Alcian Blue staining (Figure 3.4D; control in Figure 3.4A), and increased *COL2A1* and *ACAN* gene expression (Figure 3.5). Differentiation into the adipocyte lineage was apparent in >65% of cells from the intracellular accumulation of lipid-laden vacuoles that positively stained with Oil Red O (Figure 3.4E; control in Figure 3.4B). Expression of adipocytic genes, *ADIPOQ* (adiponectin) and *LPL* (lipoprotein lipase), were also greatly increased post-differentiation (Figure 3.5). Osteogenic differentiation was evident by Alkaline Phosphatase staining in >60% of the total area (Figure 3.4F; control in Figure 3.4C), as well as increased *COL1A1* and *BGLAP* (osteocalcin) gene expression (Figure 3.5).

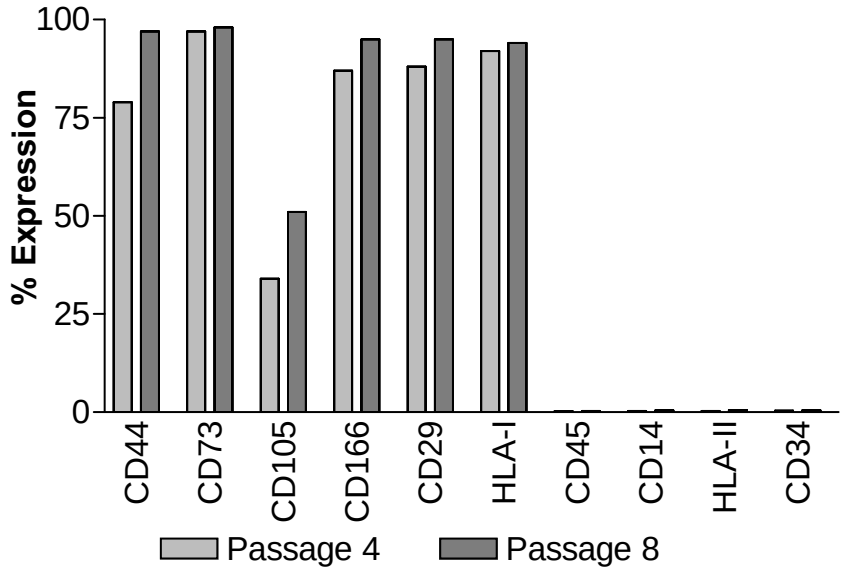

Figure 3.3 Flow cytometric detection of cell surface markers of undifferentiated hMSCs.

Representative results demonstrating that hMSCs express the expected pattern of surface antigens for human BM-derived MSCs. In addition, the pattern of expression is consistent across P4 to P8 of culture.

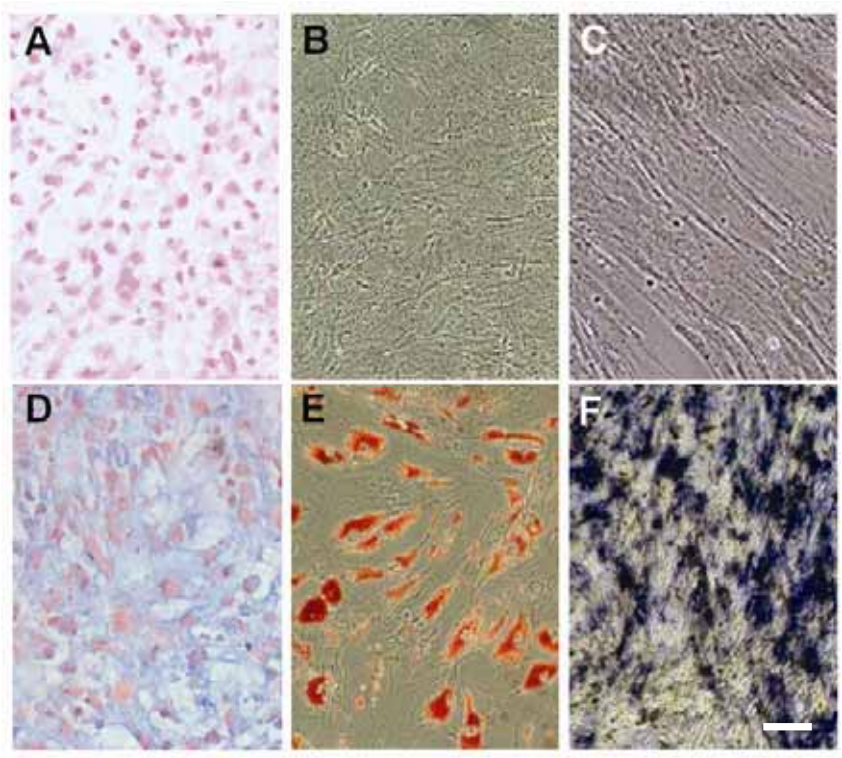

Figure 3.4 Histochemical staining of hMSCs following *in vitro* multi-lineage mesodermal differentiation.

Images shown depict undifferentiated control hMSCs (A-C) and hMSCs cultured in mesodermal differentiation conditions (D-F). (**A** and **D**) Alcian Blue staining (with Fast Red nuclear counterstain) shows evidence of proteoglycan deposition after chondrogenic differentiation. (**B** and **E**) Adipogenic differentiation of hMSCs results in accumulation of lipid-laden vacuoles that stain with Oil Red O. (**C** and **F**) Increased expression of alkaline phosphatase was detected with osteogenic induction. Scale bar: 100 μm.

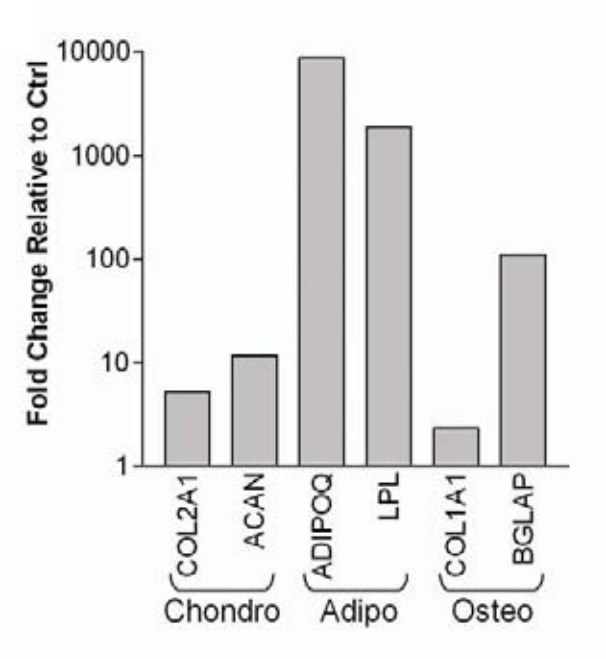

Figure 3.5 Gene expression analysis of hMSCs following *in vitro* multi-lineage mesodermal differentiation.

Representative real-time RT-PCR results showing the fold change in gene expression relative to undifferentiated control hMSCs after treatment with osteogenic, adipogenic, and chondrogenic differentiation conditions. Ctrl, control; Chondro, chondrocytic; Adipo, adipocytic; Osteo, osteocytic.

3.2.4 Expression of Pluripotency and Germ Lineage Markers

Earlier reports of MSC multi-lineage differentiation potential prompted me to examine the expression of markers of pluripotency and the three germ lineages to determine whether the expression profile of undifferentiated hMSC cultures was consistent with a 'pluridifferentiated' phenotype. Cultures were also investigated at different stages (P2, P5, P8, and P11) in order to detect any spontaneous changes in expression during long-term culture.

3.2.4.1 Gene Expression by Real-Time RT-PCR

The hMSC cultures were found to express genes typical of MSCs, pluripotent cells and mesodermal and ectodermal lineages by real-time RT-PCR. Of the markers examined, mesodermal genes *COL1A1* and *CD44* were the most highly expressed (Figure 3.6A), followed by neuroectodermal genes *CSPG4* (chondroitin sulfate proteoglycan 4) and *NES* (Figure 3.6B). The decrease in *CSPG4* expression at P8 seen in this sample was not observed across other samples. Pluripotency genes, *POU5F1* and *NANOG* (Nanog homeobox), were also detected, with *NANOG* expressed at lower levels than *POU5F1* (Figure 3.6C). Morphologically undifferentiated hMSC cultures also expressed neuronal gene *MAP-2* at low levels (Figure 3.6C); astroglial gene *GFAP* was very weakly expressed or absent, whereas endodermal genes *FOXA2* and *ALB*, and mesodermal gene *COL2A1*, could not be detected (results not shown). Expression of these genes did not appear to vary greatly during long-term serial passage over P2 to P11.

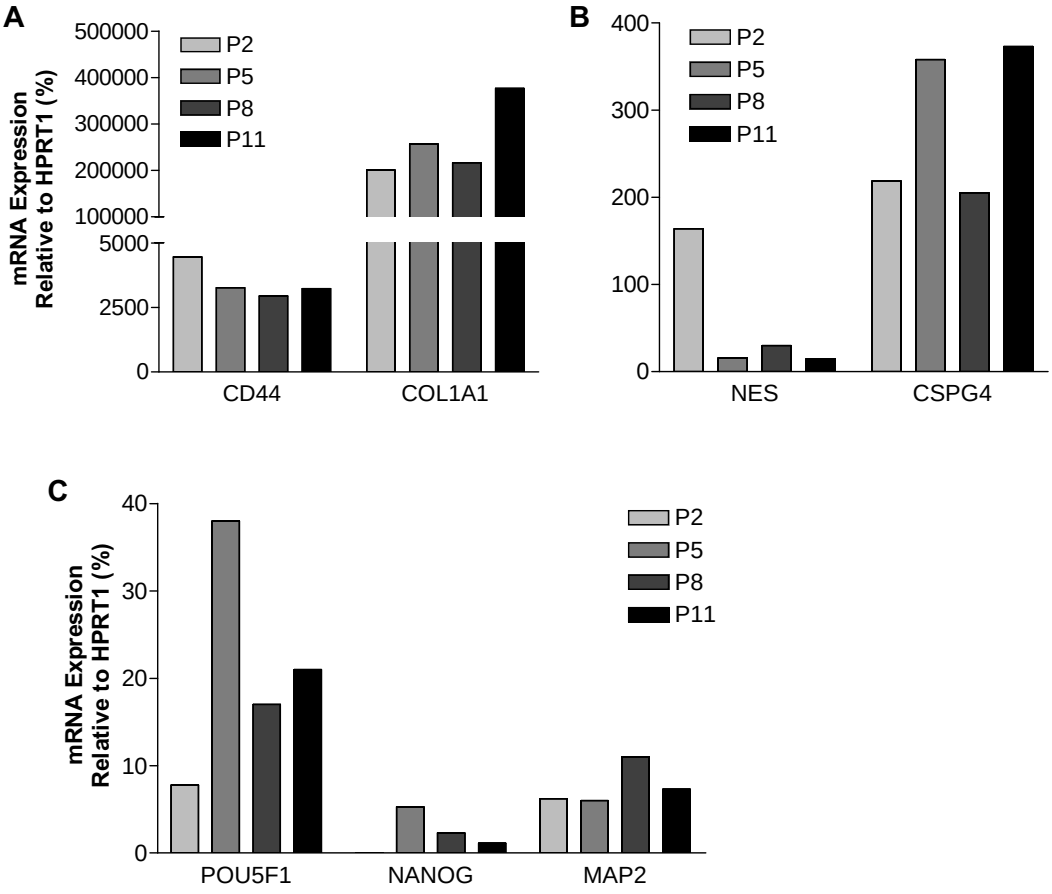

Figure 3.6 Gene expression analysis of undifferentiated hMSC cultures during long-term serial passage.

Representative results of gene expression analysis by real-time RT-PCR relative to *HPRT1* expression (normalised to 100%; $n = 4$). The most highly expressed genes were (**A**) mesodermal *COL1A1* and *CD44*, followed by (**B**) ectodermal neural genes *CSPG4* and *NES*. (**C**) Weak/medium expression was detected for pluripotency genes *POU5F1* and *NANOG*, and neuronal gene *MAP-2*.

3.2.4.2 Protein Expression by Immunofluorescence Microscopy

The results of protein expression analysis supported those obtained by real-time RT-PCR. Human MSCs strongly expressed CD44 and fibronectin, markers of MSCs and the mesodermal lineage, consistently throughout expansion in culture (Figure 3.7 and Table 3.1). Pluripotent SC transcription factor POU5F1 and ectodermal neural progenitor marker NES were weakly expressed at both early and late stages of culture (Figure 3.7); however, the proportion of cells expressing these markers decreased over time (Table 3.1). Pluripotency markers SSEA-4 and TRA-1-60, and differentiation marker SSEA-1, were not detected at any stage of culture (results not shown). Endodermal markers, FOXA2 and ALB, were also undetectable (results not shown).

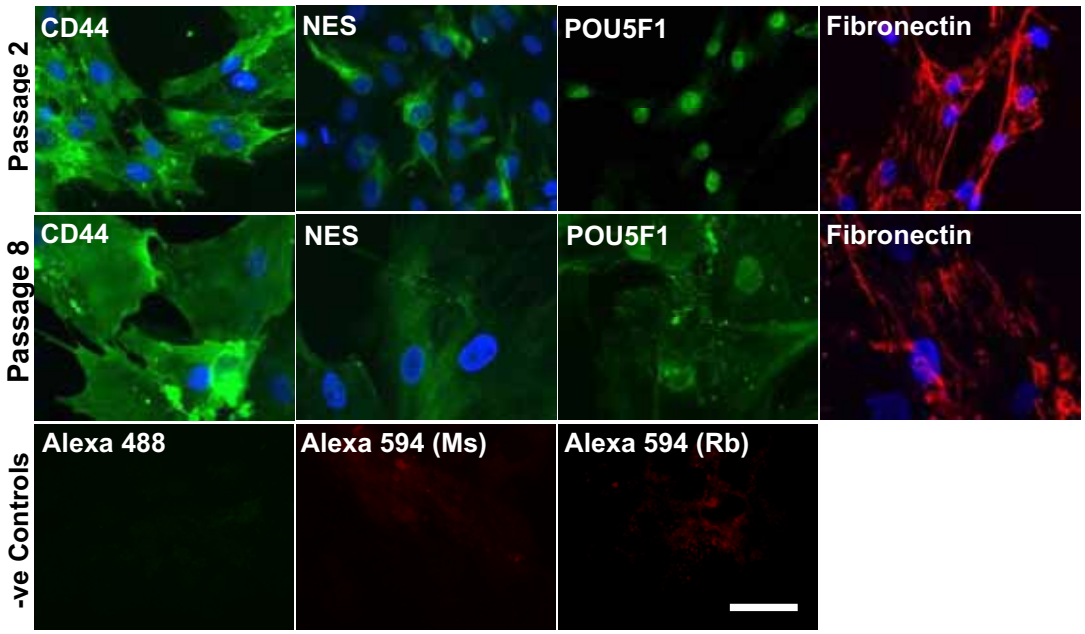

Figure 3.7 Protein expression analysis of undifferentiated hMSC cultures during long-term serial passage.

Images showing protein detection in hMSC cultures by immunofluoresence microscopy at P2 and P8 after labelling with primary antibodies against CD44, Fibronectin, NES, and POU5F1 ($n = 4$). Cells were only included as positive if pattern of staining was localised to the: cytoskeleton for NES; nucleus for POU5F1; cell surface for CD44; or extracellular and fibre-like in appearance for fibronectin. Negative controls consisted of secondary antibody application alone. Scale bar: 100 μm.

Table 3.1 Phenotypic Characterisation of Undifferentiated Human MSCs by Immunofluorescence.

Antigenic Marker	Passage 2	Passage 5	Passage 8	Passage 11
CD44	+++++	+++++	+++++	+++++
Fibronectin	+++++	+++++	+++++	+++++
POU5F1	++++	+++	+	+
NES	+/++	+/++	+	+

Data shown summarises results for all hMSC cultures (n = 4). No expression of ALB, FOXA2, A2B5, MAP-2, TRA-1-60, SSEA-4 or SSEA-1 was detected at any passage.

+++++ : Intense positive staining (greater than 91% of cells positive).

++++ : Strong positive staining (71-90%).

+++ : Intermediate positive staining (41-70%).

++ : Weak positive staining (21-40%).

+ : Very weak positive staining (0-20%).

3.3 Discussion

The findings of this study show that (1) human BM-derived MSC populations maintain a stable phenotype *in vitro* during the first 6-8 passages of expansion culture, as assessed by proliferative ability, morphological appearance and surface antigen, gene and protein expression; (2) hMSCs constitutively express mesodermal and neural lineage markers in culture, making it necessary to determine baseline expression levels prior to the commencement of differentiation; (3) long-term serial passage of hMSCs results in changes to some key phenotypic attributes of MSCs, including morphology, proliferative ability, and gene and protein expression; (4) extensive subcultivation of hMSC cultures does not result in spontaneous differentiation toward the neural lineage; and (5) hMSCs possess SC characteristics *in vitro*, with the ability to proliferate, symmetrically divide, and produce multi-lineage mesodermal derivatives.

3.3.1 Undifferentiated hMSCs Possess Characteristic Properties of MSCs

The cell population isolated in this study expressed cell surface antigens characteristic of MSCs and lacked HSC markers, in agreement with the standard MSC phenotype. As no single set of phenotypic markers is able to unequivocally identify MSCs, the cell population used in this study was shown to possess other MSC properties such as extensive proliferation, symmetric division, and multi-lineage mesodermal differentiation.

3.3.2 Undifferentiated hMSCs Express Ectodermal and Mesodermal Lineage Markers

Examination of germ lineage and pluripotency marker expression revealed that undifferentiated hMSC cultures constitutively expressed the expected mesodermal lineage markers, as well as ectodermal-specific markers of neurons (NES, *MAP-2*; also

NF-M and NSE proteins were detected in a previous study by our group (Tao *et al.*, 2005)) and glia (*CSPG4*). Other recent studies have also shown that MSCs express markers characteristic of the neural lineages (Blondheim *et al.*, 2006; Deng *et al.*, 2006; Minguell *et al.*, 2005; Phinney *et al.*, 2006; Seshi *et al.*, 2003; Tondreau *et al.*, 2004b; Tremain *et al.*, 2001; Woodbury *et al.*, 2002), providing independent support of a pluripotent MSC state, or what some have named a 'multidifferentiated' (Woodbury *et al.*, 2002) or 'pluridifferentiated' (Seshi *et al.*, 2003) phenotype. In addition, Tremain and colleagues (Tremain *et al.*, 2001) have detected the expression of several neurotrophic factor mRNAs by hMSCs, and hypothesised that MSCs produce these factors normally to promote, maintain and augment the repair of neural networks within bone and BM. Collectively, these findings emphasise the need for examining neural marker expression prior to neural differentiation as well as post-differentiation. Through this analysis we have determined the baseline expression profile of undifferentiated hMSCs from four individual donors, which will be useful for comparison in later neuronal differentiation studies.

Expression of endodermal genes (Ceruloplasmin, IPP isomerase, Lanosterol-14a-demethylase) has also been reported in undifferentiated rat MSCs by microarray analysis (Woodbury *et al.*, 2002). However, in the present study expression of representative genes or proteins (FOXA2, ALB) from the endodermal lineage were undetectable or extremely low in most hMSC cultures. Similarly, a microarray analysis of human BM-derived MSCs (Seshi *et al.*, 2003) detected simultaneous expression of transcripts from multiple lineages and cell types, including osteoblast, muscle, fibroblast, adipocyte, epithelial cell, endothelial cell and neural cell, without mention of endodermal lineage expression. Therefore, it could be possible that this discrepancy

may be due to species-to-species variation or differences in culture maintenance and isolation.

3.3.3 Undifferentiated hMSCs Express Markers of Pluripotency

Human MSC cultures were also found to express POU5F1 and *NANOG*, both of which are transcription factors present in pluripotent ESCs. Expression of pluripotency and germline-specific genes (including protamine 2, *POU5F1*, *hTERT*, and ornithine decarboxylase antizyme) was recently reported in rat and human MSCs (Pochampally *et al.*, 2004b; Roche *et al.*, 2007; Tondreau *et al.*, 2005; Woodbury *et al.*, 2002). A high degree of cooperation has been found between POU5F1 and NANOG in regulating gene expression, and it is thought that these transcription networks contain common downstream targets important for the prevention of ESC differentiation (Loh *et al.*, 2006). In the present study other pluripotent SC markers SSEA-4 and TRA-1-60 were absent from hMSC cultures, and the levels of POU5F1 and *NANOG* expression were low.

The postnatal role of POU5F1 is still unclear, with a recent study showing that terminally differentiated human peripheral blood MNCs expressed POU5F1 (Zangrossi *et al.*, 2007), raising the possibility of a separate role for POU5F1 outside of embryonic tissue. Nonetheless, increased *POU5F1* and *REX-1* expression in hMSCs by culturing in low serum concentrations has been shown to result in increased differentiation efficiency toward osteogenic and adipogenic phenotypes (Roche *et al.*, 2007), suggesting that POU5F1 may indeed have a role in the multipotency of MSCs.

Further conflicting results have been reported showing expression of both *POU5F1* transcripts as well as pseudogenes by hMSCs in the absence of detectable protein expression, suggesting that POU5F1A does not play a major role in MSC multipotency (Kaltz *et al.*, 2008). More recent studies have demonstrated that different

isoforms of POU5F1 and a number of pseudogenes may exist in adult SCs, complicating the interpretation of POU5F1 expression as a marker of pluripotency in cells derived from adult tissues (Kotoula *et al.*, 2008; Liedtke *et al.*, 2008; Mueller *et al.*, 2009). Alternative splicing of *POU5F1* has been found to result in two main variants POU5F1A and POU5F1B (also known as OCT4A and OCT4B) that share a common DNA-binding POU-homeodomain, but differ in NH_2-termini (Takeda *et al.*, 1992). Importantly, the different isoforms differ in function and subcellular localisation. Only the longer POU5F1A variant shows localisation to the nucleus and the abilities to sustain SC properties and confer self-renewal, whereas POU5F1B is mainly found in the cytoplasm and has as yet unknown function (Cauffman *et al.*, 2006; Lee *et al.*, 2006).

To date, the majority of reports in the literature have not distinguished between the two isoforms, and this is also a caveat of our present study as the primers and commercial antibody used here also detect the presence of both isoforms. Although, immunofluorescent staining did reveal some localisation of POU5F1 to the nucleus of hMSCs suggesting that some POU5F1A protein was detected. However, further confirmation of these results is required through methods suggested by Liedtke and colleagues (Liedtke *et al.*, 2008), such as application of primers specific for exon 1 of *POU5F1*, which would detect expression of *POU5F1A* alone, as well as use of a monoclonal antibody recognising a single epitope in the N-terminal part of POU5F1, to exclude recognition of POU5F1B.

3.3.4 Effects of Long-term Serial Passage on hMSCs

While multi-lineage marker expression has been demonstrated previously in MSCs, to our knowledge, no studies have investigated changes in the constitutive expression of pluripotency and lineage-associated differentiation markers by

undifferentiated hMSC populations during long-term serial passage, at both gene and protein levels. We found hMSCs to be capable of maintaining a consistent morphological appearance, proliferative rate and apoptotic level, as well as the expected antigenic pattern of MSCs between P1 and P6-8. Beyond this period, gradual changes in morphological appearance and decreased proliferative capability were noted. Protein expression of POU5F1 and NES was observed to decrease in parallel with changes in hMSC morphology and growth, suggesting that the pluridifferentiated state of hMSCs is affected by *ex vivo* expansion. However, *POU5F1, NES*, *MAP-2* and *CSPG4* gene expression levels were maintained over the duration of culture. The incongruity between gene and protein expression highlights the need to examine protein expression.

Mesodermal lineage marker expression by hMSC populations over long-term culture was maintained at a fairly constant level. A dramatic increase in *COL1A1* gene expression was detected in late stage culture, at the time corresponding with the cessation of hMSC proliferation. These findings suggest an apparent deviation away from pluripotency, and imply the commitment of hMSCs to the mesodermal lineage with long-term culture. This is also supported by findings by DiGirolamo, *et al.* (Digirolamo *et al.*, 1999) of spontaneous mineral deposition in late passage cultures, which were found to retain osteogenic differentiation capability at the expense of adipogenic differentiation. In addition, we found that long-term subculture of hMSCs did not result in spontaneous neural differentiation, in contrast to a recent study on rat MSCs (Tseng *et al.*, 2007). These differences could be attributed to variation between species and different culturing conditions.

3.3.5 Common Culture Conditions are Insufficient for hMSC Maintenance

The deviation toward asymmetric division observed in hMSC cultures during long-term *in vitro* expansion suggests that the current culture methods are insufficient

for hMSC maintenance. The culture method chosen for this study was one demonstrated to isolate and expand MSCs with pluripotent potential (Jiang *et al.*, 2003; Jiang *et al.*, 2002; Reyes *et al.*, 2001), and has also been shown to induce higher expression of genes involved in metabolism, when compared with an alternative method that upregulates genes involved in development, morphogenesis, extracellular matrix and differentiation (Wagner *et al.*, 2006). Decreased proliferative capacity has been documented in other studies (Baxter *et al.*, 2004; Bruder *et al.*, 1997; Digirolamo *et al.*, 1999; Mets *et al.*, 1981); however, little progress has been made in further optimising MSC culture conditions over the last two decades. The dependence on FBS is problematic due to the large variation in batches of FBS and potential immunogenicity of xenogeneic FBS proteins (Spees *et al.*, 2004).

These MSC culture inadequacies and the possibility of subpopulations within the culture may account for the inconsistent results between studies and inter-laboratory variation. Future prospects for improving MSC culture methods include: culturing under low oxygen conditions, replacement of FBS with defined cytokines, human serum (Shahdadfar *et al.*, 2005; Spees *et al.*, 2004) or serum substitutes (Meuleman *et al.*, 2006), and reduction of calcium and addition of antioxidants (Lin *et al.*, 2005). Further improvements in MSC culture homogeneity could arise with the identification of marker/s for the prospective isolation of MSCs. The recent finding that CD146 could be a potential marker of MSCs (Sacchetti *et al.*, 2007; Sorrentino *et al.*, 2008) may provide a much-needed and long-awaited positive selection method, which will hopefully facilitate progress in this research field.

3.3.6 Limitations Associated with Interpretation of Marker Expression

Difficulties have also been encountered in the SC field through the use of markers to represent certain cell types or tissue lineages, which may not be entirely

restricted to that cell type or tissue lineage, leading to potential misinterpretation of data. One such marker is NES, which is expressed in certain mesodermal cell types, including developing and regenerating muscle (Kachinsky *et al.*, 1994; Sejersen *et al.*, 1993; Vaittinen *et al.*, 2001), and endothelial cells (Klein *et al.*, 2003; Mokry *et al.*, 1998a; Mokry *et al.*, 1998b), as well as within neural tissue. Therefore, caution must be taken in the interpretation of these forms of data, and examination of multiple markers should be undertaken, as well as assessment of function.

3.3.7 Summary

In summary, the experiments described in this chapter have demonstrated that hMSC populations isolated from four individual donors were capable of maintaining a stable phenotype during the first 6-8 passages of culture, and also constitutively expressed pluripotency and neural lineage markers in the undifferentiated state. These results highlight the necessity of ascertaining the baseline expression profile of undifferentiated MSCs prior to commencing differentiation. Furthermore, examination of the hMSC phenotype and expression profile during long-term *in vitro* culture has enabled me to determine that optimal time frames may exist for hMSC expansion and lineage differentiation. For the hMSC populations studied here, the window for expansion and differentiation occurs before P6, and in all subsequent studies hMSCs were only used prior to this passage. In addition, the effects of long-term serial passaging on hMSCs have been investigated further as part of the neuronal differentiation studies discussed in the following chapter.

CHAPTER 4

NEURONAL DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS *IN VITRO*

4.1 Introduction

The first studies describing the *in vitro* neuronal differentiation of MSCs were reported in 2000 (Sanchez-Ramos *et al.*, 2000; Woodbury *et al.*, 2000), and utilised a cytokine and signalling molecule-based or chemical-based approach. Since then, a wide range of methods have been implemented, which have been discussed in greater depth in Section 1.3.1. Nevertheless, the current approaches used for MSC neural induction still primarily involve the use of a chemical or cytokine-based system, or a combination of both.

Recently, some concerns have been raised over the validity of chemically induced neuronal differentiation of MSCs, particularly with methods involving DMSO/BHA or IBMX/cAMP (Bertani *et al.*, 2005; Choi *et al.*, 2006; Deng *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004; Suon *et al.*, 2004; Tao *et al.*, 2005). These independent studies could not replicate the findings of the original studies, and instead found that the rapid effects of chemical-induced neuronal differentiation resulted from culture artifacts due to environmental stress and toxicity caused by the chemical agents (further details in Section 1.3.1.1 and Section 1.3.2). While these findings cannot account for all reported instances of *in vitro* and *in vivo* MSC neural transdifferentiation, serious questions surfaced regarding the actual extent of MSC plasticity, and the initial optimism associated with the application of MSCs in treatments for neurological disorders was dampened. However, it remained undetermined whether culture artifacts were also responsible for the changes observed during cytokine-based neural differentiation of MSCs.

In addition, it has not been established whether long-term serial passaging affects the neural differentiation capability of MSCs. Digirolamo and colleagues (Digirolamo *et al.*, 1999) have reported a loss of MSC multipotentiality, in terms of *in*

vitro mesodermal differentiation, following extensive proliferation in culture. Differentiation toward the adipocytic lineage failed in late passage MSCs, and this was found to occur in conjunction with the loss of CFU-F forming ability. On the other hand, osteogenic differentiation appeared unaffected, and there was some evidence of spontaneous differentiation into osteoblasts in some late passage MSCs (Digirolamo *et al.*, 1999). The results described in Chapter 3 of this thesis showed that long-term serial passaging of hMSCs did result in a number of phenotypic alterations (Khoo *et al.*, 2008). However, long-term subcultivation of hMSCs was not accompanied by spontaneous differentiation toward the neural lineage. Changes in the neural differentiation capacity of MSCs with serial passaging may have important implications for the use of *in vitro* cultured MSCs in neural differentiation procedures, and may be a factor contributing to inconsistencies in the results of different studies. Therefore, further investigations are warranted.

The generation of DA-producing neuronal-like cells from rodent and human MSCs have been reported in previous studies (Dezawa *et al.*, 2004; Fu *et al.*, 2006b; Guo *et al.*, 2005; Hermann *et al.*, 2004; Jiang *et al.*, 2003; Suon *et al.*, 2006), which were discussed in more depth in Section 1.4.4. A range of varying methodologies were employed in these studies, although a number of similarities existed in the use of extrinsic factors and manipulation of the culture environment to induce differentiation. Also, these studies only achieved fairly low efficiencies of dopaminergic differentiation, which ranged between 11% and 41%. Since the goal of directed differentiation of MSCs toward DA-producing cells is clinical application in cellular therapies for PD, it is desirable to obtain these cells through methods devoid of genetic engineering and animal-derived proteins or cellular components. Ideally, the induction procedure

employed should also cause the treated MSCs to respond in a manner that mimics *in vivo* dopaminergic neuronal development and differentiation.

This chapter aimed to investigate the abovementioned aspects of neuronal and dopaminergic neuronal differentiation of BM-derived hMSCs. Our laboratory has previously shown hMSCs to be capable of adopting a neuronal-like phenotype upon cytokine-exposure (Tao *et al.*, 2005). The present study evaluated this method using live cell microscopy to determine whether the morphological changes associated with cytokine-induced neuronal differentiation were due to a differentiation response or due to epiphenomena mediated by environmental stress similar to what occurs during chemical induction. The effect of long-term serial passaging on the neuronal differentiation capability of hMSCs was also investigated to determine whether there exists an optimal period at which to commence neuronal differentiation from our hMSC populations, and whether long-term *in vitro* culture alters hMSC neuronal differentiation potential. Furthermore, it was hypothesised that dopaminergic differentiation of hMSCs could be induced using cytokines that have been reported to be physiologically important in *in vivo* midbrain dopaminergic neuron development (see Section 1.4.3.1). In addition, since during development cells are sequentially exposed to growth factors, I investigated whether sequentially stimulating hMSCs with cytokines in a multiple-stage approach might provide advantages over a single-step protocol.

During the course of these experiments, a report by Trzaska and colleagues (Trzaska *et al.*, 2007) was published describing a single-stage cytokine-based procedure for specifying a dopaminergic phenotype from hMSCs. Therefore, in the present study I have compared a novel multiple-stage dopaminergic neuronal differentiation (MultiDA) method (sequential cytokine stimulation), and a previously described single-stage neuronal differentiation (SingleND) procedure developed in our laboratory, with the

recently published single-stage dopaminergic neuronal differentiation (SingleDA) method (Trzaska *et al.*, 2007). The three different methodologies examined are summarised in Table 2.1 and Figure 4.1.

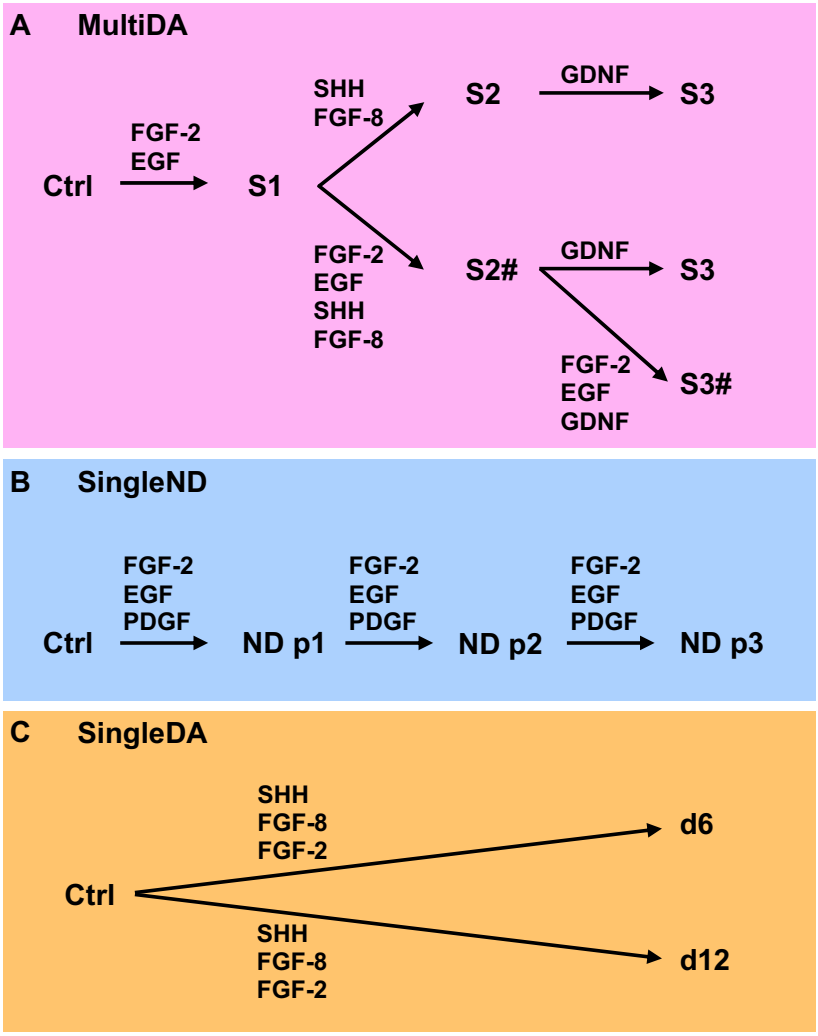

Figure 4.1 Diagram of the hMSC neuronal differentiation methods. (**A**) Novel multiple-stage dopaminergic neuronal differentiation method (MultiDA). (**B**) Previously described single-stage neuronal differentiation method developed by our laboratory (SingleND). (**C**) Recently published (Trzaska *et al.*, 2007) single-step dopaminergic neuronal differentiation method (SingleDA). Ctrl, control undifferentiated hMSCs; S, stage; #, cytokines from S1 included; ND, neuronal differentiation; p, passage; d, day.

4.2 Results

4.2.1 Live Cell Imaging of hMSC Neuronal Differentiation

Recent studies have demonstrated that the rapid effects of chemical-induced MSC neuronal differentiation result from culture artifacts due to environmental stress caused by the chemical agents (Bertani *et al.*, 2005; Choi *et al.*, 2006; Deng *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004; Suon *et al.*, 2004; Tao *et al.*, 2005). In these studies, the neuron-like morphology was found to result from shrinkage of the cell body due to chemical toxicity, with small remnants of the cell periphery left behind to form pseudo-processes, causing a pseudo-neuronal appearance. In the present study, I examined whether this was also true of the morphological changes associated with cytokine-based neuronal differentiation through the SingleND procedure. For this purpose, live cell imaging techniques were employed to provide real-time recording of changes in cell morphology to determine whether the neurite-like processes were indeed extended outward from the cells, rather than being remnants of the initial cell periphery after shrinkage.

Live cell imaging of hMSC neuronal differentiation revealed cells to undergo a considerable degree of morphological change and movement (Figure 4.2; see video footage labelled Supplementary File 1 – Part 1 and Supplementary File 1 – Part 2 on Supplementary CD). Cells developed a neuronal-like bipolar or multipolar appearance by active extension and retraction of long thin cellular processes (arrowheads), development of refractile cell bodies (black arrows), and formation of short transient branches (asterisks). Minute protuberances were also seen along the course of some long cellular processes. In addition, a degree of dynamic cell-cell interaction was observed, during which, neurite-like processes touched, appeared to interact, and then

retracted or moved away (white arrow). At the end of the 3 week differentiation period, this morphology was observed in approximately 65-80% of cells. These images were captured post-trypsinisation; however, spreading phenomena associated with re-attachment does not solely explain the observed morphology, as this was continually altered throughout the monitoring period (>18 hours) and also encompassed migration, and retraction as well as extension of cellular processes. This period of time allows for initial re-attachment as well as any subsequent, but distinct, alterations in morphology. In contrast our previous work has shown that chemically induced hMSCs failed to re-attach following trypsinisation. Furthermore, these cells showed little survival beyond 3 days post-induction, and also detached spontaneously from the culture surface due to cell death (Tao *et al.*, 2005). The morphological changes associated with hMSC neuronal differentiation using the SingleND cytokine-based system are, therefore, the result of active and dynamic processes involving the outgrowth of cellular extensions or processes.

In support of the observed morphological changes, immunofluorescence staining showed that hMSCs also expressed increased levels of neuronal progenitor marker NES and neuronal marker β tubulin III from basal levels after neuronal differentiation (>90% and >95% respectively; Figure 4.3). Weak expression of MAP-2 and GFAP was also detected in some cells (Figure 4.3).

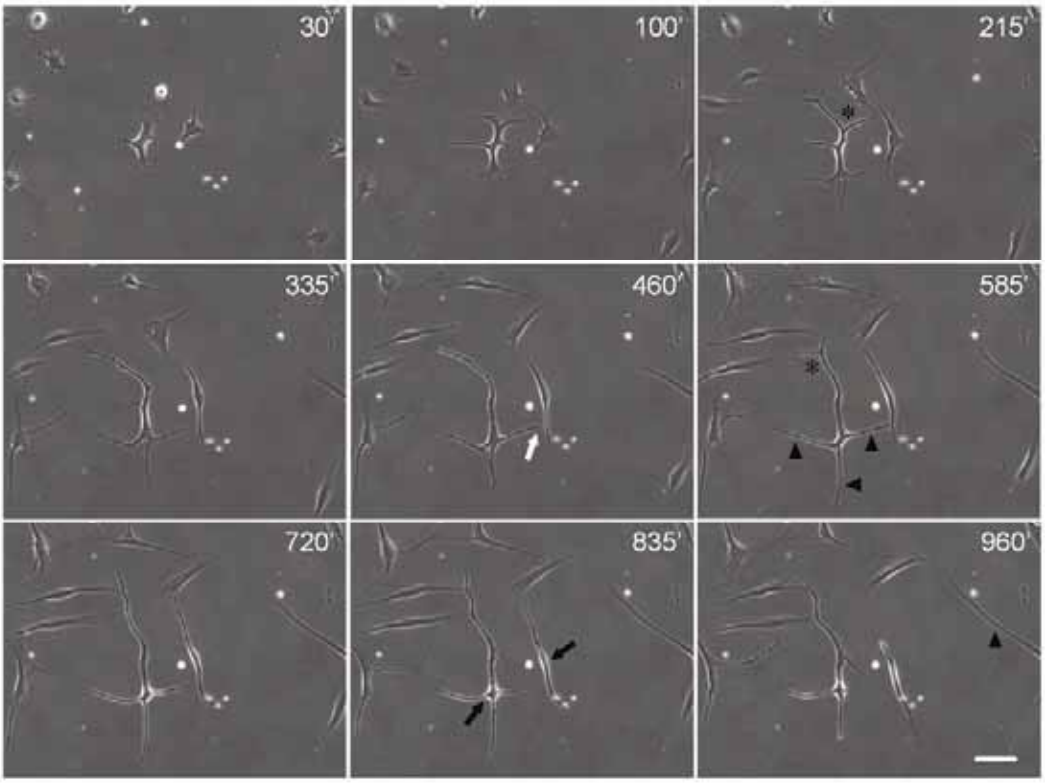

Figure 4.2 Examination of cytokine-induced hMSC neuronal differentiation by live cell imaging.

Analysis using phase-contrast live cell imaging techniques over an 18 hour period revealed cells to be active and to undergo considerable elongation and movement in neuronal differentiation conditions. Cells actively extended and retracted long thin cellular processes (arrowheads), developed refractile cell bodies (black arrows), and formed short transient branches (asterisks). A degree of dynamic cell-cell interaction was also observed, during which cellular processes touched, appeared to interact, and then retracted or moved away (white arrow). The morphological changes associated with cytokine-induced hMSC neuronal differentiation are, therefore, the result of active and dynamic processes, rather than consequences of cellular shrinkage or toxicity. ', minutes. Scale bar: 100 μm.

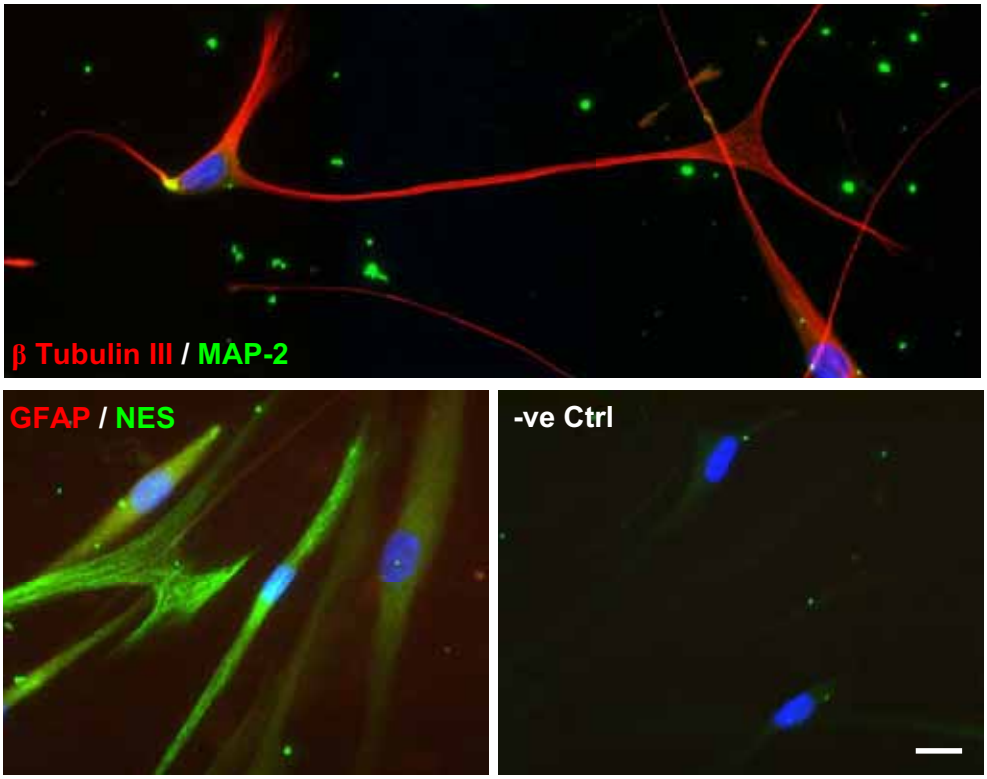

Figure 4.3 Examination of cytokine-induced hMSC neuronal differentiation by immunofluorescence microscopy.

After 3 weeks in neuronal differentiation conditions, cells strongly expressed neuronal markers NES (>90% +ve) and β tubulin III (>95% +ve). Weak expression of GFAP and MAP-2 was also detected in some cells. Representative images are shown. Nuclei counterstained with DAPI. Negative controls consisted of secondary antibody application alone. Scale bar: 20 μm.

4.2.2 Effect of Long-Term Serial Passage on hMSC Neuronal Differentiation

To assess whether prolonged culture of hMSCs *in vitro* alters the neuronal differentiation potential of hMSCs, cells from P4-5 and P11-12 were exposed to the cytokine-based SingleND procedure and compared for differences in morphology and expression of neural markers. Characteristic morphological changes were observed in both cultures as early as within 7 days of treatment with SingleND conditions (Figures 4.4B and F; control in Figures 4.4A and E, respectively), and continued over the 3 week differentiation period (Figures 4.4C, D, G, and H). Early and late passage cultures showed development of refractile cell bodies and long cellular processes/extensions with some branching. Interestingly, despite having undergone changes in the ability to proliferate and symmetrically divide as seen in the culture growth kinetics, P11-12 hMSC cultures did not appear to have a reduced propensity toward acquiring a neuronal-like morphology. However, P11-12 cultures did contain larger cells and greater amounts of cellular debris than P4-5 cultures, and also appeared to contain a higher proportion of cells that were unresponsive to the differentiation conditions, and to a degree, retained a large flat fibroblast-like appearance (Figure 4.4I). In addition, both early and late passage cultures gradually ceased proliferating upon differentiation, and had similar proportions of viable cells by trypan blue dye exclusion (consistently >85% viable; results not shown). As described earlier, long-term serial passage alone without exposure to neuronal differentiation conditions again did not result in spontaneous neural differentiation.

Analysis of gene expression by real-time RT-PCR showed similar changes after differentiation in both early and late passage cultures, with some donor variability (Figure 4.5). Both showed highly increased expression of neuronal progenitor marker *NES*, whereas other markers for the neural lineage (*CSPG4* and *MAP-2*), pluripotency

(*NANOG* and *POU5F1*), and mesodermal lineage (*COL1A1*) were not very differentially regulated. Expression of astrocyte marker *GFAP* was markedly up-regulated in late passage cultures, unlike in early passage cultures, suggesting a possible bias toward astrocytic differentiation in late passage cultures. However, although the fold change appears greatly increased in late passage hMSCs, the level of *GFAP* expression in differentiated cells was similar to the level in early passage hMSCs post-differentiation.

Neural protein expression was examined by immunofluorescence staining for neuronal markers NES, β tubulin III and MAP-2, and astrocyte marker GFAP. Similar results were obtained for both early and late passage neuronal differentiated hMSCs, with cytoskeletal expression of NES and β tubulin III, and weak/no expression of MAP-2 and GFAP. On the other hand, undifferentiated hMSCs expressed NES, but showed weak/no expression of β tubulin III, MAP-2 and GFAP (results not shown).

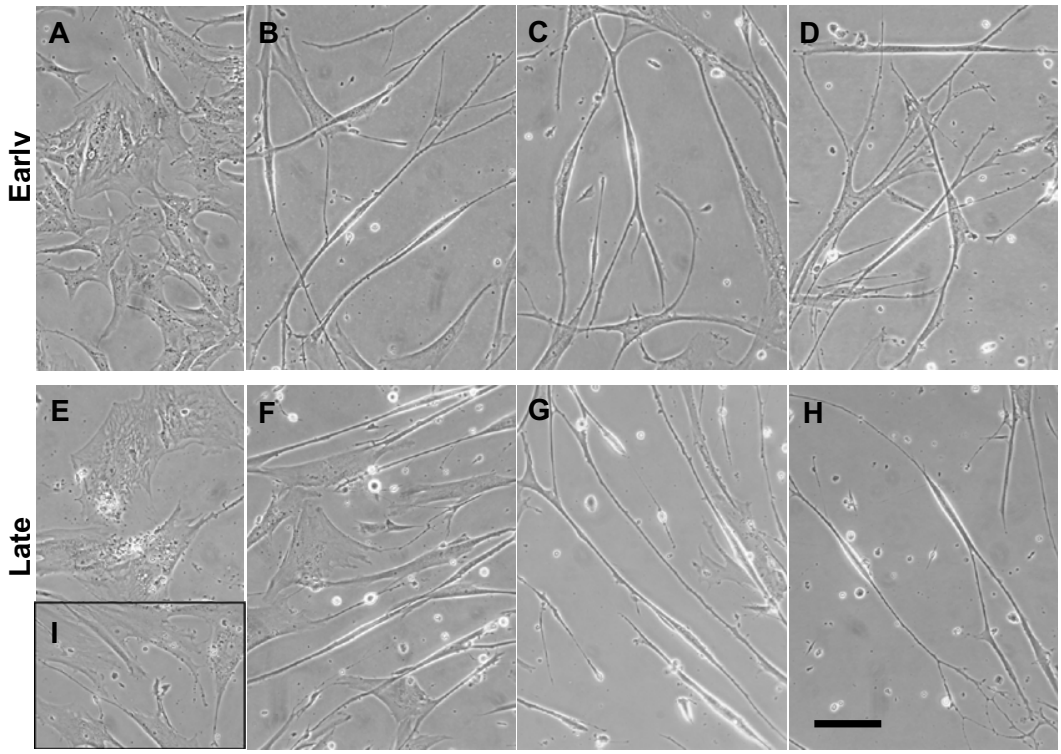

Figure 4.4 Morphology of early and late passage hMSC cultures undergoing neuronal differentiation.

Representative phase-contrast microscopy images of control hMSCs and hMSCs treated with neuronal differentiation conditions. (**A**-**D**) Early/P4-5 stage hMSCs and (**E**-**I**) Late/P11-12 stage hMSCs: (**A** and **E**) Prior to treatment, (**B** and **F**) 1 week, (**C** and **G**) 2 weeks, and (**D**, **H**, and **I**) 3 weeks post-treatment. Following treatment, both P4-5 and P11-12 hMSCs developed refractile cell bodies, retracted cytoplasm and long branching processes. Larger cells and more cellular debris were observed in P11-12 cultures, and a greater number of unresponsive large flat fibroblast-like cells were seen (I). Scale bar: 100 μm.

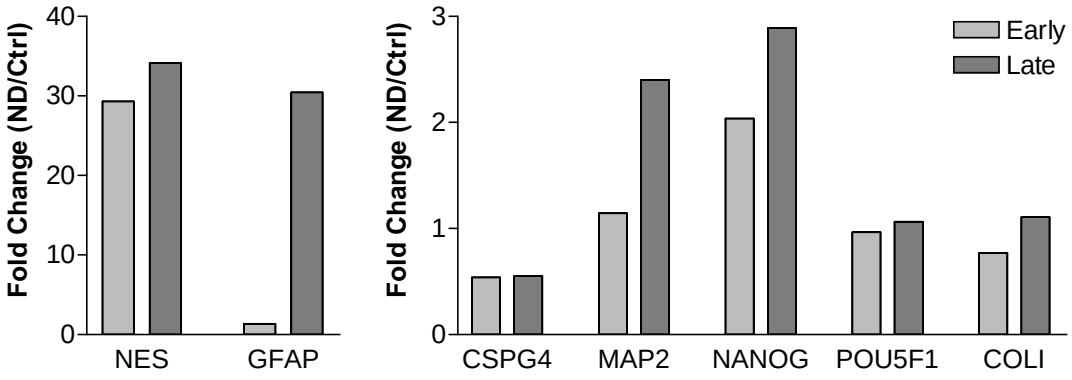

Figure 4.5 Gene expression of early and late passage hMSC cultures post-neuronal differentiation.

Representative real-time RT-PCR results from undifferentiated control hMSC cultures and cultures treated with neuronal differentiation conditions ($n = 2$). Results are depicted as mean fold change in gene expression relative to the control, with baseline set at 1.0. Ctrl, control undifferentiated hMSCs; ND, neuronal differentiated hMSCs.

4.2.3 Cytokine-Based Dopaminergic Neuronal Differentiation of hMSCs

Previous results obtained by live cell imaging (Section 4.2.1) indicated that the morphological changes induced in hMSCs by cytokine-based neuronal differentiation were the result of active and dynamic processes rather than culture artifacts. In addition, cytokines were found to drive hMSCs toward an early neuronal fate. As a result, it was hypothesised that the application of cytokines relevant to midbrain dopaminergic neuronal differentiation may direct hMSCs toward this lineage. Furthermore, sequential application of cytokines was investigated to determine whether this provided advantages over single-step protocols. Figure 4.1 shows a diagram of the steps in each differentiation method.

4.2.3.1 Morphological Analysis of hMSC Dopaminergic Neuronal Differentiation

Two methods of cytokine-based hMSC dopaminergic neuronal differentiation were employed in this study, a novel multiple-stage procedure (MultiDA) and a recently published single-step protocol (SingleDA) (Trzaska *et al.*, 2007). These techniques were also compared with the SingleND method previously published by our group (Tao *et al.*, 2005). Representative phase-contrast images of cultures treated with these different methods are shown in Figures 4.6, 4.7 and 4.8 respectively. Prior to differentiation the hMSC cultures mainly contained cells with a fibroblast-like appearance (Figures 4.6A, 4.7A and 4.8A), and with continued growth *in vitro* the cells displayed an increasingly flattened morphology. The three different cytokine-based treatments all produced cells possessing a similar neuronal-like bipolar or multipolar morphology, with the formation of refractile cell bodies and long thin cellular processes, occasionally containing branching or minute protuberances. In addition, for all three methods the changes in morphology occurred gradually over the differentiation period.

Treatment with the MultiDA protocol yielded the most distinct morphological alterations. Following the application of FGF-2 and EGF in the first stage (S) of differentiation, hMSCs developed a bipolar, refractile appearance and formed neurosphere-like cell clusters (Figure 4.6B). While bipolar refractile morphology was also common to both the single-stage differentiation methods, neither was found to result in neurosphere-like formation. With subsequent culturing in S2 (SHH/FGF-8) and S3 (GDNF) many of the cells that had acquired neuronal-like morphology were observed to return toward a flatter appearance, and thin cellular processes were also lost from some of these cells (Figures 4.6C and 4.6E). In addition, cell survival was observed to decrease in MultiDA S2 and S3 conditions, with fewer adherent cells remaining in culture. However, combining S1 cytokines with later stages (denoted by #), permitted hMSCs to maintain a neuronal-like appearance throughout the second MultiDA stage (Figure 4.6D). Continued supplementation of cultures with S1 cytokines right through S3 (S3#) allowed the bipolar and refractile morphology to be maintained (Figure 4.6F), whereas upon the removal of FGF-2 and EGF after S2 (S3(2#)), cells were again observed to gain a flattish appearance (Figure 4.6G and H). Interestingly, although the addition of FGF-2 and EGF to later culture stages appeared to promote a continued bipolar and refractile neuronal-like morphology and increased cell survival, it was also found that uninterrupted treatment resulted in cells with less complex branching of cellular processes. A considerable decrease in cell number was also observed across all stages of the MultiDA method.

Application of the recently reported SingleDA method (Trzaska *et al.*, 2007) generated a neuronal-like morphology similar to the published results (Figure 4.7). In addition, the morphology of hMSCs treated with the SingleDA method resembled that obtained through the SingleND protocol developed by our group ((Tao *et al.*, 2005);

Figure 4.8), despite differences in the cytokines used. The SingleND method also elicited reduced cell numbers, while slight increases were seen using the SingleDA method.

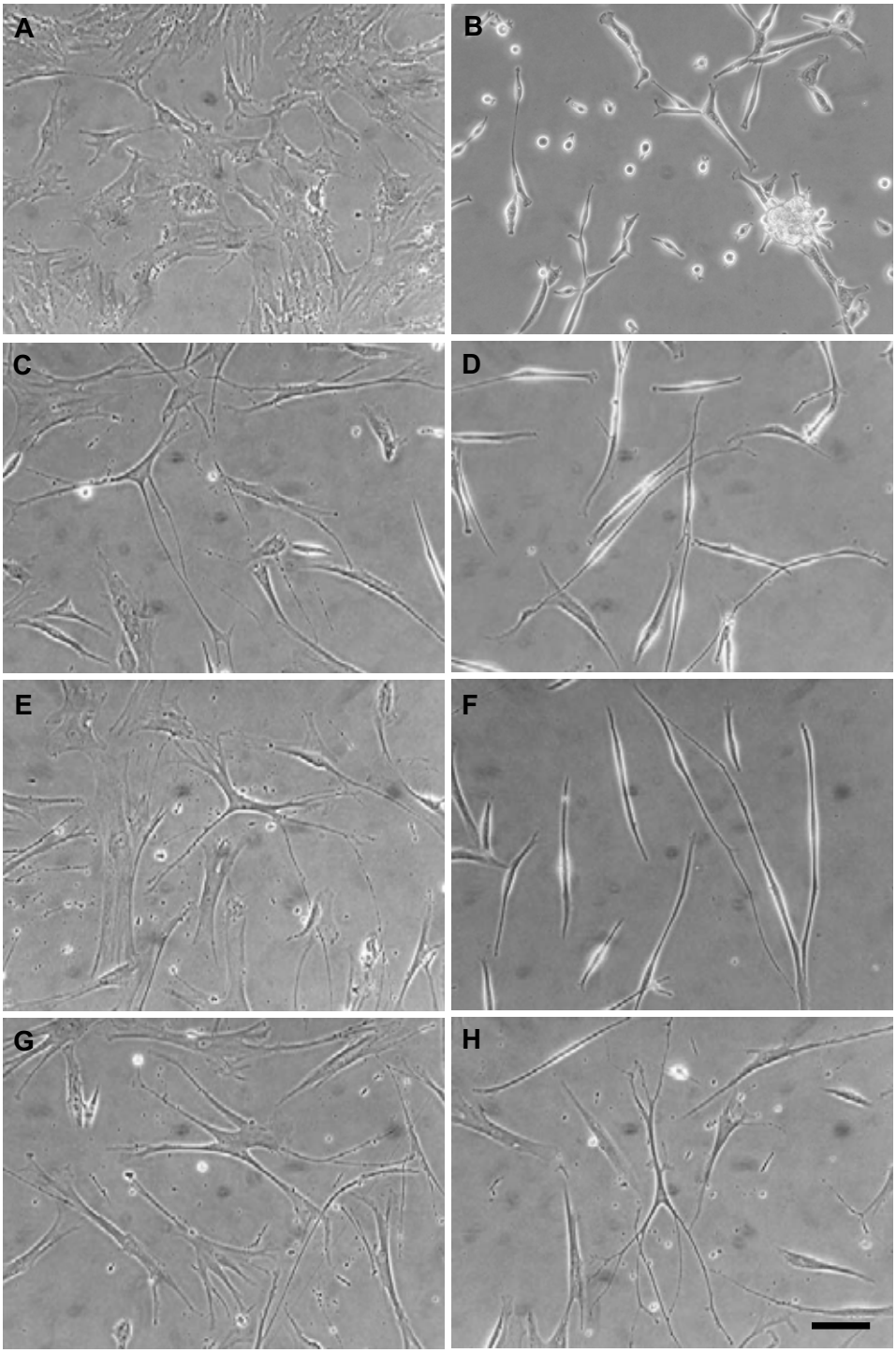

Figure 4.6 Morphology of hMSCs during *in vitro* cytokine-induced multiple-stage dopaminergic neuronal differentiation (MultiDA).

(Refer to next page for Figure 4.6 Legend text)

Figure 4.6 Morphology of hMSCs during *in vitro* cytokine-induced multiple-stage dopaminergic neuronal differentiation (MultiDA).
Images of hMSCs undergoing dopaminergic differentiation were captured at the end of each stage (1 week) of sequential cytokine treatment. Representative phase-contrast images are depicted of hMSCs: (**A**) Prior to treatment (d0); after (**B**) S1 (d7), (**C**) S2 (d14), (**D**) S2# (d14), (**E**) S3 (d21), (**F**) S3# (d21), and (**G** and **H**) S3(2#) (d21) of differentiation ($n = 3$). d, day; S, stage; #, cytokines from S1 included. Scale bar: 100 μm.

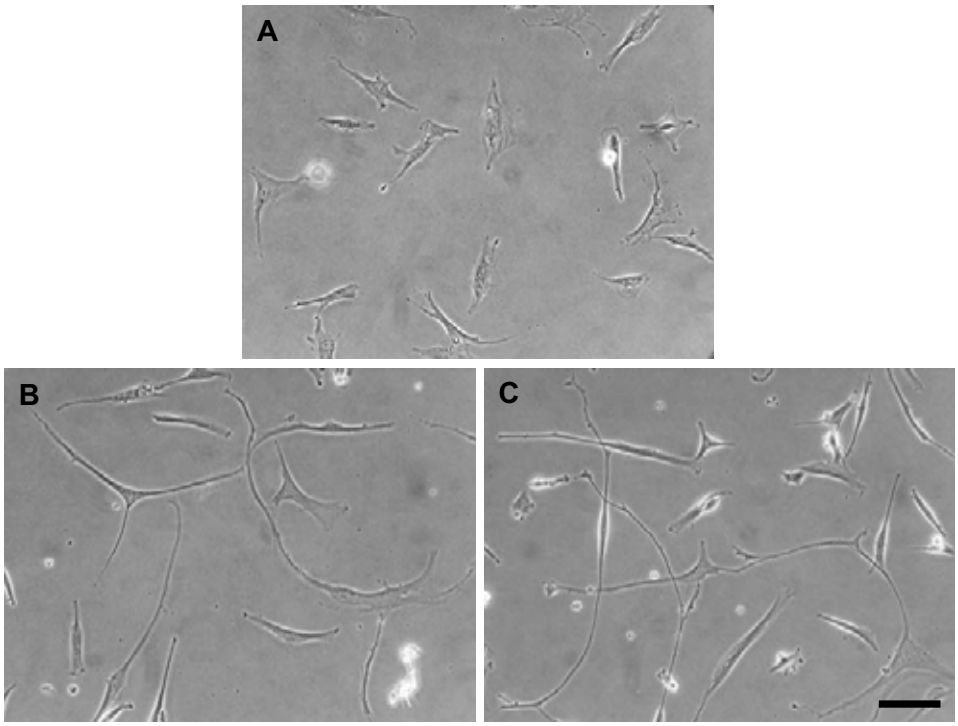

Figure 4.7 Morphology of hMSCs during *in vitro* cytokine-induced single-stage dopaminergic neuronal differentiation (SingleDA).
Dopaminergic neuronal differentiation of hMSCs was performed according to a previously published single-step method (Trzaska *et al.*, 2007) for comparison with our multiple-stage method. Representative images captured with a phase-contrast light microscope are depicted of hMSCs: (**A**) Prior to treatment, and (**B**) 6 days and (**C**) 12 days post-treatment ($n = 3$). Scale bar: 100 µm.

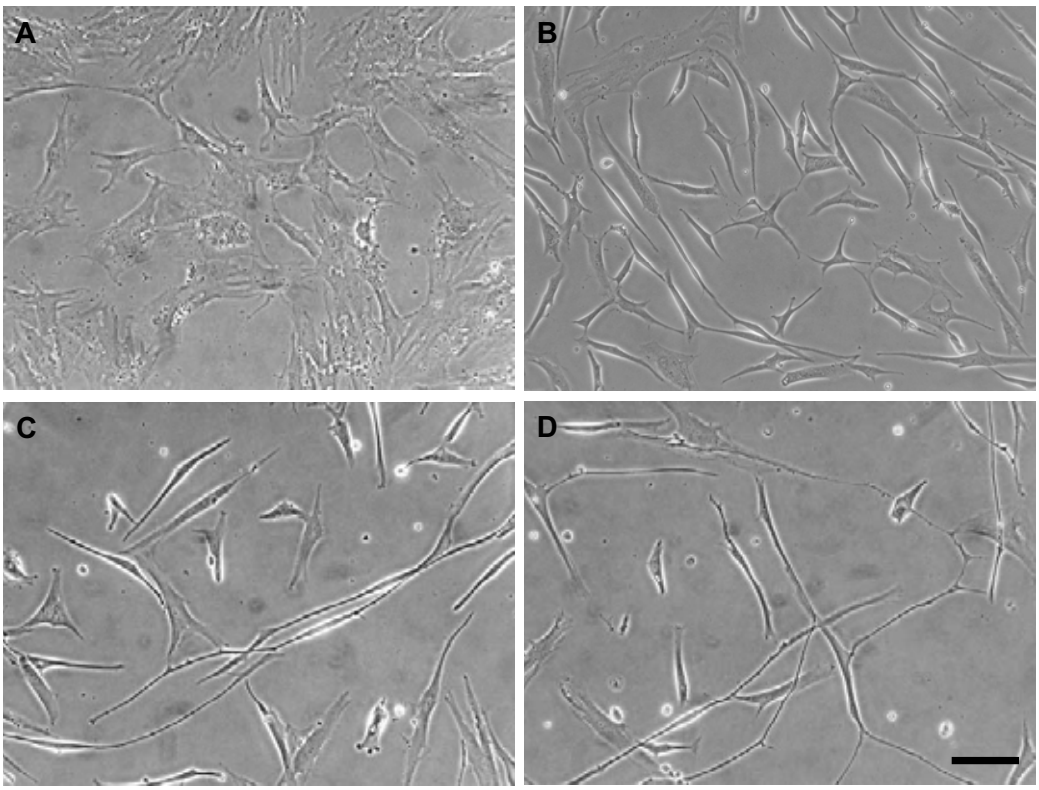

Figure 4.8 Morphology of hMSCs during *in vitro* cytokine-induced single-stage neuronal differentiation (SingleND).

Neuronal differentiation of hMSCs was performed according to a method previously published by our group (Tao *et al.*, 2005) for comparison with the dopaminergic neuronal differentiation methods. Representative phase-contrast images are shown of hMSCs: (**A**) Prior to treatment (same image shown in Figure 4.6), and at the end of (**B**) P1 (1 week), (**C**) P2 (2 weeks), and (**D**) P3 (3 weeks) post-treatment (n = 3). P, passage. Scale bar: 100 µm.

4.2.3.2 Gene Expression Analyses During In Vitro hMSC Dopaminergic Neuronal Differentiation

The gene expression of hMSCs subjected to *in vitro* neuronal and dopaminergic neuronal differentiation procedures were examined by real-time RT-PCR to examine alterations in neuronal, dopaminergic neuronal, glial, mesodermal, and pluripotency markers during cytokine-based differentiation. Cells were harvested for analysis prior to differentiation (control hMSCs) and at the end of each week of cytokine treatment for hMSCs undergoing the MultiDA and SingleND procedures. Alternatively, for the SingleDA procedure (Trzaska *et al.*, 2007), hMSCs were collected at days 6 and 12 in accordance with the time points used in the published study. Data are presented as gene expression of the sample relative to the gene expression of the control (all relative to the expression of house-keeping gene *HPRT*; $n = 3$). Statistical analysis was performed using the paired *t*-test, and significance was set at $p < 0.05$.

Expression of Neuronal Genes During *In Vitro* hMSC Dopaminergic Neuronal Differentiation: Expression of neuronal progenitor gene *NES* and neuronal genes *MAP-2* and *ENO2* were detected in all samples before and after *in vitro* cytokine-based differentiation (Figure 4.9). *NES* was found to be significantly up-regulated at all time points in all three differentiation methods, apart from S2 of the MultiDA method. The highest levels of *NES* expression were elicited by the MultiDA procedure, and the addition of S1 cytokines (FGF-2/EGF) to later differentiation stages appeared to be beneficial for *NES* expression. The first stage of the MultiDA method (S1) resulted in a 39.2-fold increase in *NES* ($p = 0.012$), while S2# resulted in a 175.5-fold increase ($p = 0.022$), and S3# showed 117.5-fold increased *NES* expression ($p = 0.023$). Whereas, the highest level of *NES* expression in the SingleND method was found after 3 weeks of differentiation (P3: 23.9-fold increase; $p = 0.025$), and the SingleDA method

yielded a similar result, with the highest *NES* expression levels seen after 12 days of induction (d12: 21.9-fold increase; $p = 0.005$). The mRNA levels of *MAP-2* were not significantly different to the control in all methods examined, except at day 12 of the SingleDA method which displayed a 1.36-fold increase ($p = 0.040$). Likewise, the expression of *ENO2* did not vary significantly from the control, with the exception of cells grown in S2 and S3 of the MultiDA method, which showed a significant decrease in *ENO2* levels of 2.9- and 3.8-fold respectively ($p = 0.040$ and $p = 0.038$ respectively). However, this decrease in *ENO2* could be prevented by the addition of FGF-2/EGF to these stages of differentiation.

Expression of Dopaminergic Neuronal Genes During *In Vitro* hMSC Dopaminergic Neuronal Differentiation: Human MSCs were also found to express *NR4A2*, a transcription factor essential for midbrain dopaminergic neuronal differentiation, as well as classical dopaminergic neuronal marker *TH*, prior to and following the three different induction protocols (Figure 4.10). A trend of increasing *NR4A2* expression could be observed in S1 and S2# of the MultiDA method (1.9- and 3.0-fold respectively); however, this did not reach a level of significance in comparison to the control. Absence of FGF-2/EGF from Stage 3 of the MultiDA method did result in significantly decreased *NR4A2* levels (S3: 18.9-fold decrease, $p = 0.00068$; S3(2#): 10.8-fold decrease, $p = 0.027$), which was not observed with FGF-2/EGF supplementation (S3#(2#): 1.4-fold decrease, $p = 0.26$). On the other hand, both single-step differentiation procedures exhibited a non-significant trend of decreasing *NR4A2* expression. For all differentiation methods investigated, no significant changes were detected in *TH* expression levels compared to control undifferentiated hMSC cultures; however, the SingleND method yielded the highest levels of *TH* expression (3.8- to 5.6-fold increase).

Expression of Glial Genes During *In Vitro* hMSC Dopaminergic Neuronal Differentiation: Expression of mRNA for glial markers *GFAP* and *CSPG4* was also detected using the real-time RT-PCR technique (Figure 4.11). Only slight expression of *GFAP* could be detected in hMSCs prior to and after the differentiation procedures, although significant up-regulation was observed in S1 and S2# of the MultiDA method (S1: 15.8-fold increase, $p = 0.026$; S2#: 20.4-fold increase, $p = 0.026$). The level of *GFAP* expression in all other differentiation conditions did not show significant difference to the control cultures; however, a trend was observed in the SingleND method, which showed initial increase in *GFAP* expression at 1 week post-differentiation, followed by a trend of decreasing expression over later time points. *CSPG4* mRNA was moderately expressed under all conditions examined, although significant changes from the control were not observed.

Expression of Mesodermal Gene *COL1A1* During *In Vitro* hMSC Dopaminergic Neuronal Differentiation: The expression of mesodermal marker *COL1A1* was also examined to determine whether a loss of mesodermal characteristic occurred in parallel with neuronal differentiation (Figure 4.12). On the contrary, significant down-regulation of *COL1A1* compared to the control could not be detected by real-time RT-PCR under any of the neuronal differentiation conditions examined. Instead, *COL1A1* expression was observed to significantly increase in hMSCs cultured in the absence of FGF-2/EGF in stages 2 and 3 of the MultiDA method (S2: 6.3-fold increase, $p = 0.0029$; S3: 14.6-fold increase, $p = 0.0012$; S3(2#): 5.6-fold increase, $p = 0.0099$), while the other conditions did not yield any significant changes from the control.

Expression of Pluripotency Genes During *In Vitro* hMSC Dopaminergic Neuronal Differentiation: Pluripotency markers *POU5F1* and *NANOG* were also

examined by real-time RT-PCR, with hMSCs prior to and following differentiation found to express weak levels of mRNA for both these markers (Figure 4.12). None of the neuronal differentiation methods investigated was found to induce significant changes in *POU5F1* expression compared to control hMSC cultures. In addition, expression of the *NANOG* gene was also unaffected by the MultiDA procedure and the SingleND procedure. However, the SingleDA method published by Trzaska and colleagues (Trzaska *et al.*, 2007), was found to result in significant up-regulation of *NANOG* expression at both day 6 (4.5-fold increase, $p = 0.0074$) and day 12 (4.8-fold increase, $p = 0.041$) post-induction.

Effect of FGF-2 and EGF Supplementation: In addition, supplementation with FGF-2 and EGF in the second and third stages of the MultiDA procedure resulted in significant differences in the gene expression of hMSCs. Expression of *NES* was found to be significantly up-regulated in S3#, when compared to hMSCs treated with S3 (GDNF alone; $p = 0.050$), or S3(2#) ($p = 0.021$). *MAP-2* expression also showed a significant increase at S3# compared to S3(2#), which did not contain FGF-2 and EGF in the final stage ($p = 0.005$). Furthermore, neuronal marker *ENO2* was also significantly up-regulated in S2# in comparison S2 ($p = 0.005$). The level of *NR4A2* transcription was also affected by FGF-2/EGF addition, with up-regulation occurring in S2# and S3# compared with the corresponding stages without further supplementation ($p = 0.006$, and $p = 0.012$ respectively). Significant increases in astrocytic marker *GFAP* were observed with FGF-2/EGF addition in stage 2 ($p = 0.027$), and in stage 3 when hMSCs exposed to continual supplementation were compared with those only receiving FGF-2/EGF in stage 2 ($p = 0.034$). The final marker exhibiting significantly increased expression with the addition of S1 cytokines to later stages was *NANOG*, which showed significant up-regulation in S2# compared to S2 ($p = 0.046$), and in S3(2#) compared to

S3 ($p = 0.003$). The only gene examined that showed significantly down-regulated expression with FGF-2/EGF supplementation was *COL1A1*. This was seen in S2# ($p = 0.045$), and also in S3# ($p = 0.047$), when compared with the corresponding stages cultured in the absence of FGF-2 and EGF. These results suggest an important role for FGF-2 and EGF in the *in vitro* neuronal differentiation of hMSCs.

Very slight mRNA expression of further dopaminergic neuronal markers and transcription factors were also detected in some hMSC cultures prior to and following differentiation. These markers included *PITX3*, *EN2*, *VMAT2*, and *DAT*. However, expression of these markers did not occur consistently in samples from different donors and the level of expression was fairly low. For these reasons, this data has not been shown here.

Overall, the real-time RT-PCR findings suggest that all three cytokine-based neuronal differentiation methods examined were only capable of eliciting an immature neuronal phenotype in hMSCs, with elevated expression of neuronal progenitor marker *NES*, and little change in mature neuronal markers *MAP-2*, *ENO2*, and *TH*. Mesodermal and pluripotency markers were not observed to be down-regulated, providing further evidence for incomplete neuronal differentiation. In addition, continued supplementation with FGF-2 and EGF was beneficial in the MultiDA procedure, as it resulted in enhancement of neural marker expression and prevented increases in mesodermal *COL1A1* expression. A summary of the gene expression changes during *in vitro* culturing of hMSCs under the three different neuronal induction protocols is shown in Table 4.1.

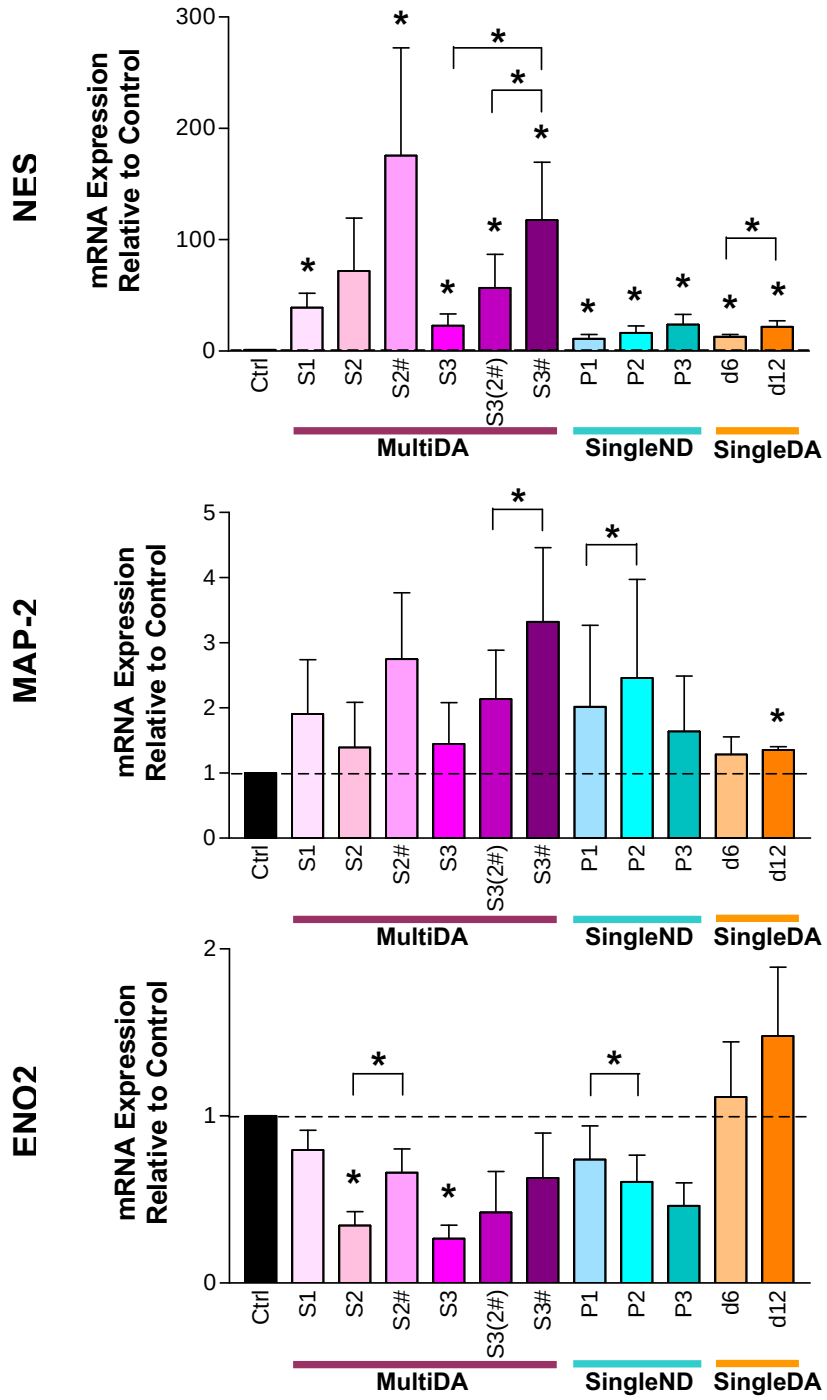

Figure 4.9 Expression of neuronal genes in hMSC cultures undergoing *in vitro* neuronal and dopaminergic neuronal differentiation.

(Refer to next page for Figure 4.9 Legend text).

Figure 4.9 Expression of neuronal genes in hMSC cultures undergoing *in vitro* neuronal and dopaminergic neuronal differentiation.

Real-time RT-PCR results from control undifferentiated hMSC cultures and cultures treated with the MultiDA method shown in purple shades, the SingleND method shown in blue shades, and the SingleDA method published by Trzaska, *et al.* (Trzaska *et al.*, 2007) shown in orange shades. Significant increases in the expression of neuronal progenitor marker *NES* was observed in all conditions, except S2, whereas *MAP-2* and *ENO2* showed similar expression levels to the control. Results are depicted as mean fold change in mRNA expression + SEM relative to control undifferentiated hMSC cultures, with baseline set at 1.0 ($n = 3$). Separate control hMSC data were used for the SingleDA method, as this was not performed in parallel with the other methods. * $p < 0.05$ compared with the control. Ctrl, control undifferentiated hMSCs; S, Stage; #, cytokines from S1 included; P, passage; d, day.

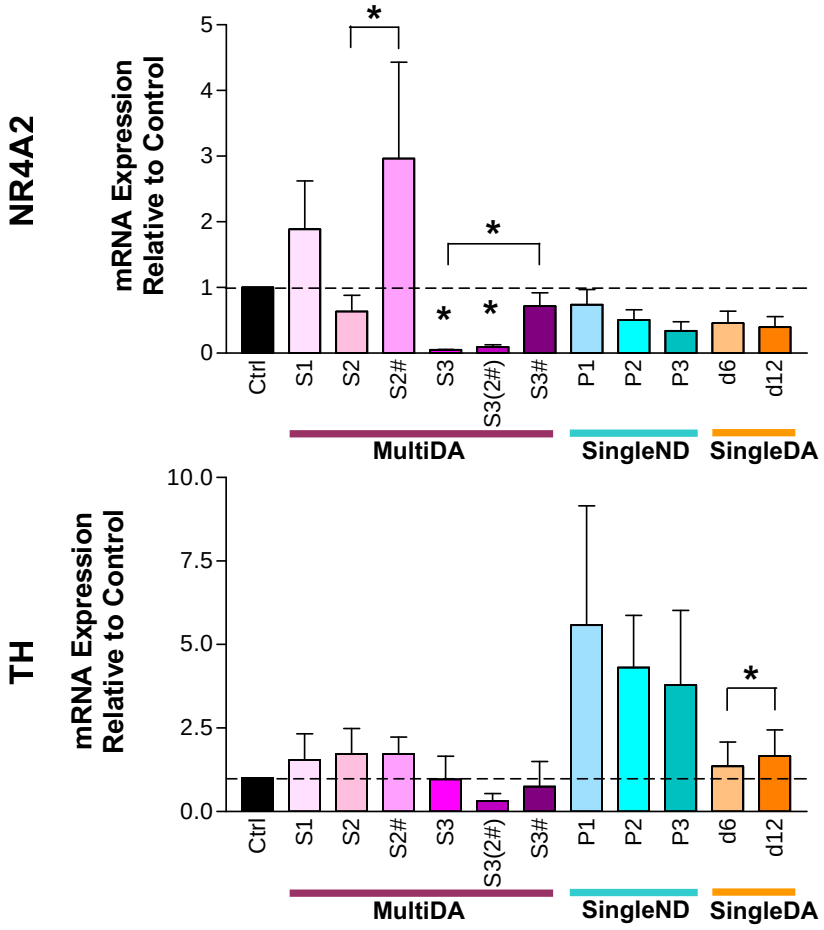

Figure 4.10 Expression of dopaminergic neuronal genes in hMSC cultures undergoing *in vitro* neuronal and dopaminergic neuronal differentiation.

Real-time RT-PCR analyses of the MultiDA (purple shades), SingleND (blue shades), and SingleDA (orange shades) methods. Significant decrease in transcription factor *NR4A2* expression was seen when S1 cytokines were absent from S3 cultures. Otherwise, *NR4A2* and *TH* expression were similar to the control. Results are depicted as mean fold change in mRNA expression + SEM relative to control undifferentiated hMSC cultures (set at 1.0; $n = 3$). Separate control hMSC data were used for the SingleDA method, as this was not performed in parallel with the other methods. * $p < 0.05$ compared with the control. Ctrl, control undifferentiated hMSCs; S, Stage; #, cytokines from S1 included; P, passage; d, day.

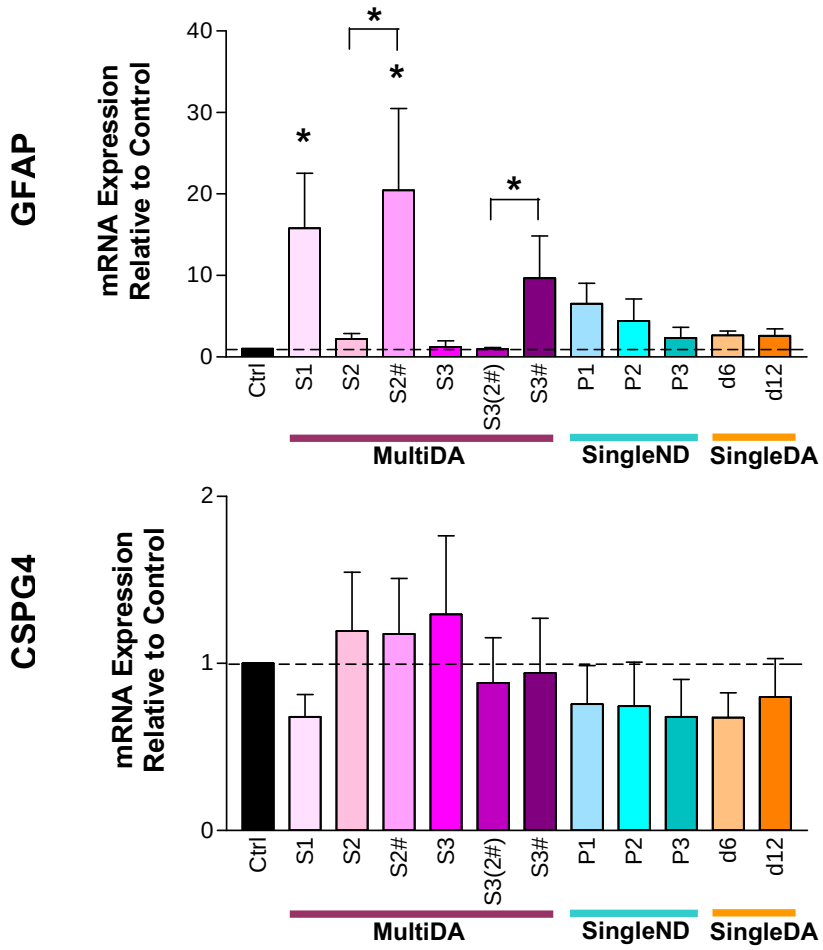

Figure 4.11 Expression of glial genes in hMSC cultures undergoing *in vitro* neuronal and dopaminergic neuronal differentiation.

Real-time RT-PCR analyses of the MultiDA (purple shades), SingleND (blue shades), and SingleDA (orange shades) methods. Significant increase in *GFAP* expression was seen in S1 and S2# cultures. Otherwise, *GFAP* and *CSPG4* expression were not significantly different from the control. Results are depicted as mean fold change in mRNA expression + SEM relative to control undifferentiated hMSC cultures (set at 1.0; $n = 3$). Separate control hMSC data were used for the SingleDA method, as this was not performed in parallel with the other methods. * $p < 0.05$ compared with the control. S, Stage; #, cytokines from S1 included; P, passage; d, day.

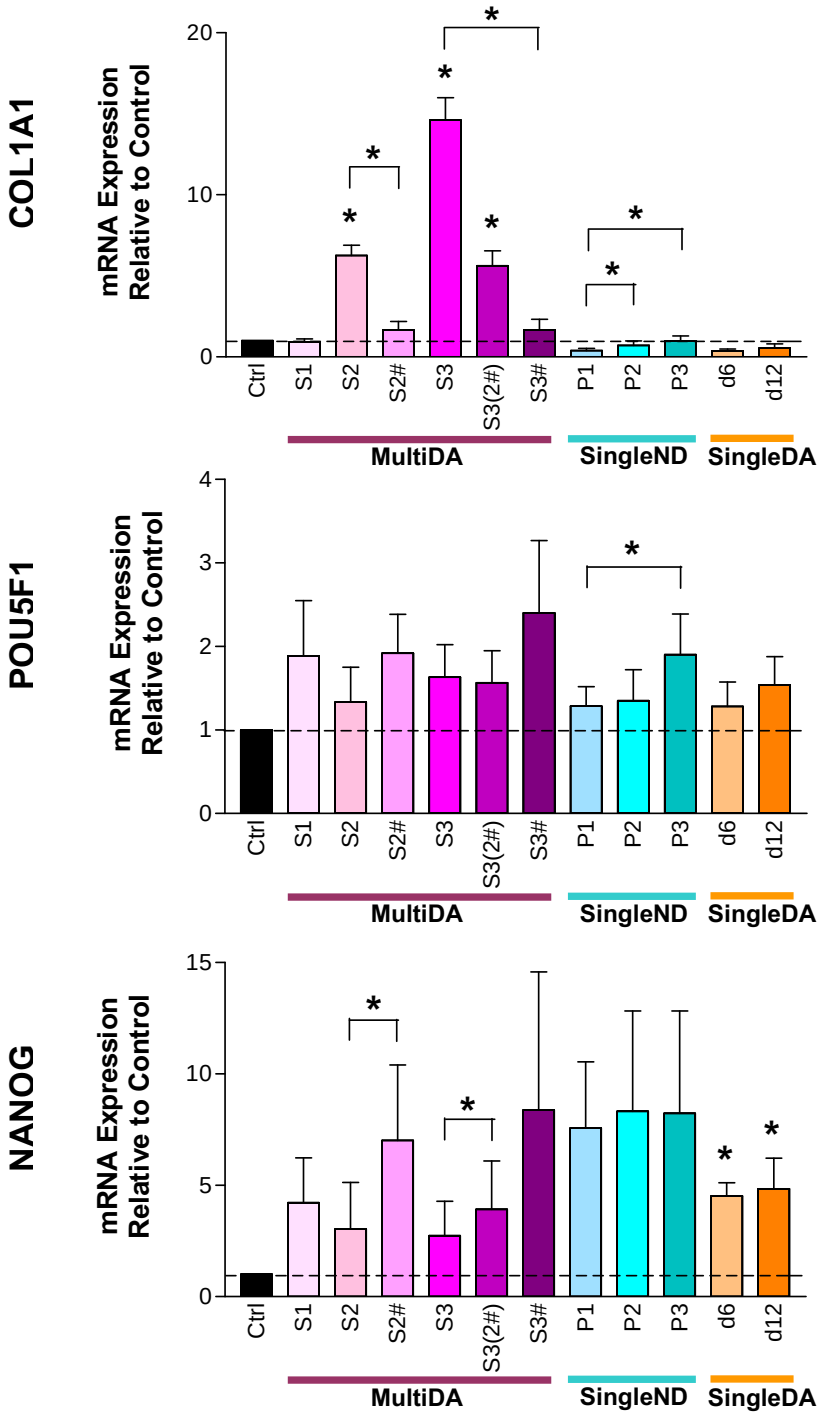

Figure 4.12 Expression of mesodermal and pluripotency genes in hMSCs undergoing neuronal & dopaminergic neuronal differentiation.

(Refer to next page for Figure 4.12 Legend text).

Figure 4.12 Expression of mesodermal and pluripotency genes in hMSCs undergoing neuronal & dopaminergic neuronal differentiation.

Real-time RT-PCR results from control undifferentiated hMSC cultures and cultures treated with the MultiDA method shown in purple shades, SingleND method shown in blue shades, and SingleDA method published by Trzaska, *et al.* (Trzaska *et al.*, 2007) shown in orange shades. Expression of mesodermal marker *COL1A1* was significantly increased when S1 cytokines were absent from S2 and S3 cultures. Pluripotency markers *POU5F1* and *NANOG* were detectable in all samples; however, expression was not significantly different from the control, except for significantly increased *NANOG* expression in SingleDA samples. Results are depicted as mean fold change in mRNA expression + SEM relative to control undifferentiated hMSC cultures, with baseline set at 1.0 ($n = 3$). Separate control hMSC data were used for the SingleDA method, as this was not performed in parallel with the other methods. * $p < 0.05$ compared with the control. Ctrl, control undifferentiated hMSCs; S, Stage; #, cytokines from S1 included; P, passage; d, day.

Table 4.1 Summary of Real-Time RT-PCR Results: Gene Expression Changes During hMSC Cytokine-Based Neuronal Differentiation

	MultiDA					SingleND			SingleDA	
Genes	**S1**	**S2**	**S3**	**S2#**	**S3#**	**P1**	**P2**	**P3**	**d6**	**d12**
NES	↑	↑↑	↑	↑↑↑	↑↑↑	↑	↑	↑	↑	↑
MAP-2	—	—	—	−/↑	−/↑	—	—	—	—	−/↑
ENO2	—	↓↓	↓↓	↓	↓	—	↓	↓	—	—
NR4A2	↑	—	↓↓	↑	—	—	↓	↓	↓	↓
TH	—	—	—	—	—	−/↑	−/↑	−/↑	—	—
GFAP	↑↑	−/↑	—	↑↑	↑↑	↑↑	↑/↑↑	↑	↑	↑
CSPG4	−/↓	—	—	—	—	—	—	—	−/↓	—
POU5F1	−/↑	—	—	−/↑	−/↑	—	—	−/↑	—	—
NANOG	↑	↑	↑	↑/↑↑	↑/↑↑	↑/↑↑	↑/↑↑	↑/↑↑	↑/↑↑	↑/↑↑
COLI	—	↑/↑↑	↑↑	—	—	−/↓	−/↓	−/↓	↓↓	↓↓
FGF-2	+	- - -	- - -	+	+	+	+	+	+	+
EGF	+	- - -	- - -	+	+	+	+	+	- - -	- - -
PDGF	- - -	- - -	- - -	- - -	- - -	+	+	+	- - -	- - -
SHH	- - -	+	- - -	+	- - -	- - -	- - -	- - -	+	+
FGF-8	- - -	+	- - -	+	- - -	- - -	- - -	- - -	+	+
GDNF	- - -	- - -	+	- - -	+	- - -	- - -	- - -	- - -	- - -

Yellow: Significant change ($p < 0.05$ compared with control)

: Cytokines from Stage 1 (FGF-2/EGF) included

↑ : Slight increase

↑↑ : Moderate increase

↑↑↑ : High increase

↓ : Slight decrease

↓↓ : Moderate decrease

— : No change

+ : Cytokine included in culture conditions

- - - : Cytokine excluded from culture conditions

4.2.3.3 Protein Expression Analyses During In Vitro hMSC Dopaminergic Neuronal Differentiation

The previous results obtained by real-time RT-PCR (Section 4.2.3.2) indicated that hMSCs were capable of up-regulating the expression of certain neural genes when exposed to all three cytokine-based neuronal differentiation procedures. So, to examine whether these changes also occurred at the protein level, immunofluorescence staining for neuronal, glial, and mesodermal markers were performed on hMSCs undergoing the MultiDA (Figure 4.13), SingleND (Figure 4.14) and SingleDA (Figure 4.15) procedures, at the same time points investigated by real-time RT-PCR ($n = 3$).

Consistent with the PCR findings neuronal progenitor marker NES was detected in both undifferentiated control hMSCs (<10% positive) and at all time points examined during cytokine-based neuronal differentiation (<20% positive). NES expression showed an intracellular cytoplasmic localisation that resembled a cytoskeletal pattern or filamentous network, and appeared to be slightly up-regulated following cytokine induction. Similar levels and patterns of NES staining were observed in cells subjected to all three neuronal differentiation methods. Some cells cultured under the neuronal differentiation conditions were noticed to be lost from the culture surface during the staining procedure, particularly those displaying a refractile cell body and possessing long thin cellular extensions, i.e. cells with a neuronal-like appearance. Therefore, it should be acknowledged that the percentage of cells positive for NES and the other markers examined here may be underestimated.

Prior to differentiation, some hMSCs expressed neuronal marker β tubulin III at low levels (<15% positive). Following exposure to neuronal differentiation conditions, the level of β tubulin III expression was dramatically increased, and a greater proportion of cells also exhibited the intracellular cytoskeletal staining pattern typical of this

structural protein. Generally, >80% of cells cultured in the neuronal differentiation conditions were positive for β tubulin III protein, with the exception of: the SingleND method, which generated >90% β tubulin III-positive cells; and S2 and S3 of the MultiDA method that excluded FGF-2/EGF, which produced a lower yield of 60-80% β tubulin III-positive cells.

As expected, extracellular matrix glycoprotein fibronectin was observed to be strongly expressed by control undifferentiated hMSCs (90-100% positive). This expression was also found to occur in a typical extracellular fibrous pattern. In addition, continued fibronectin expression was detected after implementation of the three neuronal differentiation methods, with >90% of cells generally positive for fibronectin. S3 of the MultiDA method showed slightly lower proportions of fibronectin-positive cells (>80%), and the intensity of fibronectin expression in these cells also appeared weaker.

The other markers examined by immunofluorescence staining included astroglial protein GFAP, and neuronal proteins NeuN and TH. In general, for all three cytokine-based neuronal differentiation procedures investigated, the expression of these markers was absent from the majority of cells and only occasionally could very weak expression be detected.

As a whole, the examination of protein expression by immunofluorescent staining confirmed the findings obtained by real-time RT-PCR. Following neuronal differentiation hMSCs were found to continue expressing NES and fibronectin proteins, but also up-regulated β tubulin III protein expression. Together these results indicate that the three different cytokine-based neuronal induction methods generated hMSC-derived cells possessing an immature neuronal phenotype. The findings of this chapter are summarised in Table 4.2.

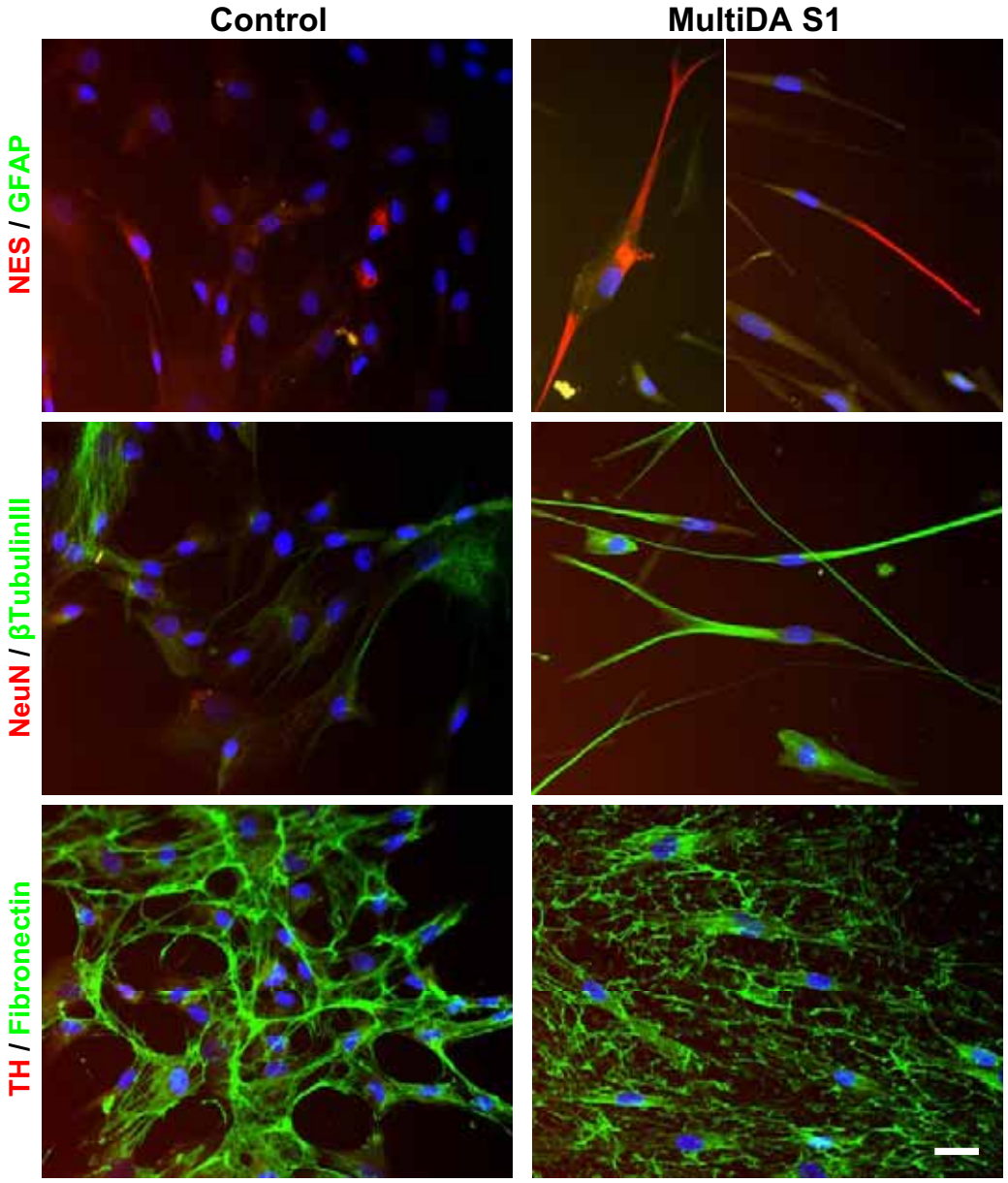

Figure 4.13 Protein expression analysis of hMSCs during *in vitro* cytokine-induced multiple-stage dopaminergic neuronal differentiation (MultiDA) by immunofluorescence microscopy (Part 1).

(Refer to the following pages for Figure 4.13 Parts 2 and 3, and Figure 4.13 Legend text).

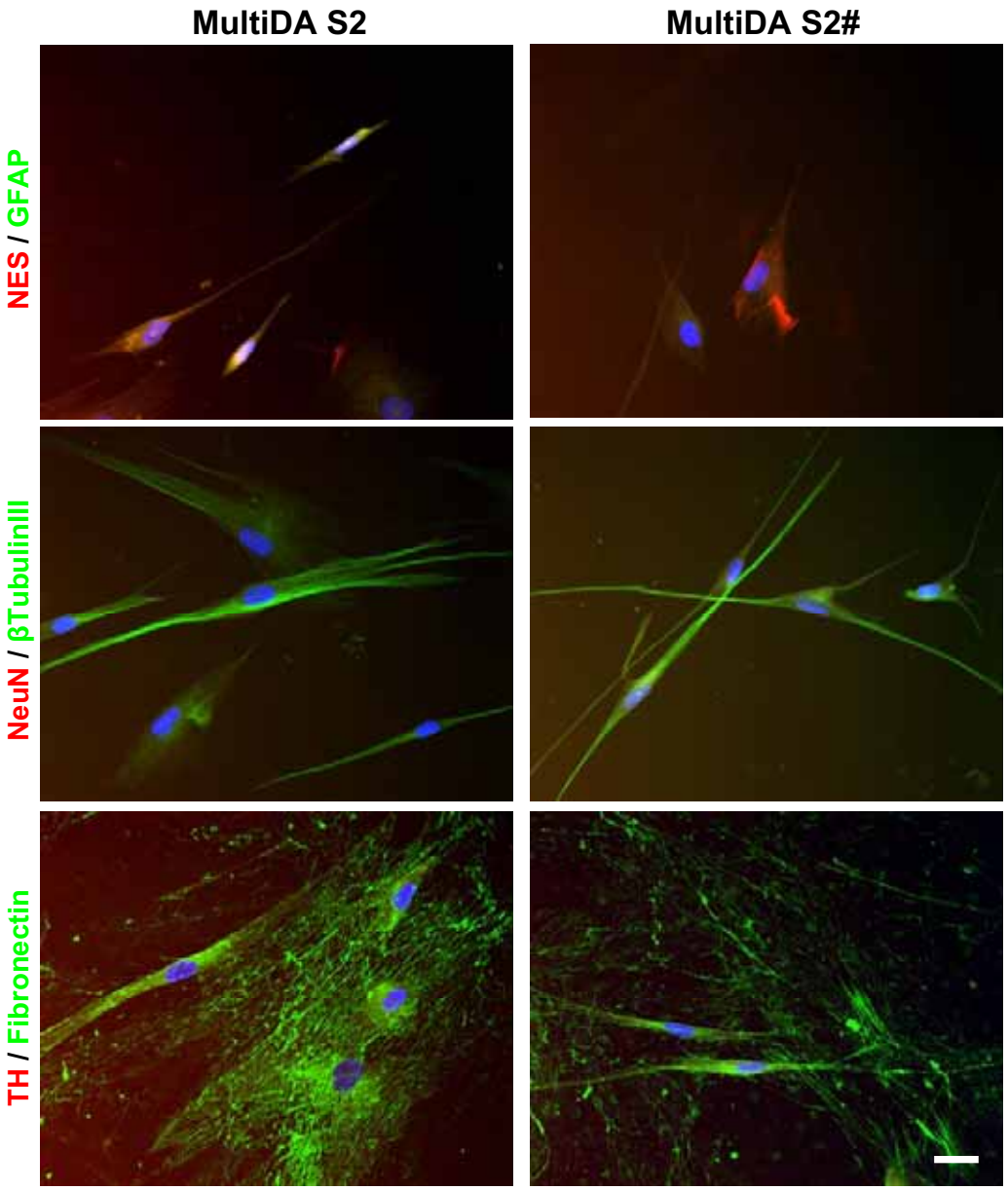

Figure 4.13 Protein expression analysis of hMSCs during *in vitro* cytokine-induced multiple-stage dopaminergic neuronal differentiation (MultiDA) by immunofluorescence microscopy (Part 2).

(Refer to the following pages for Figure 4.13 Part 3, and Figure 4.13 Legend text).

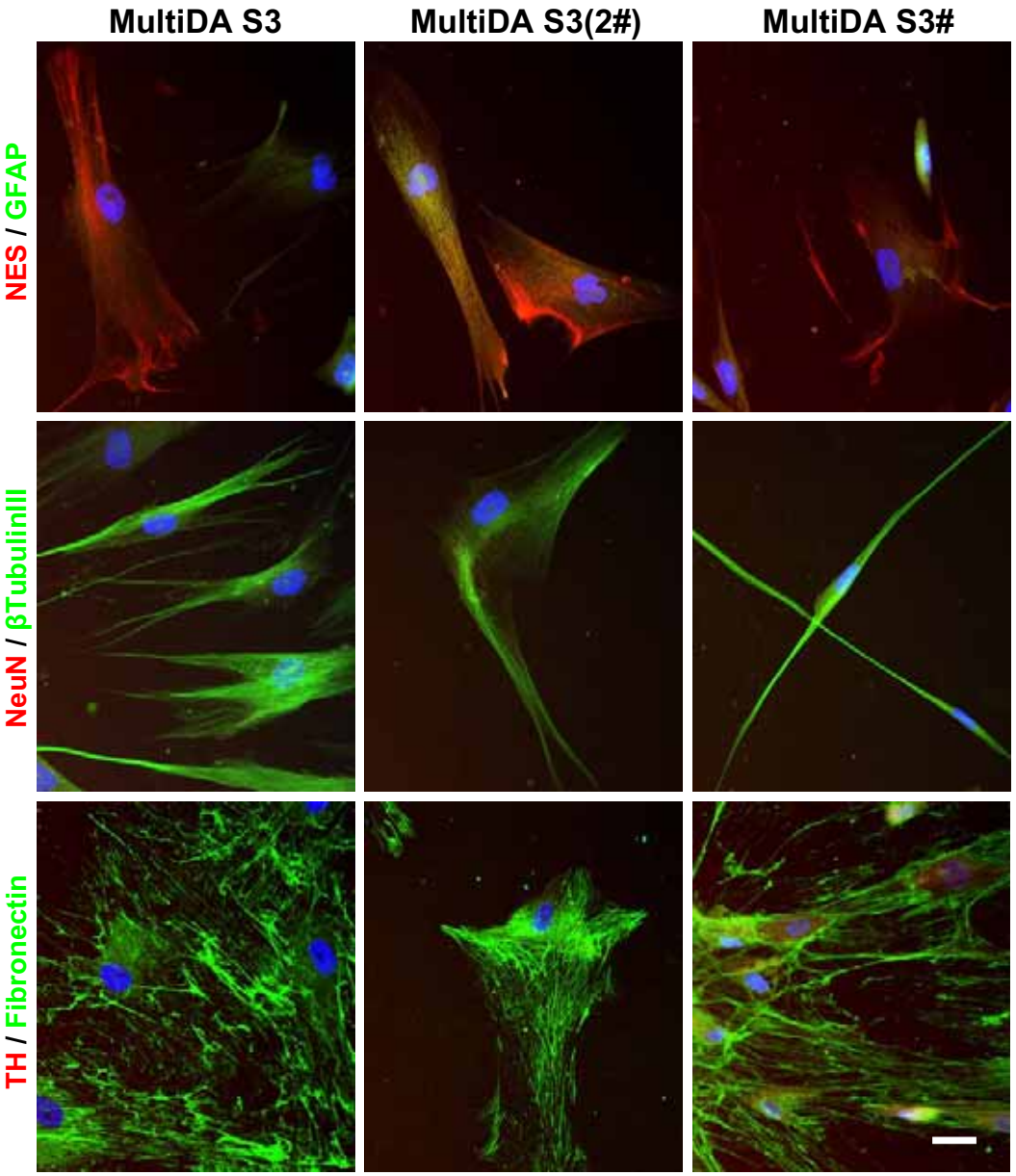

Figure 4.13 Protein expression analysis of hMSCs during *in vitro* cytokine-induced multiple-stage dopaminergic neuronal differentiation (MultiDA) by immunofluorescence microscopy (Part 3).

(Refer to the next page for Figure 4.13 Legend text).

Figure 4.13 Protein expression analysis of hMSCs during *in vitro* cytokine-induced multiple-stage dopaminergic neuronal differentiation (MultiDA) by immunofluorescence microscopy.
Representative images depicting immunofluorescence staining of control undifferentiated hMSC cultures and cultures treated with the MultiDA method ($n = 3$) at the end of each stage (1 week). Cells were examined for expression of neuronal progenitor marker NES, astroglial marker GFAP, neuronal markers NeuN and β tubulin III, dopaminergic neuronal marker TH, and mesodermal marker fibronectin. Cytokine-treated cells were observed to express NES, and up-regulated β tubulin III expression, but other neural markers were not detected. Cells also continued to strongly express fibronectin. These results suggest that only immature neuronal-like cells could be obtained through the MultiDA method. Nuclei were counterstained with DAPI. Negative controls consisted of secondary antibody application alone (not shown). S, Stage; # cytokines from S1 included. Scale bar: 20 μm.

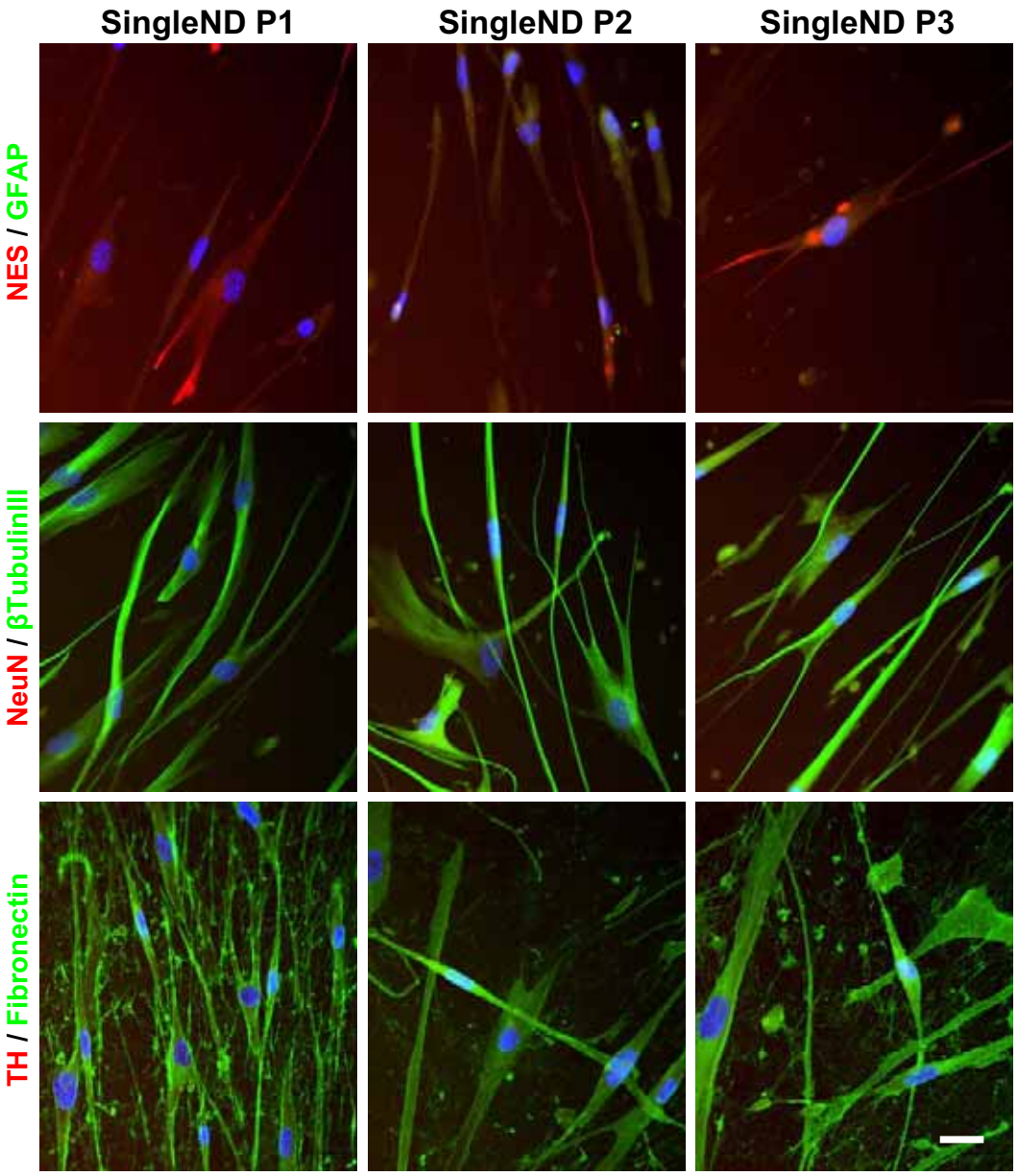

Figure 4.14 Protein expression analysis of hMSCs during *in vitro* cytokine-induced single-stage neuronal differentiation (SingleND) by immunofluorescence microscopy.

(Refer to the next page for Figure 4.14 Legend text).

Figure 4.14 Protein expression analysis of hMSCs during *in vitro* cytokine-induced single-stage neuronal differentiation (SingleND) by immunofluorescence microscopy.

Representative images depicting immunofluorescence staining of hMSC cultures treated with the SingleND method ($n = 3$) at the end of each passage (1 week). Control undifferentiated hMSC cultures are shown in Figure 4.13 (Part 1). Cells were examined for expression of neuronal progenitor marker NES, astroglial marker GFAP, neuronal markers NeuN and β tubulin III, dopaminergic neuronal marker TH, and mesodermal marker fibronectin. The protein expression observed here was similar to that obtained using the MultiDA method. Therefore, both methods only generated immature neuronal-like cells. Nuclei were counterstained with DAPI. Negative controls were performed without primary antibody application (not shown). P, passage. Scale bar: 20 μm.

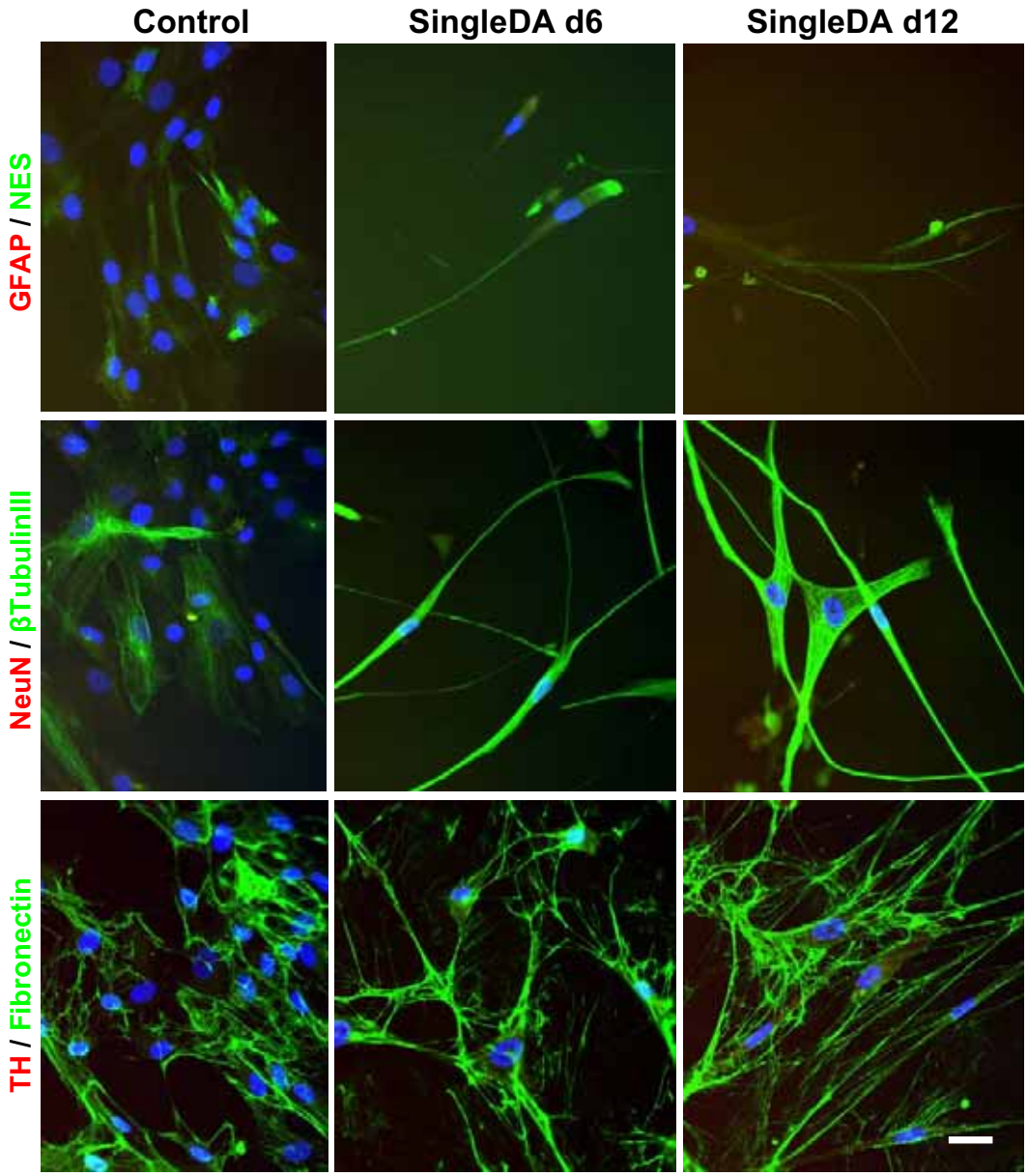

Figure 4.15 Protein expression analysis of hMSCs during *in vitro* cytokine-induced single-stage dopaminergic neuronal differentiation (SingleDA) by immunofluorescence microscopy.

(Refer to the next page for Figure 4.15 Legend text).

Figure 4.15 Protein expression analysis of hMSCs during *in vitro* cytokine-induced single-stage dopaminergic neuronal differentiation (SingleDA) by immunofluorescence microscopy.

Representative images depicting immunofluorescence staining of control undifferentiated hMSC cultures and cultures treated with the SingleDA method at d6 and d12 ($n = 3$). Cells were examined for expression of neuronal progenitor marker NES, astroglial marker GFAP, neuronal markers NeuN and β tubulin III, dopaminergic neuronal marker TH, and mesodermal marker fibronectin. Cells obtained through the SingleDA method displayed similar protein expression as those obtained using the MultiDA method and the SingleND method. Therefore, all three neuronal differentiation procedures investigated were only capable of generating immature neuronal-like cells. Nuclei were counterstained with DAPI. Negative controls were performed without primary antibody application (not shown). d, day. Scale bar: 20 μm.

Table 4.2 Summary of hMSC Cytokine-Based Neuronal Differentiation

Analysis	MultiDA	MultiDA#	SingleND	SingleDA
Morphology	+ / ++	+++	+++	+++
Gene Expression	++	+++	++ / +++	++
Protein Expression	++	++	++	++
Cell Number	~	~~	~~~	~~~

: Cytokines from Stage 1 of MultiDA method included in later stages

+ : Indicates the degree of neuronal-like characteristics obtained

~ : Indicates the degree of cell survival obtained

4.3 Discussion

The results presented in this chapter have demonstrated that (1) hMSCs are capable of acquiring an immature neuronal phenotype upon stimulation with cytokines; (2) cytokine-based neuronal differentiation of hMSCs involves active and dynamic processes in response to differentiation stimuli, including the outgrowth and motility of cellular extensions; (3) long-term subcultivation of hMSC cultures does not compromise the ability of hMSCs to differentiate toward an immature neural fate; (4) treatment of hMSCs with different cytokine combinations in the MultiDA, SingleND and SingleDA procedures elicits different effects on cellular morphology and gene expression, however, none of these methods is capable of generating a mature midbrain dopaminergic neuronal phenotype from hMSCs; and (5) continual supplementation with FGF-2, and perhaps also EGF, appears beneficial for the *in vitro* neuronal differentiation of hMSCs, at least in terms of cellular morphology and gene expression.

4.3.1 Cytokine-Based *In Vitro* Neuronal Differentiation of hMSCs Elicits Dynamic Cellular Responses

The current study demonstrates for the first time that *in vitro* neuronal differentiation of hMSCs using cytokines elicits an entirely distinct cellular response in comparison with the "simple chemical induction protocol" described by Woodbury and colleagues (Woodbury *et al.*, 2000). Cells exposed to the SingleND cytokine cocktail (FGF-2/EGF/PDGF) were highly active and motile, displaying dynamic extension and movement of neurite-like processes and structures similar to neuronal growth cones, as well as the formation of transient branching morphology, as recorded in live cell imaging videos. These observations differ greatly from recent reports describing the

changes in cellular morphology generated by chemical induction (DMSO/BHA or IBMX/cAMP) to be the result of cytotoxic effects of added chemicals leading to cell death, rapid disruption of the actin cytoskeleton and retraction of selected areas of the cell perimeter and cytoplasm, leaving behind filopodium-like processes formed from strong focal adhesions to the substrate (Bertani *et al.*, 2005; Choi *et al.*, 2006; Deng *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004; Suon *et al.*, 2004).

In addition, cytokine-induced hMSCs progressively attained neuronal-like morphology over the first week of culture and maintained these changes for at least 1 month *in vitro*. Viability of cultures was also consistently maintained at >85% over the course of cytokine-induced neuronal differentiation. The time frame of these changes was in terms of days and weeks, rather than hours, and allowed sufficient time for changes in cellular organisation and gene/protein expression, unlike the rapid conversion (within hours) observed with chemical treatment. Furthermore, MSCs could not be maintained in DMSO/BHA serum-free conditions, because of increased cell death (50% death within 72 hours) and detachment from the culture surface (Lu *et al.*, 2004; Tao *et al.*, 2005). In addition, subculturing DMSO/BHA-treated cells was not possible due to the failure of these cells to re-attach following trypsinisation. Therefore, the alterations in cell phenotype induced by cytokine-based neuronal differentiation of hMSCs are not simply due to culture artifacts, but can be attributed to active and dynamic cellular processes.

4.3.2 Long-Term Subcultivation of hMSCs Does Not Affect Differentiation Towards an Immature Neuronal-Like Phenotype

The effect of long-term serial passage on the neural differentiation capability of hMSCs has not been previously reported. From the examination of hMSCs over long-term *in vitro* culturing (results presented in Chapter 3) it was determined that extensive

subcultivation did not cause spontaneous neural differentiation (Khoo *et al.*, 2008). In this chapter, we found that late passage cultures (P11–12) were able to undergo morphological and gene expression changes toward neuronal-like cells similar to early passage cultures (P4–5), despite loss of proliferation and symmetric division abilities. However, a trend of increased *GFAP* expression in late passage cultures was seen that may affect differentiation toward a mature neural phenotype.

It is not yet clear why late passage cultures retain this ability when other SC qualities are lost, but the retention of *POU5F1* and *NANOG* expression may play a role, since it has been shown that *POU5F1* expression in mouse ESCs is involved in neurogenesis (Chen *et al.*, 2007a). Maintenance of NES expression may also be involved, since induction of NES in rat BM MSCs by prolonged culture in serum-free conditions, has been reported to be a prerequisite for neural lineage differentiation (Wislet-Gendebien *et al.*, 2003). However, this report differs from the study presented in this chapter as 10 passages, or more, in serum-free conditions were necessary for the expression of NES by rat MSCs (Wislet-Gendebien *et al.*, 2003), whereas, the hMSCs employed here were found to constitutively express NES in the presence of serum. In addition, another study reported that long-term cultivation (6 weeks without subculturing) of P1 rat MSCs resulted in spontaneous enrichment of NES-positive cells, which exhibited greater sensitivity to neuronal induction by serum deprivation and growth factor supplementation (Tseng *et al.*, 2007). A number of variations exist between these studies, including the species, isolation methods and culturing techniques employed, which may account for the differences observed.

Nevertheless, while early and late passage hMSC cultures were equally capable of undergoing cytokine-based neuronal differentiation, early passage cells have distinct advantages over late passage hMSCs. The benefits associated with early passage

hMSCs were shown in Chapter 3, and include a higher proliferative ability, and maintenance of a more stable phenotype in terms of morphology and gene/protein expression (Khoo *et al.*, 2008). Therefore, in our subsequent neuronal differentiation studies we have only utilised hMSCs harvested from early passage cultures.

4.3.3 Undifferentiated hMSCs Express Neuronal and Dopaminergic Neuronal Markers

Consistent with the data presented in Chapter 3, hMSCs were found to express neuroectodermal markers prior to the commencement of neuronal differentiation procedures. The current findings extend these previous observations that hMSCs express NES, *MAP-2*, *GFAP* and *CSPG4*, by demonstrating the expression of further neuronal (*ENO2* and β tubulin III) and dopaminergic neuronal markers (*NR4A2* and *TH*) in undifferentiated hMSCs. These results reflect those of other recent studies, which have also shown expression of key markers of the neuro-dopaminergic system by rat and human MSCs, including TH, *NR4A2*, *PITX3*, *EN1*, aldehyde dehydrogenase 1, aromatic L-amino acid decarboxylase, catechol-o-methyltransferase, GTP cyclohydrolase-1, *OTX-1*, SHH receptor components (patched (*PTC*) and smoothened (*SMO*)), and GDNF family receptor alpha 1 (*GFRA1*) (Blondheim *et al.*, 2006; Kramer *et al.*, 2006).

Expression of these neuroectodermal and dopaminergic neuronal markers demands that caution be taken in the interpretation of hMSC neural differentiation, particularly through examination of neural markers prior to differentiation as well as post-differentiation. However, these observations also provide support for the neural and dopaminergic neuronal differentiation capacity of hMSCs (Hermann *et al.*, 2006).

Furthermore, the constitutive *NR4A2* expression present in hMSCs suggests that these cells may be viable candidates for cellular replacement therapies for PD,

particularly since transfection of ESCs with *NR4A2* was considered to be crucial for the successful generation of ESC-derived dopaminergic neurons (Kim *et al.*, 2002b). This study by Kim and colleagues demonstrated that stable transfection of murine ESCs with *NR4A2* in conjunction with a five-stage dopaminergic neuronal differentiation method could generate TH-expressing neurons with electrophysiological activity and the ability to promote significant behavioural recovery in transplanted Parkinsonian rats.

4.3.4 Sequential Application of Cytokines Involved in Midbrain Dopaminergic Neuronal Development Did Not Enhance Dopaminergic Neuronal Differentiation of hMSCs

In view of my experimental findings that cytokine-based neuronal differentiation of hMSCs yields actual changes in cellular morphology (Section 4.2.1) and gene/protein expression (Section 4.2.2), I endeavoured to determine whether cytokines involved in midbrain dopaminergic neuronal development *in vivo* could induce hMSCs toward this neuronal lineage. Furthermore, a multiple-stage approach (MultiDA) was employed to mimic the *in vivo* situation, and this method was compared to two single step neuronal differentiation procedures (SingleND (Tao *et al.*, 2005) and SingleDA (Trzaska *et al.*, 2007)). Despite the different cytokines, surface coatings and culturing procedures employed, all three methods were effective to a similar extent and were only capable of generating immature neuronal-like cells. Sequential supplementation with cytokines in the MultiDA procedure did not direct hMSCs toward a mature dopaminergic neuronal fate, however, neither did the published SingleDA method.

Interestingly, slightly different responses were elicited in hMSCs by the three different methods. Examination of cellular morphology revealed the acquisition of a neuronal-like bipolar or multipolar morphology with long thin cellular extensions and occasional branching in all methods studied, which resembled the morphology obtained

during the live cell imaging analysis. The first stage of the MultiDA method was unique in the spontaneous formation of cellular aggregates resembling neurosphere-like structures. In addition, investigation of gene expression showed significant up-regulation of *NES* and *GFAP* in stages 1 and 2 of the MultiDA method, as well as a trend of higher *NR4A2* expression. However, protein expression analysis through immunofluorescent staining did not show clear distinctions between the three methods.

In contrast, a study comparing the effects of different neuronal differentiation protocols by Hermann and colleagues (Hermann *et al.*, 2006) reported that a sequential two-step protocol involving neurosphere formation could induce significant neural conversion of hMSCs, whereas other single-step direct neural conversion methods (published in (Sanchez-Ramos *et al.*, 2000; Woodbury *et al.*, 2000), and a modified method from (Hermann *et al.*, 2004)) showed no significant differences in comparison to undifferentiated hMSCs. Furthermore, a multi-step technique was also employed by Jiang, *et al*. (Jiang *et al.*, 2003) that was capable of successfully deriving a mature electrophysiologically-active neuronal phenotype from murine MAPCs following astrocyte co-culture. While the MultiDA procedure displayed promising results favouring neural conversion of hMSCs in the early phases of culture, subsequent to this the cells then appeared to revert slightly toward a MSC-like phenotype by the final MultiDA stage, in terms of morphology and gene expression.

Observations that cultures were not maintained well in the later phases of the MultiDA culture system raise possible questions regarding the responsiveness of hMSCs to SHH, FGF-8 and GDNF. Previous reports have established that addition of these cytokines is beneficial for the *in vitro* dopaminergic neuronal differentiation of MSCs (Dezawa *et al.*, 2004; Fu *et al.*, 2006b; Jiang *et al.*, 2003; Trzaska *et al.*, 2007). In addition, MSCs have been shown to express both components of the SHH receptor,

PTC and *SMO* (Kramer *et al.*, 2006; Trzaska *et al.*, 2007), as well as FGF receptors *FGFR1* and *FGFR4* (Kramer *et al.*, 2006). Furthermore, treatment of MSCs with SHH and FGF-8 revealed increased DNA synthesis indicating that these receptors were functional (Kramer *et al.*, 2006). However, it was also found that only the *GFRA1* component of the GDNF receptor was present on MSCs, whereas the *c-RET* component was absent, rendering MSCs incapable of responding to stimulation with GDNF (Kramer *et al.*, 2006).

These results suggest possible variability in MSC expression of cytokine receptors, which may account for later parts of the MultiDA method failing to elicit the expected differentiation effects. Underlying factors that should also be taken into consideration include differences in the isolation procedures, species, culturing methods, and cytokine concentrations used across the different studies.

4.3.5 Beneficial Effect of FGF-2 and EGF in hMSC Neuronal Differentiation

The reversion phenomenon observed in the MultiDA procedure could be prevented by continuous addition of S1 cytokines (FGF-2 and EGF) to all MultiDA stages, which maintained the expression of neural genes and a refractile neuronal-like morphology, as well as inhibited the up-regulation of mesodermal gene *COL1A1*. Moreover, significant differences were observed between the gene expression of cells cultured in the presence or absence of FGF-2/EGF in stages 2 and 3 of the MultiDA method, including significantly higher expression of *NES*, *NR4A2*, *MAP-2*, *NANOG*, *ENO2*, and *GFAP* in FGF-2/EGF-containing cultures, and significantly lower expression of *COL1A1*. Although continued FGF-2/EGF supplementation appeared capable of maintaining an immature neuronal-like phenotype in MultiDA-treated hMSCs, no further advancement of neuronal-like characteristics could be observed.

Many other studies have achieved neuronal differentiation of MSCs through the application of FGF-2 (Jiang *et al.*, 2002; Kim *et al.*, 2002a; Suzuki *et al.*, 2004), EGF (Sanchez-Ramos *et al.*, 2000), or FGF-2 and EGF (Hermann *et al.*, 2004; Jin *et al.*, 2003; Suon *et al.*, 2006; Tao *et al.*, 2005), in conjunction with other differentiation agents, providing independent support of the usefulness of these cytokines for this purpose (discussed further in Section 1.3.1). Nevertheless, prior to use in MSC differentiation protocols, FGF-2 and EGF were described as critical factors for stimulating survival and proliferation of rodent multipotential neural progenitor/stem cells in culture (Gritti *et al.*, 1999; Gritti *et al.*, 1996; Ray *et al.*, 1993; Reynolds *et al.*, 1992; Richards *et al.*, 1992), and *in vivo* (Kuhn *et al.*, 1997; Wagner *et al.*, 1999). This role in regulating neuronal progenitors and SCs, therefore, opens the possibility that the continued addition of these cytokines in later stages of the MultiDA method may cause the retention of cells in an immature neuronal-like or neuronal progenitor-like state.

4.3.6 Recently Published SingleDA Method Yielded Similar Immature Neuronal-Like Cells as the MultiDA and SingleND Procedures

A single-step cytokine-based procedure for inducing a dopaminergic neuronal phenotype from hMSCs was reported by Trzaska and colleagues during the course of our studies (Trzaska *et al.*, 2007). This method utilised a cocktail of cytokines similar to those which were implemented in our work, i.e. SHH, FGF-8, and FGF-2, and generated promising results including the highest reported efficiency of TH-positive cell production (~67%), expression of dopaminergic-specific genes, synthesis and secretion of DA, and the presence of electrophysiological properties comparable with immature neurons. The period of differentiation was brief and only encompassed 12 days, in comparison with the 3 week differentiation period of our MultiDA and SingleND methods. However, despite detection of DA release *in vitro*, this was not found to occur

in response to depolarisation through elevated extracellular K^+, and instead DA was found to be constitutively released into the culture medium.

Examination of the excitable properties provided further confirmation that the cells obtained were not mature neurons, with the whole-cell patch clamp technique only detecting robust outward K^+ currents, while inward Na^+ and Ca^{++} currents were only weakly exhibited in a small fraction of cells, and spontaneous or evoked action potentials were not detected. In addition, although Trzaska and colleagues demonstrated up-regulation of dopaminergic-specific markers, the examination of mesodermal and MSC markers was not conducted, and in order to show clear dopaminergic neuronal differentiation, one would expect to observe concomitant down-regulation of markers of MSCs and the mesodermal lineage.

Since this recent report still demonstrated the most promising results for the derivation of DA-producing cells from hMSCs via a cytokine-based procedure, the SingleDA method was also examined for comparison with the MultiDA and SingleND systems. Using the SingleDA method morphological changes and up-regulation of NES and β tubulin III expression similar to that reported by Trzaska, *et al*. could be obtained, and slight increases in *TH* expression were also detected; however, the majority of the results obtained were dissimilar. In addition, increased expression of dopaminergic neuronal genes (including *NR4A2*, *EN2*, and *DAT*), and neuronal proteins (including TH and NeuN) could not be detected. As a result, the SingleDA method could only generate immature neuronal-like cells from the hMSC populations studied, and did not appear to provide any significant advantages over the MultiDA and SingleND methods.

4.3.7 Transcription Factor Expression During hMSC Dopaminergic Neuronal Differentiation

According to current knowledge, the development of midbrain dopaminergic neurons *in vivo* is also dependent on cell intrinsic programs, of which several critical transcription factors have been identified (discussed further in Section 1.4.3.2). It is thought that MSCs undergoing dopaminergic neuronal differentiation would elicit transcription factor networks similar to those involved in dopaminergic neuron development *in vivo*. Therefore, I investigated the involvement of several of these crucial transcription factors (*NR4A2*, *PITX3*, and *EN2*) during the course of the MultiDA, SingleND and SingleDA procedures.

Of the transcription factors examined, *NR4A2* was the most highly and consistently expressed across the various culture methods. However, apart from S1 and S2# of the MultiDA method, all treatments showed a trend of decreased expression compared to the control. Whereas for *PITX3* and *EN2*, expression was not detected in all samples, and in general, there was little change in expression with the three different treatments, although a non-significant trend of increased expression was observed. Taken together, these results indicate that exposure to the three differentiation methods did not direct hMSCs toward a dopaminergic neuronal fate. However, of the different treatments examined the FGF-2/EGF-containing stages of the MultiDA method appeared to induce the greatest up-regulation of *NR4A2*, *EN2* and *PITX3* expression, although this did not reach a level of significance.

Since the transcription factors studied here are thought to be involved in the differentiation and survival of dopaminergic neurons, rather than the initial specification and generation of midbrain dopaminergic precursors, more information may be garnered from examining earlier transcription factors, particularly since the hMSC-

derived neuronal-like cells in the present study appear fairly immature. Earlier transcription factors that could be investigated include LMX1A, msh homeobox 1, and NEUROG2 (discussed in Section 1.4.3.2), as well as FOXA2, which has recently been described to regulate the specification, neurogenesis and differentiation of midbrain dopaminergic neurons (Arenas, 2008; Kittappa *et al.*, 2007).

4.3.8 Continued hMSC Expression of Mesodermal and Pluripotency Markers Following *In Vitro* Neuronal Differentiation

The cytokine induction methods employed in this chapter were only successful in driving hMSCs toward an early neuronal fate, with cells continuing to express neuronal progenitor marker NES and pluripotency markers *POU5F1* and *NANOG*. Furthermore, the persistence of mesodermal and glial markers, together with the pluripotency markers, suggests that additional differentiation signals are required for attaining proper neuronal differentiation and maturation. A comparative study conducted by Hermann and colleagues (Hermann *et al.*, 2006) also found continued expression of mesodermal marker fibronectin in certain neuronal differentiation methods investigated, including the initial methods reported for MSC neuronal differentiation using cytokine-based (Sanchez-Ramos *et al.*, 2000) and chemical-based systems (Woodbury *et al.*, 2000). Examination of mesodermal lineage markers was not performed in these initial reports, therefore, the findings of Hermann and colleagues highlight the importance of showing concomitant down-regulation of mesodermal and MSC markers with neuronal differentiation.

The findings of this thesis and those of Hermann, *et al*. suggest that further differentiation signals or factors may be required for the *in vitro* neural differentiation of MSCs, particularly since a complex milieu of cytokines and signalling molecules are believed to be present in neural development *in vivo*. A number of potential factors have

been identified to provide advantages for the neuronal and dopaminergic neuronal differentiation of MSCs. One such factor involves co-culturing MSCs together with neural support cells, such as astrocytes, which may function through direct cell-cell interactions, trophic factor production, or removal of glutamate from the culture environment (Jiang *et al.*, 2003). Support for this approach has also been shown in a study examining ESC and astrocyte co-culture systems, which found that the type of neurons obtained were dependent upon the regional origin of the astrocytes, with mesencephalic astrocytes strongly potentiating dopaminergic neurogenesis (Roy *et al.*, 2006). Another approach similar to the co-culturing method is the supplementation of cultures with conditioned medium from neural support cells (Joannides *et al.*, 2003).

Furthermore, other studies have described improved MSC neuronal differentiation through the addition of fatty acids, such as docosahexaenoic acid and arachidonic acid (Kan *et al.*, 2007b), and inflammatory mediator IL-1α (Cho *et al.*, 2005; Greco *et al.*, 2007a); whilst, still others have suggested that beneficial effects could be produced through additional supplementation with RA, BDNF and/or NGF (Trzaska *et al.*, 2007), or through the optimisation of culture surface nanotopography (Yim *et al.*, 2007). Aside from the improvement and optimisation of culture components, the time required for *in vitro* dopaminergic neuronal differentiation requires careful consideration, as it may differ between species and may also differ to that required for other neuronal cell types.

In an earlier study, Bjorklund and colleagues transplanted ESCs into the 6-OHDA unilaterally-lesioned Parkinsonian rat and demonstrated that the time course of behavioural recovery corresponded with the developmental rate (i.e. length of gestational period) of the donor species (Bjorklund *et al.*, 2002). Thus, if dopaminergic differentiation of hMSCs does indeed mimic that which occurs during gestation, then it

would be expected that longer periods are required for the differentiation of human cells than for rodent cells. Evidence supporting this has been shown in a meta-analysis of studies which found that behavioural recovery in Parkinsonian rats occurred approximately 5 weeks after mouse foetal ventral mesencephalon transplantation, while for the equivalent human cells, recovery only occurred after approximately 20 weeks (Isacson *et al.*, 1997).

Longer periods of *in vitro* hMSC differentiation may therefore be required, particularly since most protocols, including our own, have only been conducted over several weeks, which is considerably shorter than the 20 week period reported for *in vivo* behavioural recovery using human foetal cells. However, it should be noted that neural differentiation of hMSCs may be divergent from that occurring in normal physiological development, particularly since undifferentiated hMSCs have been shown to constitutively express some neural markers.

While the current study was being completed, a number of reports were published describing the induction of a dopaminergic phenotype in MSCs. The procedures employed included: multiple-stage application of cytokines FGF-2 (1 week), SHH+FGF-8 (1 week) followed by BDNF (1 week) (Bouchez *et al.*, 2008); treatment with FGF-2/EGF for 48 hours as step 1, then application of BHA, dbcAMP, IBMX, RA and GDNF for 96 hours as step 2 (Kan *et al.*, 2007a), and a later study by the same group, which found improved TH expression with addition of dbcAMP, IBMX and AA with BDNF alone or GDNF/RA/TGF-β3 in the second step (Barzilay *et al.*, 2008); application of $CoCl_2$ and Y-27632, to presumably cause hypoxia-inducible factor 1 activation and Rho kinase inhibition (Pacary *et al.*, 2006); and treatment with cytokines FGF-2 and GDNF, and 10% foetal calf serum, for 2 weeks (Zhang *et al.*, 2008). A varying degree of success was achieved, including depolarisation-induced secretion of

DA (Barzilay *et al.*, 2008; Kan *et al.*, 2007a; Zhang *et al.*, 2008), and reduction of behavioural deficits in a Parkinsonian rat model (Bouchez *et al.*, 2008). Furthermore, it was demonstrated for the first time that hMSCs isolated from PD patients could be induced toward a DA-secreting neuronal phenotype, with expression of TH protein in approximately 30% of cells (Zhang *et al.*, 2008).

Another study examining a subpopulation of MSCs, termed marrow-isolated adult multilineage inducible (MIAMI) cells, also described an alternative multi-step procedure for the generation of TH-positive cells (Tatard *et al.*, 2007). This method involved neural specification (step 1) with FGF-2 and 5-20% FBS for 24 hours, neuronal commitment (step 2) using BME, neurotrophin-3, SHH, FGF-8, and RA for 2 days, and finally, neuronal differentiation (step 3) with BHA, KCl, VPA, forskolin, hydrocortisone, insulin, neurotrophin-3, NGF, BDNF, and GDNF for 3-7 days. However, while research efforts are continuing to reveal new insights into the induction of a dopaminergic phenotype from MSCs, further improvements and optimisation of established techniques is still very much desired.

4.3.9 Functional Assessment of hMSC-Derived Neuronal-Like Cells is Required

The present study focused on examining hMSC neuronal differentiation *in vitro* by morphological and gene and protein expression analysis; however, true neuronal differentiation must be confirmed by investigating the electrophysiological properties and *in vivo* function of neuronal-like cells. The function of immature neuronal cells could be investigated by analysing electrophysiological function (Ambrogini *et al.*, 2004), performing further *in vitro* differentiation to attain a mature phenotype capable of firing voltage-gated action potentials and neurotransmitter-mediated synaptic communication with other neurons, or by transplantation to demonstrate functional integration in the host brain and alteration of host behaviour.

The cells described in this chapter exhibit an immature neuronal phenotype, therefore, future experiments are warranted for investigating whether the electrophysiological properties of these neuronal-like cells resembles that of immature neurons. Rat MSC-derived neuronal-like cells obtained through co-culture with mouse cerebellar granule neurons have been shown to acquire the electrophysiological properties of maturing newborn adult neurons (Wislet-Gendebien *et al.*, 2005), according to the maturation stages described by Carleton, *et al*. (Carleton *et al.*, 2003). These cells appear to progress through the first stage of neuronal maturation with cells displaying a low resting membrane potential and response to some neurotransmitters, but absence of Na^+ currents and spiking behaviour.

A second stage of maturation could also be reached with cells exhibiting both Na^+ and K^+ currents, a more negative resting membrane potential and the ability to fire single-spike action potentials. However, the cells obtained by Wislet-Gendebien, *et al*. were unable to reach the final stage of maturation, i.e. mature neurons capable of firing trains of spikes, exhibiting strong synaptic activities and possessing a very negative resting membrane potential (Wislet-Gendebien *et al.*, 2005). A number of other studies have also found evidence for the acquisition of electrophysiological properties by MSCs that supports the neuronal differentiation potential of these cells (Cho *et al.*, 2005; Dezawa *et al.*, 2004; Hung *et al.*, 2002; Jiang *et al.*, 2003; Kohyama *et al.*, 2001; Mareschi *et al.*, 2006; Song *et al.*, 2007; Tropel *et al.*, 2006).

Achievement of functional mature neurons would have potential clinical relevance for the treatment of neurodegenerative diseases, although, prior rigorous testing in animal models is necessary. In addition, the use of immature but committed neurons may prove advantageous for increasing survival and functional integration post-transplantation, particularly since terminally differentiated neurons with elaborate

axonal connections are known to poorly survive detachment and subsequent transplantation procedures (Bjorklund *et al.*, 2000; Hermann *et al.*, 2004).

4.3.10 Contaminating Cells, Culture Artifacts or Cell-Cell Fusion Events Cannot Explain the Observed hMSC Neuronal Differentiation

As described earlier, transdifferentiation has recently been met with skepticism arising from experimental findings over the past few years that have questioned the authenticity of observations of transdifferentiation, especially neuronal differentiation from MSC populations. These findings dictate the need for caution when interpreting the results of transdifferentiation studies. The main areas of concern include the possibilities that MSC neural transdifferentiation events may actually be due to: (1) contaminating cells of the neural lineage present within heterogeneous MSC populations; (2) rare culture artifacts resulting from repeated *in vitro* passaging or toxicity of differentiation agents; and (3) possible fusion of MSCs with neural cells present in the culture system or *in vivo* following transplantation (Jin *et al.*, 2003).

In the present study hMSCs were isolated by negative selection from BM aspirates and cultured under conditions favouring MSC expansion. The BM is innervated by a rich network of sympathetic autonomic nerve fibers, but the cell bodies of these neurons resides in paravertebral sympathetic ganglia (Calvo, 1968; Yamazaki *et al.*, 1990), therefore, it is unlikely that neural cells would be present in these cultures. Furthermore, hMSCs were passaged 4-6 times prior to differentiation in MSC culture medium lacking the factors necessary for *in vitro* neuronal maintenance, so that even if neuronal cells were initially present, these contaminating cells would be lost over the expansion period.

In addition, following cytokine-based differentiation neuronal-like characteristics were acquired by the majority of cells, which is inconsistent with the

possibility of a small contaminating subpopulation of cells being responsible for the detection of a neuronal phenotype. Moreover, I have also demonstrated that these findings are unlikely to be caused by culture artifacts through the real-time analysis of differentiation using live cell imaging techniques, as well as examination of long-term serial passaging, which showed no significant changes to the phenotype of MSCs during the first 6-8 passages of culture, and the absence of spontaneous neural differentiation. Finally, neural cells were not utilised in our differentiation systems, therefore, these findings cannot be the result of cell-cell fusion events.

4.3.11 Summary

In conclusion, this study has demonstrated that cytokine-based neuronal induction of hMSCs elicits changes resulting from active and dynamic processes that involve outgrowth and motility of cellular extensions, which to my knowledge has not been previously reported. These observations were found to be entirely distinct to the rapid epiphenomena of cytotoxicity and cytoskeleton disruption associated with chemical-based neuronal induction methods. Furthermore, it was shown that long-term subcultivation of hMSCs did not compromise the ability of these cells to differentiate toward an early neuronal fate. In addition, cytokine-induced neuronal differentiation of hMSCs through the MultiDA, SingleND and SingleDA procedures were associated with up-regulation of genes and proteins indicative of an immature neuronal phenotype. However, none of the methods investigated was capable of generating mature midbrain dopaminergic neurons, although, the MultiDA method was found to yield the highest increases in NES and *NR4A2* expression. As a result, further analysis was performed in Chapter 5 using hMSCs exposed to the first stage of the MultiDA method (FGF-2/EGF). Next, the functional capacity of these phenotypically immature hMSC-derived

neuronal cells, as well as undifferentiated hMSCs, were assessed through transplantation into the 6-OHDA unilaterally-lesioned rat model of PD.

CHAPTER 5

IN VIVO STUDIES IN AN ANIMAL MODEL OF PARKINSON'S DISEASE

5.1 Introduction

In vitro and *in vivo* studies describing the neurogenic potential and disease ameliorating effects of MSCs have stimulated interest and support for applying MSCs in cellular treatments of CNS diseases and injuries. The use of MSCs for cell-based therapies is associated with further advantages, such as, overcoming of ethical and logistical limitations tied to the use of ESCs and foetal tissue-derived cells, and also avoidance of possibilities of transplant rejection and teratoma formation. However, the fate and function of MSCs following transplantation *in vivo* remains a topic of much debate and investigation.

The initial reports establishing the engraftment of MSCs and restoration of neural deficits following intracerebral, intravenous or intra-arterial transplantation were reviewed in Section 1.2.3. Engraftment and migration of MSCs was observed in neonatal mice and embryonic rats following injection into the lateral ventricles (Kopen *et al.*, 1999; Munoz-Elias *et al.*, 2004), as well as in adult rats after direct injection into the uninjured corpus striatum (Azizi *et al.*, 1998). Furthermore, these studies demonstrated efficacy of MSC transplantation in promoting neurological recovery in rodent models of cerebral ischemia (Chen *et al.*, 2001; Li *et al.*, 2000; Zhao *et al.*, 2002) and traumatic brain injury (Lu *et al.*, 2001a; Mahmood *et al.*, 2003; Mahmood *et al.*, 2002; Mahmood *et al.*, 2001). Initially it was believed that neurological recovery occurred as a result of conversion of MSCs to a neural phenotype and integration into host circuitry, although more recently it has been suggested that recovery may instead be related to the secretion of trophic factors by MSCs, or interaction of MSCs with host cells (Chen *et al.*, 2001; Li *et al.*, 2001a; Zhao *et al.*, 2002). In addition, further mechanisms have been proposed to explain observations of MSC-mediated functional improvement in animal disease models, including modulation of immune responses,

mitochondrial transfer and stimulation of endogenous host responses (Phinney *et al.*, 2007; Prockop, 2007).

Cellular transplantation therapy has long been thought to hold great potential for the treatment of PD, since this chronic neurodegenerative disorder results from the selective loss of dopaminergic neurons in a distinct region of the brain (see Section 1.4.1 and 1.4.2 for further details). A variety of cell types have been examined in the search for a potential renewable source of DA-producing cells, including human foetal brain tissue (Brundin *et al.*, 1986; Brundin *et al.*, 1988; Stromberg *et al.*, 1986), ESCs (Bjorklund *et al.*, 2002; Kim *et al.*, 2002b; Lee *et al.*, 2000), and adult NSCs and neural progenitors (Ostenfeld *et al.*, 2000; Studer *et al.*, 1998; Svendsen *et al.*, 1997; Svendsen *et al.*, 1996). These studies have yielded encouraging findings in animal models including graft survival, DA production and alleviation of lesion-induced motor deficits, providing evidence supporting the concept that cellular transplantation therapy may be viable for PD. Furthermore, while clinical trials examining transplantation of human foetal mesencephalic tissue in PD patients have produced conflicting results in the past, the findings of recent studies have restored hope in the possibility of future cell-based therapies for PD (Kordower *et al.*, 2008; Li *et al.*, 2008; Mendez *et al.*, 2008; Piccini *et al.*, 1999).

As described earlier, MSCs appear to have great potential for application in cellular therapies. Support for the potential utility of MSCs in PD therapy has been shown through studies demonstrating the acquisition of dopaminergic neuronal traits by MSCs *in vitro*. The findings of our *in vitro* neuronal differentiation studies presented in Chapter 4 suggest that MSCs are capable of acquiring markers typical of immature dopaminergic neurons, and further reports discussed in Section 1.4.4 have also described the expression of a range of dopaminergic specific markers, presence of

electrophysiologically active voltage-gated sodium channels, and/or DA secretion in response to depolarising stimuli (Dezawa *et al.*, 2004; Fu *et al.*, 2006b; Guo *et al.*, 2005; Hermann *et al.*, 2004; Jiang *et al.*, 2003; Suon *et al.*, 2006; Trzaska *et al.*, 2007). In addition, engraftment and functional improvement have been demonstrated following transplantation of undifferentiated MSCs (Hellmann *et al.*, 2006; Li *et al.*, 2001b) and differentiated MSCs (Dezawa *et al.*, 2004; Fu *et al.*, 2006b) in rodent models of PD. These studies have been reviewed in Section 1.4.4.

However, in these reports a range of approaches have been taken with some conflicting results obtained, and furthermore the studies are not agreed on which methodology produces the optimal outcomes. As a result, a number of pertinent issues remain to be resolved, including the engraftment and survival capability of undifferentiated and partially dopaminergic-differentiated MSCs post-transplantation, the optimal site for intracerebral transplantation, the potential immunological responses to undifferentiated and differentiated MSC grafts, and whether undifferentiated MSCs or partially dopaminergic-differentiated MSCs are optimal for transplantation into an experimental PD model. The present study aims to extend the findings discussed above by examining these issues.

Once considered as having the unique ability to suppress immune responses both *in vitro* and *in vivo*, MSCs are now recognised to possess immunomodulatory properties with the capacity to suppress and/or activate immune responses (Locatelli *et al.*, 2007; Stagg, 2007), as discussed in Section 1.1.5. In addition, while the brain has been traditionally viewed as an immunologically privileged organ, evidence suggesting otherwise is now apparent and reveals routine and effective immunological surveillance of the CNS (Hickey, 2001). Consequently, transplanted cells and tissues are exposed to host immunological responses, particularly since the transplantation procedure elicits

inflammatory processes due to surgical trauma (Barker *et al.*, 2004; Widner *et al.*, 1988). Contrary to earlier findings, recent evidence has also been reported during the course of this study that suggests lack of MSC survival following intracerebral transplantation into normal or hemiparkinsonian rats (Coyne *et al.*, 2006; Suon *et al.*, 2006), raising further questions concerning the ability of MSCs to engraft and mature after transplantation into the CNS.

Another cell type that has demonstrated potential for application in autologous cellular therapies for injuries and diseases of the CNS are OECs, which can be easily obtained through biopsy of the nasal olfactory mucosa, as described by investigators including my co-supervisor (Bianco *et al.*, 2004; Mackay-Sim, 2005; Ruitenberg *et al.*, 2006). OECs are known to possess unique properties that are advantageous in the transplantation setting, including the secretion of a variety of growth factors, such as NGF, BDNF, GDNF, and neuregulin (Boruch *et al.*, 2001; Fairless *et al.*, 2005; Woodhall *et al.*, 2001), which are beneficial for increasing neurite formation and exerting neuroprotective effects. OECs also express extracellular matrix proteins with axon and neurite growth promoting properties, such as laminin, fibronectin, and cell adhesion molecules like neural cell adhesion molecule and L1 cell adhesion molecule (Doucette, 1990; Ramon-Cueto *et al.*, 1992). Furthermore, transplantation of OECs has been shown to promote axonal regeneration and behavioural recovery in animal models of spinal cord injury (Lu *et al.*, 2001b; Lu *et al.*, 2002). In addition, co-transplantation of OECs with foetal ventral mesencephalic cells into the lesioned striatum of hemiparkinsonian rats resulted in significant functional restoration, in terms of amphetamine-induced rotational behaviour, spontaneous locomotor activity, DA levels, and TH immunoreactivity (Agrawal *et al.*, 2004; Johansson *et al.*, 2005). Therefore, in

light of these findings the present study also examined an OEC co-transplantation strategy.

The present study aimed to investigate the function of hMSCs through *in vivo* transplantation studies rather than examining *in vitro* function by electrophysiological testing, since the differentiation procedures employed in Chapter 4 were only capable of generating immature neuronal-like cells, and the CNS environment *in vivo* may provide additional signals allowing further maturation. The experimental model employed in this study was the 6-OHDA unilaterally-lesioned rat (discussed further in Section 1.4.1.5), as this is the accepted universal standard model for assessing PD therapies, and enables quantifiable assessment of behavioural function through amphetamine-induced rotational asymmetry. Initially, this study aimed to examine the effects of transplanting undifferentiated MSCs into the striatum of hemiparkinsonian rats, in terms of amphetamine-induced rotational behaviour and graft phenotype over 3 months post-transplantation (referred to as Part I; see Section 2.2.7.2). The experimental results generated from Part I led to a change in experimental approach. Therefore, Part II of this study was performed to examine the survival, differentiation, and anatomical integration of neuronal-primed hMSCs over a short-time course post-intracerebral transplantation (see Section 2.2.7.2). In addition, neuronal-primed hMSCs were transplanted into the striatum and substantia nigra to determine the optimal site for transplantation, and co-transplantation with OECs was also examined to determine if this provides an advantage or synergistic effect. Furthermore, potential neurotrophic and immunomodulatory effects of hMSCs on endogenous host cells were investigated, since it has been suggested that these mechanisms may play a role in MSC-mediated improvement or restoration of neural deficits.

5.2 Results – Part I

5.2.1 Long-Term Transplantation of Undifferentiated hMSCs in PD Model

Initially, undifferentiated hMSCs were transplanted intrastriatally into 6-OHDA unilaterally lesioned Parkinsonian rats obtained from Taconic Farms Inc. (NY, USA), for assessing behavioural function and graft phenotype over 3 months post-transplantation. However, due to the poor health status of the animals the study was adjusted to have an earlier experimental endpoint at slightly over 2 months. This was most likely the result of a number of factors, including the impact of transportation from the USA, differences in animal strain, size and sex, and also the high severity of the lesion, which resulted in additional loss of VTA dopaminergic neurons together with the expected loss of nigral dopaminergic neurons (discussed in Section 1.4.1.1). In combination, these factors appear to have resulted in weaker than expected animals. Consequently, in Part II of this study the rat PD model was sourced from Integrative Neuroscience Facility (Melbourne, AUS) to minimise the impact of transportation.

Nevertheless, some information was obtained from these experiments. Immunofluorescent analysis of the graft regions revealed the absence of engraftment of undifferentiated hMSCs in the lesioned striatum of hemiparkinsonian rats. Instead, only evidence of astrocytic infiltration and/or proliferation could be seen at the graft site and along the needle tract (Figure 5.1). Furthermore, the core of the graft and needle tract was found to contain autofluorescent matter resembling cell debris and lipofuscin granules (Figure 5.2), suggesting that cellular destruction had occurred. Since the hMSCs had been cultured in phenol red-containing culture medium prior to transplantation, and phenol red uptake by cells can cause autofluorescence, confocal three-dimensional reconstruction analysis was performed to investigate this possibility.

Analysis of the autofluorescent core using confocal 3-dimensional reconstruction confirmed that the autofluorescent signal did not co-localise with DAPI-stained nuclei, therefore establishing that phenol red uptake was not responsible for the observed autofluorescence (see 'Supplementary File 2' video on Supplementary CD). This finding also provided additional support for the absence of hMSC engraftment. Examination of behavioural function by amphetamine-induced rotational testing showed no improvement at 1 month post-transplantation (data not shown). Assessment of later time points (2 months and 3 months post-transplantation) were intended, but could not be performed due to the early experimental endpoint.

These data suggested that the loss of donor hMSCs could be the result of: 1) the inability of hMSCs to survive in the host striatal environment, resulting in cell death and phagocytosis, or 2) rejection of hMSCs through a host-mediated immune response. Additionally, the health of the rats and high severity lesion may have contributed to the absence of engraftment.

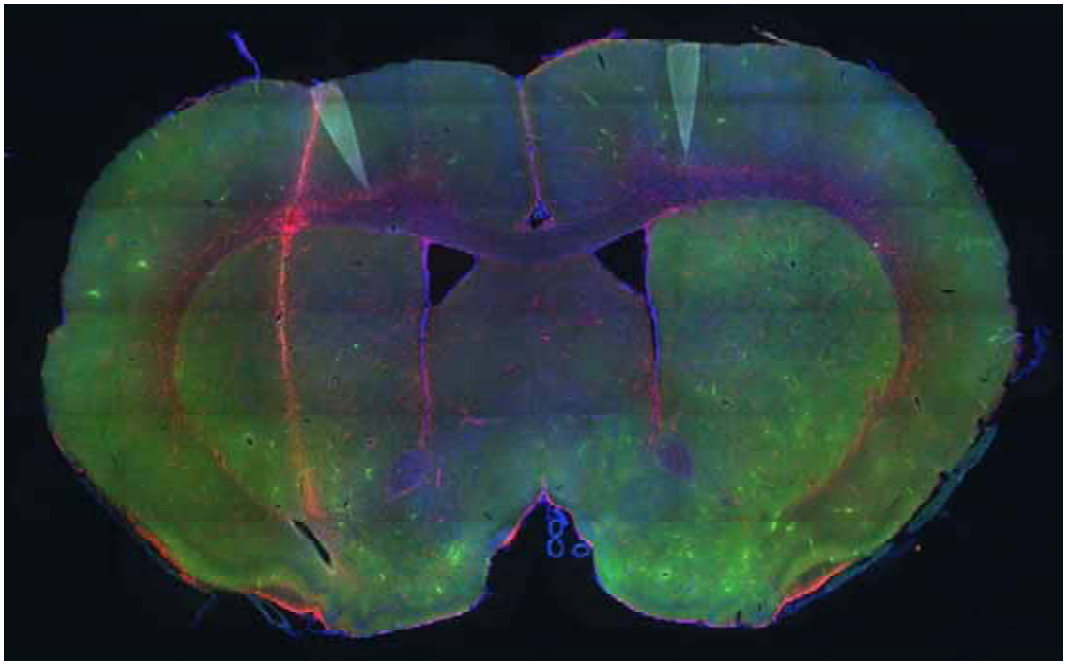

Figure 5.1 Presence of astrogliosis around undifferentiated hMSC grafts and needle tract regions at 2 months post-transplantation.
Representative mosaic tile scan showing immunohistological examination of the graft site following intrastriatal transplantation into the lesioned hemisphere of the 6-OHDA-induced hemiparkinsonian rat ($n = 8$). GFAP-positive astrocytes (red) were found to be concentrated around the graft site and along the length of the needle tract, while this pattern of astrocytic distribution was absent from the contralateral hemisphere. Autofluorescent material could also be observed (green). Nuclei were counterstained with DAPI (blue).

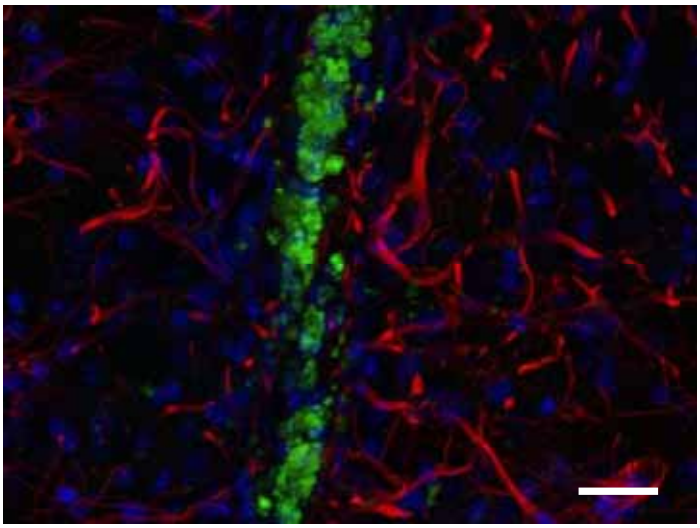

Figure 5.2 Evidence of autofluorescent cellular debris and lipofuscin in undifferentiated hMSC grafts at 2 months post-transplantation. Immunohistological analysis revealed the presence of autofluorescent material (cellular debris and lipofuscin granules; shown in green) within graft cores and along needle tracts, suggesting that cellular destruction had occurred. The representative image depicted here is a maximum projection z-stack of the graft site containing an autofluorescent core and surrounded by GFAP-positive astrocytes (red). Nuclei were counterstained with DAPI (blue). Scale bar: 25 μm. (For confocal 3-dimensional reconstruction see 'Supplementary File 2' video on Supplementary CD).

5.3 Results – Part II

As a consequence of the findings obtained in Part I, a new experimental approach was taken for Part II in order to evaluate the survival, engraftment and differentiation of neuronal-primed hMSCs within the initial 3 weeks post-intracerebal transplantation. The concerns raised in Part I were addressed through purchasing the rat PD model from a source within Australia, and also using older, male rats of a different strain. Other approaches taken in attempts to increase the likelihood of hMSC engraftment, included (1) priming hMSCs toward the neuronal lineage prior to transplantation; (2) injection into the substantia nigra as well as the striatum; and (3) co-transplantation with OECs.

5.3.1 Graft Site Identification

For the identification of graft sites, regions of the striatum and substantia nigra were screened for expression of astrocyte marker, GFAP, and neuronal progenitor marker, NES, using immunohistological techniques. In OEC co-transplanted animals, the GFP signal from the OECs also aided graft identification. A marked astrogliosis was observed in graft regions and along the needle tract as early as 7 days post-transplantation (Figure 5.3), similar to the observations in Part I at the much later time-point of 2 months post-transplantation. However, at day 1 the human cell grafts only appeared as a dense mass or cluster of cells (DAPI-stained nuclei) along the needle tract, sometimes containing rare NES-positive cells. In addition, autofluorescent cell debris and lipofuscin-like material were found within the graft sites, as seen previously in Part I. Also, it was found that transplantation of neuronal-primed hMSCs into the striatum or substantia nigra in the presence or absence of OECs yielded comparable results.

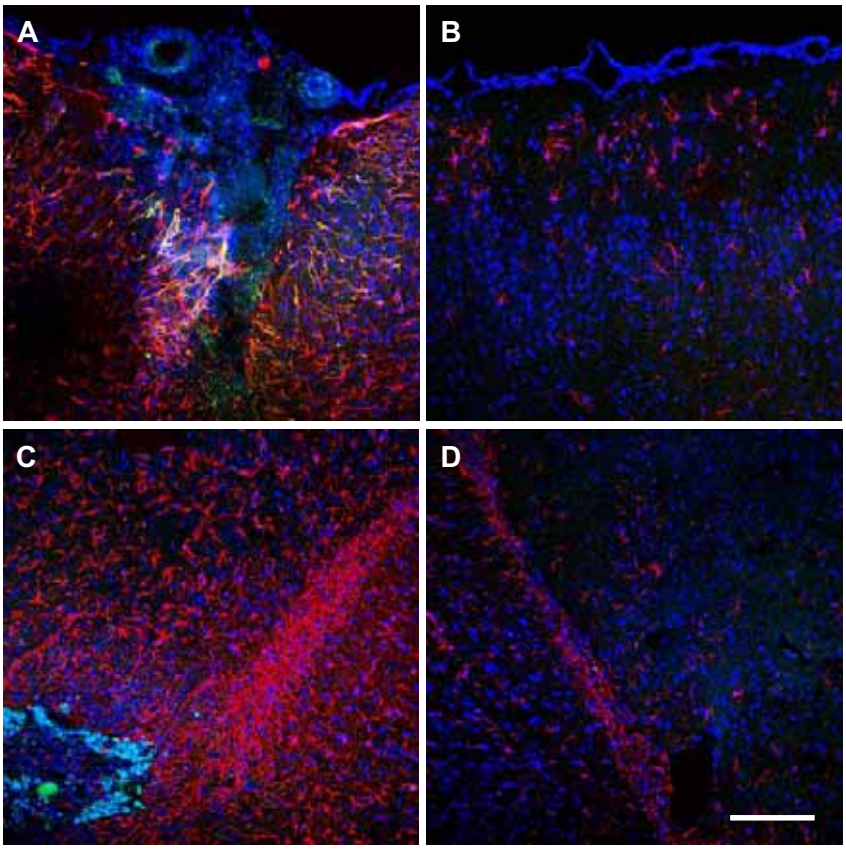

Figure 5.3 Identification of neuronal-primed hMSC graft sites and comparison with the contralateral hemisphere.

Representative results of immunohistological screening of the striatum and substantia nigra for identification of graft sites, using expression of GFAP (red) and NES (green), as well as GFP signal detection for OEC co-transplanted recipients ($n = 3$). (**A**) Needle tract entry site in Day 7 nigral graft and (**B**) corresponding region in contralateral hemisphere. (**C**) Striatal graft site at Day 7, and (**D**) corresponding region in contralateral hemisphere. Similar to results in Part I, graft sites contained autofluorescent material and were surrounded by astrogliosis, which were absent in the contralateral hemisphere. Images are maximum projection z-stacks. Similar findings were obtained with transplantation into either site in the absence or presence of OECs. Nuclei were counterstained with DAPI (blue). Scale bar: 150 μm.

5.3.2 Limited Survival of Neuronal-Primed hMSCs and OECs Post-Transplantation

Apart from the autofluorescent material detected within graft sites, the presence of intact healthy nuclei was also found suggesting the possible survival of transplanted cells. Therefore, the survival and engraftment of neuronally-primed hMSCs and OECs following transplantation in the striatum and substantia nigra were examined by immunohistological staining for human nuclear antigen (HNA; Figure 5.4). Cells displaying co-localisation of HNA and DAPI signals were found within striatal and nigral graft sites at day 1 post-operatively (Figures 5.4A and G). Surviving human cells were typically visible as a mass of cells toward the end of the needle tract region, with few HNA-positive cells detected at the needle tract entry point (Figure 5.4F), and none seen in the contralateral hemisphere (Figure 5.4E). Over time, a decrease in HNA staining was apparent with day 7 grafts showing little remaining HNA signal (Figure 5.4B and H), and complete absence by day 21 (Figure 5.4C and I). In addition, autofluorescence within the graft core increased in parallel with HNA loss, and shrinking graft size and cellular density. Surprisingly, little differences were observed between grafts containing neuronal-primed hMSCs alone or in combination with OECs. Concomitant loss of the GFP signal from OECs provided further support for the inability of transplanted human cells to persist within the host environment.

Confocal z-series analysis of graft sites was also performed to verify the co-localisation of HNA and DAPI staining, and to exclude possible misinterpretation due to overlaying fluorescent signals. Through this analysis HNA signals were indeed found to be co-localised with DAPI-stained nuclei (arrows in Figure 5.5) for both hMSC only and OEC co-transplanted grafts. Therefore, the presence of surviving human cells was

confirmed; however, only limited survival could be detected following intracerebral transplantation.

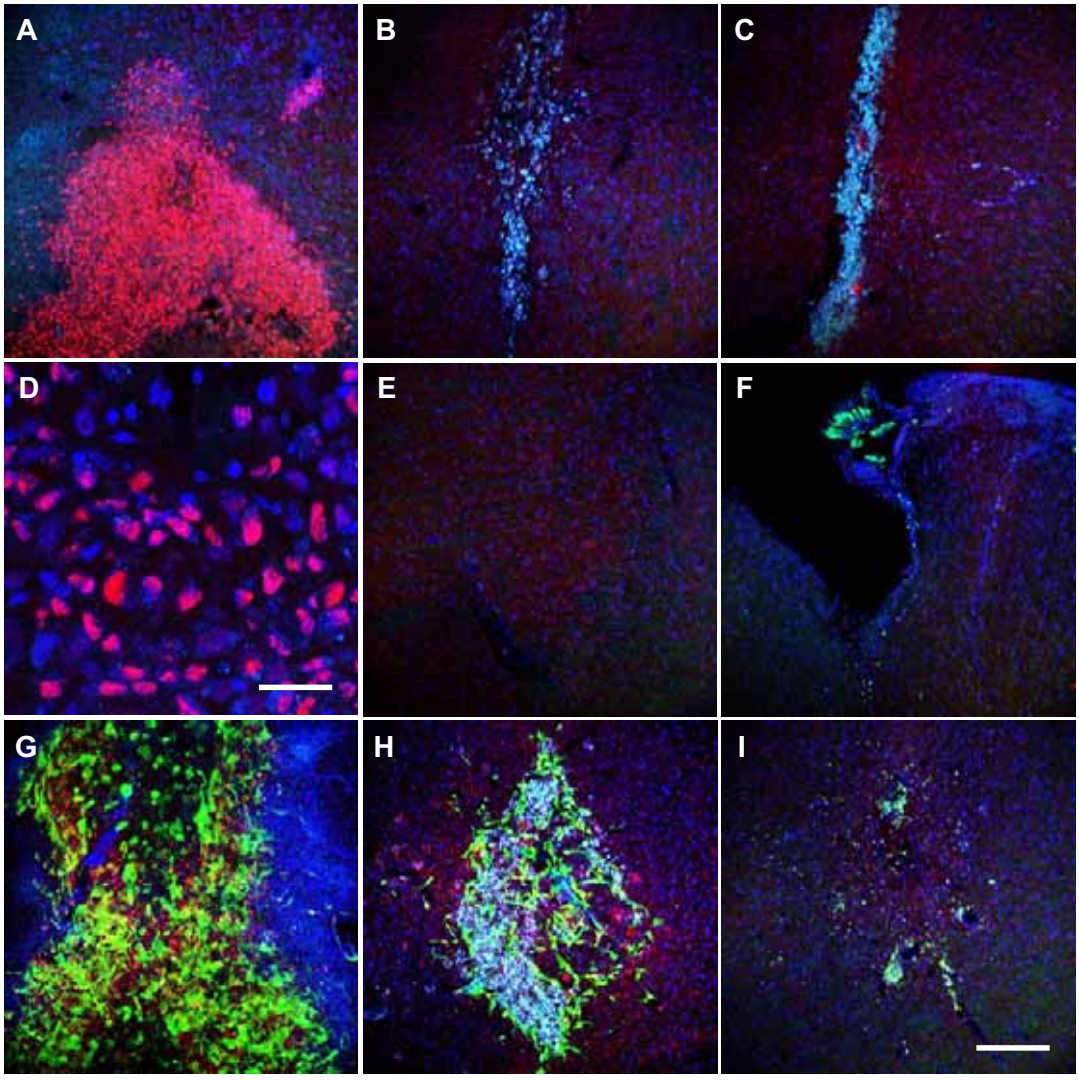

Figure 5.4 Evidence of limited survival of neuronal-primed hMSCs and OECs in the lesioned hemisphere of the hemiparkinsonian rat.

(Refer to next page for Figure 5.4 Legend text)

Figure 5.4 Evidence of limited survival of neuronal-primed hMSCs and OECs in the lesioned hemisphere of the hemiparkinsonian rat.
The survival and engraftment of neuronal-primed hMSCs and GFP-positive OECs (green) were examined by immunohistological staining using an antibody against HNA (red). Representative images following transplantation of (**A-F**) neuronal-primed hMSCs alone, and (**G-I**) neuronal-primed hMSCs co-transplanted with OECs ($n = 3$). Surviving human cells could be detected at day 1 post-transplantation (A and G). However, by day 7 little HNA signal remained (B and H), and this could no longer be detected by day 21 (C and I). Higher magnification view of (A) is shown in (D), with human nuclear staining seen to be co-localised with DAPI-stained nuclei. Increasing presence of autofluorescent matter within the graft core was observed in parallel with the loss of human cells. This autofluorescence and human nuclear staining were not observed in the corresponding regions in the contralateral hemisphere (representative image shown in (E), which is contralateral to (A)). The majority of human cells were concentrated in the needle tract with few, if any, detected at the needle tract entry site (F). Similar findings were observed in both striatal and nigral graft sites. Images shown are maximum projection z-stacks. Nuclei were counterstained with DAPI (blue). Scale bar: 150 µm in (I) for all images, except (D) which has scale bar: 30 µm.

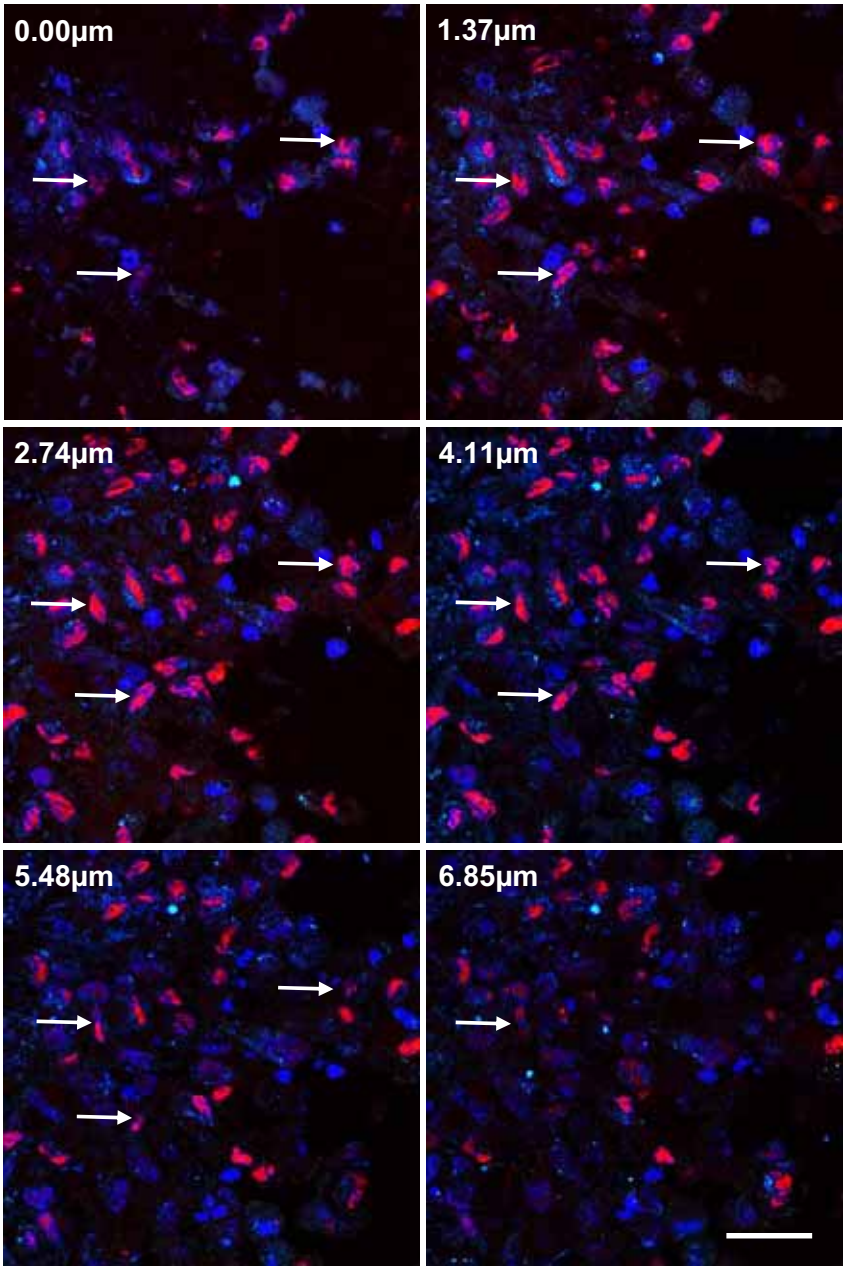

Figure 5.5 Confocal z-series identification of human cells at graft sites.

Immunohistological examination of graft sites with antibodies to HNA (red) demonstrates co-localisation with DAPI-stained nuclei (blue) within the nigral graft site at day 1 (arrows). Similar results obtained for striatal graft sites and OEC co-transplanted animals, as well as at the day 7 time point. Scale bar: 30 µm.

5.3.3 Accumulation of Astrocytes and Microglia/Macrophages at Graft Sites

To investigate the loss of neuronal-primed hMSCs and OECs following transplantation, graft sites from both the striatum and substantia nigra were characterised for the presence of glial cells by immunohistological analysis (Figure 5.6). Microglia and monocytes/macrophages were identified using the Iba-1 marker and astrocytes were detected by GFAP expression.

Both striatal and nigral grafts displayed massive infiltration by Iba-1-positive microglia/macrophages and GFAP-positive astrocytes that increased over the 3 week examination period (Figure 5.6 A-C). Initially, little glial accumulation was detected at day 1 post-operatively. However, by day 7 microglia/macrophages could be detected in a radial pattern around the graft core, which contained autofluorescent material. Increased astrocytic presence was also found surrounding the graft site and extending beyond the microglia/macrophage layer. Glial accumulation was observed to persist until 21 days post-transplantation, which was the last time point examined. Furthermore, the microglia/macrophages found at graft sites displayed an activated appearance, with increased intensity of Iba-1 immunoreactivity (Ito *et al.*, 1998), and alterations in morphology including retraction and thickening of processes, and increased cytoplasmic area (Ito *et al.*, 1998; Streit *et al.*, 1988).

The corresponding regions in the contralateral hemisphere did not exhibit glial accumulation (Figure 5.6 D-F). The microglia here possessed a quiescent morphology with small cell bodies and fine cytoplasmic ramifications. Interestingly, examination of injection sites in the sham controls also revealed evidence of increasing glial accumulation over the 3 week period (Figure 5.6 G-I), suggesting that this may be a response to injury caused by needle insertion during surgery. These results suggest that

intracerebral transplantation of hMSCs and OECs may have elicited an inflammatory response that led to the destruction of donor cells.

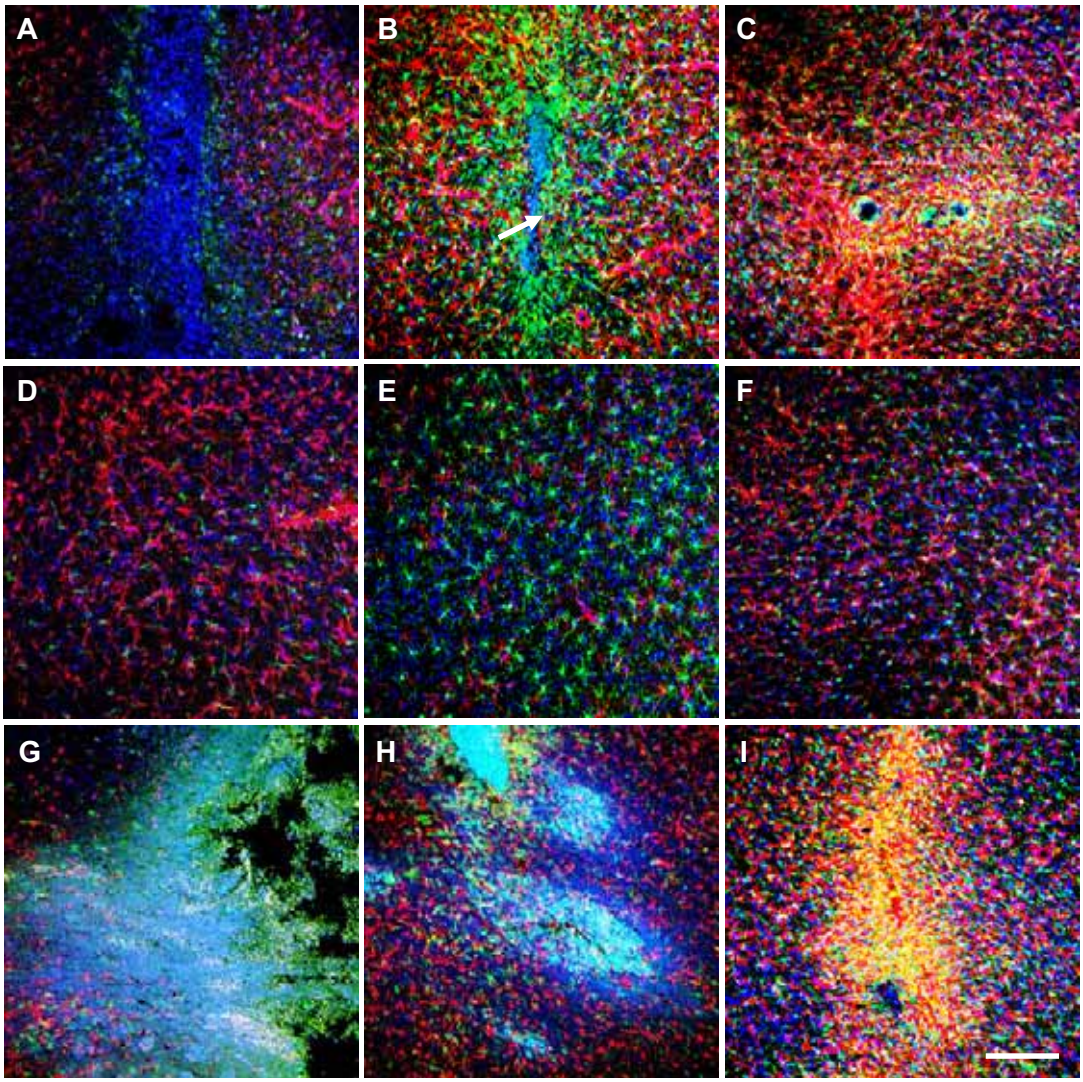

Figure 5.6 Increasing glial accumulation at graft sites during the initial 3 weeks post-intracerebral transplantation in the hemiparkinsonian rat.

(Refer to next page for Figure 5.6 Legend text)

Figure 5.6 Increasing glial accumulation at graft sites during the initial 3 weeks post-intracerebral transplantation in the hemiparkinsonian rat.

Immunohistological analysis revealed the accumulation of GFAP-positive astrocytes (red) and Iba-1-positive microglia/macrophages (green) at graft sites ($n = 3$). Representative images depicting (**A-C**) neuronal-primed hMSC graft sites in the striatum, (**D-F**) corresponding regions in the contralateral hemisphere, and (**G-I**) striatal injection sites in sham controls, at day 1 (left column), day 7 (middle column) and day 21 (right column). (A) Initially, at day 1 post-transplantation little GFAP or Iba-1 was detected. (B) By day 7, grafts were densely surrounded by a layer of GFAP-positive and Iba-1-positive cells, and contained an autofluorescent core (white arrow). (C) At the end of the 21 day period a marked astrogliosis was present in the graft region. Both striatal and nigral grafts exhibited this glial accumulation, which suggests the presence of an inflammatory response at the graft sites. (D-F) This pattern of glial distribution was absent from the contralateral hemisphere. (G-I) Whereas, a similar response is observed in the sham controls, which also exhibited autofluorescent cellular debris at injection sites (aqua colour). Images shown are maximum projection z-stacks. Nuclei have been counterstained with DAPI (blue). Scale bar: 150 μm.

5.3.4 Endogenous Host Neuronal Differentiation Post-Transplantation

The current study has also characterised the graft regions for host neuronal progenitor activity, since transplantation of hMSCs has been proposed to elicit endogenous host responses that may account for the functional improvements observed in animal models of neurological disease. Immunohistological analysis of graft sites for the expression of neuronal progenitor marker NES (Figure 5.7) revealed little, if any, NES immunoreactivity in the graft regions at any period. The predominant host-derived cells that were found to express NES were GFAP-positive astrocytes located immediately surrounding the grafted cells. Expression of NES and GFAP by astrocytes is an indicator of activation (Clarke *et al.*, 1994), which is consistent with the increased accumulation of astrocytes around the graft sites. A few rare NES-positive cells could be detected within the graft core and at the astrocytic border of the graft at day 7 (Figure 5.7D). However, these NES-positive cells were not detected often, and were typically found in the graft core, therefore, it is unlikely that these NES-positive cells represented endogenous host neuronal progenitor involvement. Instead, this NES expression may be derived from grafted neuronal-primed hMSCs, which have been shown to express the NES gene and protein (see Chapter 4). Furthermore, this explanation is consistent with the lack of NES expression at day 21, since the transplanted neuronal-primed hMSCs were absent from this final time point. In addition, no differences were observed between striatal and nigral grafts of neuronal-primed hMSCs, or with grafting in the presence or absence of OECs. Interestingly, similar to previous findings in Part I, cavitations and autofluorescence were seen within the graft cores suggesting the presence of cell destruction.

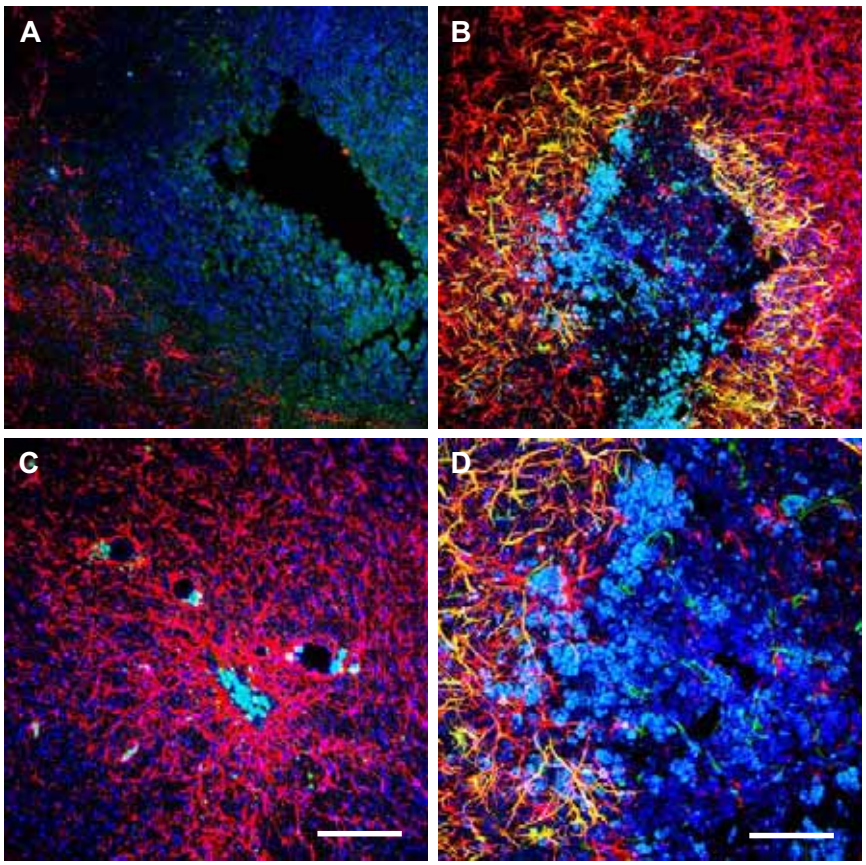

Figure 5.7 Absence of endogenous neuronal differentiation in host brain after transplantation of neuronal-primed hMSCs.

Striatal and nigral grafts were also examined for the presence of NES (green) and GFAP (red) at (**A**) day 1, (**B**) day 7 and (**C**) day 21 post-transplantation ($n = 3$). Higher magnification view of (B) shown in (**D**). Rare NES-positive cells were only detected within the graft core or in the immediate astroglial boundary at day 7, suggesting that neuronal-primed hMSCs do not elicit host neuronal differentiation, but express some NES protein. NES-positive activated astrocytes could also be detected. Similar results were observed in OEC co-transplanted recipients. Representative images shown are maximum projection z-stacks. Nuclei were counterstained with DAPI (blue). Scale bar: 150 μm in (C) for all images, except (D) which has scale bar: 75 μm.

5.3.5 Characterisation of Graft Sites for Neuronal Marker Expression

Since neuronal progenitor marker NES was detected in a small proportion of cells within the graft site, further immunohistological characterisation of grafts for the expression of neuronal markers TH and β tubulin III was also conducted. Staining for dopaminergic neuronal marker TH confirmed the unilateral expression of TH in the unlesioned hemisphere of the 6-OHDA-induced hemiparkinsonian rats. Dopaminergic neurons of the VTA were also found to be intact. TH immunoreactivity was predominantly absent from the graft sites (Figure 5.8 A-C), regardless of whether neuronal-primed hMSCs were transplanted alone or in combination with OECs, and regardless of the site of transplantation. Although, strong TH signal could be detected in the corresponding unlesioned contralateral hemisphere (Figure 5.8 D-F). Sham-treated animals also did not exhibit TH expression at injection sites (Figure 5.8 K and L). However, it was observed that the human cell grafts were not always administered into the correct regions, particularly for the substantia nigra. This could be due to the small size of the substantia nigra, and the difficulty in accessing this region of the CNS in stereotactic surgical procedures.

Striatal and nigral graft sites containing neuronal-primed hMSCs were also found to lack expression of neuronal marker β tubulin III (Figure 5.9 A-C), and similar results were observed with OEC co-transplantation. In addition, β tubulin III immunoreactivity was absent from the entire transplantation region, whereas the corresponding contralateral region showed consistent and evenly distributed expression of β tubulin III (Figure 5.9 D), suggesting that trauma associated with the surgical procedures caused loss of endogenous host β tubulin III protein. The findings obtained through immunohistological analysis of TH and β tubulin III expression indicate that neuronal-primed hMSCs do not undergo further differentiation or maturation *in vivo*

after intracerebral transplantation into the striatum or substantia nigra of hemiparkinsonian rats.

5.3.6 Characterisation of Graft Sites for Extracellular Matrix Deposition

The presence of fibronectin deposition at striatal and nigral graft sites was also examined using immunohistological techniques (Figure 5.8). A dense fibronectin-positive matrix was observed surrounding neuronal-primed hMSC grafts as early as day 1 post-transplantation, however, immunoreactivity appeared to decrease over time with very little fibronectin remaining at day 21 (Figure 5.8 A-C). Similar findings were obtained with the transplantation of neuronal-primed hMSCs in combination with OECs (Figure 5.8 G-I), and with both striatal and nigral transplantation. Only slight fibronectin expression could be detected in the corresponding contralateral regions, at much lower levels than that present around and within graft sites (Figure 5.8 D-F). Furthermore, analysis of striatal and nigral injection sites in sham controls revealed very little, if any, fibronectin matrix deposition at all periods examined (Figure 5.8 J-L). These results suggest that the dense fibronectin matrix present at graft sites was produced by the transplanted human cells, rather than being deposited by host cells as part of a scar formation process. Observations that fibronectin was drastically decreased in parallel with the loss of hMSCs and OECs provide further support for this explanation, and also suggest a potential role for microglia/macrophages in removing fibronectin deposits. Additional evidence supporting graft-derived fibronectin production can be found in the results of Section 4.2.3.3, which demonstrate continued expression of fibronectin by neuronal-primed hMSCs *in vitro*.

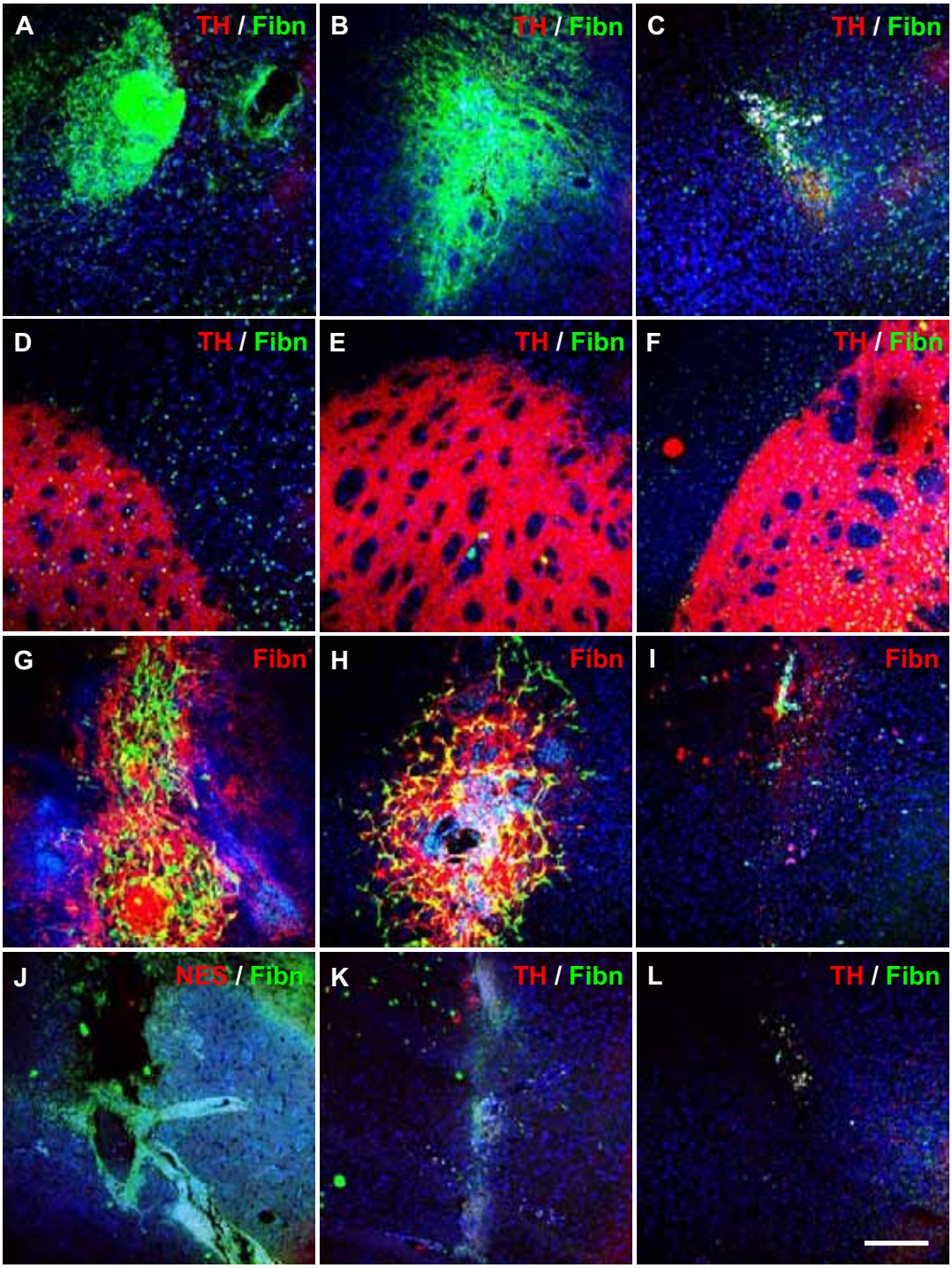

Figure 5.8 Matrix deposition and lack of dopaminergic neuronal differentiation after transplanting neuronal-primed hMSCs and OECs.

(Refer to next page for Figure 5.8 Legend text)

Figure 5.8 Matrix deposition and lack of dopaminergic neuronal differentiation after transplanting neuronal-primed hMSCs and OECs.

Fibronectin and TH expression were assessed in striatal and nigral grafts at day 1 (left column), day 7 (middle column) and day 21 (right column). Representative images are shown of (**A-C**) neuronal-primed hMSC graft sites in the striatum, (**D-F**) corresponding regions in the contralateral hemisphere, (**G-I**) co-transplanted hMSC and OEC nigral graft sites, and (**J-L**) injection sites in sham controls ($n = 3$). Immunohistological analysis revealed a fibronectin-positive matrix surrounding hMSC and OEC-containing graft sites from day 1 post-transplantation (A, B, G, and H), however, this was drastically reduced by day 21 (C and I). The concomitant loss of transplanted cells suggests that the fibronectin matrix was deposited by the graft. Furthermore, sham injected animals did not exhibit dense fibronectin expression (J-L). TH expression was not detected in any graft sites or sham-injected sites, and was only expressed in the unlesioned hemisphere (D-F). Images shown are maximum projection z-stacks. Nuclei were counterstained with DAPI (blue). Scale bar: 150 μm.

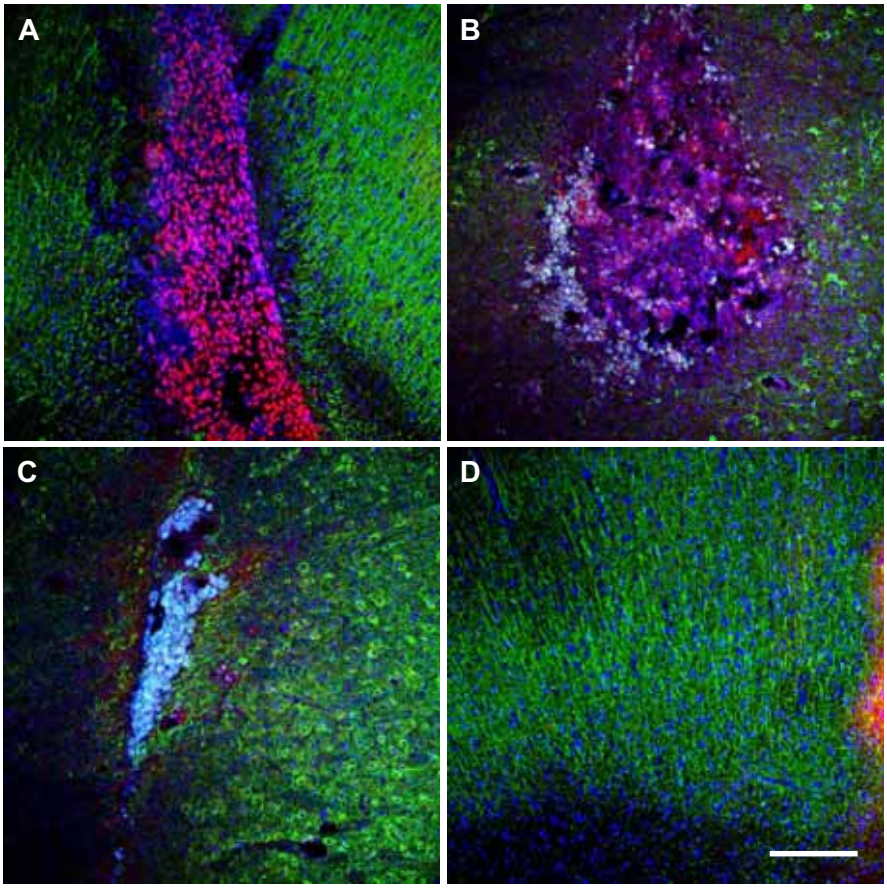

Figure 5.9 Absence of neuronal marker expression within graft sites following transplantation of neuronal-primed hMSCs.

Striatal and nigral grafts were also examined for the presence of β tubulin III (green) and HNA (red) at (**A**) day 1, (**B**) day 7 and (**C**) day 21 post-transplantation ($n = 3$). Expression of β tubulin III was missing from the region containing the graft sites, and was primarily found surrounding the graft core. While, the corresponding regions in the contralateral hemisphere exhibited evenly distributed β tubulin III expression (representative image shown in (**D**), which is contralateral to (A)). Similar results were also obtained in OEC co-transplanted recipients. The representative images shown are maximum projection z-stacks. Nuclei were counterstained with DAPI (blue). Scale bar: 150 μm.

5.4 Discussion

The central findings of the present study have shown (1) evidence of limited survival and engraftment of undifferentiated and neuronal-primed hMSCs in the lesioned hemisphere of the 6-OHDA-induced hemiparkinsonian rat, with loss of hMSCs as early as 7 days post-grafting; (2) absence of further differentiation of neuronal-primed hMSCs *in vivo* in both the striatum and substantia nigra, suggesting that neither site contains factors involved in promoting the survival and dopaminergic neuronal differentiation of hMSCs; (3) accumulation of activated astrocytes and microglia/macrophages around graft sites, indicating the presence of an inflammatory response; (4) co-transplantation of OECs in combination with neuronal-primed hMSCs does not confer graft survival or differentiation advantages, and instead, OECs are also lost early after transplantation; (5) absence of endogenous host neuronal differentiation in response to hMSC transplantation; and (6) evidence suggesting continued production of fibronectin extracellular matrix by hMSCs after intracerebral transplantation.

The majority of published reports in this field have demonstrated some level of MSC-induced restoration of neurodegeneration in Parkinsonian animal models, whether it be renewed presence of immunoreactivity to dopaminergic markers within the lesioned hemisphere or functional improvement in behavioural asymmetry (reviewed in Section 1.4.4). In contrast, the current study has found limited survival and engraftment of hMSCs together with the continued absence of TH-positive neurons from the lesioned hemisphere following hMSC transplantation. A number of possible explanations may account for these observations, including the rejection of hMSCs by a host immune response, lack of appropriate factors for supporting hMSC survival and engraftment within the environment of the adult lesioned brain, insufficient differentiation of hMSCs toward a midbrain dopaminergic neuronal phenotype,

inadequate techniques for cellular transplantation, and misleading results of previous studies that employed thymidine analog labelling of donor cells as the sole method for graft identification.

5.4.1 Activation of Innate Immune Responses with Intracerebral hMSC Transplantation

In the present study evidence was found of vigorous microglia/macrophage infiltration in response to grafted neuronal-primed and undifferentiated hMSCs in an animal disease model of PD. This response occurred in the presence of cyclosporine immunosuppression and was also detected in sham control animals. In contrast, MSCs have long been considered to possess the ability to suppress adaptive immune responses. More recently, however, evidence to the contrary has arisen, which indicates that MSCs may not be intrinsically immune privileged (discussed in greater depth in Section 1.1.5). Furthermore, the CNS is now understood to be capable of effective immunological surveillance (Barker *et al.*, 2004; Hickey, 2001).

In the wake of these findings, reports published during the course of this study have described similar findings to that presented in this chapter, that is the rejection of transplanted MSCs following intracerebral transplantation in rodents (Camp *et al.*, 2009; Coyne *et al.*, 2006; Suon *et al.*, 2006). Coyne and colleagues demonstrated that transplantation of MSCs into the intact adult rat hippocampus or striatum elicited an immediate inflammatory response involving massive infiltration of activated microglia/macrophages and astrocytes in the presence of cyclosporine-mediated immunosuppression. This led to the rapid rejection of hMSC grafts with near complete rejection by 7 days, a much briefer period than rejection through adaptive immune responses, which generally commence 10-14 days post-transplantation (Coyne *et al.*, 2006). The inflammatory response was ascribed to the mechanical trauma produced by

intracerebral transplantation. Prior to the findings from this thesis and from Coyne and colleagues, the inflammatory response elicited during MSC transplantation had not been evaluated. Subsequently, Camp and colleagues (Camp *et al.*, 2009) also demonstrated the generation of robust cellular immune responses (MHC class I- and class II-expressing cells and $CD4^+$ and $CD8^+$ lymphocytes) following the injection of undifferentiated rat MSCs into the striatum of allogeneic hemiparkinsonian recipients. However, despite the presence of a marked immune response, some grafted MSCs were still detectable at 22-24 days post-transplantation; although MSC administration was not found to prevent behavioural deficits or DA depletion, and MSCs did not acquire the ability to synthesise TH (Camp *et al.*, 2009).

Furthermore these findings are in accordance with the current understanding of the role of microglia/macrophages in orchestrating the inflammatory response of CNS innate immunity (Giulian, 1987; Perry *et al.*, 1995). Previous studies have reported that mechanical injury to the brain provoked rapid and focal microglial activation to shield the injured area, with possible immediate phagocytic engulfment and removal of damaged tissue (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Microglia-mediated inflammation has also been associated with astrogliosis (Giulian *et al.*, 1994; Matsumoto *et al.*, 1992), and Giulian and colleagues have described the production of pro-inflammatory cytokine IL-1 by microglia, which is capable of inducing astrogliosis (Giulian *et al.*, 1994). In addition, the pattern of microglial and astrocytic distribution observed in the present study bore resemblance to the pattern of microglial and astroglial reactions to inflammatory lesions in experimental autoimmune encephalomyelitis described by Matsumoto and colleagues (Matsumoto *et al.*, 1992). This pattern consisted of (1) early response of microglia to inflammatory lesions; (2) proliferation of microglia close to lesions, with astrocytes encasing the lesions; and

(3) formation of micro-astroglial scars at later stages that were composed of residual inflammatory cell aggregates and dense microglial and astrocytic gliosis (Matsumoto *et al.*, 1992). Additional evidence for the induction of a microglial-mediated innate inflammatory response with SC transplantation was found in a recent study examining transplantation of Müller SCs into an experimental model of retinal degeneration (Singhal *et al.*, 2008). Extensive microglial accumulation was found in this study, in association with poor migration, integration and survival of Müller SCs (Singhal *et al.*, 2008).

In the current study, microglial- and astroglial-reactivity were found to coincide with fibronectin-reactivity. However, the majority of fibronectin deposition was observed to occur at hMSC graft sites with very little fibronectin present at sham injection sites, suggesting that the primary source of fibronectin may be the transplanted hMSCs. Similarly, Coyne, *et al*. have described the presence of fibronectin-reactivity in association with the marked astrogliosis surrounding MSC graft sites in the intact rat striatum and hippocampus (Coyne *et al.*, 2006). On the contrary, the authors ascribed the presence of the dense fibronectin-positive matrix at the transplantation site to be solely indicative of scar formation. Another study by Singhal and colleagues also reported the deposition of chondroitin sulfate proteoglycans at Müller SC transplantation sites, which were thought to have been released by microglia and possibly contributed to the inhibition of cell migration and integration (Singhal *et al.*, 2008). However, in the context of this thesis, the deposition of fibronectin at graft sites by neuronal-primed hMSCs may have also contributed to the lack of survival and engraftment, and may have resulted from the incomplete neuronal differentiation of hMSCs.

Further support for the rejection of hMSC transplants by an inflammatory response in this thesis is found in another study examining the early cellular responses mediating rejection of xenogeneic porcine endothelial cells and foetal neurons transplanted into the rat striatum (Remy *et al.*, 2001). Surprisingly, the work by Remy, *et al*. demonstrated rapid rejection of endothelial cell grafts within 11 days through an inflammatory response mediated by microglia/macrophages, whereas rejection of foetal mesencephalic neurons occurred after 3 weeks and was typical of an adaptive immune response involving T cells. The differences in rejection kinetics were suggested to arise from the intrinsic characteristics of the xenogeneic donor cells, such as differences in MHC-I surface expression and production of pro-inflammatory cytokines. These findings also indicate the possibility that transplantation of non-neural cell types into neural environments elicits an immunological response distinct to that resulting from transplantation of neural cell types. In addition, since MSCs are known to secrete a wide variety of growth factors, including GM-CSF and others that support granulocyte and macrophage proliferation and maturation (Guba *et al.*, 1992; Kittler *et al.*, 1992), Coyne and colleagues have commented that the production of these factors by MSCs post-transplantation could augment post-operative inflammation, as well as aid graft destruction (Coyne *et al.*, 2006). Further to this, GM-CSF has been described to be a potent mitogen for microglia, with the ability to induce reactive microglia capable of scavenging and removal of damaged tissue (Giulian *et al.*, 1994).

In the present study, and in the study by Coyne, *et al*. (Coyne *et al.*, 2006), immunosuppression using cyclosporine was employed yet MSC graft rejection still occurred. Czech, *et al*. have also found that cyclosporine administration could not prevent or delay the rejection of xenogeneic bovine endothelial cells grafted into the rat CNS (Czech *et al.*, 1997). Since cyclosporine treatment acts primarily to inhibit

T lymphocyte-mediated immunity (Borel *et al.*, 1976; Borel *et al.*, 1977), these findings indicate the involvement of other arms of the immune system, such as innate immunity, in MSC graft rejection. In the current study xenogeneic hMSCs were transplanted into the rat CNS, and other studies have reported that cyclosporine treatment for neural xenotransplantation is suboptimal (Larsson *et al.*, 2000; Larsson *et al.*, 2001). Furthermore, reports have suggested the involvement of complement factors, immunoglobulins and macrophages in immune responses against discordant xenografts, which are not affected in a crucial way by cyclosporine treatment (Barker *et al.*, 2000; Larsson *et al.*, 1999; Wallgren *et al.*, 1995). Therefore, in this thesis the observations of rapid loss of transplanted xenogeneic neuronal-primed hMSCs, massive microglial/macrophage and astrocyte accumulation at the graft site, and inability of cyclosporine treatment to avert these outcomes, together support the notion of graft rejection through host innate immune responses.

Administration of further immunosuppression may therefore be required for improved MSC graft survival. Enhanced microglial suppression was employed by Singhal and colleagues for Müller SC transplantation into degenerated retina through application of oral cyclosporine A, azathioprine, prednisolone, and indomethacin (Singhal *et al.*, 2008). This immunosuppressive regimen yielded decreased microglial accumulation and increased survival of Müller SCs. Other studies investigating the improvement of xenograft survival in the CNS have found promising outcomes through the use of triple drug therapy (cyclosporine, methylprednisolone, azathioprine), complete complement blockade, and absence of an induced primate anti-mouse antibody response (Cicchetti *et al.*, 2003); tacrolimus or cyclosporine treatment with additional inductive treatment using prednisolone or mycophenolate mofetil (Wennberg

et al., 2001); or local immunosuppression by co-transplantation of liposomal tacrolimus (Alemdar *et al.*, 2007).

Another potential factor affecting neuronal-primed hMSC engraftment *in vivo* is the immunogenicity of hMSCs following *in vitro* differentiation. At present, the majority of studies have examined the immunomodulatory properties of MSCs in the undifferentiated state, and little is known of whether this is affected by *in vitro* differentiation. Recently, *in vitro* chondrogenic differentiation has been reported to increase the immunogenicity of rat MSCs, resulting in the stimulation and maturation of human DCs, possibly due to MSC up-regulation of costimulatory B7 molecules after chondrogenic differentiation (Chen *et al.*, 2007b). Furthermore, the presence of stimulated DCs was required for chondrogenic-differentiated MSC induction of human peripheral blood lymphocyte proliferation and cytotoxicity. On the other hand, osteogenic and adipogenic differentiation did not appear to alter the immunological properties of MSCs (Chen *et al.*, 2007b). Therefore, the immunogenicity of MSCs following *in vitro* differentiation may depend on the resulting cell type obtained through differentiation.

Another recent study investigated the effects of *in vitro* cytokine-based neuronal differentiation on hMSC expression of immunogenic molecules and stimulation of allogeneic peripheral blood lymphocyte proliferation (Liu *et al.*, 2006). While, increased hMSC expression of HLA molecules and costimulatory CD80 molecules was detected over the course of neuronal differentiation, the proliferation of allogeneic peripheral blood lymphocytes could not be induced. Instead, neuronal differentiated hMSCs suppressed the proliferation of peripheral blood lymphocytes induced by allogeneic lymphocytes or mitogens, through mechanisms involving cell-cell contact and soluble factors, such as TGF-β1 and IL-10. In addition, IFN-γ treatment enhanced the

expression of HLA molecules by neuronal differentiated hMSCs, suggesting that an inflammatory environment *in vivo* could elicit increased immunogenicity of hMSCs. However, IFN-γ exposure was unable to significantly up-regulate the expression of CD40, CD80 or CD86, indicating that functional costimulatory signalling on neuronal differentiated hMSCs was incomplete. Also, peripheral blood lymphocyte proliferation was suppressed to a similar extent even after IFN-γ treatment of hMSCs cultured in neuronal differentiation medium (Liu *et al.*, 2006). Further studies are required to more clearly elucidate the immunogenicity and immunomodulatory properties of hMSCs after neuronal differentiation, and it will be of interest to evaluate this in the neuronal-primed hMSCs in the present study to determine whether these factors contributed to the rapid demise of hMSC grafts after *in vivo* transplantation.

5.4.2 Absence of Further hMSC Neuronal or Dopaminergic Neuronal Differentiation at Both Striatal and Nigral Graft Sites Post-Transplantation

Examination of striatal and nigral graft sites containing neuronal-primed hMSCs with or without OEC co-transplantation revealed the absence of further neuronal or dopaminergic neuronal differentiation and maturation. In the same way, comparable absence of neural maturation was described by Coyne and colleagues following transplantation of undifferentiated MSCs into the hippocampus and striatum of normal adult rats (Coyne *et al.*, 2006). This was attributed to the intact adult brain possessing limited capacity to direct the differentiation of transplanted SCs. It is understood that the survival and establishment of functional connections by neuronal grafts is dependent on host variables, occurring maximally during the foetal period and diminishing with post-natal development (Bjorklund *et al.*, 2000). Functional integration of donor neurons can still occur in the adult brain, however, the capacity for this is increased by damage to the CNS (Bjorklund *et al.*, 2000).

Similar lines of thought have also been applied to the transplantation of SCs into the CNS. Suon and colleagues transplanted hMSC-derived neural progenitor spheres into the lesioned striatum of 6-OHDA-induced hemiparkinsonian rats, but were unable to detect graft survival beyond 4 weeks post-transplantation (Suon *et al.*, 2006). In addition, although *in vitro* differentiation systems had yielded cells capable of expressing dopaminergic and GABAergic traits, after *in vivo* transplantation hMSC-derived neuroprogenitor spheres were unable to differentiate further into TH-expressing cells, although many were found to express GABA. The authors proposed that hMSCs transplanted into the striatum had preferentially differentiated into GABA-expressing neurons due to the local differentiation factors present in the striatum, which is intrinsically comprised of 90% GABA neurons (Suon *et al.*, 2006). In the present study, neuronal-primed hMSCs were also transplanted into the striatum as this is the region requiring DA provision, and analogous results of poor graft survival and absence of TH expression were also observed. We also performed transplantation into the substantia nigra to determine whether graft survival and differentiation could be improved, since the cell bodies of midbrain dopaminergic neurons are contained in the substantia nigra and the specific cues for dopaminergic differentiation may be present here rather than in the striatum. However, no differences were seen between nigral grafts and striatal grafts, and neuronal-primed hMSCs were lost from both regions by 21 days post-transplantation.

In contrast, evidence supporting the *in vivo* and *in vitro* transdifferentiation of hMSCs into functional dopaminergic neurons was reported in a recent study by Shetty and colleagues (Shetty *et al.*, 2009). In this study, undifferentiated hMSCs (2-3 x 10^5) cultured under xenofree conditions were transplanted into the substantia nigra of 6-OHDA unilaterally-lesioned rats. Significant motor improvement was observed from

4 weeks post-transplantation onwards and reduced apomorphine-induced rotations were seen after 12 weeks. Furthermore, histological analysis revealed survival of grafted cells and differentiation into dopaminergic neurons. *In vitro* differentiation of hMSCs using a 2-step procedure (pre-induction with 10% cord blood serum, 2% B27, 2 ng/mL FGF-2, 100 ng/mL NGF, and 50 ng/mL Noggin for 1 week, followed by 200 μM BHA for 4-5 hours) also yielded cells with neuronal morphology and gene and protein expression, which were capable of secreting DA into the culture medium (Shetty *et al.*, 2009). Therefore, despite difficulties encountered in this field, some hope remains in the ability of MSCs to be implemented in cell-based therapies for PD.

5.4.3 Further Commitment of hMSCs to the Neuronal Lineage Prior to Transplantation May Be Necessary for Intracerebral Engraftment

Both the present study and that by Suon, *et al*. (Suon *et al.*, 2006) had utilised partially neuronal-differentiated or neuronal progenitor-like cells for transplantation, since it is believed that immature neurons are more suitable for transplantation (Bjorklund *et al.*, 2000; Hermann *et al.*, 2004), and confirmation of this was seen in pilot studies conducted by Suon and colleagues (Suon *et al.*, 2006). Yet, neither study could obtain long-term graft survival. The stage of SC differentiation that is optimal for transplantation purposes remains to be determined, although, in general it is still thought that immature neuronal cells possess the greatest potential for successful engraftment and integration into the host circuitry.

Continued deposition of fibronectin matrix was observed at graft sites in the current study, perhaps indicating that hMSCs still possessed primitive properties and had not been directed far enough along the neuronal differentiation pathway. It is possible that excessive production of fibronectin by hMSCs may have elicited a stronger inflammatory reaction at graft sites by stimulating the removal of unphysiological

amounts of fibronectin by microglia/macrophages. Furthermore, transplantation of undifferentiated MSCs into the hemiparkinsonian rat model resulted in graft loss by 3 weeks, presumably due to the non-neural/foreign nature of these cells within the CNS, whereas MSC-derived mature dopaminergic neurons were capable of engraftment and functional improvement (personal communication, M. Dezawa). Nevertheless, other studies have also reported fibronectin production by hMSCs following transplantation into the ischemic and normal rat brain, without adverse effects on survival and engraftment (Azizi *et al.*, 1998; Zhao *et al.*, 2002).

A narrow window of opportunity may exist during the neuronal differentiation of SCs that permits successful transplantation and integration into the CNS, but outside of this period cells may be either too mature to survive transplantation or not yet committed enough to complete differentiation *in vivo*. Therefore, additional studies are necessary to further define this optimal window for transplantation.

5.4.4 Unresolved Technical Aspects of hMSC Transplantation into the CNS

A number of technical aspects remain undetermined concerning the transplantation of MSCs into experimental models of CNS diseases. One of the primary concerns is the improvement of cell survival and differentiation post-transplantation. Graft survival can be linked to a number of factors, including the quantity of SCs used for transplantation. In the present study, undifferentiated and neuronal-primed hMSCs were transplanted at a dose of 100,000 cells per site, in accordance with the majority of reported studies examining MSC transplantation in Parkinsonian rodent models (Dezawa *et al.*, 2004; Fu *et al.*, 2006b; Hellmann *et al.*, 2006). However, the absence of engraftment we observed was not seen in these studies. For NSCs, transplantation of between 150,000 and 300,000 cells per site has been reported to be adequate for yielding graft survival, however, it was still suggested that use of higher numbers of

SCs may result in higher survival rates (Svendsen *et al.*, 1996). Whereas, a separate report comparing the transplantation of 200,000, 1 million or 2 million human neural precursors into 6-OHDA hemiparkinsonian rats found that smaller grafts elicited greater neuronal fibre extension and less immunological rejection in comparison with the larger grafts (Ostenfeld *et al.*, 2000). For MSCs, it was found that primate MSC engraftment into the primate CNS was enhanced when low doses (500,000) rather than high doses (2.5 million) of MSCs were injected intracerebrally (Isakova *et al.*, 2007). Therefore, the results of the present study may be improved through increasing the number of hMSCs transplanted to a quantity more similar to that used in successful neural SC transplantation or primate MSC transplantation.

Few transplanted hMSCs or OECs were observed to migrate out of graft sites and needle tract regions raising the question of whether migration and integration were prevented or inhibited. The glial scar formed at graft sites contains an inhibitory proteoglycan component, and the presence of inhibitory proteins such as the chondroitin sulfate proteoglycans ACAN, versican, and neurocan has been reported to inhibit axon guidance and prevent neurite outgrowth and axon regeneration (Fitch *et al.*, 1997; Horner *et al.*, 2002; Jones *et al.*, 2003; McKeon *et al.*, 1999). Recent studies have attempted to improve graft survival and integration by creating a more permissive microenvironment at graft sites through chondroitinase ABC treatment to reduce inhibitory proteoglycans (Fouad *et al.*, 2005; Singhal *et al.*, 2008). Singhal and colleagues employed combined transplantation of Müller SCs together with chondroitinase ABC for promotion of matrix degradation and cell migration in the degenerated retina (Singhal *et al.*, 2008). Chondroitinase ABC treatment in conjunction with enhanced microglial suppression (oral cyclosporine A, azathioprine, prednisolone, indomethacin) was found to result in dramatic improvement in migration of Müller SCs

into the retina, decreased microglial accumulation, significantly higher numbers of transplanted Müller SCs, and a characteristic neuronal morphology in the migrating cells. These results suggest that abnormal extracellular matrix deposition and activation of innate inflammatory responses may constitute major barriers to retinal SC transplantation (Singhal *et al.*, 2008). Furthermore, combination of OEC and Schwann cell transplantation with chondroitinase ABC resulted in improved axonal regeneration and locomotor recovery after complete transection of the spinal cord (Fouad *et al.*, 2005). Alternatively, MSCs have been transplanted in the presence of heparin and DNase to improve cell survival and engraftment in experimental murine models of acute myocardial infarction (personal communication, K. Atkinson). Difficulties with migration through inhibitory proteoglycans and extracellular matrices may hold true for the current study, and as a consequence, the application of these treatments may be beneficial for the transplantation of undifferentiated and neuronal-primed hMSCs into the 6-OHDA hemiparkinsonian rat model. In addition, the findings of a recent study suggested that manipulation of the graft niche to facilitate cross-talk between transplanted SCs and the diseased brain may be beneficial for more efficacious transplantation in chronic lesions or advanced stages of neurodegenerative diseases in the aged CNS (Ourednik *et al.*, 2009). Evidence of this was observed through the over-expression of neural cell adhesion molecule L1 in donor NSCs and in recipient transgenic MPTP-lesioned mice (over-expression in host astrocytes under the GFAP promoter), which resulted in rapid and extensive distribution of donor NSCs, rescue of dysfunctional host dopaminergic neurons, and enhanced differentiation of donor NSCs into TH-expressing neurons (Ourednik *et al.*, 2009). These findings offer further options for more efficacious transplantation of SCs into the diseased CNS, and further studies are warranted.

Other strategies aimed at improving the survival and differentiation of SC and neuronal progenitor grafts have been suggested, including the addition of trophic factors, such as FGF-2 (Bhang *et al.*, 2007; Takayama *et al.*, 1995), or antioxidants (Nakao *et al.*, 1994) to the cell suspension prior to transplantation. Furthermore, little is known of the optimal timing or therapeutic window for SC transplantation post-injury. A study examining systemic infusion of MSCs for the treatment of liver fibrosis found that immediate MSC transplantation post-injury was able to significantly reduce liver damage and collagen deposition, whereas delaying MSC infusion by 1 week hampered the prevention of disease progression (Fang *et al.*, 2004). Unfortunately, no conclusions have been reached on which techniques or combinations of techniques provide the best outcomes and further investigation into these aspects is necessary.

5.4.5 Controversies Associated with Cell Identification Techniques

Further confusion has emerged through recent unexpected findings indicating that commonly used cell-labelling techniques were not as reliable as originally thought, and could result in the transfer of donor labels to host cells (Burns *et al.*, 2006; Coyne *et al.*, 2006). Burns and colleagues were the first to describe the *in vivo* release of thymidine analogs, BrdU, chlorodeoxyuridine and tritiated thymidine, from transplanted MAPCs and subsequent incorporation into the DNA of dividing host neural cells (Burns *et al.*, 2006). Added confirmation of thymidine analog transfer was demonstrated through the transplantation of labelled dead cells (by repeated freeze-thawing) or fibroblasts, leading the authors to suggest that rigorous controls or alternative cell identification techniques are required when transplanting into highly proliferative environments, such as the developing, neurogenic or injured brain (Burns *et al.*, 2006). Shortly thereafter Coyne, *et al.* also reported the transfer of BrdU and bis benzamide from donor MSCs to host phagocytes, astrocytes and neurons, which falsely suggested

MSC engraftment and differentiation up to 12 weeks, despite histological evidence of graft destruction by 14 days (Coyne *et al.*, 2006). Therefore, these cell-labelling techniques may provide misleading indication of donor cell survival and differentiation, and cast doubts on the findings of earlier studies that solely used these labelling techniques for graft identification.

In the present study, we avoided the use of these cell labels, and instead employed techniques of immunohistological detection through human-specific antibodies, as well as GFP-transgene fluorescence in OECs. Furthermore, our results were consistent with those reported by Burns, *et al*. and Coyne, *et al*. in that only limited survival of hMSCs could be detected post-transplantation. The discrepancy in results between these studies and the earlier studies demonstrating MSC engraftment and integration may therefore be explained by the methods of cell detection utilised in the various studies.

Although GFP fluorescence was not found to be transferable to host cells (Burns *et al.*, 2006), caution must also be taken in interpreting cell identification through GFP fluorescence techniques alone, since loss or reduction of transgene expression can also lead to false negative results. Further difficulties with the detection of donor cells post-transplantation have been discussed in Section 1.2.4, and include issues arising from the presence of endogenous autofluorescence, particularly from dying cells, which can be observed at similar wavelength as used for GFP detection (Goodell, 2003). Such autofluorescence was detected in the present study.

5.4.6 Transplantation of hMSCs Did Not Elicit Endogenous Neurotrophic Effects

An increasing number of studies have now described the ability of MSCs to promote tissue repair and functional improvement, despite little evidence of differentiation and only low or transient levels of *in vivo* engraftment (Phinney *et al.*,

2007; Prockop, 2007). These studies have suggested that MSCs may not repair tissues solely through differentiation and replacement of lost cells, but may possess a more prominent role in the secretion of factors with the ability to alter the host tissue microenvironment. The absence of engraftment observed in this thesis reflects certain aspects of this recent perspective. However, in the current study investigation of endogenous host NSC activation by immunohistological detection of NES expression found no response of host NSCs to hMSC transplantation. Staining indicative of NSCs could not be detected around graft regions, and host NES expression could only be detected in activated GFAP-positive astrocytes surrounding the needle tract and graft site (see Figure 5.7). In addition, transplantation of hMSCs did not result in differences in microglia/macrophage and astrocyte accumulation around the needle tract and graft regions when compared with the sham-treated controls (see Figure 5.6).

While we were unable to find a neurotrophic response in host cells following intracerebral hMSC transplantation, this has been reported in a number of recent studies. Munoz and colleagues demonstrated that hMSC transplantation into the murine hippocampus stimulated the proliferation, migration and differentiation of endogenous NSCs into neural precursors and mature neural cells (Munoz *et al.*, 2005). The mechanism underlying the increased neurogenesis was suggested to involve secretion of NGF, VEGF, CNTF, FGF-2, and other chemokines by hMSCs, which may have acted directly on NSCs, or could have activated astrocytes that then produced the increase in neurogenesis (Munoz *et al.*, 2005).

Another recent study showed that hMSC transplantation in an experimental model of transient forebrain ischemia led to improved neurologic function through hMSC expression of anti-immune, anti-inflammatory and anti-apoptotic-related factors and establishment of a Th2-immune bias through alternative activation of microglia

and/or macrophages by hMSCs (Ohtaki *et al.*, 2008). These studies, together with findings from many different sources have indicated the involvement of multiple mechanisms in MSC-mediated tissue repair, including the stimulation of survival and proliferation of endogenous host cells, modulation of immune responses, induction of angiogenesis and reduction of apoptosis (Phinney *et al.*, 2007; Prockop, 2007).

Although difficulties were encountered in the present study with the application of hMSCs in PD cellular therapy, efforts in this field have continued and promising results have been reported during the course of our study. The strategy employed in this thesis of *in vitro* dopaminergic neuronal differentiation of MSCs prior to transplantation has also been examined in 2 recent studies, which implemented *in vitro* differentiation procedures containing cytokines similar to those utilised in our methods (Bouchez *et al.*, 2008; Levy *et al.*, 2008). Apart from differences in the *in vitro* differentiation techniques employed, the *in vivo* studies in this thesis also differed from these two reports in that higher numbers of MSCs were transplanted in both these studies (180,000 and 500,000 respectively). In addition, unlike these reports we were unable to detect graft survival even with neuronal-priming of hMSCs prior to transplantation.

The study by Bouchez and colleagues employed a 3 week sequential culture protocol involving FGF-2, FGF-8, SHH and BDNF, which had been adapted from a previous study (Jiang *et al.*, 2002).The differentiation method resulted in incomplete engagement of MSCs through the neural pathway, but intrastriatal transplantation of these cells still yielded a stable 50% reduction in rotational asymmetry, and partial restoration of dopaminergic markers and the vesicular striatal pool of DA. Surprisingly, it was also found that transplantation of undifferentiated MSCs resulted in equivalent behavioural improvement. Recovery was observed to occur rapidly by 1 week post-grafting, and was maintained during the 6 week study period. This finding, together

with the detection of BDNF, GDNF, FGF-2 and FGF-8 expression *in vitro* by undifferentiated and neuronal-differentiated MSCs, suggested that neurotrophic factor secretion could be the mechanism behind recovery (Bouchez *et al.*, 2008).

The study conducted by Levy, *et al*. utilised a 2-stage method of neuronal differentiation containing growth factors (FGF-2, EGF), chemicals (BHA, dbcAMP, IBMX), RA, and docosahexaenoic acid (Levy *et al.*, 2008). Transplantation of these differentiated hMSCs also resulted in improvement in rotational behaviour, and was of superior benefit when compared to grafting of undifferentiated hMSCs. In contrast to the previous study by Bouchez, *et al*., the authors of this study suggested that recovery was due in part to dopaminergic cell replacement, since donor hMSCs were found to survive more than 130 days post-transplantation and migrated to the damaged nigra, with a small proportion of cells also expressing TH (Levy *et al.*, 2008).

As previously mentioned, the majority of recently published studies have concentrated on the growth factor secretory effects of MSCs in Parkinsonian rodents *in vivo* (Bahat-Stroomza *et al.*, 2009; Sadan *et al.*, 2009; Shintani *et al.*, 2007), or in neural co-culture systems *in vitro* (Jin *et al.*, 2008; Sadan *et al.*, 2009; Shintani *et al.*, 2007). Shintani and colleagues reported the release of BDNF, GDNF and FGF-2 by murine MSCs into culture media, and found that treatment of embryonic dopaminergic neurons with MSC-conditioned medium prior to intrastriatal transplantation yielded significantly enhanced graft survival and more rapid recovery from rotational deficits in the 6-OHDA hemiparkinsonian rat model (Shintani *et al.*, 2007).

Two studies conducted by the Offen laboratory used a different strategy of directing hMSCs toward an astrocyte-like, neurotrophic factor-secreting phenotype (Bahat-Stroomza *et al.*, 2009; Sadan *et al.*, 2009). These cells were then found to be capable of eliciting behavioural improvement in pharmacologically-induced rotation

tests and non-pharmacological motor tests, as well as enhanced striatal DA levels, and increased TH immunoreactivity after intrastriatal transplantation. Interestingly, a different 6-OHDA-induced hemiparkinsonian experimental model was used in these studies. Instead of the complete lesion model used in our studies and in the majority of published reports, a partial lesion model with residual surviving DA terminals was employed, which was chosen as the authors were interested in examining the protection-restoration effect of trophic factor-secreting MSCs. The authors also commented that neurotrophic factor-secreting cells are not expected to be beneficial in models with complete lesioning, and that these models may only show improvement with dopaminergic cell replacement (Bahat-Stroomza *et al.*, 2009). Similar to our findings, absence of graft survival was observed by Sadan, *et al.*, with the majority of transplanted cells rejected within a week post-transplantation, despite cyclosporine immunosuppression and transplantation of higher numbers of cells than used in our study (Sadan *et al.*, 2009).

In vitro effects of trophic factor secretion by MSCs have also been described, with findings of increased expression of TH and DA by ventral mesencephalic cells co-cultured with undifferentiated rat MSCs (Jin *et al.*, 2008). Also, treatment with MSC-conditioned medium was found to increase the survival of embryonic mesencephalic dopaminergic neurons in *in vitro* neuronal injury models of serum deprivation and exposure to 6-OHDA (Shintani *et al.*, 2007). Furthermore, marked protection was demonstrated when conditioned medium from astrocyte-like, neurotrophic factor-secreting MSCs or undifferentiated MSCs were applied to neuroblastoma cell line, SH-SY5Y, one hour prior to exposure to neurotoxin 6-OHDA (Sadan *et al.*, 2009).

Together these studies provide support for another potential mechanism by which MSCs may effect functional improvement in Parkinsonian models. Further

evaluation of both the neuroprotective and cell regenerative/replacement properties of MSCs will be required in order to harness these effectively in cellular therapies for PD and other neurological diseases.

5.4.7 Co-Transplantation of OECs Did Not Yield Improved Engraftment of Neuronal-Primed hMSCs

In this thesis, OEC co-transplantation with neuronal-primed hMSCs was, to our knowledge, investigated for the first time, to determine whether OECs could aid hMSC engraftment into the lesioned hemisphere of the 6-OHDA-induced hemiparkinsonian rat. Since the commencement of this work, several studies examining OEC transplantation for PD and nigrostriatal dopaminergic axon transection have been reported (Dewar *et al.*, 2007; Shukla *et al.*, 2009; Teng *et al.*, 2008). Overall, these recent studies indicate that co-grafting with OECs is beneficial in cellular therapies for these disorders.

OEC co-transplantation with olfactory nerve fibroblasts was shown to promote axonal regeneration, and prevent fibrotic scar formation and type IV collagen deposition at lesions caused by unilateral transection of nigrostriatal dopaminergic axons (Teng *et al.*, 2008). Furthermore, Shukla and colleagues found that co-grafting of OECs significantly enhanced the survival of NSC-derived dopaminergic neurons and striatal reinnervation in 6-OHDA lesioned hemiparkinsonian rats (Shukla *et al.*, 2009). Co-transplanted animals also displayed significant reduction in amphetamine-induced rotational behaviour, increased spontaneous locomotor activity, and neurochemical recovery of DA and its metabolite DOPAC (Shukla *et al.*, 2009). Whereas, Dewar, *et al*. demonstrated that transplantation of OECs alone into the striatum of hemiparkinsonian rats is insufficient for the promotion of tissue repair and functional recovery (Dewar *et*

al., 2007), suggesting that in cellular therapies for PD, OECs play a supportive role for neuronal progenitors or SCs that act to replace damaged or lost dopaminergic neurons.

In contrast to these reports, co-transplantation of OECs in the present study did not appear to provide advantages for the survival or differentiation of neuronal-primed hMSCs in the striatum or substantia nigra of 6-OHDA lesioned hemiparkinsonian rats. Furthermore, transplanted OECs also did not survive or engraft into the host brain. A major difference in our study is the use of xenogeneic hMSCs and OECs for transplantation into the rat CNS, and although immunosuppression with cyclosporine was implemented strong glial accumulation was observed at graft sites, which may account for the graft loss. Other potential factors contributing to the loss of hMSCs have been discussed previously, and these may also have influenced the survival of OECs. Therefore, further studies are required to determine the capabilities of hMSC and OEC transplantation into experimental models of PD.

5.4.8 Summary

In conclusion, this chapter has shown that transplantation of hMSCs into the lesioned hemisphere of hemiparkinsonian rats results in limited graft survival and absence of differentiation *in vivo*, regardless of the differentiation state of hMSCs (undifferentiated or neuronal-primed), the transplantation site (striatum or substantia nigra), the presence or absence of OECs, and the use of cyclosporine immunosuppression. In addition, hMSC transplantation was not found to result in neurotrophic or immunomodulatory effects on endogenous host cells. These findings are contradictory to the majority of initial reports describing functional improvement in neurological diseases resulting from cellular replacement by MSCs. The discrepancies may be related to differences in the differentiation procedures, transplantation techniques and cell labelling/identification methods implemented. On the other hand,

similar findings have been reported in a recent publication describing limited MSC engraftment and the presence of inflammatory responses and glial accumulation around graft sites post-transplantation (Coyne *et al.*, 2006). The mechanisms underlying the rapid loss of transplanted hMSCs and OECs remain to be determined, but may involve innate immune response targeting of xenogeneic cells, inadequate neuronal differentiation of hMSCs prior to transplantation, insufficient immunosuppression, and the possible need for improved transplantation strategies. While improvements to our current understanding of MSC-based cellular therapy for PD are still required, the progress in this field to date validates the significance of this work and warrants continued research efforts in this field.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Concluding Remarks

The results of this thesis illustrate the complexity of the cellular therapy discipline, and emphasise the need for a greater understanding of each facet of this field. Promising outcomes and continued research progress have fuelled enthusiasm and hope within the scientific community and the general public, however, much remains to be understood concerning the biology of the donor cell population, the optimal procedures for directed differentiation toward the desired cell phenotype, effective strategies for transplantation into sites of injury/degeneration, the relevancy of animal models to the disease in humans, and techniques for accurate analysis of transplantation outcomes. This thesis aimed to investigate the baseline characteristics of BM-derived hMSCs over long-term subcultivation, the potential of hMSCs to differentiate *in vitro* toward the neuronal lineage under the influence of cytokines, and the effects of intracerebral hMSC transplantation in the 6-OHDA lesioned hemiparkinsonian rat model.

Analysis of undifferentiated hMSCs confirmed that the cell populations exhibited the expected characteristics of MSCs, in terms of proliferative ability, morphological appearance, expression of surface antigens, genes and proteins, and multi-lineage mesodermal differentiation capacity. Furthermore, through examination of these characteristics over the course of long-term serial passaging, it was found that hMSCs do not spontaneously differentiate toward the neural lineage, and optimal periods for the commencement of neuronal differentiation were determined. Interestingly, hMSCs cultured beyond the optimal time frame (first 6-8 passages of expansion culture) were still able to acquire an immature neuronal-like phenotype. Nevertheless, obvious differences were seen between neuronal-differentiated early passage and late passage hMSCs, with late passage cultures containing larger cells,

greater amounts of cellular debris, and a higher proportion of cells retaining a flat, fibroblast-like appearance that were unresponsive to cytokine treatment.

Through this work evidence was provided supporting the *in vitro* generation of an immature neuronal-like phenotype in hMSCs through the application of cytokines and culture conditions that are known to have neural-inducing capabilities. It was demonstrated for the first time that cytokine-based neuronal differentiation of hMSCs is the result of active and dynamic cellular processes, involving migration, as well as outgrowth, motility and retraction of cell extensions. These observations showed clear differences to chemical-based neuronal differentiation systems, which have been found to induce toxicity-related morphological changes causing a false pseudo-neuronal appearance (Bertani *et al.*, 2005; Choi *et al.*, 2006; Deng *et al.*, 2006; Lu *et al.*, 2004; Neuhuber *et al.*, 2004; Suon *et al.*, 2004). It was also established that prior to differentiation hMSCs expressed neural and pluripotency markers together with mesodermal markers, providing support for MSC neural differentiation capacity and also confirming the need to ascertain baseline expression levels in order to accurately assess the outcomes of differentiation procedures.

Since cytokine-based neuronal differentiation was able to generate immature neuronal properties in hMSCs, this study also endeavoured to induce a dopaminergic neuronal phenotype through the use of cytokines known to be important in midbrain dopaminergic neuronal development *in vivo*. In addition, a strategy of sequential cytokine application was also investigated, as this would more closely mimic the *in vivo* situation. However, none of the differentiation strategies investigated was capable of eliciting enhanced dopaminergic neuronal differentiation from hMSCs. Although, of the 3 methods examined, the first stage of the MultiDA method yielded the greatest increase in expression of neuronal progenitor marker *NES* and dopaminergic neuronal

transcription factor *NR4A2*. Furthermore, through evaluating the effects of the different cytokine combinations it was determined that FGF-2 and EGF exerted the greatest benefits for the *in vitro* neuronal differentiation of hMSCs.

In view of the fact that immature neuronal cells survive intracerebral transplantation procedures better than mature neurons, the present thesis investigated the effects of transplanting hMSCs that had undergone neuronal-priming (first stage of the MultiDA method), as well as undifferentiated hMSCs, on the lesioned hemisphere of hemiparkinsonian rats. Grafted hMSCs were found to survive transplantation and could be detected initially at days 1 and 7 post-transplantation through immunohistological staining for HNA expression. However, only limited graft survival could be detected, with loss of HNA signal apparent at day 7 post-transplantation and complete absence of grafted cells by day 21. Moreover, strategies that were implemented to improve engraftment and differentiation, such as administration into the substantia nigra as well as the striatum, neuronal-priming *in vitro* prior to transplantation, and co-transplantation with OECs, were unable to provide any significant advantages. The observed absence of engraftment may be explained by: the presence of an innate inflammatory response at graft and sham sites, which may have been initiated by surgical trauma during transplantation; the xenogeneic nature of the graft, since both MSCs and OECs were lost from graft sites; the 'foreign' nature of the hMSCs, which had not gained a completely mature neuronal phenotype as evidenced by the continued production of fibronectin extracellular matrix; or the lack of appropriate factors within the lesioned adult rat striatum and substantia nigra for supporting hMSC survival, engraftment and differentiation. Additionally, transplantation of hMSCs and OECs did not appear to elicit neurogenic effects on endogenous host NSCs or neural progenitors. These findings contradict those predominantly reported in the literature,

however, shows similarity with recent reports describing absence of MSC engraftment and presence of glial accumulation at graft sites (Coyne *et al.*, 2006; Suon *et al.*, 2006). The results of this thesis also show for the first time the surprising presence of microglial/macrophage accumulation, suggesting activation of innate inflammatory responses after hMSC transplantation into the lesioned hemiparkinsonian rat brain.

Therefore, taken together, the study presented in this thesis demonstrates that cytokine-based neuronal differentiation of hMSCs elicits authentic and dynamic cellular responses, and induces the acquisition of an immature neuronal phenotype. However, difficulties were encountered in achieving *in vivo* engraftment and differentiation after intracerebral transplantation into the lesioned brain. The results obtained in the *in vivo* studies of this thesis differ from some published reports; nevertheless, the results of those reported studies also display conflicting data and remain a topic of ongoing debate. Central to this controversy is the mechanism by which MSCs exert functional improvements *in vivo* in experimental animal models of disease. Pioneering studies suggested that tissue repair occurred via the broad developmental plasticity and transdifferentiation capacity of MSCs; however, an emerging line of evidence has arisen suggesting that MSCs exhibit low or transient levels of *in vivo* engraftment and transdifferentiation, and instead promote tissue repair through enhancing the regeneration of injured cells, preserving surviving cells, or stimulating endogenous host SC activity (Chen *et al.*, 2006; Phinney *et al.*, 2007; Prockop, 2007). In the context of this thesis, transient survival post-transplantation may therefore be expected. It is also possible that the absence of an endogenous host response to hMSC and OEC transplantation may be related to the severe, complete lesion hemiparkinsonian disease model employed in this study, in which there is no chance for rescuing damaged dopaminergic neurons since the process of neuronal death has been completed.

However, a MSC-induced protection-restoration effect on endogenous host neural cells may be possible in a less severe PD model, such as the partial lesion model used by Bahat-Stroomza and colleagues (Bahat-Stroomza *et al.*, 2009).

It is unclear whether the discrepancies observed between studies result from actual differences in outcome, or are representative of variations in experimental techniques. Interpretation of data across the different studies is also complicated by the vast array of procedures that have been utilised for the *in vitro* neuronal differentiation and *in vivo* transplantation and identification of MSCs. Furthermore, these diverse methodologies often result in supposedly similar outcomes, which is even more surprising given that different measures of that outcome have been used for assessment purposes. Taken together, this has led to little consistency across the field and difficulties in comparing data obtained by different studies. A meticulous and systematic approach is therefore required for the proper development of reproducible and reliable methods for MSC neuronal differentiation and application in cellular therapies for neurological diseases. Inherent to this situation is the reliance of the majority of studies on the use of heterogeneous populations of MSCs, raising the question of whether different studies are possibly using 'different' MSC populations, which could potentially account for the diverse findings. Nevertheless, current data still suggests that MSC-based cellular therapies for neurological diseases and disorders are possible, although very much more complicated than initially believed with potential involvement of a number of distinct underlying mechanisms.

The issues discussed above emphasise the present need for a more comprehensive understanding of the biology and identity of the bona fide MSC. Considering the potential significance of this research, further studies are necessary to achieve a better understanding of this field and to identify the optimal *in vitro* and *in*

vivo procedures for directed neuronal differentiation and effective transplantation of hMSCs.

6.2 Future Directions

In order to advance the experimental observations related to the cytokine-based neuronal differentiation of hMSCs and the absence of *in vivo* engraftment found in this thesis, further studies are required. Efforts directed towards advancing our understanding of the isolation and expansion of undifferentiated hMSCs *in vitro* would allow the establishment of consistent methods across different studies and may lead to less variation between results from different laboratories. CD146 has been proposed as a potential positive selection marker for MSCs (Sacchetti *et al.*, 2007), therefore, $CD146^+$ MSCs could be characterised according to the parameters examined in the present study to determine any similarities or differences with the MSC population utilised in our study.

To confirm the immature neuronal-like phenotype obtained in hMSCs through cytokine induction, *in vitro* functional analysis could be performed to detect the presence of electrophysiological activity. In addition, this analysis would reveal whether the hMSC-derived neuronal-like cells possess functional properties that resemble those of immature neurons.

Since hMSCs appeared to respond poorly to the second (SHH/FGF-8) and third (GDNF) stages of the MultiDA method, it would be interesting to examine the growth factor receptors present on hMSCs during the different stages of neuronal differentiation. Furthermore, the functionality of these receptors could be confirmed through performing DNA synthesis studies by incubating hMSCs with [^{3}H] thymidine following cytokine exposure to examine stimulation of cells into the S-phase of the mitotic cycle (as performed by Kramer and colleagues (Kramer *et al.*, 2006)).

The *in vitro* neuronal differentiation procedure for hMSCs could be improved through the addition of neural support cells, such as astrocytes. In particular, it has been shown that the regional origin of astrocytes was the determining factor for the neuronal fate of co-cultured ESCs (Roy *et al.*, 2006), therefore potential lies in the co-culturing of hMSCs with midbrain astrocytes.

Further characterisation of the immune response to hMSCs after intracerebral transplantation should be undertaken to assess the involvement of other inflammatory cell types, as well as T and B lymphocytes. This investigation of innate and adaptive immune responses should also be conducted for the period between 1 and 7 days post-transplantation, to further elucidate the mechanisms involved in hMSC and OEC graft loss.

Since microglia/macrophages appear to be activated in the regions surrounding the graft site and needle tract, additional immunosuppression could be implemented as reported by Singhal and colleagues, who utilised a combination of oral cyclosporine A, azathioprine, prednisolone and indomethacin for enhanced immunosuppression (Singhal *et al.*, 2008).

Immunofluorescence staining could also be performed to examine the deposition of inhibitory proteoglycans, such as CSPGs, at the graft site and needle tract to reveal whether proteoglycan deposition encases the grafted cells from host brain tissue and prevents hMSC migration and integration. If this is indeed the case, then transplantation can be performed in the presence of chondroitinase ABC, or heparin and DNase to facilitate graft survival, engraftment and integration.

Additional improvements could be attempted for the *in vivo* neural transplantation of MSCs. Transplantation of a greater number of hMSCs may provide improved donor cell survival, since recent studies have used higher MSC dosages and

obtained promising results (Bouchez *et al.*, 2008; Levy *et al.*, 2008). Difficulties relating to the xenogeneic nature of the grafts employed in this study can be confirmed and avoided through the use of rat MSCs and OECs when transplanting into the hemiparkinsonian rat model. Furthermore, transplantation into a partial lesion hemiparkinsonian model could be performed to allow examination of endogenous host NSC responses and protection-restoration effects of MSC transplantation. Alternate routes of MSC delivery to the CNS could also be investigated, including intravenous or intrathecal administration of MSCs, which would avoid most of the trauma associated with direct intracerebral transplantation.

Finally, little is known of the effects of cytokine-based neuronal differentiation procedures on the immunomodulatory properties of hMSCs. To address these concerns, it will be of benefit to examine the hMSC surface antigen expression profile, capability for stimulating peripheral blood lymphocyte proliferation and cytotoxicity, and potential for interacting with DCs (rosette binding assay and chemotaxis assay) following the *in vitro* neuronal and dopaminergic neuronal differentiation procedures investigated in this thesis.

CHAPTER 7

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CHAPTER 8

SUPPLEMENTARY FILES

The Supplementary CD is attached to the back cover of this thesis, and contains 'Supplementary File 1 – Part 1' and 'Supplementary File 1 – Part 2' video footage discussed in Section 4.2.1, and 'Supplementary File 2' video discussed in Section 5.2.1.