Mesenchymal stem cells as trophic mediators of neural differentiation

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MESENCHYMAL STEM CELLS AS TROPHIC MEDIATORS OF NEURAL DIFFERENTIATION

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Abstract

Intense excitement and optimism surrounds the rapidly-expanding field of stem cell research, owing to their high capacity for self-renewal and intrinsic ability to differentiate into mature cell lineages. Although it may be envisioned that embryonic stem cells will be of significantly greater therapeutic value than their adult stem cell counterparts, the use of embryonic stem cells is fraught with both technical and ethical challenges and, as such, significant impetus has been placed on adult stem cell-based research. In particular, mesenchymal stem cells (MSCs) present as exciting candidates for potential use in cellular therapies and tissue engineering strategies.

MSCs are defined at the functional level in terms of their ability to differentiate into mesodermal derivatives such as bone and fat. However, this functional definition is evolving, and there is considerable evidence to suggest that MSCs have a key role within their niche involving the release and/or uptake of soluble factors and cytokines, significantly influencing the behaviour of other cell types within the niche. Both facets of MSC behaviour are valuable from a clinical perspective, and have been examined in the present thesis.

The most obvious and realistically-achievable clinical application of MSCs at present is in the treatment of osseous and adipose tissue defects. However, before the use of MSCs in the clinic becomes more commonplace, it is crucial to gain a more comprehensive understanding of the complex molecular and cellular mechanism(s) by which MSCs commit to a given fate and undergo differentiation to produce mature, fully-functional derivatives. Much of our present knowledge is derived from studies performed on the highly unnatural, 2D environment of tissue culture plastic. The present study assessed the behaviour of MSCs cultured on Alvetex^{TM}, a novel, 3D scaffold manufactured by ReInnervate, with particular emphasis on the ability of MSCs to undergo osteogenic and adipogenic differentiation. Results obtained suggest that Alvetex^{TM} may provide a more realistic and physiologically-relevant system in which to study osteogenesis and adipogenesis, in a manner more pertinent to that which occurs *in vivo*.

Furthermore, the ability of MSCs to influence the behaviour of other cell types *via* the release of trophic factors and cytokines was examined, with particular emphasis on the nervous system. An *in vitro* conditioned media model was developed in order to investigate the influence(s) of MSC-derived soluble factors/cytokines on neural development and plasticity, using the adult rat hippocampal progenitor cell (AHPC) line as a model system. Results obtained suggest that, under defined conditions, MSCs secreted a complement of soluble factors/cytokines that induce AHPCs to commit to and undergo astrogenesis. This effect was characterised at both the cellular and molecular level. The specific complement of bioactive factors secreted by MSCs has been investigated using a combination of targeted transcriptional profiling and shotgun proteomics, and several putative candidate factors have been identified for further investigation.

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Declaration

This thesis is entirely the result of my own work. It has not been submitted for any other degree.

Publications arising from this thesis

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Abbreviations

2D	Two dimensional
3D	Three dimensional
5-HT	5-hydroxytryptamine
AChE	cAMP-acetylchotinesterase
AHPC	Adult hippocampal progenitor cell
AIM	Aggregate induction medium
ALP	Alkaline phosphatase
AMPA3	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 3
APC	Allophycocyanin
ATRA	All *trans*-retinoic acid
BDNF	Brain-derived neurotrophic factor
bFGF	basic Fibroblast growth factor
BHA	Butylated hydroxyanisole
bHLH	Basic helix-loop-helix
BME	β-mercaptoethanol
BMP	Bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CCM	Complete culture medium
cDNA	Complementary DNA
CFU-F	Colony forming unit-fibroblasts
CM	Conditioned medium
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
CRE	cAMP-response element
CREB	cAMP-response element binding protein
Cy3	Cyanine 3
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOPA	Dihydroxyphenylalanine
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EC	Embryonal carcinoma
ES	Embryonic stem
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FL 1-4	Fluorescence 1-4
FLT3	Foetal liver tyrosine kinase 3
FSC	Forward scatter
GABA	Gamma aminobutyric acid
GalC	Galactocerebroside
GAP	Growth-associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM-SCF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft versus host disease
HCl	Hydrochloric acid
HEK 293	Human embryonic kidney
HGF	Hepatocyte growth factor
HIPE	High internal phase emulsion
HPLC	High pressure liquid chromatography
HSC	Haematopoietic stem cell
HSV	Herpes simplex virus
IBMX	3-isobutyl-1-methylxanthine
Id	Inhibitor of differentiation
IGF	Insulin growth factor

IL	Interleukin
IVF	*In vitro* fertilization
JAK-STAT	Janus-activated kinase signal transducer and activator of transcription
LC-MS/MS	Liquid chromatography-mass spectrometry
LIF	Leukaemia inhibitory factor
LSEC	Liver sinusoidal endothelial cells
MALDI-TOF MS	Matrix-assisted laser desorption time-of-flight mass spectrometry
Map2ab	Microtubule-associated protein
MBP	Myelin basic protein
MCAo	Middle cerebral artery occlusion
MEF	Mouse embryonic fibroblast
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MONO-CM	Monolayer conditioned medium
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MSC-CM	Mesenchymal stem cell-conditioned medium
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
NEPAF	North East Proteome Analysis Facility
NeuN	Neuronal nuclei
NF200	Neurofilament 200
NGF	Nerve growth factor
Ngn	Neurogenin
NGS	Natural goat serum
NMDA	*N*-methyl *D*-aspartate

NOD-SCID	Non-obese severe combined immunodeficient
npMSC	Nestin positive MSC
NSC	Neural stem cell
NSE	Neuron-specific enolase
NT-3	Neurotrophin-3
NTC	No template control
O4	Oligodendrocyte marker 4
P0	Passage 0
PBS	Phosphate-buffered saline
PC	Proliferation control
PC 12	Pheochromocytoma 12
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PEDF	Pigment epithelial-derived growth factor
PES	Phenazine ethosulphate
PFA	Paraformaldehyde
PI	Propidium iodide
PKA	Protein kinase A
PLO	Poly-L-ornithinne
pNNP	*p*-nitrophenyl phosphate
PPARγ	Peroxisome proliferator-activated receptor γ
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse-transcription PCR
RNA	Ribonucleic acid
RT	Reverse transcription
SAGE	Serial analysis of gene expression
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGZ	Subgranular zone
SEM	Scanning electron microscopy
SFI	Sciatic function index
α-SMA	α-smooth muscle actin

SSC	Side scatter
STAT	Signal transducer and activator of transcription
SVZ	Subventricular zone
TCTP	Translationally controlled tumour protein
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
Tuj-1	Neuron-specific class III β-tubulin
UC	Unconditioned medium
UV	Ultra violet
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

Chapter 1

LITERATURE REVIEW

1.1 Introduction

Intense excitement and optimism surrounds the rapidly-expanding field of stem cell research where it is envisioned that these primitive cells hold the potential to revolutionise modern medical approaches against a wide range of clinically-important disorders, principally *via* cellular-based regenerative therapies and tissue engineering strategies (Bianco and Robey, 2001; Giannoudis and Pountos, 2005; Martino and Pluchino, 2006). The potential role of stem cells in modern medicine has garnered much media interest to date and it is anticipated that this interest will only serve to intensify in the future.

One aspect of stem cell behaviour that is overlooked by the media, however, is the somewhat paradoxical role played by stem cells in disease aetiology. Although the obvious therapeutic potential of stem cells in the clinic cannot be questioned, it is has been demonstrated in several systems that stem cells present as the main targets for neoplastic transformation in oncology, where their persistence throughout the lifespan of the organism means they exist long enough to accumulate the numerous genetic alterations required for transformation, in keeping with Knudson's 'multi-hit' hypothesis (Knudson *et al.*, 1973; Bonnet and Dick, 1997). Additionally, the maintenance of stem cell populations within tumours has been demonstrated in numerous systems, where it proposed that such 'cancer stem cells' have the potential to give rise to new tumours (Reya *et al.*, 2001; Al-Hajj et al., 2003; Collins et al., 2005; Florek et al., 2005; Kim et al., 2005; Singh et al., 2003).

From an academic perspective, stem cells are also invaluable models in developmental biology and it is envisioned that a greater understanding of the complex cellular and molecular mechanisms by which stem cells commit to and undergo differentiation will reveal new facets in how development proceeds from embryo to organism (Rohwedel *et al.*, 2001; Rodda *et al.*, 2002).

Stem cell research therefore has many different facets and potential applications in both the laboratory and the clinic; however, our current understanding of stem cell behaviour is too basic at present in order to recognise their true potential. Consequently, translation of stem cells from the laboratory bench to the clinic has been

slow and somewhat disappointing, despite continued claims from the media that stem cell breakthroughs are imminent. Improved knowledge regarding the behaviour/function of stem cells and how such behaviour can be controlled/manipulated appropriately is the first major step towards achieving this goal.

1.2 Basic principles in stem cell biology

1.2.1 Stem cell definition, sources and potency

Stem cells are characterised at the functional level in terms of their high capacity for self-renewal and intrinsic ability to differentiate into mature cell lineages (Weissman, 2000). 'Embryonic stem (ES) cells' are derived from the inner cell mass of the blastocyst, a structure formed during the early stages of embryogenesis in mammals, and demonstrate the potential to differentiate into all the specialised tissues of an organism. This potential for human ES cells was initially demonstrated both *in vitro* and *in vivo* by Thomson and colleagues (1998). 'Adult stem cells', conversely, are found in the vast majority of adult tissues, albeit in relatively small numbers (approximately 1-2%), where they contribute to tissue repair and general cell turnover (Presnell *et al.*, 2002). The capacity for self-renewal extends throughout the adult life of an organism, permitting tissue repair and regeneration from damage encountered throughout the lifespan of an organism (Preston *et al.*, 2003). Despite this high capacity for self-renewal, stem cells generally exist in a non-dividing, quiescent state *in vivo*. This serves to protect the stem cell from accumulative DNA replication errors, which would be deleterious to the survival of an organism. Previously, the majority of adult stem cell research has focussed on tissues characterised by a rapid cell turnover, such as the skin, gut and haematopoietic systems; however, there has since been a shift in interest to include tissues characterised by a much slower cell turnover rate, including the prostate (Hall and Watt, 1989; Isaacs and Coffey, 1989).

Although stem cells can be classified at the broadest level as either 'embryonic' or 'adult', they can be further classified according to their differentiative potential (potency).

- *Totipotency (omnipotency)*

  Describes the ability of a stem cell to differentiate into specialised cell types representative of all three germ layers *and* form extraembryonic tissue such as the trophoblast of the placenta (Thomson *et al.*, 1998).

  By definition, totipotent (omnipotent) stem cells are the only cell type with the potential to generate a new, viable organism; as such, use of this term is usually restricted to description of the zygote and cells produced by the first few zygotic divisions (Mitalipov and Wolf, 2009).

- *Pluripotency*

  Describes the ability of a stem cell to differentiate into specialised cell types representative of all three germ layers but not extraembryonic tissue; as such, pluripotent stem cells do not have the potential to generate a new, viable organism and use of this term is usually restricted to description of ES cells derived from the inner cell mass of the blastocyst (Ulloa-Montoya *et al.*, 2005).

- *Multipotency*

  Describes the ability of a stem cell to differentiate into a range of closely-related specialised cell types appropriate to their physiological location. This term can be used in the description of most adult stem cell types, including mesenchymal and haematopoietic stem cells.

- *Unipotency*

  Describes the ability of a stem cell to differentiate into one specialised cell type e.g. spermatogonodal cells.

1.2.2 Embryonic stem cells versus adult stem cells

The obvious differences between pluripotent embryonic stem cells and multipotent, lineage-restricted adult stem cells reflects the different functions of the two cell types in development. Embryonic stem cells contribute to the formation of all the specialised tissue that will eventually give rise to a new organism, whereas adult stem cells are predominantly involved in tissue maintenance and general cell turnover; as such, the

differentiative potential of adult stem cells is more restricted in keeping with this function.

From a clinical perspective, it may therefore be envisioned that embryonic stem cells will be of significantly greater therapeutic value than their adult counterparts, owing to their potential to generate all specialised tissues found throughout organisms, including germ cells as well as somatic cells. However, the use of embryonic stem cells in both an academic and clinical environment is fraught with both technical and ethical challenges. These will be considered very briefly below.

1.2.2.1 Source of material

This is perhaps the most ethically-controversial aspect of embryonic stem cell biology. Thomson and colleagues isolated human embryonic stem cells from 'spare' embryos that had been donated from couples undergoing *in vitro* fertilisation (IVF). Similarly, Zhang and colleagues isolated human embryonic stem cells from arrested embryos produced during IVF treatment (Thomson *et al.*, 1998; Zhang *et al.*, 2006). At present, public opinion is largely divided over the ethical issues concerning the derivation of stem cells from human embryos. Conversely, adult stem cells can be easily and routinely isolated from a wide variety of readily-accessible sources, including bone marrow, adipose tissue, adult blood, umbilical cord blood, amniotic fluid, tendon and ligaments, chorionic villi of the placenta, synovial membranes and deciduous teeth, amongst many others (Kuznetsov et al., 2001, Rosada et al., 2003, Vandenabeele et al., 2003, Miura et al., 2003, Gronthos et al., 2001, Igura et al., 2004, Tsai et al., 2004, Anker et al., 2003, Salingcarnboriboon et al., 2003, Seo et al., 2004). As such, the use of adult stem cells both in the laboratory and the clinic is more ethically-acceptable at present.

1.2.2.2 Technical challenges of in vitro research

Culturing human ES cells in the laboratory is technically challenging for several reasons.

- Isolation of cells from the inner cell mass is technically challenging due to the presence of a protective layer of tissue surrounding the blastocyst known as the trophoectoderm (Stojkovic *et al.*, 2004).

- Requires a feeder layer of mitotically-inactive mouse embryonic fibroblasts, though leukaemia inhibitory factor (LIF) can be used for mouse ES cells (Evans and Kaufman, 1981).

- Difficult to maintain embryonic stem cells in an undifferentiated state as they are highly prone to spontaneous differentiation.

1.2.2.3 Teratoma formation

Direct systemic administration of undifferentiated embryonic stem cells leads to teratoma formation in the host organism (Thomson *et al.*, 1998).

The numerous ethical and technical disadvantages/limitations of embryonic stem cell behaviour have resulted in increased interest in adult stem cell biology, as such cells present as promising alternatives for potential therapeutic applications in the clinic.

1.2.3 Stem cells undergo asymmetric division to produce two phenotypically-distinct daughter cells

Since stem cells only account for approximately 1-2% of total cellular content of adult tissues, a number of interesting issues are raised:

a) How can such a small proportion of stem cells (1-2%) contribute to total tissue turnover and repair throughout the lifespan of an organism?

b) How are stem cell populations maintained throughout the lifespan of an organism and how do they avoid depletion?

Although there are currently no satisfactory explanations to account for these phenomena, one facet of stem cell biology which could potentially account for these observations is the ability of stem cells to undergo asymmetric division, as demonstrated in Figure 1.1. It is widely accepted that stem cells are able to undergo asymmetric division to produce two phenotypically-distinct daughter cells – one corresponding to a quiescent, immature stem cell (self-renewal) and the other corresponding to highly-proliferative, lineage-restricted progenitor cell (transit-

amplifying progenitor). Following a limited number of replications, transit-amplifying progenitors will exit the cell cycle and undergo terminal differentiation, thus contributing to tissue maintenance/repair whilst maintaining the stem cell population (Hall and Watt, 1989; Preston *et al.*, 2003).

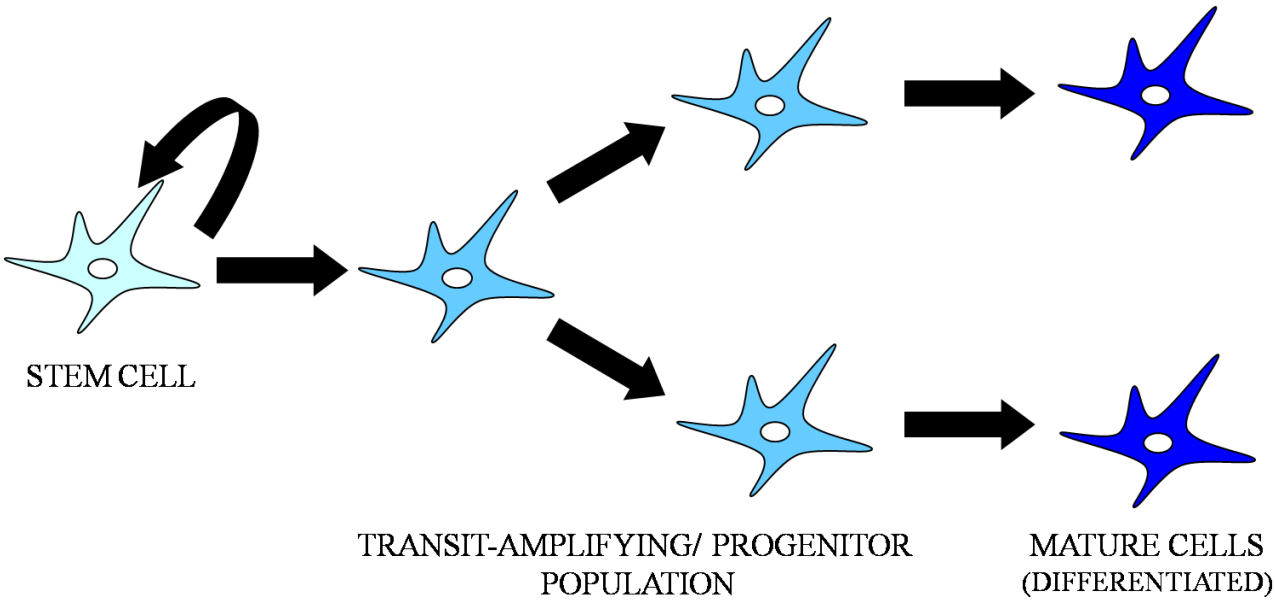

Figure 1.1. Stem cells undergo asymmetrical division to produce two phenotypically-distinct daughter cells. Under normal physiological conditions it is hypothesised that stem cells undergo asymmetrical division to produce two phenotypically-distinct daughter cells, one of which corresponds to an identical stem cell (self-renewal) and the other of which corresponds to a rapidly-cycling, lineage-restricted transit-amplifying progenitor. The proliferative potential of transit-amplifying progenitors is limited and, following a limited number of replications, these cells exit the cell cycle and undergo terminal differentiation. (Adapted from Preston *et al.*, 2003).

As demonstrated in Figure 1.1, the relationship between stem cell, transit-amplifying progenitor and terminally-differentiated cell can be considered a hierarchical organisation; however, this is likely to represent an oversimplification of the biological situation. For example, it is highly probable that there are numerous intermediate stages in the transition from stem cell to terminally differentiated cell. Similarly, it is not known with any certainty whether stem cell division is always asymmetrical or whether the reported equality in the proportion of stem and transit-amplifying progenitors is a consequence of an overall net effect encompassing both symmetrical

and asymmetrical divisions (Hall and Watt, 1989). Distinction between symmetrical and asymmetrical division at the molecular level has been achieved by analysis of the composition of cell membrane proteins between daughter cells (Beckmann *et al.*, 2007). Regardless, the hierarchy highlighted in Figure 1 must be stringently regulated in order to maintain correctly-balanced tissue turnover.

1.2.4 Stem cell niche

The concept of the 'stem cell niche' was first proposed by Schofield over 30 years ago to describe the specialised microenvironment in which a stem cell resides *in vivo* (Schofield, 1978). At the physiological level, the stem cell niche consists of cellular and extracellular matrix components which form a functioning unit of tissue. At a more molecular level, however, the stem cell niche can be considered a dynamic entity in which complex interactions occur between stem cells and neighbouring cells, typically involving direct cell-cell interactions and/or the release of diffusible soluble factors/cytokines (produced by identical cells (autocrine) or different cells (paracrine) within the niche), extracellular matrix, oxygen tension and pH, amongst many other crucial parameters (Preston, 2003; Fuchs *et al.*, 2004; Scadden, 2006). The complex and highly dynamic relationship between the stem cell and various components of its surrounding microenvironment play a crucial role in regulation of stem cell behaviour, with niche-specific signals serving to maintain the stem cell in its quiescent state until appropriate, at which point niche-specific signals can be modified accordingly to induce an appropriate response in the stem cell e.g. proliferation, migration and/or differentiation (Fuchs *et al.*, 2004; Scadden, 2006).

The concept of the stem cell niche and its importance in maintenance/regulation of stem cell behaviour/function has remained a largely theoretical concept following its original postulation by Schofield in 1978. However, it is now widely accepted that the influence of niche-specific signals on stem cell behaviour/function are equally as important as the intrinsic properties of the stem cells themselves; indeed, Scadden (2006) states, 'The niche is as critical as stem-cell-autonomous functions in shaping our understanding of basic stem-cell biology, and how it contributes to health and disease'. Evidence in support of niche-specific signals governing stem cell behaviour/function is perhaps best demonstrated by one study in which germ line cells were transplanted into male mice lacking a stable germ line stem cell population

(Wang and Zhao, 1999). Following transplantation, it was observed that the majority of transplanted cells occupied the empty niches along the basal layer of the testes and underwent differentiation into mature sperm, restoring fertility in the recipient. As such, there is rejuvenated interest in the stem cell niche, its various components, and how these interplay to shape stem cell behaviour/function *in vivo*.

Stem cell niches have since been described in considerable detail for numerous stem cell populations *in vivo*; however, it is perhaps the niches contained with epithelial tissues such as skin and the intestinal crypt that have been characterised the most extensively (Figure 1.2).

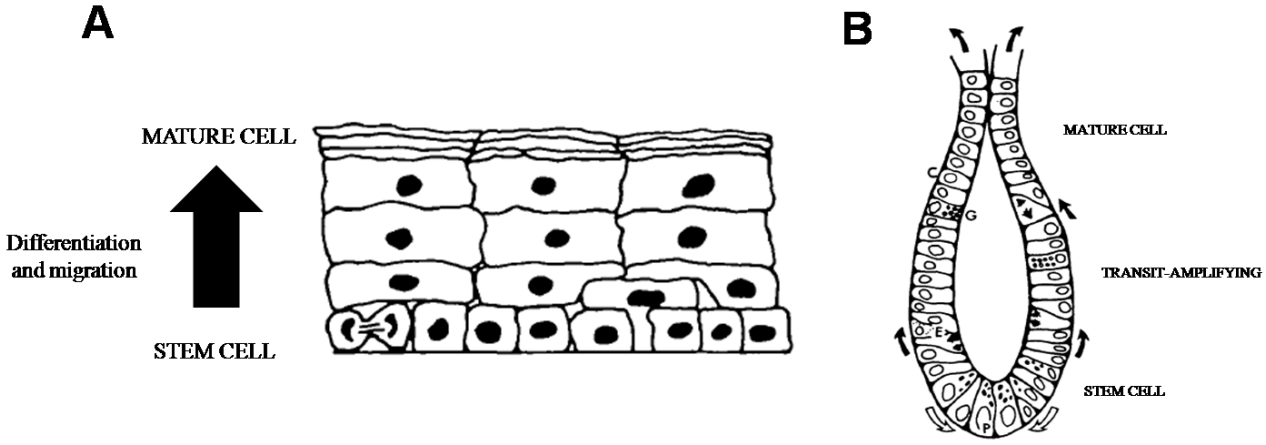

Figure 1.2. Organisation of the highly-characterised stem cell niches within the skin epidermis (A) and intestinal crypt (B). A: The putative stem cell population within the skin epidermis is housed in the basal layer. During differentiation, stem cells migrate from the basal layer in an upward direction towards the upper layers of the epidermis. **B:** The intestinal crypt demonstrates a similar organisation, wherein the putative stem cell population is housed deep within the crypt and undergoes migration in an upward direction towards the villi during differentiation. (Adapted from Hall and Watt, 1989).

Cells within the skin epidermis are arranged within multiple layers, as demonstrated in Figure 1.2A, with the putative stem cell population being housed within the basal layer (Hall and Watt, 1989). During differentiation, stem cells undergo migration from the basal layer in an upward direction through the various other layers of the niche, such that terminally-differentiated cells exist as a squame at the uppermost region of the epidermis (Withers, 1967, Potten and Hendry, 1973). A similar phenomenon has been

described in other epithelial-lined tissues such as prostate (Isaacs and Coffey, 1989). Confinement of the stem cell population to the basal layer in the skin epidermis was demonstrated following radiological damage to the mouse epidermis and subsequent cell cycle analysis. Cell proliferation could only be detected within the basal layer, and within a small subpopulation at that, accounting for approximately 10% of total cellular content of the basal layer (Withers, 1967).

A second example of a highly-characterised stem cell niche is provided by the intestinal crypt (Hall and Watt, 1989). The gross morphology of the small intestine is such that its epithelial layer is organised into crypts and villi, as demonstrated in Figure 1.2B, with the putative stem cell population being housed deep within the crypts (Hall and Watt, 1989; Potten *et al.*, 1987). As described for the skin epidermis, stem cells in the intestinal crypt undergo migration in an upward direction during differentiation, replacing those terminally-differentiated cells that are continually shed from the tips of villi (Potten *et al.*, 1987). It was originally estimated that the number of stem cells contained with each crypt averaged between 4 and 16; however, further studies have since proven that crypts are derived and maintained by a single stem cell (Potten *et al.*, 1987; Potten and Loeffler, 1987; Griffiths *et al.*, 1988; Schmidt *et al.*, 1988).

Although the structural organisation of the stem cell niches in the skin epidermis and intestinal crypt have been extensively characterised, a more comprehensive understanding of the effects of niche-specific signals and how they combine to modulate stem cell behaviour/function is desirable (Coffey *et al.*, 1987; Akhurst *et al.*, 1988; Barnard *et al.*, 1989). Such signals may include growth factors released by underlying stromal cells and other cell types within the niche.

1.3 Mesenchymal stem cells (MSCs)

1.3.1 Overview of MSC biology

Mesenchymal stem cells (MSCs) are exciting candidates for use in cellular therapies and tissue engineering strategies. They are an adult stem cell population and derive their name from an intrinsic ability to differentiate into multiple cell lineages of

mesodermal origin such as bone, fat and cartilage (Pittenger et al., 1999). This is shown diagrammatically in Figure 1.3. Given these differentiative capabilities, MSCs can be described as ***multipotent***, meaning they are able to differentiate into closely-related cell types of the same germ layer.

1.3.2 Historical perspective

MSCs were first identified in 1976 in a fundamental work by Friedenstein (Friedenstein et al., 1976). At the time of this work, much research had been done and emphasis placed on haematopoietic cells present within the bone marrow. Friedenstein identified the presence of a second cell population within the bone marrow, distinct from the haematopoietic lineage, and these cells displayed two key properties: 1) colony-forming potential; and 2) ability to differentiate into bone and cartilage (both mesodermal tissues). Both the ability to form colonies (self-renew) and differentiate to produce progeny displaying a more mature phenotype confirmed that the cell population identified by Friedenstein were stem cell in nature. Morphologically, these cells appeared fusiform and fibroblastic, and as such were initially described as *colony forming unit-fibroblasts (CFU-F).* This original terminology proposed by Friedenstein has generally been replaced and nowadays these cells are typically referred to as mesenchymal stem cells, or marrow stromal cells.

1.3.3 Sources of MSCs and their niche

MSCs can be found ubiquitously throughout the body and are routinely isolated from sources such as bone marrow, adipose tissue, adult blood, umbilical cord blood, amniotic fluid, tendon and ligaments, chorionic villi of the placenta, synovial membranes, deciduous teeth and foetal liver, lung and spleen (Kuznetsov et al., 2001, Rosada et al., 2003, Vandenabeele et al., 2003, Miura et al., 2003, Gronthos et al., 2001, Igura et al., 2004, Tsai et al., 2004, Anker et al., 2003, Salingcarnboriboon et al., 2003, Seo et al., 2004). In fact, the distribution of MSCs throughout the body may be even more widespread, as alluded to in a recently published study by da Silva Meirelles and colleagues entitled ‘Mesenchymal stem cells reside in virtually all post-natal organs and tissues’ (da Silva Meirelles et al., 2006). In this study, it was found that MSCs could be isolated and subsequently cultured *in vitro* from all of the organs and tissues examined in a murine system, including brain, spleen, liver, kidney, lung, muscle, thymus, pancreas and, of course, bone marrow. In doing so, the authors

convincingly showed that MSCs can be isolated from tissues arising from all three germ layers. Morphologically, MSCs isolated from the different tissues were very similar and marker expression profiles were indicative of an MSC population, although slight differences in marker expression profiles were observed between MSCs isolated from different sources. In terms of their capacity to differentiate into the mesodermal derivatives bone and fat, differences were more apparent between the cells depending on their site of origin, although all cells examined had the capabilities to undergo osteogenic and adipogenic differentiation (and hence may be correctly termed MSC). Such differences aside, the cells isolated from these various sources of tissue were similar enough in biological terms to suggest a close relationship between them.

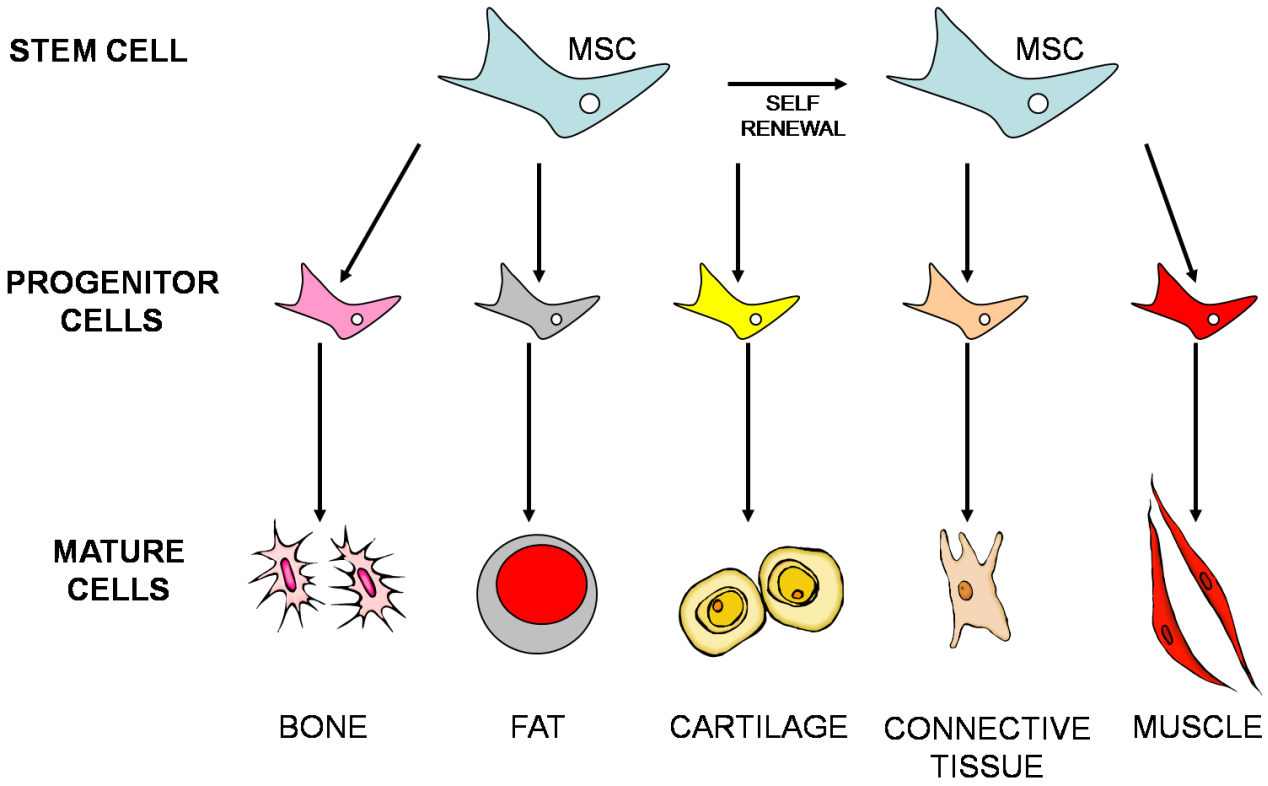

Figure 1.3. A schematic diagram showing the differentiative capabilities of MSCs. Following cell division, MSCs may form one of two different progeny: they may produce a daughter stem cell, in a process known as self-renewal, or may give rise to a progenitor cell that is more rapidly proliferating but more restricted in terms of its proliferative capacity and differentiative capabilities. MSCs have the capability to differentiate into a wide range of mesodermal cell types, including bone, fat, cartilage, connective tissue and muscle.

The ability to isolate and successfully propagate MSCs from such a wide range of tissues, both mesodermal and non-mesodermal in origin, raises the interesting issue of whether these tissues house a common MSC niche or whether MSCs are able to function in a manner that is independent of their environment, meaning that niche-specific signals are not important determinants of their behaviour (Kolf *et al.*, 2007). Da Silva Meirelles and colleagues shed further light on this subject, examining the isolation and culture of MSCs from vascular tissue, both complex (aorta and vena cava) and simple (capillaries of the kidney glomerulus). The successful isolation and propagation of MSC populations from such sources is in keeping with previous reports claiming that MSCs may arise from perivascular cells (Farrington-Rock et al., 2004, Shi and Gronthos, 2003). These data therefore suggest that MSCs occupy a perivascular niche *in vivo*, which could also account for the widespread distribution of MSCs throughout the body (da Silva Meirelles et al., 2006). The perivascular niche of MSCs and its putative structure is shown diagrammatically in Figure 1.4.

However, despite improved understanding of the structural organisation of the MSC perivascular niche, current knowledge pertaining to the complex and dynamic interactions occurring between MSCs and neighbouring cells/extracellular matrix within this niche is somewhat limited. In one study, Kaigler and colleagues (2005) examined the influence of endothelial cells on the osteogenic potential of MSCs, wherein they observed that endothelial cells could enhance the osteogenic differentiation of MSCs but only when in direct contact. This effect could not be reproduced following culture of MSCs with conditioned media or following culture of MSCs on extracellular matrix laid down by endothelial cells (Kaigler *et al.*, 2005). Another important parameter to consider within the MSC niche is oxygen tension, as cells are exposed to much lower oxygen tensions *in vivo* compared to tensions encountered during routine *in vitro* culture. Indeed, culturing of cells under lowered oxygen tension *in vitro* has been demonstrated in numerous systems to significantly influence cellular behaviour (Csete, 2005; Sullivan *et al.*, 2006).

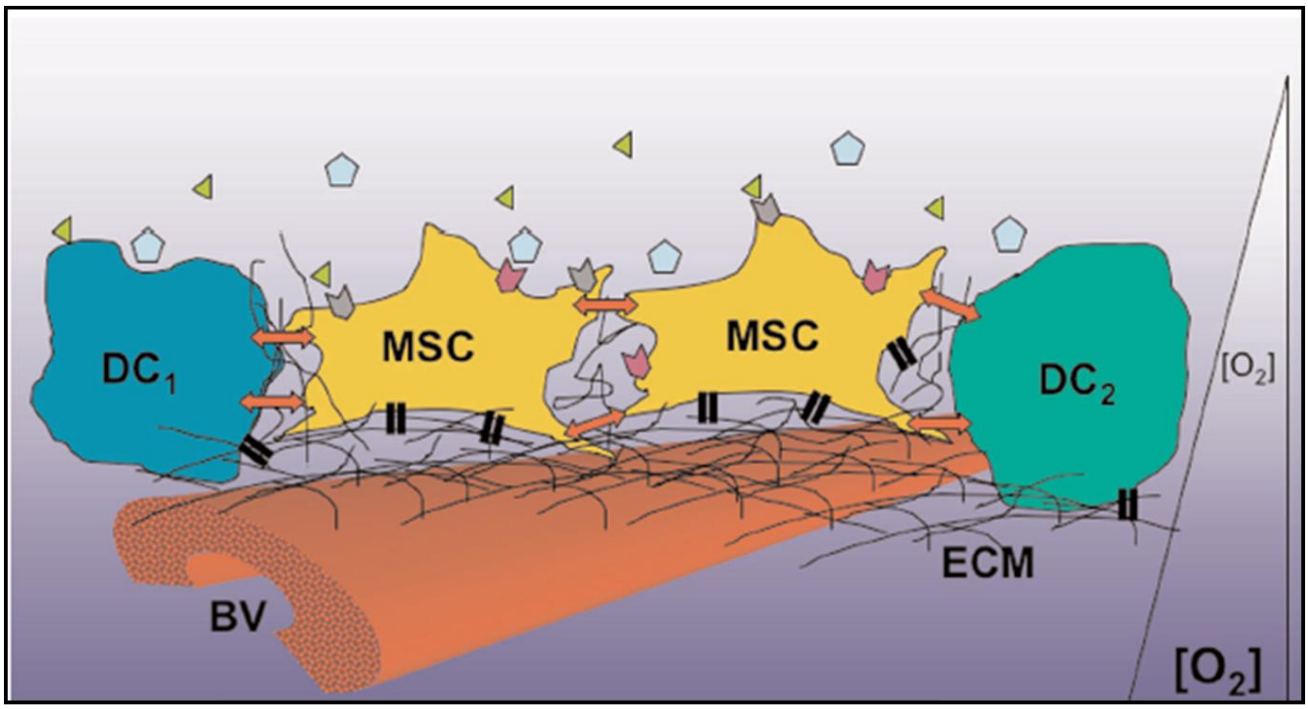

Figure 1.4. The hypothesised perivascular niche of MSCs and its organisation. It is postulated that MSCs occupy a perivascular niche *in vivo*, which accounts for their widespread distribution throughout the body. MSCs interact with various components of this niche, including blood vessels (BV), differentiated cell types within the niche (DC_1 and DC_2), extracellular matrix (ECM) components and numerous diffusible soluble factors and cytokines, both self-derived (autocrine behaviour) and derived from other cell types (paracrine behaviour). (Reproduced from Kolf *et al.*, 2007).

1.3.4 Isolation of MSCs from bone marrow

Despite the wide distribution of MSCs throughout the body, the bone marrow remains the most commonly used source for MSC isolation to date, even though MSCs represent only a tiny fraction of the total marrow population, around 0.01% (Pittenger et al., 1999). Commonly used sources of bone marrow for the isolation of MSCs include the iliac crest of the pelvis, femur, tibia and thoracic and lumbar spine (Pittenger et al., 1999, Digirolamo et al., 1999, Murphy et al., 2002, D'Ippolito et al., 2002). The iliac crest of the pelvis is the most commonly used human source of MSCs, whereas the most predominant source in rodents are the longer bones (femurs and tibiae).

A typical protocol for the isolation of MSCs from rodent femurs and tibiae is described below and shown in Figure 1.5. Firstly, the rodent must be euthanized and their femurs and tibiae extracted, cleaned of all connective tissue and placed in complete culture

medium (CCM) on ice. CCM typically consists of Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS), non-essential amino acids, L-glutamine, penicillin and streptomycin, although the exact components of CCM can vary somewhat between different laboratories. The ends of the bones must then be cut in order to expose the bone marrow, and the marrow aspirated with CCM by inserting a 21-gauge needle into the shaft of the bone and flushing with CCM. Bone marrow aspirates must then be plated into a tissue culture flask with more CCM and incubated, typically at 37^{o}C and 5% CO_2 in a humidified incubator. Aspirates must be left untouched for 48 hours to allow the stromal component of the marrow (MSCs) to adhere to the tissue culture plastic. The second cell population present within bone marrow – the haematopoietic population – should remain in suspension and not attach to the cellware. Adherence to tissue culture plastic is the major criterion to discriminate between and separate the haematopoietic and stromal components of the bone marrow. The resulting cultures of MSCs are therefore highly heterogeneous, consisting of a mixture of cells at various stages of development. Alternative isolation methods exploiting marker expression are currently being sought in order to isolate a more homogenous starting population of MSCs, however, adherence to tissue culture plastic remains the most highly used method to date. After 48 hours, non-adherent cells (haematopoietic cells) should be removed from the cultures and the adherent cells maintained in CCM. These cells are designated as *passage 0* (*P0*).

1.3.5 Cell-surface and intracellular markers indicative of a MSC phenotype

Presently, there is no single definitive marker to define an MSC population. This is largely due to the heterogeneity observed within MSC cultures. MSCs are heterogeneous not only in terms of marker expression, but also cell size, morphology and potency. It is therefore necessary to build up an expression profile i.e. examine expression of a range of markers, none of which purely exclusive to MSCs, in order to determine the phenotype of the population. Positive markers include CD29, CD44, CD54, CD55, CD73 (SH3 and SH4), CD90 (Thy-1), CD105 (SH2, or endoglin), CD106 (vascular cell adhesion molecule, VCAM-1), CD117 and CD166, while negative markers include CD11b, CD14, CD31, CD33, CD34 and CD45 (Pittenger et al., 1999; Shyu et al., 2006). Negative markers are largely typical for the haematopoietic contingent within the bone marrow. In addition to these cell surface

antigens, MSCs also show expression of intracellular mesenchymal markers, including fibronectin, vimentin and α-smooth muscle actin (α-SMA) (Romanov et al., 2005).

There are several examples in the literature where groups have attempted to exploit marker expression to produce a more homogeneous starting population of MSCs. By far, the most prominent method of MSC isolation exploits the property of plastic adherence, which assumes that the stromal population (MSC) of the bone marrow will adhere to tissue culture plastic, whereas the haematopoietic contingent (haematopoietic stem cells or HSCs) will not (Figure 5). Consequently, contaminating HSCs will be removed with continual media changes and passage, and what should remain in culture is a heterogeneous mixture of stromal cells at various stages of development. Efforts are currently underway to utilise cell surface markers to further purify specifically-labelled MSCs from these heterogeneous pools, for example by fluorescence-activated cell sorting (FACS) (Alsalameh et al., 2004; Dennis et al., 2002).

1.3.6 Differentiation of MSCs into mesodermal derivatives

Analysis of marker expression is not the only factor to consider in confirming the isolation of a true MSC population. As the definition of a stem cell is a functional one, it is perhaps functional characterisation rather than simple marker analysis that is more compelling in proving the isolation of a true MSC population. MSCs have the potential to differentiate into a wide range of mesodermal cell types, including osteoblasts (bone), adipocytes (fat), chondrocytes (cartilage) and myoblasts (muscle) (Pittenger et al., 1999). Such differentiation can be easily achieved *in vitro* by addition of specific inducing factors to the cell's culture medium. For osteogenic differentiation, MSCs must be treated with a cocktail of dexamethasone, ascorbic acid 2-phosphate and β-glycerophosphate. Adipogenesis, on the other hand, is slightly more complex, requiring a cyclic treatment with dexamethasone, indomethacin, insulin and 3-isobutyl-1-methylxanthine (IBMX) (induction medium) for three days, followed by treatment with insulin alone (maintenance medium) for two days. Although slight variations in the differentiation protocols are inevitable between different laboratories e.g. concentrations of differentiation factors, frequency of media changes etc., the inducing factors used are highly standardised.

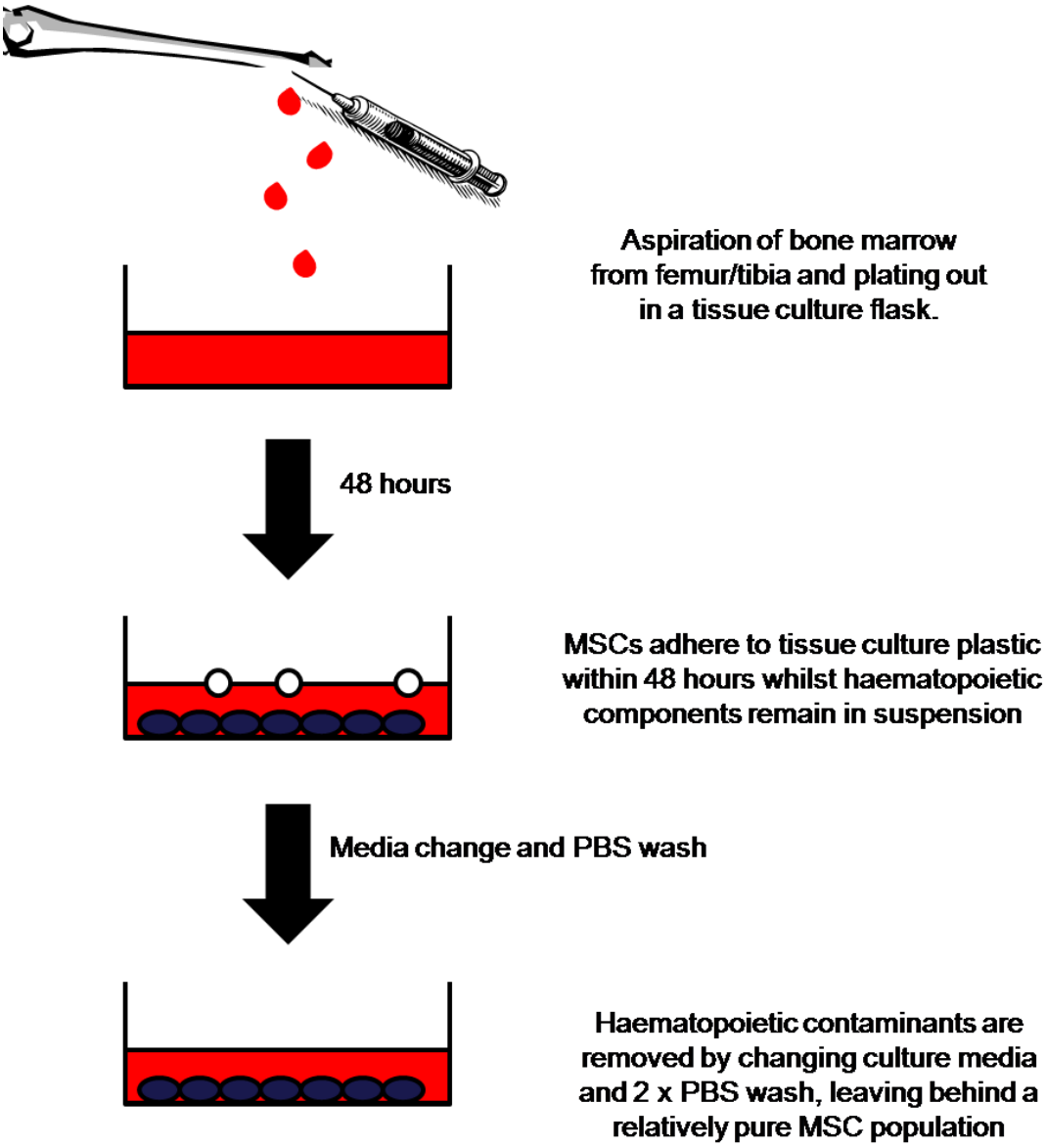

Figure 1.5. Overview of the isolation of MSCs from bone marrow. Schematic representation of the isolation of MSCs from bone marrow *in vitro*. Briefly, bone marrow is aspirated and plated out into a tissue culture flask and cultured for 48 hours at 37^{o}C and 5% CO_2 to allow adherence of MSCs to tissue culture plastic. Haematopoietic components remain in suspension and do not adhere to tissue culture plastic. Following 48 hours, haematopoietic contaminants are removed by changing the culture media and PBS washes, resulting in a relatively pure MSC population.

To assess the phenotype of MSCs induced to undergo osteogenic and adipogenic differentiation, a range of techniques must be employed. These techniques are largely histological and require the cells to be stained with solutions that allow identification of various structures associated with either lineage. Demonstration of a mature

osteoblastic phenotype can be achieved using standard procedures involving staining for deposition of mineralised matrix using von Kossa and staining to visualise calcium and collagen deposition using Masson's trichrome (Pittenger et al., 1999). Similarly, demonstration of a mature adipogenic phenotype can be achieved using standard procedures involving staining for the accumulation of intracellular lipid droplets within the cytoplasm of a cell using oil red O staining (Pittenger et al., 1999). In both instances, it is necessary to counterstain cells with haematoxylin, a basic dye that stains cell nuclei a blue-purple colour. Examples of von Kossa, Masson's trichrome and oil red O staining are demonstrated in Chapter 3.

1.3.7 Differentiation of MSCs into non-mesodermal derivatives

Established dogma in stem cell biology has held that adult stem cells possess a more limited differentiation potential than their embryonic counterparts, being restricted to only form lineages specific to their tissue of origin. Consequently, the potency of MSCs was considered to be such that they could only form tissues of mesodermal origin. However, in recent years this paradigm has been challenged, with numerous reports of stem cells 'crossing' the germ layer boundary and forming tissues representative of a different lineage, a process termed *'trans'-differentiation*. The concept that adult stem cells may possess a far wider potential for differentiation than previously anticipated has led to much excitement with respect to their therapeutic application, since the use of adult stem cells is not met with the same criticism or ethical issues associated with the use of embryonic stem cells. Reports of '*trans*'-differentiation are especially rife with regard to MSCs, with evidence of MSCs (mesodermal in origin) forming tissues of both endodermal and ectodermal origins (Krause et al., 2001; Petersen et al., 1999; Sanchez-Ramos et al., 2000; Sato et al., 2005; Woodbury et al., 2002; Woodbury et al., 2000). If such reports hold true, then it is perhaps more accurate to describe MSCs as a pluripotent stem cell population as opposed to a multipotent one though this term should be used with caution. This chapter will focus particularly on the formation of neural-like cells from MSCs and address the issue of whether or not they represent an alternative source to neural stem cells for use in neurological therapies.

1.4 Potential therapeutic applications of MSCs

1.4.1 MSCs demonstrate several properties which render them promising candidates for therapeutic application

MSCs are promising candidates for clinical use in cellular therapies and tissue engineering strategies. Cellular therapies are medical processes that involve the administration of healthy, fully-functioning cells to replace dysfunctional cells that have been damaged or destroyed in disease. The principle of tissue engineering is largely the same, though the situation is somewhat more complex as it involves culturing cells on three-dimensional structures *in vitro*, rather than tissue culture plastic, in order to form functional units that more closely resemble the *in vivo* target tissue. Tissue engineering brings together expertise from a range of fields, including cell biology, biochemistry and polymer chemistry. A variety of different cell types may be used in cellular therapies and tissue engineering, including mature functional cells, genetically-modified cells or even cells from different species (xeno-transplantation). However, the focus of this literature review will be the potential applications of stem cell-based therapies, in particular, MSC-based therapies.

Although adult stem cells can be isolated from a variety of sources throughout the body, MSCs are perhaps the most promising for use as potential therapeutic agents. There are a number of reasons as to why this is the case:

a) MSCs can be found and isolated from a wide range of autologous sources, (Kuznetsov et al., 2001, Rosada et al., 2003, Vandenabeele et al., 2003, Miura et al., 2003, Gronthos et al., 2001, Igura et al., 2004, Tsai et al., 2004, in 't Anker et al., 2003, Salingcarnboriboon et al., 2003, Seo et al., 2004).

b) Many of these sources, particularly bone marrow and adipose tissue, are clinically accessible, meaning that MSCs can be easily isolated using robust, well-established techniques (da Silva Meirelles et al., 2006, Gronthos et al., 2001, Pittenger et al., 1999).

c) MSCs have a high proliferative potential and can be readily and rapidly expanded *ex vivo*, whilst crucially maintaining their multipotentiality to differentiate into clinically-significant derivatives such as bone, fat and cartilage. This property is essential as MSCs represent such a low proportion of the total cellular content isolate from bone marrow (approximately 0.001%).

d) MSCs are suitable for use in allogeneic transplantation as well as autologous transplantation (Aggarwal and Pittenger, 2005).

The latter point is attributable to the unique immunological properties of MSCs. Allogeneic transplantation can be clinically problematic, as the recipient's body will recognise the allogeneic material as foreign, resulting in an immune response and possible rejection of the transplanted material. As such, allogeneic transplantation must be accompanied by rigorous immune suppression. MSCs are immunologically unique in that they express intermediate levels of MHC Class I antigens and negligible levels of MHC Class II antigens (Le Blanc et al., 2003). It has also been reported that MSCs do not express co-stimulatory molecules (Majumdar et al., 2003). Crucially, it is not just undifferentiated MSCs that lack expression of MHC Class II antigens, but their differentiated derivatives also. Le Blanc and colleagues examined expression of MHC Class I and II antigens in undifferentiated MSCs and MSCs that had been differentiated into their gold-standard derivatives bone, fat and cartilage. They reported that both undifferentiated MSCs and their differentiated counterparts lacked expression of MHC Class II antigens, strongly suggesting that MSCs are not immunogenic and would not evoke an immune response upon transplantation (Le Blanc et al., 2003). In support of these observations, Aggarwal and colleagues reported that, following allogeneic transplantation, MSCs evaded immune recognition and were readily detectable in recipients at extended time points (Aggarwal and Pittenger, 2005). A common complication of allogeneic transplantation is graft-versus-host-disease (GVHD), in which functional immune cells carried over from the donor in the transplanted material recognise the recipient as foreign and launch an immune response. Transplantation of MSCs has been reportedly associated with reduced incidence of GVHD, and there are even reports of MSC transplantation being used in the treatment of GVHD (Le Blanc et al., 2004). The potential of MSCs for use in

allogeneic transplantation as well as autologous transplantation has major clinical significance, since material could be transplanted between mismatched individuals.

1.5 MSC-based therapy in the treatment of osseous and adipose tissue defects

1.5.1 Overview of the potential use of MSCs in disorders of bone and fat tissue

As MSCs are defined functionally owing to their ability to differentiate into mesodermal derivatives such as bone and fat, the most obvious clinical application of MSCs at present is in the treatment of osseous and adipose tissue defects, where their intrinsic differentiative capabilities could be exploited for the replacement of diseased bone and fat tissue (Barry and Murphy, 2004, Pittenger et al., 1999, Prockop, 1997).

Adipose tissue defects, commonly referred to as soft tissue defects, can be defined as substantial voids within the subcutaneous fat layer. Such defects can be a consequence of numerous conditions, such as traumatic injury e.g. burns, hereditary and congenital conditions e.g. Poland syndrome and Romberg's disease, tumour resection e.g. breast mastectomies, facial reconstructions and lipodystrophy associated with type II diabetes (Gomillion and Burg, 2006, Patrick, 2001, Langstein and Robb, 1999, Katz et al., 1999, Tzikas, 2004, Rooney and Ryan, 2006). Currently, treatment options for adipose tissue defects consist mainly of plastic and reconstructive surgical procedures. Logic suggests that autologous adipose tissue from alternative sources on the patient's body would provide the best and most obvious source of material for such surgeries, as adipose tissue is usually present in excess, providing a readily-available yet readily-expendable source of material (Mauney et al., 2005). The implementation of autologous fat transplantation has been trialled in many cases. However, the outcome of these procedures has unfortunately been unpredictable and somewhat inconsistent (Gomillion and Burg, 2006, Patrick, 2000). There are several reasons for this. Due to inadequate revascularization of transplanted adipose tissue, graft volume can be reduced by up to 60%, whilst the viable tissue that remains is unable to function appropriately (Patrick, 2000).. Furthermore, adipocytes are extremely vulnerable to mechanical/pressure damage during aspiration procedures due to the high lipid content

of their cytoplasm (up to 90% cytoplasm volume is lipid) – up to 90% of adipocytes are damaged during aspiration (Mandrup and Lane, 1997). Even if only a small number of cells could be isolated, *ex vivo* expansion would allow sufficient numbers to be gained for clinical use. However, mature adipocytes are terminally differentiated, rendering them unsuitable for *ex vivo* expansion. Because of such limitations, the use of autologous adipose tissue in cellular therapies for the treatment of soft tissue defects is not ideal and, as such, there is a search for alternative cell sources. MSCs in particular present themselves as promising candidates for use in the cellular treatment of soft tissue defects, as they can be easily isolated and rapidly expanded in the tissue culture laboratory, and under appropriate culture conditions can be manipulated to undergo adipogenic differentiation to form pre-adipocytes and adipocytes.

Perhaps the most obvious and realistically-achievable clinical use of MSCs at present is in bone tissue engineering strategies, designed to exploit the intrinsic osteogenic ability of MSCs as a means of replacing damaged bone tissue in osseous defects. New cellular therapies and tissue engineering strategies in the treatment of osseous defects are a major clinical goal and this impetus is set to significantly increase in the future as improved quality of life leads to increased life expectancy and therefore increased incidences of age-related disorders such as osteoporosis. A number of factors can contribute towards the development of osseous defects and bone trauma, such as fractures, tumour invasion or genetic disorders e.g. osteogenesis imperfecta (Hsu et al., 2005, Zeitlin et al., 2003). Current treatment strategies consist predominantly of autologous bone grafts, and to a lesser extent allogeneic bone grafts, metallic implants and bioceramics (Hatano et al., 2005, Salgado et al., 2004). Unfortunately, as is the case for adipose tissue defects, the success of these current treatment methods is somewhat inadequate, not to mention distressful for the patient and extremely painful due to their invasive nature. Reasons for limited success include limited autograft supply/volume, infection due to pathogen transfer/immunological rejection following allografts, loss of function/donor site morbidity and, in some cases, even nerve damage (Damien and Parsons, 1991). It is therefore hardly surprising that alternative strategies in the treatment of osseous defects are currently sought, with a particular clinical goal being the development of bone tissue engineering technologies to replace tissue damaged as a result of disease. MSCs are highly appealing candidates for use in the

cellular treatment of osseous defects and in bone tissue engineering, due to their intrinsic osteogenic abilities upon appropriate stimulation.

1.6 MSC-based therapy in the treatment of neurological and neurodegenerative disorders

1.6.1 Overview of the potential of use of MSCs in disorders of the nervous system

Besides the more generally accepted therapeutic applications of MSCs as mentioned above, MSCs are also attracting much interest as a potential resource for the treatment of neurological and neurodegenerative disorders. The remainder of this literature review will focus of the recent developments in this area, starting with a consideration of the use of MSC transplantation as a means of treating such deficits.

Neurological disorders result from disruption in the structure and/or function of nervous tissue, either in the central nervous system (CNS), peripheral nervous system (PNS), or both. Neurodegenerative disorders are a further sub-classification of neurological disorders, and this term is used to describe disorders that result from a loss and/or degeneration of nervous tissue. Examples of neurodegenerative disorders include Huntington's disease, Parkinson's disease, multiple sclerosis and Alzheimer's, amongst many others. Current therapies for the treatment of neurological and neurodegenerative disorders are largely ineffective and, as such, inadequate. The development of effective therapies in this area is further complicated owing to the significantly varying pathologies between different disorders and even between individuals suffering from the same disorder. Consequently, the development of a standardised therapy in the treatment of such conditions has eluded us to date, as patients require treatments that are tailored specifically to their unique clinical symptoms. With the major impetus in stem cell research over the last few decades, the very real possibility of a cellular therapy involving the administration of stem cells to replace damaged and/or degenerated nervous tissue is a highly desirable and realistic clinical goal (Li and Chopp, 2009).

With evidence to suggest that MSCs display a greater plasticity than previously anticipated, concomitant with their unique immunological properties which hold them

with such promise for both autologous and allogeneic transplantation, MSCs present themselves as extremely promising candidates in the treatment of neurological and neurodegenerative deficits. The following paragraphs will consider some of the key studies in this area.

A number of factors need to be taken into consideration in order to ascertain whether MSCs are suitable candidates in the cellular treatment of neurological and neurodegenerative disorders. The first of these considerations is whether or not MSCs remain viable and functional upon transplantation into the nervous system, as the microenvironment of nervous tissue is far-reached from the native niche of MSCs in the bone marrow. It is becoming increasingly more apparent that the stem cell niche plays a crucial role in the maintenance and function of stem cells, providing a delicate balance between cell-cell interactions, extracellular matrix contact, oxygen tension, pH and exposure to growth factors etc. (Fuchs et al., 2004). These are just a handful of the factors which interplay within the niche to contribute towards stem cell maintenance and function. As such, it is necessary to investigate whether the microenvironment of the nervous system is permissive to the survival and engraftment of transplanted MSCs.

1.6.2 MSC transplantation results in functional recovery in various animal models of neurological disease

A number of studies have demonstrated that, upon transplantation into the brain, MSCs survive and undergo successful engraftment and migration. A report published in 1998 by Azizi and colleagues demonstrates this concept. In this study, Azizi and colleagues examined the transplantation of both rat and human MSCs, administering them directly into the corpus striatum of rat brains. The corpus striatum consists predominantly of the basal ganglia and its primary function is in the coordination of movement. Periodically up to 72 days post-transplantation, brain sections were examined in order to ascertain whether transplanted MSCs had a) successfully engrafted and b) undergone migration. Azizi *et al* also injected rat astrocytes in the corpus striatum, which served as a suitable control, as astrocytes have been previously shown to successfully engraft and migrate throughout the CNS following transplantation, in an analogous manner to neural stem cells (Andersson et al., 1993, Zhou and Lund, 1992). Two weeks post-transplantation, an engraftment success rate of

20% for human MSCs, and a comparable 30% for rat MSCs, was observed. This observation is of particular interest, with the group reporting that the success rate of human MSC transplantation into rat brain (essentially a model of xeno-transplantation) was only 10% less than that observed for rat MSC transplantation (essentially a model of autologous transplantation). If such observations hold true, then this would suggest that the unique immunological properties of MSCs, as previously discussed, would render them suitable for xeno-transplantation as well as allogeneic and, of course, autologous transplantation. However, successful engraftment of MSCs into the host tissue is only part of the story. Azizi's study went on to address another fundamental question, that is, whether engrafted MSCs were capable of undergoing migration throughout the brain. Recruitment of MSCs to neurologically damaged areas will be a crucial event if they are to elicit any kind of beneficial effect that would ultimately lead to functional recovery. Azizi and colleagues demonstrated that MSCs were in fact capable of undergoing migration following engraftment, following a migration pathway comparable to the astrocyte control and, by extension, neural stem cells. MSCs were found in several successive layers of the brain up to 72 days post-transplantation, and this was mirrored in the pattern of astrocyte migration. Such observations only serve to intensify the excitement surrounding MSCs as potential candidates in cellular therapies of neurological and neurodegenerative disorders.

In this chapter we have only examined a single study demonstrating the successful transplantation of MSCs into the nervous system, as the work by Azizi *et al* provides a logical and progressive example that clearly highlights the key principles involved. However, many other studies have been performed, and successful transplantation of MSCs into the nervous system has been reported in numerous other models, including mouse and even higher order primates (Deng et al., 2006b, Kopen et al., 1999, Isakova et al., 2006, Phinney et al., 2006, Ankeny et al., 2004). Such observations served to intensify the promise surrounding MSCs as potential candidates in cellular therapies of neurological and neurodegenerative disorders.

Numerous studies have allowed us to establish that MSCs are capable of undergoing successful engraftment and migration following transplantation into the nervous system. The next question to address, and perhaps the most pivotal of all, is whether MSC transplantation elicits any degree of functional recovery in models of

neurological and neurodegenerative disorders. There is much evidence at present to suggest that MSC transplantation does indeed elicit functional recovery to various degrees, and this has been reported in many models of neurological and neurodegenerative disorders including, but by no means limited to, Huntington's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, stroke, traumatic brain injury and also damage to the spinal cord and peripheral nerves (Chen et al., 2003, Li et al., 2001a, Mahmood et al., 2001, Lescaudron et al., 2003, Mazzini et al., 2007, Dezawa et al., 2004, Chopp et al., 2000).

Restoration of functional activity can be assessed in many ways, and these can range from simple behavioural tests to more complex cell function assays. These proof of principle studies in animal models suggest that MSC transplantation may be a promising therapy for neurological and neurodegenerative disorders. However, to date, the large majority of work in this area has been performed using animal models, and before this can be significantly trialled in humans a number of technical issues must be addressed. It is perhaps useful to end this section of the literature review with a consideration of these technical questions. In terms of the cellular material to be transplanted, should whole bone marrow be transplanted or should attempts be made to culture a more homogeneous starting population of MSCs? Once this has been addressed, how should the cells be administered? Should they be administered directly into the damaged nervous tissue, or should they be administered systemically? If MSCs were administered systematically, would they be homed to the neurologically damaged areas in sufficient quantity to elicit a beneficial effect? In terms of timescale, when should the MSCs be administered? Does administration need to be immediate following injury, or can a longer timescale be employed for the treatment of more chronic disorders? What numbers of cells should be transplanted? Is it possible to predict the success rate of engraftment? These questions represent just a handful of the technical issues that must be considered.

1.6.3 Mechanisms via which MSCs elicit functional recovery in models of neurological and neurodegenerative disorders

Before MSC-based cellular therapies and tissue engineering strategies become serious propositions for use in the clinic, a significant advance in our knowledge and understanding of the complex mechanism(s) via which MSCs commit to specific

lineages and undergo differentiation to demonstrate mature, functioning phenotypes is required. A more thorough understanding of these biological phenomena will not only benefit from a clinical perspective, but will also be of major significance in a research setting, as MSCs provide an ideal cellular system in which to study the complex events of cell differentiation. In terms of MSC transplantation in the therapy of neurological and neurodegenerative disorders, proof of principle studies in animal models demonstrate that MSC transplantation can result in significant functional improvements. However, the mechanism(s) by which MSCs elicit neurological recovery remain largely unknown. At present, opinion is divided between three main hypotheses:

a) MSCs undergo '*trans*'-differentiation to produce neural cell types, thereby directly replacing neural tissue that has been damaged as a result of disease.

b) MSCs undergo spontaneous fusion events with host neural cells, and in doing so acquire the phenotypic properties of the host neural cells.

c) MSCs migrate to neurologically damaged areas but, rather than undergoing any phenotypic alterations, release a complement of trophic factors and cytokines that promote endogenous repair mechanisms, predominantly by the instructive, neurogeneic effects of these factors on resident neural stem and progenitor populations

The relationship between these mechanisms is shown in Figure 1.6.

1.6.3.1 'Trans'-differentiation of MSCs into neural cell types in vitro

Reports of MSCs undergoing '*trans*'-differentiation *in vitro* to form neural cell types are numerous within the current literature and, as such, it is not possible to provide a thorough discussion of these studies here; instead, the two seminal studies demonstrating this concept will be considered. These are the studies of Sanchez-Ramos and Woodbury (Sanchez-Ramos et al., 2000, Woodbury et al., 2000, Woodbury et al., 2002). A summary of the numerous other studies is given in Table 1.1.

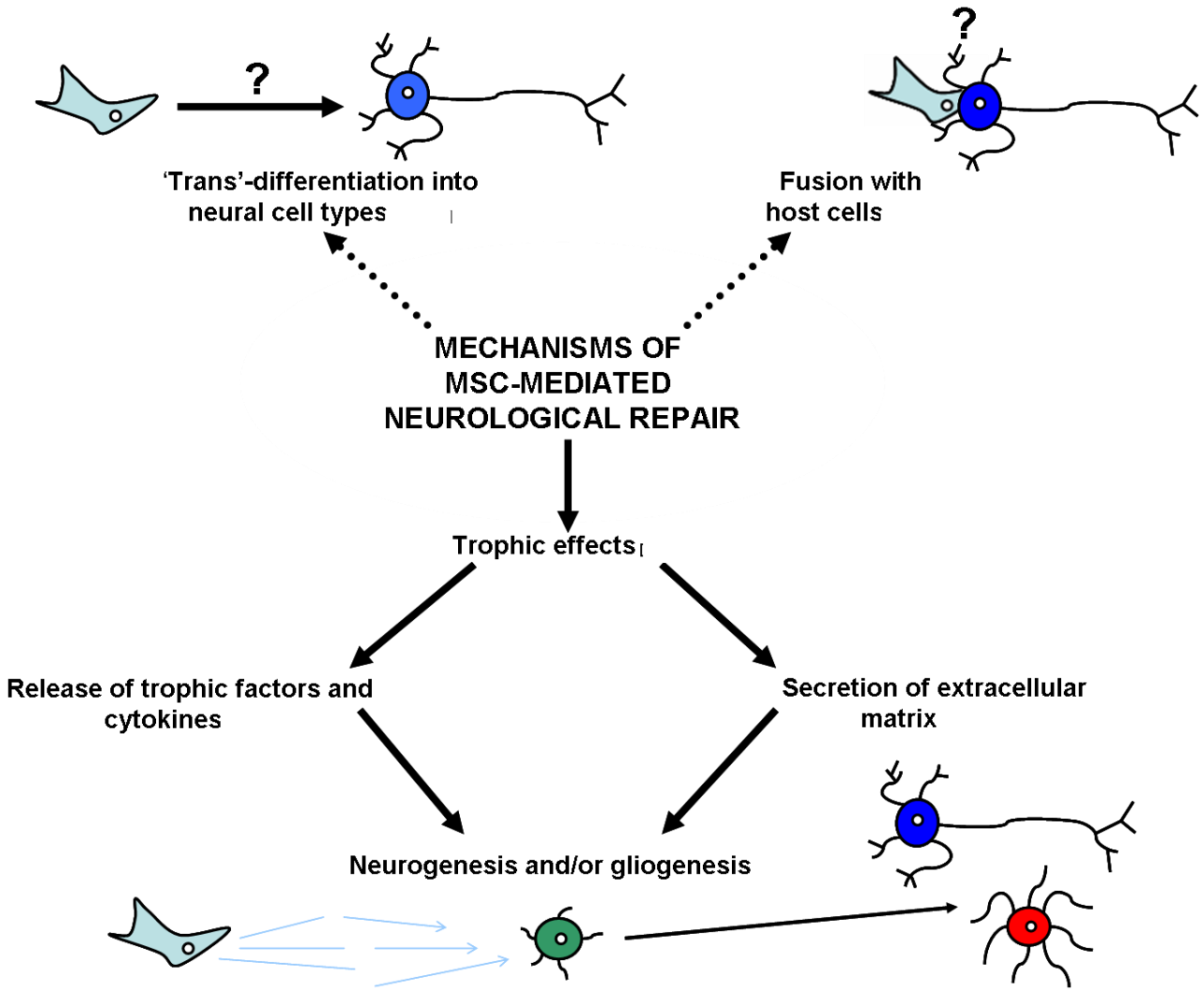

Figure 1.6. A diagrammatic representation of the mechanisms by which MSCs may elicit functional recovery following transplantation into models of neurological and neurodegenerative disorders. Three mechanisms are postulated to account for therapeutic benefits of MSC transplantation. Although '*trans*'-differentiation and spontaneous fusion events are likely to contribute to a certain extent, the low frequency at which both phenomena occur means that they are unlikely to fully account for the benefits observed. It is gaining considerable momentum that the third, and predominant, mechanism by which MSCs contribute to functional recovery is *via* the release of diffusible soluble factors/cytokines which promote endogenous repair processes. (Adapted from Hardy *et al.*, 2007).

Sanchez-Ramos and colleagues (2000) reported that MSCs had the potential to demonstrate neural differentiation when cultured in 'differentiation medium', consisting of DMEM supplemented with a cocktail of factors that had been previously reported to exert a neurogeneic effect, including all-*trans*-retinoic acid (ATRA) and

brain-derived neurotrophic factor (BDNF). Following treatment of MSCs with this cocktail of neurogeneic factors, Sanchez-Ramos and colleagues examined cellular morphology and expression of cell-specific markers. When cultured under standard conditions, MSCs display a very characteristic, stromal-like morphology, with cultures consisting predominantly of large, flat, fusiform cells. However, MSCs cultured in 'differentiation medium' underwent significant radical changes and the distinctive stromal morphology of MSCs was replaced by a structure resembling that of neural cell types whereby cells became spindle-shaped and possessed several long processes. Marker analyses revealed that MSCs cultured in 'differentiation medium' expressed the neural progenitor marker, nestin, the neuronal marker, NeuN, and the glial marker, GFAP. These data support the hypothesis that MSCs may be able to cross the germ layer boundary to form non-mesodermal cell types such as neural cells, which are ectodermal in origin. Adding more strength to the argument that MSCs were undergoing '*trans*'-differentiation to form neural cell types was the observation that the up-regulation of these neural antigens was concomitant with a down-regulation of fibronectin expression, a marker of MSCs. However, despite the observed down-regulation in fibronectin expression, the percentage of cells expressing neural markers was proportionately lower than the percentage that retained fibronectin expression (<1% compared with 30% fibronectin).

Woodbury and colleagues (2000) adopted a slightly different approach to that of Sanchez-Ramos, by culturing MSCs with a cocktail of chemicals as opposed to well-characterised neurogeneic factors. In their chemical induction protocol, Woodbury and colleagues removed MSCs from their standard culture conditions, containing 10% FCS, and subsequently cultured them under serum-free conditions followed by exposure to antioxidants. However, immediately prior to the removal of serum from their culture medium, MSCs were first 'pre-induced' for 24 hours by the addition of β-mercaptoethanol (BME) to their standard media. 'Pre-induction' was followed by 'induction', which involved removal of serum from the culture media and supplementation with the antioxidants butylated hydroxyanisole (BHA) and dimethylsulfoxide (DMSO). Similarly to the observations of Sanchez-Ramos, MSCs underwent radical changes in both cell morphology and marker expression in response to the chemical induction protocol. However, these changes occurred over an extraordinarily short timescale. Within a matter of hours following induction, MSCs

lost their typical stromal morphologies and exhibited tight, multipolar soma with many processes and cytoplasmic extensions emanating from the cell body, as is typical of neural cell types. During the very early stages of the experiment, the cytoplasmic processes were very simple, though by the end of the 5 hour time course their morphology had become somewhat more complex, with processes demonstrating secondary branching and terminating in growth cones. These morphological observations suggested that MSCs were acquiring an exclusively neuronal phenotype, at the expense of a glial phenotype. Subsequent marker analysis supported these morphological observations, as the development of complex neuronal morphologies were accompanied by an up-regulation in expression of the neuronal marker, neuron-specific enolase (NSE).

There are a substantial number of reports demonstrating the ability of MSCs to '*trans*'-differentiate and adopt neuronal and/or glial fates *in vitro*. Whilst these studies are too numerous to discuss in detail here a summary of some of these additional studies is provided in Table 1.1. Of interest are the widely varying culture conditions employed by different groups in order to induce neural differentiation of MSCs. For convenience, the various differentiation protocols have been grouped into three broad categories, as listed below and demonstrated in Figure 1.7:

a) *Chemical induction protocols*

Involving supplementation of MSC culture medium with cocktails of various chemicals in order to induce differentiation – Woodbury's chemical induction protocol would fall into this category.

b) *Neurotrophic factor-based protocols or transfection with neurogeneic genes*

Involving supplementation of MSC culture media with various combinations of known neurotrophic agents such as ATRA and BDNF – as in the Sanchez-Ramos study (2000) – or transfection of MSCs with neurogeneic genes.

c) <u>*Co-culture-based protocols*</u>

Involving co-culture of MSCs with neural cell types, in which MSCs are induced to adopt neural phenotypes as a consequence of direct cell-cell interactions and/or the interplay of soluble factors produced by each cell type.

1.6.3.1.2 'Trans'-differentiation of MSCs into neural cell types in vitro: true 'trans'-differentiation or an artifact of cell culture conditions?

It is not surprising that the concept of MSCs displaying a greater plasticity than previously anticipated such that they undergo '*trans*'-differentiation into neural cell types is somewhat controversial. MSCs have long been held as a multipotent stem cell population which, by definition, means that they are restricted in their plasticity and form cell types specific to their germ layer of origin. Since MSCs are mesodermal in origin, it was readily accepted that MSCs should possess an intrinsic ability to differentiate into mesodermal cell types, such as bone, fat and cartilage. As discussed above, however, there are some data which suggest that MSCs are able to cross the germ layer boundary and differentiate to acquire a neural phenotype, indicative of ectoderm. Whilst this holds major promise for therapeutic applications, it also challenges the central dogma of adult stem cell biology.

Regarding the *in vitro* '*trans*'-differentiation of MSCs into neural lineages, recent studies raise considerable doubt over the authenticity of the neural derivatives that result from these differentiation protocols, in particular those that result from chemical induction protocols such as those employed by Woodbury *et al* (2000). These studies raised initial concern owing to the remarkably short timescale over which differentiation occurred. According to the published data, MSCs could differentiate into neural cell types, literally a matter of hours (Woodbury et al., 2000). Neural differentiation is a highly complex and multifaceted process, and *in vivo* neurogenesis requires spatial and temporal regulation of the expression of a wide array of genes. Similarly, neuritogenesis is a dynamic process involving regulation of microtubules.

TREATMENT	NEURAL PHENOTYPE	REFERENCE
CHEMICAL		
Isobutylmethylxanthine(IBMX) + dibutyryl cAMP	Neuronal	(Deng et al., 2001, Rismanchi et al., 2003)
β-mercaptoethanol (BME), then butylated hydroxyanisole (BHA) and dimethylsulfoxide (DMSO)	Neuronal	(Woodbury et al., 2000, Woodbury et al., 2002, Munoz-Elias et al., 2004, Rismanchi et al., 2003, Jori et al., 2005)
5-azacytidine	Neuronal	(Kohyama et al., 2001)
BME + retinoic acid (RA)	Neuronal	(Hung et al., 2002)
BHA + dibutyryl cAMP + IBMX + RA + ascorbic acid	Neuronal	(Levy et al., 2003, Hellmann et al., 2006)
bFGF for 24 hours, then BHA + KCl + valproic acid + forskolin	Neuronal/glial	(Krampera et al., 2007)
NEUROTROPHIC/GLIAL FACTORS OR GENE TRANSFECTION		
GDNF + PACAP + dibutyryl cAMP	Neuronal	(Tzeng et al., 2004)
Generation of neurospheres, then induction with RA and Shh	Neuronal	(Locatelli et al., 2003)
Transfection with Notch intracellular domain (NICD), then treatment with neurotrophic factors	Neuronal	(Dezawa et al., 2004, Kamada et al., 2005)
Transfection with *noggin*	Neuronal	(Kohyama et al., 2001)
Glial growth factor (GGF)	Glial (astrocytic)	(Tohill et al., 2004)
Culture conditions as for neural stem cells (NSCs)	Neurosphere formation	(Hermann et al., 2004)
bFGF, then BME+NT-3, then BHA + valprolic acid + forskolin + hydrocortisone + insulin + NT-3 + NGF + BDNF	Neuronal	(Tatard et al., 2007)
bFGF for 24 hours, then Shh + FGF-8 + RA, then BHA + valprolic acid + forskolin + hydrocortisone + insulin + NT-3 + NGF + BDNF + GDNF	Neuronal (dopaminergic)	(Tatard et al., 2007)
Shh + FGF-8 + bFGF	Neuronal (dopaminergic)	(Trzaska et al., 2007)

EGF + FGF for 72 hours, then cAMP + IBMX for 72 hours, then GDNF + TGFβ3 + RA	Neuronal (dopaminergic)	(Barzilay et al., 2008)
BDNF + EGF + NGF	Neuronal	(Choong et al., 2007)
CO-CULTURE WITH NEURAL CELL TYPES		
Co-culture with fetal midbrain cultures	Neuronal	(Sanchez-Ramos et al., 2000)
Co-culture with astrocytes	Neuronal	(Jiang et al., 2002, Jiang et al., 2003)
Co-culture with NSCs	Glial (astrocytic)	(Wislet-Gendebien et al., 2003)
Co-culture with cerebellar granule neurons	Neuronal/ glial (astrocytic)	(Wislet-Gendebien et al., 2005)
Co-culture with Schwann cells	Glial (Schwann cells)	(Krampera et al., 2007)

Table 1.1. A table summarising the main studies reporting the *in vitro* neural differentiation of MSCs. Current *in vitro* protocols can be grouped into three broad categories – culture of MSCs with chemical-inducing agents, culture of MSCs with neurotrophic/glial-derived factors and co-culture of MSCs with neural cell types. Table updated from (Hardy et al., 2008), originally updated from (Chen et al., 2006).

Therefore, the concept that MSCs display the ability to undergo the complex molecular events associated with neural differentiation within a matter of hours to form mature, functional neural derivatives, is highly questionable and improbable. Indeed, for MSCs to differentiate and form mesenchymal derivatives such as bone or fat occurs over a timescale of several days and weeks, with the earliest phenotypic indications of differentiation appearing only after 5 days post-induction of differentiation. Osteogenic and adipogenic differentiation represent processes for which MSCs have an intrinsic ability, and it is therefore not unlikely that MSCs will have much of the protein complement required for these processes already in place, prior to induction of differentiation. It is therefore a reasonable assumption that '*trans*'-differentiation of MSCs into neural cell types, which involves MSCs crossing the germ layer boundary to form a cell type originating from the ectoderm, would require a greater change in protein complement to bring about the events required for differentiation, and therefore would occur over a longer timescale than differentiation into mesodermal derivatives, rather than the 5 hours reported by Woodbury and colleagues. It was thought to be

highly unlikely that true neural derivatives could be formed in this short timescale and, as such, the precise mechanism(s) by which chemical induction protocols were causing 'neurogenesis' became the subject of intense investigation.

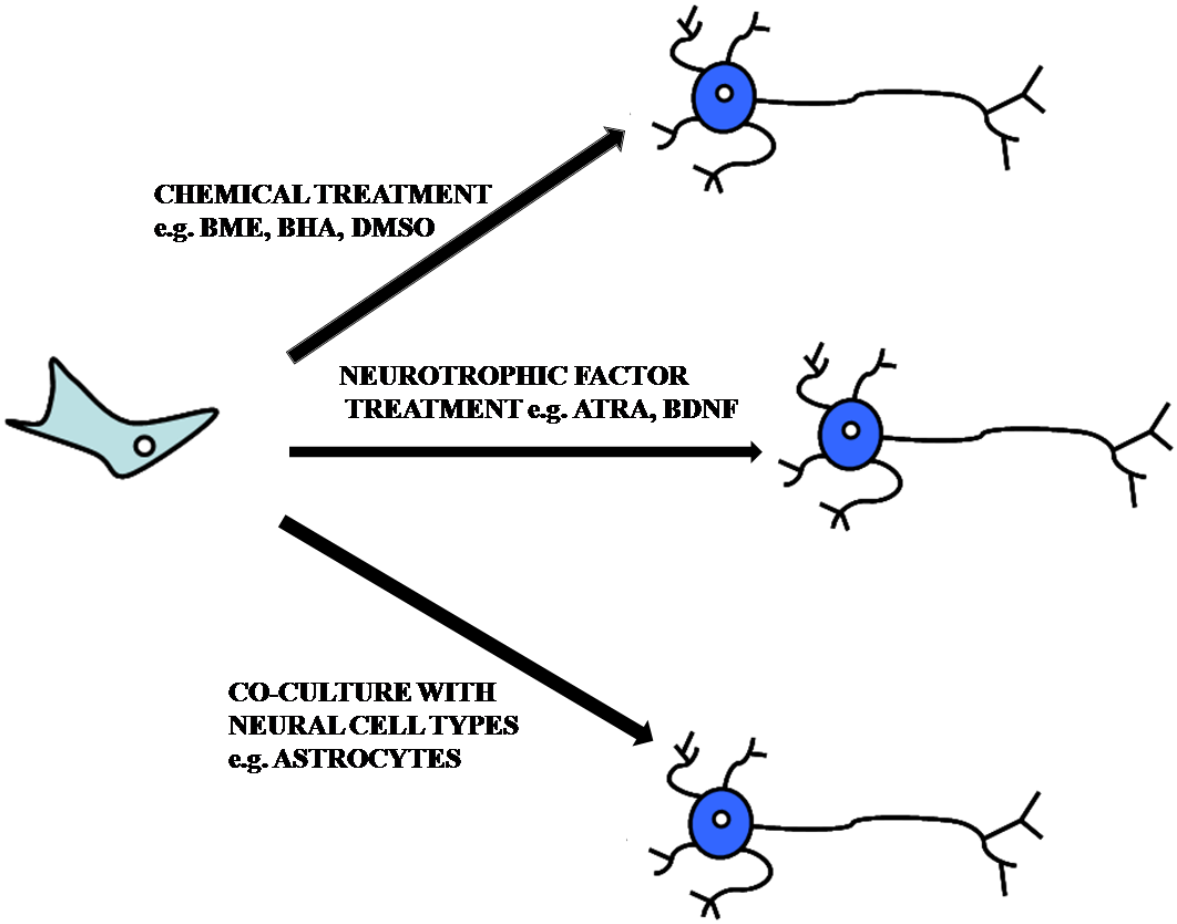

Figure 1.7. A diagrammatic representation of the predominant *in vitro* protocols used to induce the apparent neural differentiation of MSCs. Current *in vitro* protocols can be grouped into three broad categories – culture of MSCs with chemical-inducing agents, culture of MSCs with neurotrophic/glial-derived factors and co-culture of MSCs with neural cell types.

A number of studies were published between 2004 and 2006 that provide convincing and highly compelling evidence that '*trans*'-differentiation of MSCs into neural cell types did not represent a pathway of true neural differentiation and was in fact an artifact of the culture conditions employed, with the acquirement of a neural morphology by MSCs being attributable to a cell-stress response to these culture conditions (Lu et al., 2004, Neuhuber et al., 2004, Bertani et al., 2005, Croft and Przyborski, 2006). The observations of these studies will be considered here. Firstly,

the quite radical changes in MSC morphology, from a stromal to a highly distinctive neural morphology, were considered. A principal event that occurs during neuronal differentiation is that of neurite outgrowth which is a highly dynamic process involving microtubule re-modelling and the outgrowth of neurites from a central cell body, or soma. This phenomenon was examined using sophisticated techniques such as time-lapse microscopy and video, over the short timescale reported by Woodbury and colleagues (2000). The transformation of MSCs to cells possessing a neural-like structure did not follow the traditional process of neurite extension; indeed, the opposite effect was observed. Rather than the outgrowth of neurites and processes, as would be expected during neural differentiation, the cell cytoplasm retracted from its periphery towards the centre of the cell, forming a tight, spherical cell body around the nucleus, resembling a neural soma (Neuhuber et al., 2004, Bertani et al., 2005, Croft and Przyborski, 2006). What were originally perceived to be neurites were in fact cytoplasmic extensions that remained following cytoplasmic retraction, a result of strong focal contacts made between the cells and the underlying substrata. These cytoplasmic extensions morphologically resembled neurites, hence indicating the acquirement of a neural phenotype. Further studies confirmed that retraction of the cytoplasm, causing MSCs to acquire an apparent neuronal morphology, was in fact a consequence of cytoskeletal disruption and/or cytoskeletal collapse. The morphological changes in MSCs treated with the chemical induction protocol could be mimicked by treating MSCs with pharmacological agents, such as cytochalasin B and D, which disrupt the cytoskeletal structure, with the resulting morphology being indistinguishable from that induced by chemical culture (Bertani et al., 2005, Croft and Przyborski, 2006, Neuhuber et al., 2004). Specifically, disruption and/or collapse of the actin cytoskeleton brought about the observed change in morphology.

However, whilst these studies proved that culturing MSCs with the chemical induction protocol did not induce the formation of a true neural phenotype, they did not discount the possibility of MSCs possessing an intrinsic neurogeneic potential that may have contributed in part to the acquirement of a neural phenotype. To address this issue, several independent groups applied the chemical induction protocol to a range of other cell types in addition to MSCs. When treated with the chemical induction protocol, primary fibroblasts, HEK293 cells (a human embryonic kidney cell line) and PC-12 cells (a human pheochromocytoma cell line) all underwent morphological changes that

were indistinguishable from the morphological changes in control MSCs (Lu et al., 2004, Bertani et al., 2005, Croft and Przyborski, 2006, Neuhuber et al., 2004). These data indicated that the morphological changes observed in MSCs following chemical induction were most likely to be attributable to an aberrant response by the cell to the artificial culture conditions rather than any intrinsic neurogeneic potential possessed by MSCs.

It appears therefore that under certain culture conditions, MSCs adopt a neural-like morphology as a result of cytoskeletal collapse. However, in addition to structural changes Woodbury and colleagues also reported regulated expression of known neural markers (Woodbury *et al.*, 2000). Whilst such data may partly support the concept of neural differentiation by MSCs, it must be recognised that analysis of marker expression is insufficient in itself to conclusively prove true differentiation and cell identity. Functional data provides the strongest evidence that a cell has differentiated into a mature, functional derivative and, in terms of assessing neural differentiation, this classically involves measurement of electrical activity. MSCs have been shown to spontaneously express neural proteins under their standard culture conditions, that is, even before induction to undergo neural differentiation (Lamoury et al., 2006, Deng et al., 2006a, Tondreau et al., 2004). Furthermore, in a study published by Lu *et al*, MSCs were able to acquire a neuronal morphology even when protein synthesis was totally blocked, which casts further doubts that neural morphology of MSCs relates to true neural differentiation (Lu et al., 2004). It is a highly unlikely scenario that a cell would be able to undergo differentiation without modifying its protein complement, let alone the changes in protein complement that would be required to cross the germ layer boundary to differentiate into a cell type of a different germ layer. An extension of this argument is provided in a study by Bertani *et al* who, using a microarray to examine the transcriptome of induced and uninduced MSCs, reported that chemical induction of MSCs did not significantly alter the transcriptome compared to uninduced controls (Bertani et al., 2005). Of particular interest, the expression of neural markers did not significantly differ between induced and control MSCs.

Analysis of differentiation in cell biology and discrimination between stem cells and their differentiated counterparts can sometimes be more complicated than originally anticipated. Morphological observations, although useful, are insufficient on their own

to conclude the identification of a particular cell type in culture. By far the most common method of discriminating between different cell types involves analysis of cell-specific marker expression. Whilst data provided from such cell marker analyses is usually informative, it should nonetheless be treated with caution, as has been highlighted above and careful validation of results is required before drawing a solid conclusion. If possible, gene and protein expression data are best complemented by functional analysis of the differentiated cell phenotype to provide the best indication of cell type. Only when all such information is brought together can a firm view on cell identity be determined.

1.6.3.2 'Trans'-differentiation of MSCs into neural cell types in vivo

Thus far, '*trans*'-differentiation of MSCs into neural cell types in an *in vitro* environment has been considered. The findings of these studies are somewhat controversial, particularly those regarding chemical induction protocols, such as that described by Woodbury and colleagues (2000). In the absence of functional data to support marker analysis in the majority of cases, it is therefore necessary to view *in vitro* '*trans*'-differentiation assays with an air of caution. It is generally accepted that the growth of cells outside the body in the culture environment is artificial and cells may respond abnormally to such conditions. For example, culturing cells on the two-dimensional (2D) environment of tissue culture plastic places them under immense stress by causing cells to adopt a highly unnatural conformation which in turn influences their behaviour and function. It is therefore also appropriate to study MSC behaviour in an *in vivo* setting to gain a more physiologically-relevant understanding of whether MSCs can '*trans*'-differentiate into neural cell types.

Using the work of Azizi *et al* as an example, we have previously demonstrated that MSCs are not only capable of surviving transplantation into the unfamiliar environment of the nervous system, but are also capable of undergoing successful engraftment and migration (Azizi et al., 1998). Numerous reports have stated that MSCs demonstrate marker expression profiles indicative of neural differentiation, and therefore the acquirement of a neural phenotype, following transplantation into the nervous system. Kopen *et al* proposed MSCs as potential alternatives to neural stem cells in the cellular therapy of neurological and neurodegenerative disorders (Kopen et al., 1999). In this study, MSCs proliferated and underwent migration throughout the

forebrain and cerebellum, in a manner pertinent to that of neural stem and progenitor cells following administration into the lateral ventricle of neonatal mice. By labelling MSCs it was possible to distinguish between transplanted cells and host neural cells. Transplanted MSCs demonstrated strong expression of GFAP, indicative of astrocytic differentiation, and to a lesser degree expression of neurofilament, indicative of neuronal differentiation. Interestingly, since these observations were based on the transplantation of undifferentiated MSCs that had not been manipulated *in vitro* prior to transplantation, Kopen and colleagues proposed that the acquirement of a neural phenotype by MSCs was a consequence of exposure to the brain microenvironment alone. If such findings hold true, this would be of major interest from a clinical perspective, as it would permit MSCs to be administered to patients in their undifferentiated state, without any requirement for prior *in vitro* manipulation to produce the desired neural phenotype prior to transplantation.

Another study, by Li *et al*, reported that MSCs had the potential to undergo '*trans*'-differentiation *in vivo* to produce dopaminergic neurons, following transplantation into a mouse model of Parkinson's disease (Li et al., 2001). The acquirement of a dopaminergic phenotype was supported by the expression of tyrosine hydroxylase (TH), a marker of dopaminergic neurons, in MSCs post-transplantation. If Li's findings hold true and MSCs are in fact able to undergo '*trans*'-differentiation into TH-expressing dopaminergic neurons, this is of major clinical significance in the therapy of Parkinson's disease. The main causative mechanism of Parkinson's disease is insufficient action and/or efficacy of dopamine, which is a neurotransmitter produced predominantly by dopaminergic neurons. If MSCs can adopt a dopaminergic phenotype following transplantation and express functional TH, an enzyme required for the conversion of tyrosine to dihydroxyphenylalanine (DOPA, a precursor of dopamine), then MSC transplantation may be able to counteract the deficits causative of Parkinson's disease. However, it was not fully established whether transplantated MSCs adopted a bona fide dopaminergic phenotype following transplantation. The same group reported in a later study that MSCs were able to demonstrate expression of two mature neuronal markers, NeuN and MAP2, and the mature astrocytic marker, GFAP following transplantation (Li et al., 2002). The number of MSCs induced to express markers of neural differentiation, as percentage of the total number of MSCs that successfully engraft following transplantation is very low. Only 1% expressed

neuronal markers and only 5% of these expressed glial markers (Li *et al.*, 2002). Whilst it is not possible to discount the possibility that the cells expressing markers of neural differentiation are functional, what is recognized is that such a rate of '*trans*'-differentiation would be unable to fully account for the neurological improvements reported following administration of MSCs in models of neurological and neurodegenerative disorders. There are several other studies reporting the '*trans*'-differentiation of MSCs into neural cell types *in vivo*; however, the exact mechanism(s) by which such observations occur remains to be fully elucidated. (Mezey and Chandross, 2000, Lu et al., 2006, Munoz-Elias et al., 2004, Deng et al., 2006a). In the absence of sufficient functional data, the formation of true neural derivatives from MSCs *in vivo* cannot be confirmed, nor can it be discounted.

1.6.3.3 Spontaneous cell fusion of MSCs with host neural cells

As '*trans*'-differentiation of MSCs into neural cell types *in vivo* occurs at too low a frequency to fully account for the improvements observed following transplantation of MSCs into models of neurological and neurodegenerative deficits, alternative mechanisms to explain these observations are required. A second mechanism that has been proposed to explain how MSC transplantation results in such beneficial effects is that of spontaneous cell fusion events with host neural cells following transplantation.

Two independent studies by Ying *et al* and Terada *et al*, both published in Nature in 2002, examined these spontaneous cell fusion events (Terada et al., 2002; Ying et al., 2002). In their studies they proposed that the ability of different cell types to demonstrate alternative phenotypes, originally perceived as '*trans*'-differentiation, could be attributable to an alternative mechanism of spontaneous cell fusion. They proposed that cells acquiring alternative phenotypes was not necessarily the result of '*trans*'-differentiation, and that similar phenomena were observed following spontaneous fusion of two different cell types, resulting in the formation of hybrid cells that displayed phenotypic traits of the recipient cell. Detailed cytogenetic analysis would therefore be required to distinguish between cells that had truly undergone '*trans*'-differentiation and cells that had undergone spontaneous fusion. Bringing us to MSCs, it has been reported that bone marrow cells can spontaneously fuse with other cell types in co-culture systems and, in doing so, acquire phenotypic traits of these cells (Ying et al., 2002, Terada et al., 2002). Interestingly, acquirement of these

phenotypic properties can extend to plasticity. However, it is necessary to stress that cell fusion events cannot be used to completely disregard the phenomena of '*trans*'-differentiation, as Crain and colleagues have demonstrated that MSCs can '*trans*'-differentiate into neural cell types by mechanisms independent of cell fusion (Crain et al., 2005). Alvazrez-Dolado and colleagues have shown that MSCs spontaneously fuse with neural cell types, as well as liver and heart (Alvarez-Dolado et al., 2003). Therefore, by applying the principles introduced by Terada and Ying in their respective studies, spontaneous fusion of MSCs with host neural cells following transplantation into the nervous system could cause MSCs to undergo genetic reprogramming and subsequently adopt phenotypic traits of the recipient neural cell. As such, spontaneous fusion events provide a potential mechanism by which MSC transplantation contributes towards functional recovery in neurological and neurodegenerative systems. However, as is the case for '*trans*'-differentiation of MSCs, spontaneous cell fusion occurs at an extremely low frequency. In Terada's study, a fusion rate of 2-11 clones per million cells was reported (Terada et al., 2002). Together, the low frequency of both '*trans*'-differentiation and spontaneous cell fusion suggest that both of these phenomena are unlikely to fully account for the observed neurological improvements. A third possibility is gathering momentum as the mechanism most likely to be the major player in MSC-mediated neurological recovery.

1.6.3.4 MSCs as trophic mediators of neural differentiation

The low frequency at which '*trans*'-differentiation and cell fusion events occur means that they are unlikely to account for the neurological recovery observed following MSC transplantation in models of neurological and neurodegenerative disorders. However, that is not to discount these mechanisms completely. It is possible that both may contribute in part towards observed functional recovery but the low frequency with which they occur suggests there may also be other mechanisms at play.

By definition, stem cells are cells that that a) display the capacity for self-renewal and b) display the potential to differentiate into more mature cell types. This functional definition of a stem cell continues to evolve, and there is much evidence at present to suggest that stem cells have an important role within their niche that involves the release and/or uptake of trophic factors and cytokines. This intercellular communication occurs between cells in close proximity to one another and may be

paracrine or autocrine. The term 'paracrine' is used to describe signalling that occurs between two different cell types i.e. a cell-derived soluble factor acting upon a different cell type to that which secreted it. Conversely, the term 'autocrine' describes signalling that occurs between two cells of the same type i.e. a cell-derived soluble factor acting upon a cell of the same type as that which secreted it. The concept of stem cells having a trophic role in addition to their 'foremost' role in differentiation have been described for both embryonic and adult stem cell populations, including haematopoietic and neural stem cells, amongst others (Lu et al., 2003, Guo et al., 2006, Cabanes et al., 2007). Regarding MSCs, there are several studies demonstrating trophic roles *in vivo*, and perhaps the best characterised of these is the trophic role MSCs perform in the regulation of haematopoiesis (Haynesworth et al., 1996). It is proposed that the third, and predominant, mechanism by which MSCs promote functional recovery from neurological deficits is not from cell differentiation or fusion events, but instead via the release of trophic factors and cytokines. The cocktail of factors secreted by MSCs ameliorate neural damage by a paracrine mechanism, exerting a neurogeneic effect on resident neural stem and progenitor cells, thereby promoting endogenous repair processes in the host nervous system (Chen et al., 2007, Chen et al., 2005). Factors secreted by MSCs can be loosely categorised into two groups: neurotrophic factors and non-neurotrophic factors. Both will be considered here.

1.6.3.4.1 Secretion of neurotrophic factors by MSCs

There are numerous reports suggesting that MSCs secrete a wide array of neurotrophins, growth factors, cytokines and other soluble factors. Work from both *in vitro* and *in vivo* settings indicate that MSC-derived factors promote cellular events such as proliferation, survival and differentiation. Several of the soluble factors secreted by MSCs are known to exert neurogenic effects on neural stem and progenitor cells and include molecules such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and ciliary neurotrophic factor (CNTF) (Chen et al., 2005, Chen et al., 2002, Chen et al., 2007). In addition to these highly documented neurotrophic factors, the 'secretome' of MSCs also consists of a wide variety of interleukin (IL) molecules, including IL-6, -7, -8, -11, -12, -14 and 15,

and this is in keeping with their housekeeping role as trophic mediators of haematopoiesis. It has long been established that a complement of IL molecules play key roles throughout the process of haematopoiesis, though it is now becoming increasingly apparent that IL molecules also play key roles during neural development, indicating that there maybe crosstalk between haematopoietic and neural differentiation pathways, further supporting MSCs as trophic mediators in both capacities (Majumdar et al., 1998, Mehler et al., 1993).

As MSCs are essentially a population of stromal cells it comes as no surprise that, in addition to the array of soluble factors and cytokines, they also secrete a wide range of extracellular matrix proteins, including collagen, laminin and fibronectin, that may modify the niche of neural stem cells (Chen et al., 2007). Such extracellular matrix proteins may promote events that would facilitate neurological recovery e.g. neurite extension. The secretion of non-soluble factors will be subject to discussion in Section 1.6.3.4.2.

Proteomic analysis of the MSC secretome suggests that it is likely to be a cocktail of soluble factors and cytokines, rather than the activity of any single factor, that is responsible for neurological recovery following MSC transplantation (Schinkothe et al., 2008). The beneficial effects of MSC-derived factors in mediating neurological recovery are highly documented in the literature, and the effects of these factors can be broadly classified into five main categories (Fiore et al., 2002, Schanzer et al., 2004, Palmer et al., 1999, Hsu et al., 2007, Hagg, 2005, Chopp and Li, 2002, Chen and Chopp, 2006).:

- *Angiogenic effects*
  Involving the formation of vasculature and new blood vessels

- *Neurogeneic effects*
  Involving the formation of neural tissue

- *Neuroprotective effects*

  Involving the protection of neural tissue from apoptosis and degeneration.

- *Synaptogenic effects*

  Involving the formation of synapses and synaptic contacts.

- *Inhibition of scarring*

  Involving the prevention of scarring, which could potentially prevent reconstruction of neural circuitry following injury or damage.

A few of the key studies demonstrating MSCs as trophic mediators of neurological recovery will be considered.

One of the most interesting studies investigating the role of MSCs as trophic mediators of neural differentiation was that published by Chen and colleagues in 2002 (Chen et al., 2002). In this study, it was demonstrated that the major mechanism behind MSC-mediated repair of neural tissue in an *in vitro* model of stroke was a result of cytokine and soluble factor secretion by MSCs. Even more intriguing was the convincing argument that the MSC secretome was significantly influenced by the microenvironment in which MSCs were cultured, suggesting that MSCs are capable of modifying their secretory profile in response to various environmental cues. Demonstration of this latter point involved an *in vitro* model system in which Chen and colleagues collected supernatant media that had been exposed either to normal rat brain tissue or to rat brain tissue that had been subjected to middle cerebral artery occlusion (MCAo), a model of stroke in rat. Human MSCs in culture were exposed to both supernatants and allowed to condition their media, which was subsequently collected and analysed using a human-specific ELISA to determine levels of the neurotrophic factors BDNF, NGF, bFGF, VEGF and HGF. However, the authors were keen to stress that, such was that nature of the ELISA technique used in their study, a degree of cross-reactivity with rat-derived cytokines could not be discounted (potentially carried over in the supernatants). Following exposure to ischemic brain supernatant compared to normal brain supernatant, Chen and co-workers observed that

MSCs produced and secreted elevated quantities of the neurotrophic factors BDNF, NGF, VEGF and HGF, suggesting that perhaps the main mechanism *in vivo* by which MSCs ameliorate neural damage is the result of production and secretion of neurotrophic factors and cytokines that stimulate endogenous recovery. Results regarding bFGF levels were inconclusive. This study was successful in demonstrating that not only do MSCs secrete neurotrophic factors and cytokines but, perhaps more importantly in terms of cellular therapy of neurological deficits, that MSCs are able to alter their secretory profile in response to specific microenvironmental cues i.e. increased secretion of neurotrophic factors in response to neural damage. Of additional interest from a clinical perspective, the secretome of MSCs not only varied in response to different environmental cues, but also varied depending on the time point at which the brain tissue was extracted following MCAo. This is a crucial factor requiring further investigation further if MSC-based cellular therapies are to be used successfully in the treatment of neurological and neurodegenerative disorders, as delayed administration of MSCs post-injury may not be as efficacious as when administered immediately.

In a more recent study by the same group, Chen and colleagues used sciatic nerve injury in rat as a model to ascertain whether MSC transplantation could be used as a means of treating peripheral nerve damage in addition to CNS damage (Chen et al., 2007). The sciatic nerve is one of the most commonly used in such studies, and is perhaps the most useful, as it is one of the longest nerves in the body, found in the lower limbs. Functional recovery following MSC transplantation was assessed using the sciatic function index (SFI). Whilst a detailed description of SFI is superfluous for the purposes of this chapter, it is useful to point out the scale on which SFI is measured, which runs from 0 to -100, where a value of 0 is indicative of normal nerve function and a value of -100 is indicative of complete sciatic nerve dysfunction. Sciatic nerve injury was induced, followed by MSC administration, to ascertain whether MSC transplantation could mediate functional recovery following damage to peripheral nerves. To assess functional recovery, SFI was determined periodically for up to 10 weeks post-transplantation. Higher SFI values were reported in animals subjected to MSC transplantation compared to controls, and these differences were highly significant at the statistical level from the fifth week onwards. Chen and co-workers had therefore successfully demonstrated that MSC transplantation resulted in clear

beneficial effects following peripheral nerve damage. In terms of the potential mechanism(s) by which MSC transplantation mediated functional repair, an increase in the levels of NGF, BDNF, GDNF and CNTF, and similarly increased expression of the extracellular matrix proteins, collagens I and IV, fibronectin and laminin, were observed. This suggested that not only were MSCs secreting soluble neurotrophic factors and cytokines that potentially stimulated endogenous repair mechanisms *via* their effects on immature neural stem and progenitor cells, but they were also secreting molecules that modified the microenvironment within the neurologically damaged area. This phenomena may also have been key in mediating functional recovery by encouraging beneficial events, such as neurite outgrowth, that would aid in the reconstruction of neural circuitry.

These data support the notion that transplanted MSCs release trophic factors and cytokines that mediate functional recovery following neurological damage in the CNS and to peripheral nerves. However, there is little evidence in these studies to demonstrate that these MSC-derived factors act directly on neural stem and progenitor cells. A study by Munoz *et al* in 2005 addressed this issue (Munoz et al., 2005). In this study, MSCs were administered directly into the dentate gyrus of the hippocampus, an area known to be rich in neural stem and progenitor cells. Munoz *et al.*, (2005) observed a substantial increase in the proliferation and migration of host neural stem cells, at the expense of MSC proliferation and migration. The proliferation and migration of resident neural stem and progenitor cells was attributed to increased levels of NGF, VEGF, CNTF and bFGF in the hippocampus following MSC transplantation, strongly suggesting that MSCs were at least partly responsible for their secretion. In this study, Munoz and colleagues make a very interesting observation, claiming that MSC-derived soluble factors not only influence neural stem and progenitor cells to undergo proliferation and differentiation events, but also function by 'activating' resident astrocytes within the niche (Munoz *et al.*, 2005). A contribution by 'activated' astrocytes towards the increased neurotrophin levels observed in the hippocampus following MSC transplantation cannot be discounted, nor can the possibility of 'activated' astrocytes directly influencing neurogenesis of resident neural stem and progenitor cells. An astrocytic contribution to neural recovery concomitant with the direct effects of MSC-derived factors is not unlikely, as there are

numerous reports that astrocytes have a trophic role in neurogenesis (Rudge et al., 1992, Nakayama et al., 2003).

1.6.3.4.2 Secretion of non-neurotrophic factors by MSCs

The discussion in Section 1.6.3.4.1 focussed predominantly on MSCs as trophic mediators of neural recovery in terms of the secretion of soluble neurotrophic factors and cytokines that directly act upon and influence signalling pathways and gene expression in resident neural stem and progenitor cells. However, MSCs also secrete a wide array of extracellular matrix proteins, in keeping with their stromal origins. These proteins can modify the niche in which stem cells reside, and thereby influence the behaviour of themselves and their neighbours. Perhaps the best study to date examining the expression of extracellular matrix and adhesion molecules by MSCs, particularly with reference to the effects of these molecules on the behaviour of neural cell types, is that by Crigler *et al* in 2006 (Crigler et al., 2006, Tremain et al., 2001). By analysing the transcriptome of a clonal population of MSCs using a serial analysis of gene expression (SAGE) approach, Crigler and colleagues demonstrated (and validated) that MSCs express a range of extracellular matrix and adhesion molecules that may contribute to MSC-mediated recovery of neural injury *in vivo*. Examples of such molecules expressed by MSCs include netrin-4 and reticulon-4 (previously reported to be involved in axonal guidance), ninjurin-1/2 and astrotactin (previously reported to be involved in neural cell adhesion), and prosaposin and pleiotrophin (previously reported to be neurite-inducing factors), amongst others. Hence, expression and secretion of extracellular matrix and/or adhesion molecules may also contribute to the mechanism of MSC-mediated recovery of neural damage. In reality, MSC-mediated amelioration of neural damage is likely to occur by a combination of neurotrophic factors acting directly on resident neural stem and progenitor cells, and also supporting extracellular proteins that exert a chemotactic effect on axonal and neurite outgrowth, which would aid in the reconstruction of neural circuitry.

A general overview of MSCs as trophic mediators of neural differentiation is illustrated in Figure 1.8.

1.6.4 Studying MSC-derived soluble factors in an in vitro setting: a conditioned media approach

One of the primary methods that has been used to analyse the secretome of various cell types involves the production and subsequent analysis of conditioned media (CM) produced by such cells under certain growth conditions. CM is a term used to describe media in which cells have already been cultured, and thus may contain a mixture of trophic factors and cytokines that have been actively secreted by the cells. A cocktail of cell-derived soluble factors may be useful in the subsequent culture of other cell types whether this be for routine growth or to induce other events such as cellular differentiation. Accordingly, the study of the effect(s) of MSC-conditioned media (MSC-CM) on cell fate decisions by neural stem and progenitor cells coupled with the appropriate analysis of MSC-CM media, may lead to the identification of the key combination of factors responsible for such activity.

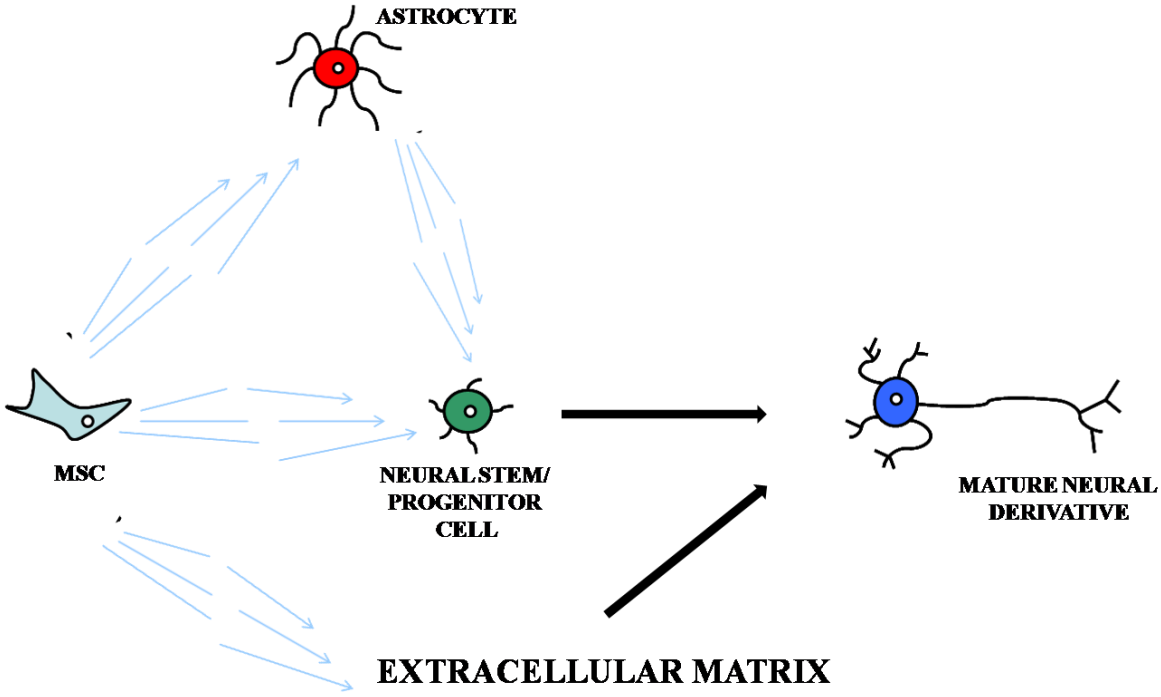

Figure 1.8. A diagrammatic representation of the predominant mechanisms by which MSCs may function as trophic mediators of neural differentiation. Molecules secreted by MSCs can be classified as either neurotrophic soluble factors/cytokines, which may act directly on neural stem/progenitor cells to induce cellular processes such as proliferation, migration and differentiation or may function indirectly *via* their influence on astrocytes within the niche, or extracellular matrix, which may promote neurogenic processes such as neurite extension.

In 2006, Rivera and colleagues demonstrated that MSC-derived soluble factors and cytokines induced neural stem cells to acquire a predominately oligodendrocytic phenotype, at the expense of an astrocytic one (Rivera et al., 2006). In this study, rat MSCs were cultured under standard culture conditions i.e. in the presence of 10% FCS, and were allowed to condition the medium for three days. The presence of serum in conditioned media can sometimes be problematic in subsequent analysis, as serum is a complex mixture of unknown nutrients, hormones and growth factors that are often necessary for routine cell culture. It can be extremely difficult to distinguish between proteins that have been secreted by the cells in culture and extraneous protein from the serum, unless sophisticated proteomic techniques are employed to distinguish between species-specific proteins. Additionally, high levels of proteins such as albumin and transferrin in serum may dominate subsequent analysis, masking cell-specific factors that have been secreted, which are likely to be present in much lower concentrations. After allowing MSCs to condition their standard, serum-containing media for 3 days, this MSC-CM was subsequently used for the culture of adult rat neural stem cells for a period of 7 days, after which the differentiative state of the cells was assessed by examining expression of mature neural markers. To serve as an appropriate control, neural stem cells cultured in unconditioned medium were also analysed. Neural stem cells cultured in MSC-CM for 7 days demonstrated a significant increase in expression of the oligodendrocytic markers galactocerebroside (GalC) and myelin basic protein (MBP), and this was concomitant with a significant decrease in the expression of the astrocytic marker, GFAP. These findings suggest that the cocktail of soluble factors secreted by MSCs into the MSC-CM induce adult neural stem cells to undergo gliogenesis, but particularly oligodendrogenesis at the expense of astrogenesis. Interestingly, in terms of neuronal differentiation, Rivera *et al* (2006) reported low levels of the neuronal marker, microtubule-associated protein 2ab (Map2ab), in both conditions, suggesting that MSC-derived soluble factors functioned independently of neurogenesis.

The main mechanistic question addressed by Rivera and colleagues (2006) in this study was whether MSC-derived soluble factors were exerting an instructive or selective effect on the NSCs. If functioning by an instructive mechanism, then the soluble factors in MSC-CM could be said to cause immature, non-committed neural stem cells to undergo oligodendrocytic differentiation. Conversely, if operating via a

selective mechanism, then these factors would be providing conditions that select for the enhanced growth of precursors already committed toward an oligodendrocytic fate and/or providing sub-optimal growth conditions for precursors committed to astrocytic or neuronal fates. To address this issue, Rivera and colleagues evaluated the effect of MSC-CM on the rates of cell proliferation and cell death within different subpopulations of the neuro-progenitor cells that differentially expressed cell type specific markers of glia and neurons. These experiments concluded that the acquirement of an oligodendrocyte phenotype produced by the neural stem cells occurred independently of rates of cell proliferation and cell death in the different progenitor subpopulations, suggesting that MSC-derived soluble factors were functioning by an instructive rather than selective mechanism.

In a separate study conducted in 2004, Wislet-Gendebien and co-workers investigated the effects of conditioned media derived from rat MSCs on the differentiation of mouse neuro-progenitor cells from the embryonic (E16) mouse (Wislet-Gendebien *et al.*, 2004). In order to compare the observations between Wislet-Gendebien's study and that by Rivera detailed above, a consideration of the differences in basic experimental setup is required. In addition to using embryonic neural cells as opposed to adult neural stem cells, the Wislet-Gendebien group produced and analysed media that had been conditioned by nestin-positive rat MSCs (npMSCs). Under standard growth conditions, MSCs are largely negative for expression of neural antigens such as nestin. However, appropriate manipulation of the culture conditions can induce MSCs to express nestin, namely by the removal of serum from the culture medium followed by a minimum of 25 population doublings; in crude terms, a minimum of 10 passages (Wislet-Gendebien et al., 2003). As MSCs have been reported in numerous studies to demonstrate neural antigen expression following transplantation *in vivo*, it may be considered more relevant to examine media conditioned by neural antigen-positive MSCs rather than neural antigen-negative MSCs.

In their study, Wislet-Gendebien and colleagues allowed npMSCs to condition media for 3 days (npMSC-CM), followed by subsequent culture of mouse embryonic neural progenitors in this npMSC-CM for a period of 5 days. During this time, expression of the astrocytic marker, GFAP, increased whilst there was a concomitant decrease in the percentage of cells expressing neuronal (Tuj1) and oligodendrocytic (O4) markers

(Wislet-Gendebien *et al.,* 2004). Similarly, the rates of cell proliferation and cell death were examined in the appropriate subpopulations of the mouse embryonic progenitor cultures following culture with npMSC-CM. Although no changes in cell proliferation were noted, a significant decrease in the rate of cell death of the GFAP-positive subpopulation was recorded after the 48 hours treatment with npMSC-CM. It is possible that a component of the npMSC-CM inhibited the death of GFAP-positive cells. In an attempt to identify the factor(s) responsible for influencing the differentiation of astrocytes, npMSCs were screened for a number of candidate factors. It was found that npMSCs showed an up-regulation of the biologically-active form of bone morphogenetic protein 4 (BMP4) at both the mRNA and protein level. MSCs that were negative for nestin expression did not express active BMP4. Accordingly, it was concluded that the effect of npMSC-CM on astrocyte differentiation by embryonic neuro-progenitor cells was attributable, at least in part, to the secretion of active BMP4 (Wislet-Gendebien *et al.,* 2004).

It is now becoming increasingly accepted that the likely predominant mechanism by which MSC transplantation results in functional recovery following neural injury is via the release of trophic factors and cytokines that act on resident neural stem and progenitor cells, thereby promoting endogenous repair processes. Whilst MSCs have been reported to secrete a wide range of neurotrophic factors and cytokines, our knowledge in this area remains at an early stage. The two studies discussed above demonstrate that the effect of MSC-derived soluble factors on the cell fate decisions of neural stem and progenitor cells can vary significantly, depending on a range of factors, including the developmental state of the MSC population and the source of neural stem cells (embryonic versus adult). However, despite the obvious gaps in our understanding, there have already been several efforts to exploit the natural beneficial properties of MSCs, by genetically engineering them to over-express the neurotrophic factors and cytokines that promote functional recovery from neurological deficits. There have recently been two studies using herpes simplex virus (HSV) as a vector to deliver target genes that enhance the expression and secretion of the growth factors HGF and FGF (Zhao et al., 2006, Ikeda et al., 2005). Results achieved in these studies have been extremely promising, with high rates of transfection efficiency reported, and the efficacy of genetically-engineered MSCs in mediating functional recovery from neural injury being enhanced above that of control MSCs. As such, MSCs may provide

useful vehicles for the delivery of highly-efficacious levels of neurotrophic factors and cytokines to areas of neurological and neurodegenerative damage.

1.7 MSCs as trophic mediators in alternative biological systems

1.7.1 Haematopoietic system

In addition to their intrinsic ability to undergo differentiation into a range of mesodermal derivatives such as bone, fat and cartilage, MSCs also demonstrate a crucial role within their niche involving the release and/or uptake of diffusible soluble factors/cytokines, which serves to modulate the behaviour of other cell types within proximity such as haematopoietic cells and endothelial cells. Although trophic roles have been described for other stem cell types within their niches, including haematopoietic and neural stem cells amongst others, trophic roles are perhaps best characterised in MSCs and, as such, will be considered here (Lu et al., 2003, Caplan and Dennis, 2006; Guo et al., 2006, Cabanes et al., 2007).

Although a major function of MSCs within their niche is to provide a solid, structural framework which is supportive of other cell types in the vicinity, such as haematopoietic cells, there is also significant evidence to suggest that an equally-important function of MSCs within their niche involves the release of diffusible soluble factors/cytokines that can function *via* autocrine and paracrine mechanisms to modify the behaviour and function of other cell types within the niche appropriately (Bobis *et al.*, 2006; Dennis and Caplan, 2006). For example, osteogenic and adipogenic differentiation of MSCs is significantly influenced by autocrine mechanisms *in vivo*, whereby MSCs secrete a range of soluble factors and cytokines that directly influence the differentiative process (Baylink *et al.*, 1993; Spiegelman and Flier, 1996; Mohamed-Ali *et al.*, 1998; Pages *et al.*, 2000; Sykara and Opperman, 2003). Furthermore, as differentiation proceeds and the MSC phenotype transforms accordingly, it has been reported that MSCs alter their secretory profile in keeping with this, serving to regulate their own behaviour *via* an autocrine feedback mechanism (Caplan and Dennis, 2006).

Stem cell bifunctionality in terms of differentiative potential and trophic activity is not a novel concept; indeed, this paradigm has been acknowledged in MSCs since the mid-

1980s, where a dual-role in both differentiation and regulation of haematopoiesis was reported (Owen, 1985). MSCs constitutively secrete a plethora of haematopoietic cytokines including, but by no means limited to, IL-1a, IL-1b, IL-6, IL-7, IL-8, IL-11, IL-14, IL-15, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-SCF), LIF, foetal liver tyrosine kinase 3 (flt-3), thrombopoietin, HGF and stem cell factor (SCF), all of which are highly documented as regulators of vascular and haematopoietic stem cell function (Dexter, 1989; Dexter *et al.*, 1990; Colter *et al.*, 2001; Gronthos *et al.*, 2003; Boiret *et al.*, 2005; Katz *et al.*, 2005; Bobis *et al.*, 2006; Dazzi *et al.*, 2006; Brooke *et al.*, 2007). MSC-mediated regulation of haematopoiesis has been demonstrated both *in vitro* and *in vivo* (Majumdar *et al.*, 1998; Maitra *et al.*, 2004; Mourcin *et al.*, 2005). One particularly interesting study by Muguruma and colleagues demonstrated that GFP-labelled MSCs integrated into the haematopoietic microenvironment following transplantation into immune-deficient NOD-SCID mice, resulting in an increase in the number of haematopoietic cells; this was a consequence of soluble factor/cytokine activity (Muguruma *et al.*, 2006). The intrinsic ability of MSCs to modulate haematopoiesis is of clinical significance; for example, in a study involving 28 breast cancer patients who had undergone extensive chemotherapy, haematopoietic recovery was greatly facilitated following administration of autologous MSCs (Koc *et al.*, 2000).

The ability of MSCs to modulate the behaviour of haematopoietic stem cells and regulate the process of haematopoiesis *via* the release of diffusible soluble factors and cytokines has been considered above. A similar phenomenon has been described in the nervous system, where MSC-derived soluble factors and cytokines promote endogenous recovery in numerous models of neurological and neurodegenerative disorders, presumably *via* their influence on neural stem and progenitor cell behaviour. Both scenarios are of significant clinical relevance. The remainder of this literature review will focus on current evidence supporting a trophic role for MSCs in other biological systems and their potential clinical significance; in particular, emphasis will be placed on MSC-mediated effects in the cardiac system, where many of the principles discussed for the nervous system hold true.

1.7.2 Cardiovascular system

Diseases of the cardiovascular system are the most significant contributors to morbidity and mortality in the Western world, claiming approximately 17 million lives worldwide in 1999 and affecting many more (Bonow *et al.*, 2002). As these figures demonstrate a propensity to increase – mirroring increasing rates of obesity and other significant risk factors – there is, consequently, a demand for more effective and satisfactory therapies. In particular, regenerative cell-based therapies using autologous stem cells remain a highly desirable therapeutic goal as adult cardiac tissue cannot regenerate following ischemic injury (Toma *et al.*, 2002).

MSCs present as ideal candidates for regenerative cell-based therapies against cardiovascular disease and, indeed, most clinically-important degenerative disorders, for the reasons detailed in Section 1.4.1. In addition to the general facets of MSC biology detailed in Section 1.4.1, their intrinsic ability to commit to and undergo myogenic differentiation makes them even more appealing as potential therapeutic agents in the cardiovascular system (Saito *et al.*, 1995; Gussoni *et al.*, 1999). Perhaps most promising are the numerous independent studies that have reported that MSC transplantation results in functional recovery, albeit to varying degrees, following myocardial infarction (MI), ischemic heart failure and dilated cardiomyopathy, as determined by measurement of functional parameters such as systolic/diastolic blood pressure, echocardiograms, blood flow and systolic wall thickness/function (Itescu *et al.*, 2003; Perin *et al.*, 2003; Stamm *et al.*, 2003; Wollert *et al.*, 2004; Amado *et al.*, 2005; Nagaya *et al.*, 2005; Caplan and Dennis, 2006).

However, a similar scenario to that observed in the nervous system presents, in that the precise mechanism(s) by which MSCs elicit functional recovery in the cardiovascular system are not known with any certainty. Numerous studies have reported the apparent differentiation of MSCs into cardiomyocytes and vascular endothelial cells both *in vitro* and *in vivo* (Makino *et al.*, 1999; Wang *et al.*, 2001; Toma *et al.*, 2002). However, several studies question the authenticity of such derivatives and, similarly, no study has successfully demonstrated functional engraftment of putative MSC-derived cardiomyocytes into cardiac tissue (Balsam *et al.*, 2004; Murry *et al.*, 2004; Brooke *et al.*, 2007). The lack of evidence regarding functional engraftment of MSC-

derived cardiomyocytes means a direct contribution of MSC differentiation to functional recovery cannot be claimed with any certainty.

It is now widely accepted that the functional recovery and therapeutic benefits observed following transplantation of MSCs in numerous models of cardiovascular disease are predominantly the result of a paracrine mechanism, mediated by MSC-derived soluble factors and cytokines such as HGF, VEGF and FGF, which promote beneficial processes such as angiogenic, anti-apoptotic, anti-fibrotic and anti-inflammatory events (Rehman *et al.*, 2003; Kinnaird *et al.*, 2004a; Kinnaird *et al.*, 2004b; Rehman *et al.*, 2004; Gnecchi *et al.*, 2005; Nagaya *et al.*, 2005; Gnecchi *et al.*, 2006; Miyahara *et al.*, 2006; Ohnishi *et al.*, 2007). Additionally, following the recent identification of a putative stem cell population within cardiac tissue, demonstrating the potential to differentiate into cardiomyocytes, smooth muscle and endothelial cell types, it can be envisioned that MSC-derived soluble factors promote endogenous repair processes *via* their influence(s) on host stem cell behaviour (Beltrami *et al.*, 2003). This is analogous to the scenario observed in the nervous system. Under normal physiological conditions, adult cardiomyocytes are largely under the influence of autocrine-derived growth factors and cytokines, including IGF, TGF and EGF, amongst others, which contribute towards many varied aspects of cardiomyocyte growth, survival and development (Perrella *et al.*, 1994; Engelmann *et al.*, 1996; Zhao *et al.*, 1998). A greater understanding of the role(s) of soluble factors and cytokines in cardiomyocyte function and development will ultimately aid in the derivation of novel therapeutic strategies targeted against clinically-important diseases of the cardiovascular system.

1.8 Concluding remarks and future directions

In terms of their potential clinical use in cellular therapies and tissue engineering strategies, adult stem cells are garnering much interest at present as they are generally easily accessible and circumvent the significant ethical issues surrounding the use of embryonic stem cells. Although there are many different types of adult stem cell that can be isolated from many sources, MSCs present as perhaps the most promising alternative to embryonic stem cells, owing to unique properties which make them potential candidates for a number of clinical applications. At present, the most obvious

and perhaps most realistically achievable of these involves exploiting the intrinsic ability of MSCs to differentiate into mesodermal derivatives, such as bone and fat, for the treatment of osseous and adipose tissue defects. There is a huge niche in the clinic for alternative treatment strategies for these disorders, due to the considerable limitations of current treatment methods, and it is in this field that MSC-based cellular therapies initially become commonplace in the clinic.

However, perhaps more exciting is the accumulating evidence to suggest that MSCs may be suitable candidates for use in a wider range of therapeutic applications than previously anticipated, and that they may be used in the treatment of disorders arising in tissues from alternative germ layers i.e. endoderm and ectoderm. The main crux of this literature review has therefore focussed on the potential use of MSCs in the treatment of neurological and neurodegenerative deficits. MSC transplantation in numerous models of neurological and neurodegenerative disorders can result in significant functional improvements. However, the mechanism(s) by which MSC transplantation mediates neurological recovery still eludes us to date. In the present literature review, the three most commonly-described hypotheses have been discussed, though it is now generally accepted that MSCs act predominantly as trophic mediators of neural differentiation involving the release of trophic factors and cytokines that act on resident neural stem and progenitor cells, promoting endogenous repair mechanisms. The other two proposed mechanisms – '*trans*'-differentiation and spontaneous cell fusion events – occur at far too low a frequency to be able to fully account for neurological improvements observed following MSC transplantation. This suggests that these are minor mechanisms involved in MSC-mediated neural recovery. The most compelling argument against a significant input from '*trans*'-differentiation and spontaneous cell fusion towards functional recovery centre around the timescale in which MSC-mediated recovery has been observed. Such mechanisms would require MSCs to successfully home to the neurologically-damaged area in the first instance, then successfully engraft into the tissue, differentiate into mature neural derivatives/acquire a mature neural morphology following fusion with host neural cell types and finally, and perhaps most complicated of all, form active neural connections. Only after all of these events have occurred successfully would any improvements in neurological function be observed. However, functional improvements following MSC transplantation have been observed as early as a few days following MSC

transplantation, which is an unrealistic period in which all of these events could occur (Chopp and Li, 2002). On the other hand, the ability of resident neural stem and progenitor cells to contribute to functional recovery during this timescale is far more likely, as these cells are already integrated within the host nervous system and, equally as importantly, already have much of the necessary cellular machinery in place to undergo differentiation. However, it must be stressed that a direct contribution of '*trans*'-differentiation and spontaneous cell fusion events towards neurological recovery cannot be discounted. It is therefore likely that the initial, short-term therapeutic benefits observed following MSC transplantation are a consequence of the action of MSC-derived soluble factors/cytokines and their direct effect(s) on host neural tissue, namely neural stem and progenitor cells, whilst longer-term recovery may indeed be contributed to the integration of '*trans*'-differentiated MSCs within the nervous system and/or MSCs that have spontaneously fused with host neural cell types and acquired their phenotypic traits.

MSCs have already received much attention as potential candidates for use in cellular therapies and tissue engineering strategies, and it is anticipated that this interest will only intensify in the near future. For the purposes of this literature review, the potential to exploit the intrinsic ability of MSCs to undergo mesodermal differentiation for regenerative cell-based therapies against osseous and adipose tissue defects has been considered; similarly, potential exploitation of MSCs in the treatment of neurological and neurodegenerative disorders has also been considered. However, these are by no means the only potential therapeutic applications of MSCs in the clinic or, indeed, the only areas in which MSC-based research is thriving. There is much interest in the potential use of MSCs in regenerative cell-based therapies of many other disorders, particularly those of the haematopoietic and cardiovascular systems, which have also been discussed above. There is accumulating evidence to suggest that MSCs also contribute to recovery from acute kidney injury, fulminant hepatic failure and pancreatic disorders, where MSC-derived soluble factors/cytokines promote survival and prevent apoptosis (Parekkadan *et al.*, 2007; Togel *et al.*, 2007; Park *et al.*, 2010). It is therefore envisioned that a greater understanding of the paracrine effect(s) of MSCs will lead to more targeted therapies which promote endogenous recovery from organ failure. Ultimately, the unique properties of MSCs may allow their use in patient-specific treatments for a whole range of clinical disorders. Identification of the

bioactive factors released by MSCs will also be important for the development of new pharmacological approaches to disease treatment and may also be of use from an industrial perspective in the development of new cell culture reagents. A general overview of the multifunctionality of MSCs is shown diagrammatically in Figure 1.9.

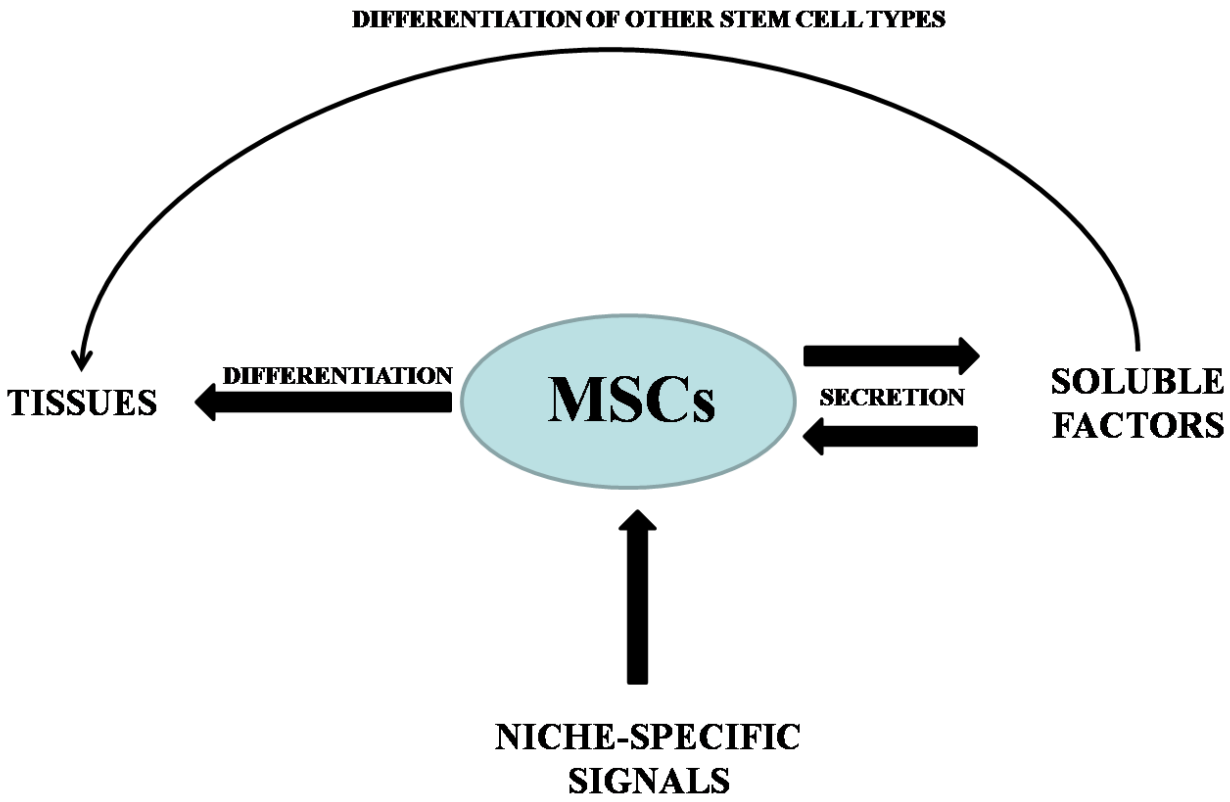

Figure 1.9. A diagrammatic representation of the dual functionality of MSCs. In addition to their intrinsic potential to differentiate into mesodermal derivatives such as bone and fat, MSCs also have a predominant role within their niche involving the release and/or uptake of soluble factors and cytokines that may influence the behaviour of other cell types within the niche. (Adapted from Caplan and Dennis, 2006).

1.9 Aims of current study

MSCs demonstrate dual functionality in terms of their intrinsic ability to differentiate into mesodermal derivatives such as bone and fat, concomitant with their ability to influence the behaviour of other cell types *via* the release and/or uptake of soluble factors and cytokines. Both facets of MSC behaviour are valuable from a clinical perspective. However, a more comprehensive understanding of the complex molecular and cellular mechanisms underlying these phenomena is necessary in order to facilitate the translation of MSCs from the laboratory bench to a clinical environment, and this will most likely involve the use of 3D cell culture systems that more accurately mimic the *in vivo* niche of MSCs. The major aims of the current study were therefore:

a) To investigate the ability of MSCs to differentiate into the mesodermal derivatives bone and fat when cultured in two- and three-dimensional polystyrene substrates

b) To investigate the ability of MSCs to mediate neural differentiation *via* the release of soluble factors/cytokines when cultured as three-dimensional aggregates, using a conditioned media approach

c) To investigate the complement of bioactive soluble factors/cytokines secreted by three-dimensional MSC aggregates, using a combination of targeted transcriptional profiling and shotgun proteomics

Chapter 2

MATERIALS AND METHODS

2.1 Isolation of MSCs from the bone marrow of postnatal rats

Rat mesenchymal stem cells (rMSCs) were isolated from the femurs and tibiae of 6-8 month old Wistar rats using established procedures (Croft and Przyborski, 2006). Rats were euthanised by CO_2 asphyxiation and their femurs and tibiae extracted, cleaned of connective tissue and placed on ice in 20ml complete culture medium (CCM). CCM consists of Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FCS, non-essential amino acids (1x), L-glutamine (12μM), penicillin (100 U/ml) and streptomycin (100 μg/ml). The ends of the bones were cut to expose the bone marrow (BM), and aspirated with 10ml of CCM by inserting a 21-gauge needle into the shaft of the bone and flushing with the CCM. The aspirates from each bone were plated into a single T75 cm^2 flask, and incubated at 37^oC and 5% CO_2 in a humidified incubator. The aspirates were left to allow stromal cells to adhere to the tissue culture plastic. After 2 days, non-adherent cells were removed, washed in phosphate-buffered saline (PBS) x 2 and maintained in CCM, with media changes every 3-4 days. Cultures were allowed to reach confluency. These cells were designated as *passage 0* (*P0*).

To passage cultures, MSCs were washed in PBS x 1 and detached from the cellware by incubation with 0.25% trypsin and 0.1% EDTA for 5-10 minutes at 37^oC. Trypsin was inactivated by the addition of CCM. Cells were centrifuged at 250 x *g* for 5 minutes, the supernatant removed, and cells resuspended in 1ml of CCM to form a single cell suspension. For routine expansion, rMSCs were passaged in a 1:3 ratio. MSCs between passages 2 and 8 were used for all the experiments described herein.

2.2 Adult hippocampal progenitor cell (AHPC) culture

The rat adult hippocampal progenitor cell (AHPC) line was originally obtained from the Salk Institute as a generous gift from Professor Fred Gage. AHPCs were cultured in cellware coated with poly-L-ornithine (10μg/ml) and laminin (5μg/ml), in N2 media. N2 media consists of DMEM:F12 (high glucose) supplemented with N2 supplement (1x), L-glutamine (12μM) and human recombinant fibroblast growth factor (FGF; 20ng/ml). Cells were routinely expanded in T75 cm^2 flasks and incubated at 37^oC and 5% CO_2 in a humidified incubator, with media being replenished every 2-3 days or until a confluency of 70-80% was reached. To passage cultures, media was removed and AHPCs detached from the cellware by quickly rinsing with 0.25%

trypsin and 0.1% EDTA for 1-2 minutes at 37^{o}C. Rinsing with PBS prior to the addition of trypsin (as for passaging of MSC cultures) was not necessary due to the lack of serum in N2 media. Trypsin was diluted and cells were resuspended by the addition of N2 media, followed by centrifugation at 1200 x rpm for 3 minutes to pellet the cells. The supernatant was removed and cells resuspended in 1ml of N2 media to form a single cell suspension and, for routine expansion, were passaged in a 1:5-1:10 ratio. AHPCs between passages 15-30 were used for all the experiments described herein.

2.3 33B cell culture

The rat 33B oligodendrocytoma (Schwannoma) cell line was originally obtained from Leeds University as a generous gift from Professor Eric Blair. For routine expansion, 33B cells were cultured in CCM as described for MSCs previously. 33B cells were also passaged using the same protocol as for MSCs, though at a much higher splitting ratio (1:5-1:10 for 33B cells, as opposed to 1:3 for MSCs).

2.4 Flow cytometry: general principles

As the name implies, flow cytometry is a general laboratory method involving measurement of cells in a flow system. Cells are delivered through a beam of light and measurement is achieved following detection of their fluorescence and the light scattered by them. Flow cytometry is commonly used in laboratories due to its wide range of applications and the ability to measure thousands of cells at the individual level within only a few minutes. Examples of applications of flow cytometry include immunofluorescence, DNA analysis, cell cycle analysis, apoptosis analysis and cell sorting (FACS). Six parameters are measured using the FACSCalibur flow cytometer (www.bdbiosciences.com) used in studies throughout this thesis:

- *Forward scatter (FSC)*

  Measures the size of a cell.

- *Side scatter (SSC)*

  Measures the granularity of a cell.

- *Fluorescence 1 (FL1)*

  Measures light emitted in the green wavelength range, produced by excitation of fluorophores including AlexaFluor-488 and FITC.

  The detection range of the FL1 filter is 515-545nm.

- *Fluorescence 2 (FL2)*

  Measures light emitted in the yellow wavelength range, produced by excitation of fluorophores including PE, AlexaFluor-546 and PI.

  The detection range of the FL2 filter is 564-606nm.

- *Fluorescence 3 (FL3)*

  Measures light emitted in the red wavelength range, produced by excitation of fluorophores including AlexaFluor-700, PerCP and PI.

  The detection range of the FL3 filter is $\geq$ 670nm.

- *Fluorescence 4 (FL4)*

  Measures light emitted in the red wavelength range, produced by excitation of fluorophores including APC and AlexaFluor-647.

  The detection range of the FL4 filter is 656-667nm.

An obvious advantage of flow cytometry, in addition to analysis of thousands of individual cells within a short timescale, is the ability to measure multiple parameters within a single assay e.g. dual- and triple-labelling of cells.

2.5 Analysis of cell surface antigen expression using flow cytometry

Flow cytometry was used to analyse the expression of several cell surface antigens on isolated MSCs. Briefly, cells were trypsinised as described in Section 2.1, washed in PBS x 1, pelleted by centrifugation at 250 x *g* for 5 minutes and resuspended in FACS

buffer (2% natural goat serum (NGS) in PBS) to a single cell suspension. Cells were subsequently distributed into the appropriate number of wells of a 96 well plate, centrifuged at 250 x *g* for 5 minutes, supernatant removed and subsequently incubated with 50µl of the following rat-specific primary antibodies for 1 hour at 4^{o}C: CD9, CD13, CD15, CD34, CD44, CD45, CD58, CD71, CD90, CD105 and CD138. All antibodies were used at a dilution of 1:50. Staining with *P3X* antibody was used as a negative control, to determine any levels of background/non-specific fluorescence and determine the threshold against which positive and negative events would be measured. Following this, cells were washed in FACS buffer x 3 and subsequently incubated in 50µl of the appropriate fluorochrome-conjugated secondary antibody for 30 minutes at 4^{o}C. All samples were stained with either an Alexa fluor 488-conjugated donkey anti-mouse secondary antibody or a FITC-conjugated goat anti-rabbit secondary antibody (both at 1:600 dilution). After 30 minutes, unbound secondary antibody was removed by washing in FACS buffer x 3. Following these final washing steps, cells were resuspended in 500µl FACS buffer and transferred to FACS tubes in preparation for running through the flow cytometer. To exclude non-viable/dead cells from our analyses, 10µl of propidium iodide (PI) (2.5mg/ml) was added to samples immediately prior to flow cytometric analysis. PI is excluded from viable cells, allowing us to gate for and only select viable cells in our analyses. Cell samples were passed through a FACSCalibur flow cytometer (www.bdbiosciences.com) and analysed using CellQuest Pro software. Both Alexa fluor 488- and FITC-fluorescence were detected in the FL1 band pass filter. Ten thousand events per sample were determined.

2.6 Analysis of intracellular antigen expression using flow cytometry

Flow cytometry was used to analyse the expression of several intracellular antigens in MSC and AHPC cultures. Briefly, cells were trypsinised as described in Section 2.1, washed in PBS x 1 and pelleted by centrifugation for 5 minutes (250 x *g* for MSCs, 2000rpn for AHPCs). Cell pellets were fixed in 2% PFA at 4^{o}C overnight by resuspension in 0.875ml cold PBS to form a single cell suspension, followed by addition of 0.125ml cold 2% PFA. Following overnight fixation, cells were processed for intracellular staining as follows. Cells were spun down at either 250 x *g* (MSCs) or 2000rmp (AHPCs) for 5 minutes, and the supernatant removed. Pellets were resuspended gently in 1ml permeabilising solution (0.2% Triton-X in PBS) and

incubated at 37°C for 30 minutes. Then, 1ml FACS buffer (2% NGS and 0.2% Tween 20 in PBS) was added, the cells spun down as described and the supernatant removed. The pellet was resuspended in FACS buffer to a single cell suspension and subsequently distributed into the appropriate number of wells of a 96-well plate, centrifuged at either 250 x *g* (MSCs) or 2000rpm (AHPCs) for 5 minutes, supernatant removed and subsequently incubated with 50µl of the following rat-specific primary antibodies (at the following dilutions) for 30 minutes at room temperature: nestin (1:200), Tuj-1 (1:600), GFAP (1:200) and GalC (1:200). Staining with *P3X* antibody was used as a negative control, to determine any levels of background/non-specific fluorescence and determine the threshold against which positive and negative events would be measured. Following this incubation, unbound primary antibody was removed by washing in FACS buffer x 3, and cells were subsequently incubated in 50µl of the appropriate fluorochrome-conjugated secondary antibody for 30 minutes at room temperature. For MSCs, Tuj-1 was detected with a FITC-conjugated goat anti-rabbit secondary antibody (1:600), whilst nestin, GFAP and GalC were detected with an Alexa fluor 488-conjugated donkey anti-mouse secondary antibody (1:600). For AHPCs, Tuj-1 was detected with an APC-conjugated goat anti-rabbit secondary antibody (1:300), whilst nestin, GFAP and GalC were detected with an APC-conjugated rabbit anti-mouse secondary antibody (1:600). After 30 minutes, unbound secondary antibody was removed by washing in FACS buffer x 3 and, following these final washing steps, cells were resuspended in 500µl FACS buffer and transferred to FACS tubes in preparation for running through the flow cytometer. Cell samples were passed through a FACSCalibur flow cytometer (www.bdbiosciences.com) and analysed using CellQuest Pro software. Alexa fluor 488- and FITC fluorescence were detected in the FL1 band pass filter, whereas APC-fluorescence was detected in the FL4 band pass filter. 10,000 events were determined per sample.

2.7 Immunofluorescence

Immunofluorescence staining was used to determine the expression of intracellular antigens. Briefly, cell cultures were washed in PBS x 1 and fixed directly in their cellware by incubating them in 4% PFA for 30 minutes at room temperature. Following this, cells were further washed in PBS x 1 and permeabilised by incubation with blocking solution (2% NGS, 0.1% sodium azide, 1% bovine serum albumin (BSA) and 0.2% Triton-X in PBS) for 30 minutes at room temperature. After

permeabilisation, cells were incubated with appropriate rat-specific primary antibodies (diluted in blocking solution) for one hour at room temperature. The following dilutions were used for each primary antibody: α-smooth muscle actin (α-SMA) (1:400), β-actin (1:400), fibronectin (1:200), GalC (1:200), GFAP (1:200), nestin (1:400), Tuj-1 (1:600) and vimentin (1:400). Staining with no antibody (i.e. blocking solution alone) was used as a negative control, to determine any levels of background/non-specific fluorescence. Following this incubation, unbound primary antibody was removed by washing in PBS x 3, and cells were subsequently incubated with appropriate fluorescently-labelled secondary antibodies for 45 minutes at room temperature. For MSCs, Tuj-1 was detected with a FITC-conjugated goat anti-rabbit secondary antibody (1:600), whilst nestin, GFAP and GalC were detected with an Alexa fluor 488-conjugated donkey anti-mouse secondary antibody (1:600). For AHPCs, Tuj-1 was detected with a Cy3-conjugated mouse anti-rabbit secondary antibody (1:300), whilst nestin, GFAP and GalC were detected with a Cy3-conjugated goat anti-mouse secondary antibody (1:600). After 45 minutes, unbound secondary antibody was removed by washing in PBS x 3, and nuclei counterstained with Hoechsst-33342 in PBS.

2.8 Viable cell number assay (MTS assay)

Determination of the number of viable cells in culture was achieved using the commercially available CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit from Promega. The reagent adopted in this assay contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (PES), which combines with MTS to form a stable solution. Cells bioreduce this MTS compound to form a coloured formazan compound, and the amount of formazan product formed can be quantified by measuring absorbance at 490nm. Absorbance is directly proportional to the number of viable cells in culture. The structures of MTS and the resultant formazan product are shown in Figure 2.1

200µl MTS reagent was added directly to the culture well for every 1ml of media, and incubated for 1-4 hours at 37^{o}C. A control well containing no cells was also set up, to take into account background absorbance of the MTS reagent in the absence of any cells. Absorbance at 490nm was subsequently recorded using a Nanodrop

spectrophotometer. Each assay was performed in triplicate, and the mean calculated and subsequently normalised against background absorbance (control well).

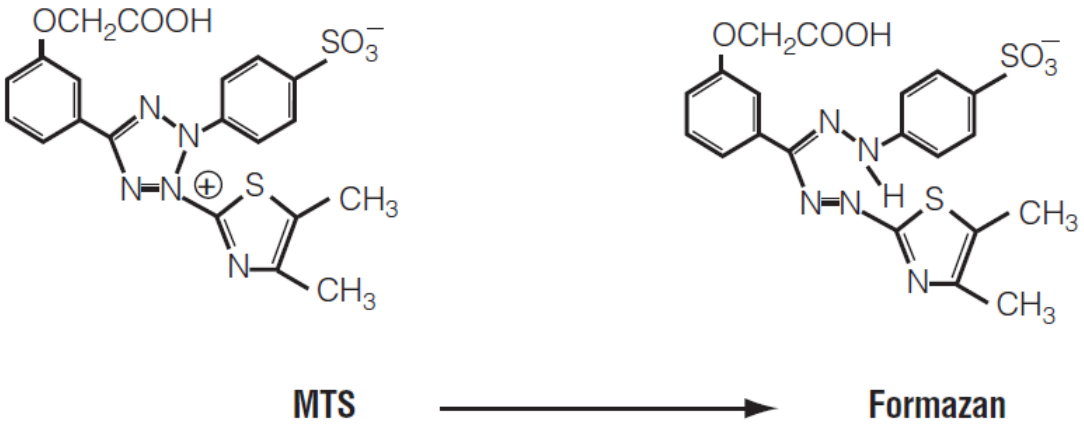

Figure 2.1. The structures of the MTS tetrazolium compound and its formazan product. Viable cells in culture bioreduce the MTS compound to form a coloured formazan product, the amount of which can be quantified spectrophotometrically by determining absorbance at 490nm. Absorbance values are directly proportion to the amount of formazan product formed, which is itself directly proportional to the number of viable cells in culture. Taken from http://www.promega.com/uk/

2.9 Isolation of RNA in preparation for gene expression analysis

Total cellular RNA was isolated using the TRIreagent method, following the manufacturer's protocol. Briefly, cells were lysed directly in their culture dish by the removal of media and washing in PBS x 1, followed by the addition of TRIreagent to the dish. Typically, 1ml of TRIreagent was added to a T25 and 3ml of TRIreagent added to a T75. Cells were incubated in TRIreagent for 5-10 minutes at room temperature, and homogenised by passing through a pipette several times. The homogenate was subsequently transferred from the culture dish to a 1.5ml Eppendorf tube for further manipulation. 0.2ml chloroform was added for every 1ml of TRIreagent used in the initial homogenisation step. Following this, samples were centrifuged at 12,000 x *g* for 15 minutes at 4^{o}C, during which time the mixture separated into layers. RNA remained exclusively in the upper, aqueous phase, whereas DNA and protein remained in the lower, organic phase. The RNA-containing aqueous layer (approximately 400-500µl) was transferred to a fresh 1.5ml tube, and the organic phase was either stored at -80^{o}C for DNA/protein extraction at a later date or disposed of appropriately. RNA precipitation was achieved by the addition of 0.5ml isopropanol (propan-2-ol) for every 1ml TRIreagent used in the initial homogenisation step.

Samples were incubated for 10 minutes at room temperature, followed by centrifugation at 12,000 x *g* for 10 minutes at 4°C, after which a pellet of RNA should be visible on the side of the tube. The supernatant was then removed and the pellet washed in 1ml 75% ethanol for every 1ml of TRIreagent initially used. An additional step of centrifugation at 10,000 x *g* for 10 minutes at 4°C was used to increase RNA yield. The supernatant was once again removed, and the RNA pellet air-dried for 5-10 minutes at room temperature. In a final step, the RNA was subsequently dissolved in 50µl RNase-free H_2O by mixing and incubation at 65°C for 5 minutes. RNA was subsequently stored at -80°C until necessary.

RNA concentration and purity was determined by using a Nanodrop spectrophotometer. 2µl of sample RNA was loaded onto the Nanodrop, and the RNA concentration and 260/280 ratio determined. Concentration was measured as ng/ml, whilst the ratio of the absorbances obtained for wavelengths of 260nm and 280nm gave an indication of the purity of the isolated RNA. A relatively pure sample of RNA has a 260/280 ratio of between 1.7 and 2. A ratio below 1.7 is indicative of protein contamination, whereas a 260/280 ratio above 2 is indicative of DNA contamination. Only RNA with a 260/280 ratio between 1.7 and 2 were used in subsequent downstream applications. RNA integrity was also assessed by running an RNA gel. Briefly, 10µg total RNA was loaded to a 1% RNAse-free agarose gel (1% agarose in TBE running buffer and 0.02% ethidium bromide) and visualised using a GelDoc EQ DNA visualisation machine and QuantityOne image analysis software. Pure, intact total RNA gives a characteristic two-band pattern, corresponding to 18S and 28S ribosomal RNA (rRNA). Only intact RNA was used for subsequent studies. An example of a non-degraded RNA is shown in Figure 2.2

2.10 Reverse Transcription

2µg total purified RNA was reverse transcribed using a commercially available reverse transcription (RT) kit. Each individual 20µl reverse transcription reaction consisted of the following components: 2µg total RNA, 2µl TaqMan RT buffer (10x), 4.4µl $MgCl_2$ (25mM), 4µl dNTPs (2.5mM), 1µl oligo d(T) (50µM), 0.4µl RNase inhibitor (20U/L), 0.5µl RT enzyme (50U/µl) and made up to a total volume of 20µl with RNase-free water. The reaction mixture was vortexed and centrifuged at 12,000 rpm to ensure complete mixing of the reaction components, and the reverse transcription reaction

performed on a thermal cycler. Thermal cycling parameters were as follows: 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 10 minutes. The newly-synthesised cDNA was subsequently stored at 4°C for short-term storage, or -20°C for longer term storage.

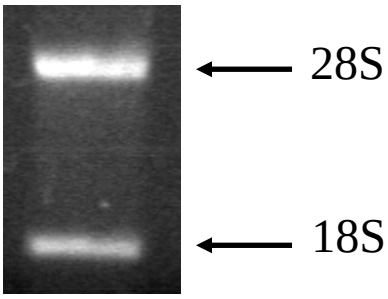

Figure 2.2. An example of a non-degraded RNA. A characteristic two-band appearance is shown, corresponding to 18S and 28S rRNA respectively. Degraded RNA does not have the two band appearance, instead appearing as a smear down the gel.

2.11 Quantitative PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed using an Applied Biosystems 7500 Fast Real Time PCR system (Applied Biosystems), using the SYBR Green method.

2.11.1 Overview of SYBR Green chemistry

SYBR Green is a dye that binds specifically to double-stranded DNA. It is ideal for use in qPCR as it binds to double-stranded DNA as it is formed during the PCR reaction (PCR product) and this resultant SYBR Green-DNA complex emits fluorescence in the green spectra (λ_{max} = 552nm). The intensity of fluorescence emitted by this complex is directly proportional to the amount of double-stranded DNA formed throughout the PCR reaction. Therefore, the amount of fluorescence detected allows absolute quantitation of PCR product present in our reaction and, by extension, the amount of original mRNA in our samples (for qRT-PCR). Fluorescence was detected at the end of each cycle during the exponential phase of the PCR reaction. A general overview of SYBR Green-based detection of PCR product is shown in Figure 2.3. The main advantage of SYBR Green over Taqman-based technologies is that the probe required in Taqman reactions is not necessary in SYBR Green reactions, reducing

setup time and running costs considerably. Paradoxically, the main disadvantage of SYBR Green technology is actually due to the lack of probe in the reaction, which can lead to a lack of specificity because SYBR Green will bind to any double-stranded DNA present in the reaction mix, including non-specific double-stranded products such as primer dimers.

2.11.2 Quantitative reverse transcription PCR (qRT-PCR)

Quantitative reverse transcription PCR (qRT-PCR) was performed using an Applied Biosystems 7500 Fast Real Time PCR system (www.appliedbiosystems.com), using the SYBR Green method, as introduced above. Each individual 10µl PCR reaction consisted of the following components: 5µl SYBR green mastermix (2x), 3.2µl MQ water, 0.4µl forward primer (diluted 1:40), 0.4µl reverse primer (diluted 1:40) and 1µl target cDNA (diluted 1:5). All reactions were performed in triplicate, for technical error. QRT-PCR plates were centrifuged at 2000rpm for 20-30 seconds to ensure complete mixing of the reaction components, and the reaction performed on an Applied Biosystems 7500 Fast Real Time PCR system. Thermal cycling parameters were as follows: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, 95^{o}C for 15 seconds followed by 60^{o}C for 1 minute (x40), followed by a dissociation step of 95^{o}C for 15 seconds, 60^{o}C for 15 seconds and 95^{o}C for 15 seconds. Data are collected during the exponential phase of the PCR at the end of each cycle and Figure 2.4 shows the formation of PCR product against cycle number for a typical multi-well plate. Specific primer sequences for specific genes are shown in Table 2.1. All primers were synthesised by VH Bio (www.vhbio.com) and received as solid oligonucleotides. Immediately upon receipt, primers were reconstituted in MQ water to make a 1µg/µl stock solution, aliquoted, and stored at -20^{o}C until required. Standard curves were constructed for each primer pair using a positive cDNA control, to allow absolute quantitation of sample cDNA. Rat Universal RNA was reverse transcribed into cDNA and used in the construction of all standard curves herein, as Universal RNA contains gene transcripts isolated from a wide range of tissues and organs, and therefore serves as a good positive control for use in qRT-PCR. A minimum of 5 points were used to construct each standard curve, covering a wide range of dilutions as follows: undiluted, 1:5, 1:10, 1:50, 1:100 and 1:1000. Only standard curves with an R2 value of 0.97 or greater were used for quantitation of sample cDNA.

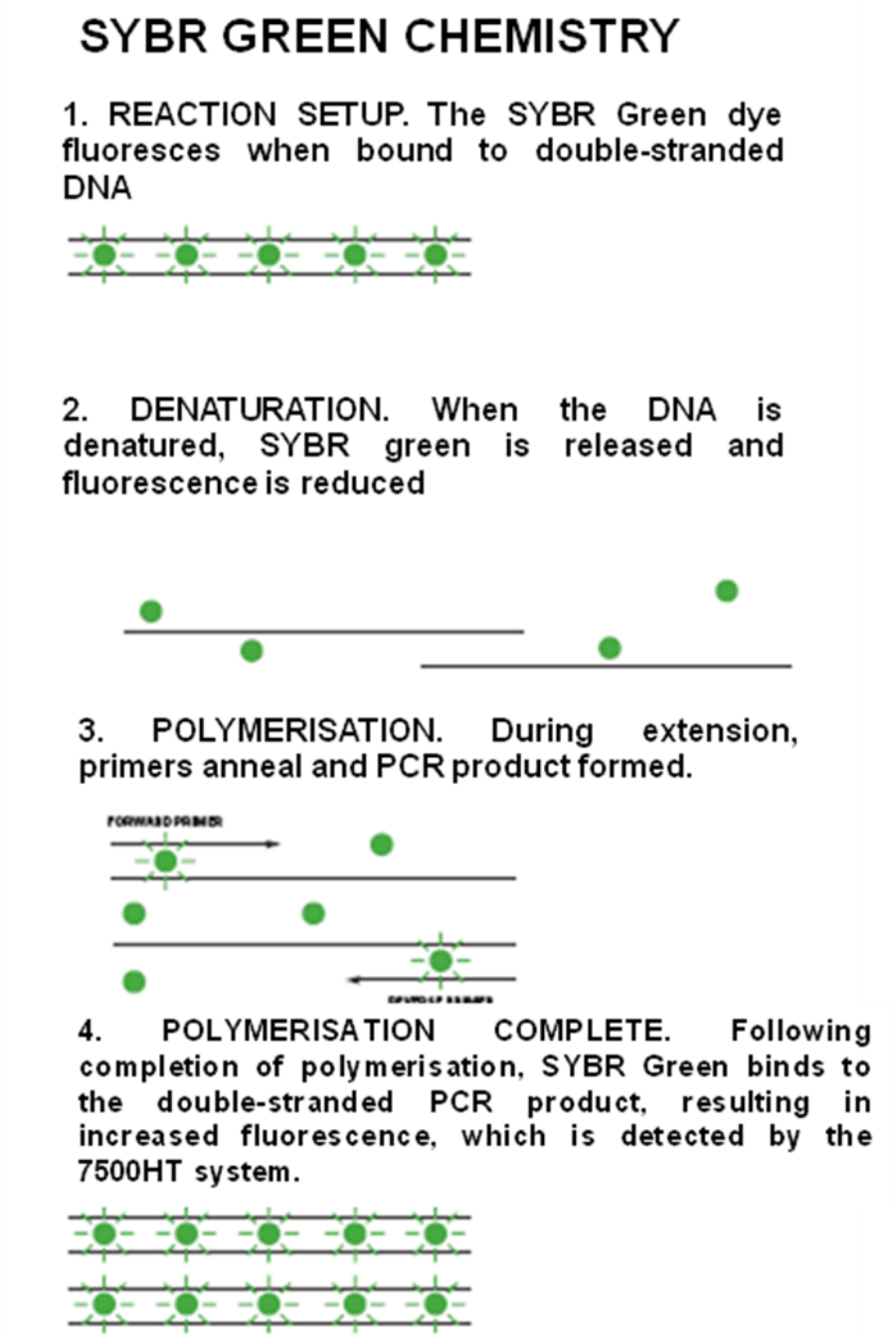

Figure 2.3. An overview of SYBR Green-based detection of PCR product formed during a qPCR reaction. SYBR Green dye binds to double-stranded DNA formed throughout the PCR reaction, emitting a fluorescence that can be detected at 552nm. During the denaturation stage of the PCR, double-stranded DNA are separated into single strands and, consequently, fluorescence intensity is massively reduced. As the PCR reaction continues through the annealing and polymerisation stages, double-stranded DNA is formed and SYBR Green once again binds to these duplexes, emitting a fluorescence which can be readily detected. (Adapted from www.appliedbiosystems.com).

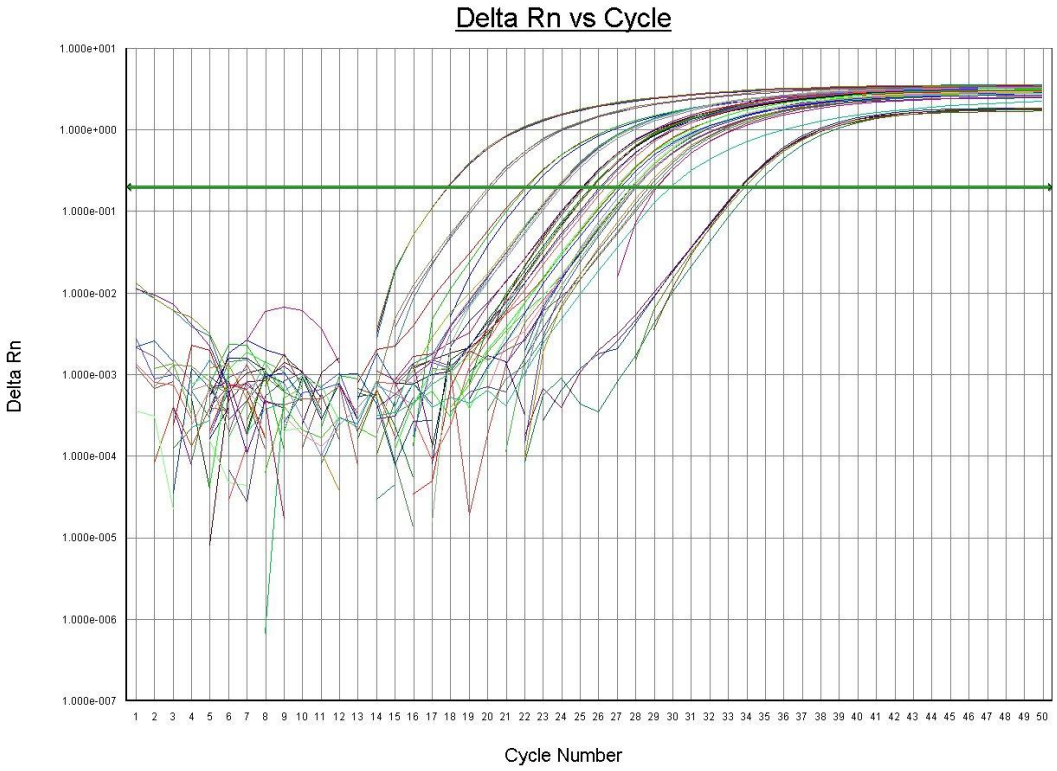

Figure 2.4. A typical trace showing the formation of PCR product against cycle number. For highly-expressed transcripts, detection of PCR product can be as early as 10-20 cycles. As the PCR progresses, the amount of product detected increases in an exponential manner until it eventually reaches a maximum, where rate of product formation remains constant. The above trace is a highly typical example of the rate of an enzyme-catalysed reaction versus time.

An example standard curve is shown in Figure 2.5. A no template control (NTC), containing all the components of the PCR reaction but no target cDNA, was used as a negative control, to ensure that no non-specific amplification/formation of secondary products (such as primer dimers) was occurring.

A dissociation step (95^{o}C for 15 seconds, 60^{o}C for 15 seconds and 95^{o}C for 15 seconds) was performed after all cycles of the PCR reaction had finished, in order to determining the melting temperature of products formed during the PCR reaction. This is an important quality control measure to determine the specificity of the reaction. A tight peak as shown in Figure 2.6A indicates that only one product has been formed during the reaction., indicating the primers used are specific and only amplifying the target region of interest. Multiple peaks, as shown in Figure 2.6B indicate that multiple

products are being formed in the PCR reaction and that the primers are not specific for the region of interest and/or forming secondary products such as primer dimers.

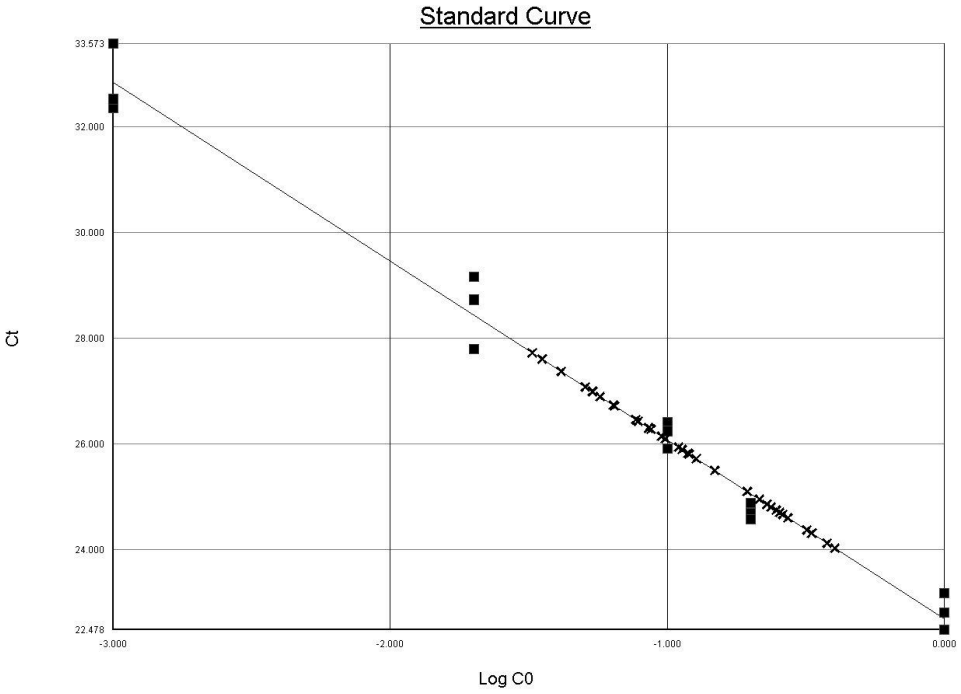

Figure 2.5. An example of a typical standard curve, constructed using cDNA reverse-transcribed from rat Universal RNA. Five points were used in the construction of this curve (each in triplicate), using the range of dilutions detailed in Section 2.11.2 above. The R2 value for this curve was 0.984769.

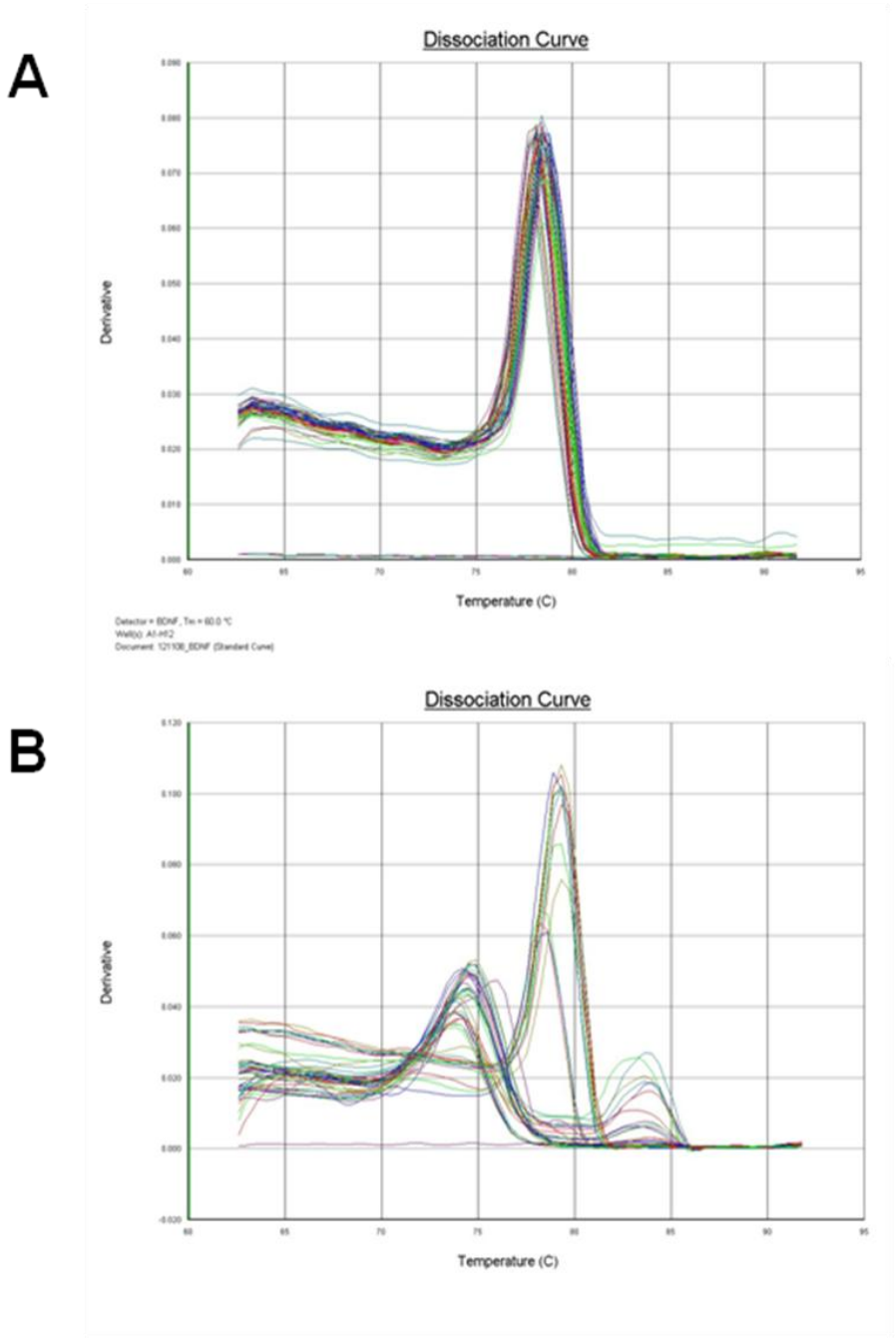

Figure 2.6. Typical examples of dissociation curves demonstrating the formation of specific (A) and non-specific (B) double-stranded products during the PCR reaction. For every primer pair used in this thesis, a dissociation step was performed in order to confirm the specificity of the primers and the target region amplified during the PCR reaction. The above traces show the melting temperature of the double-stranded products formed during the PCR. A tight peak as shown in A indicates a high degree of primer specificity and the formation of a single PCR product. Multiple peaks as shown in B demonstrate a lack of specificity and the formation of multiple double-stranded products during the PCR, most probably primer dimers.

GENE	FORWARD (SENSE) PRIMER 5' – 3'	REVERSE (ANTISENSE) PRIMER 5' – 3'
5-HT-1A	TCTGTTGCTGGGTACTCTCA	AGGAGCCGATGAGATAGTTG
Adipsin	CCGATGTCCTGCAGCAACT	GTCCCTGCGGTTGCTCTCT
Adipophilin	CTGAACCAGCCAACATCTGA	AGTAACTGCTCCTTTGGTCT
AMPA3	GCTCCATGGGCTCTCAAGTA	AGGGTCACACTTGGGAAATG
BDNF	CACTTTTGAGCACGTGATCGA	CGTTGGGCCGAACCTTCT
BMP4	ATGTGACACGGTGGGAAACTTTC	ACCTCAATGGCCAGCCCATA
CNTF	CTTCAAGAGCTCTCACAGTG	TGCTTATCTTTGGCCCCATAAT
FGF2	GACGGCTGCTGGCTTCTAAGT	TTTCCGTGACCGGTAAGTGTT
GABA-A	GCCATTGCCTTTTTAACACA	CTAAAGCAACCAGGCCAGAG
GAPDH	AGTTCAACGGCACAGTCAAG	TACTCAGCACCAGCATCACC
Hes1	TACCCCAGCCAGTGTCAACA	TTCATTTATTCTTGCCCGGC
Hes5	ACCGCATCAACAGCAGCATT	AGGCTTTGCTGTGCTTCAGGT
Id1	TGGACGAACAGCAGGTGAAC	TCTCCACCTTGCTCACTTTGC
Id2	TTTCCTCCTACGAGCAGCAT	CCAGTTCCTTGAGCTTGGAG
Id3	TGCTACGAGGCGGTGTGCTG	AGTGAGCTCAGCTGTCTGGATCGG
IGF1	ACCCCACCCACAAAACAACA	CGTCCCGGGTCGTTTACAC
LIF	TCTGTGCAACAAGTACCATGT	GCAGCCCAACTTCTTCCTT
Mash1	GATGAGCAAGGTGGAGACGC	CGGAGAACCCGCCATAGAGT
NGF	CCAGCCTGCGGACATCAC	GGCTCCTCAATGGGCAGAT
Ngn1	CAGTAGTCCCTCGGCTTCAG	AAGCAGGGTGTCGTATGGAG
Ngn2	CGGGTCAGACGTGGACTACT	GAGGACGAGAGAGGGAGACC
Ngn3	AAGAGCGAGTTGGCACTGAGC	AAGCTGTGGTCCGCTATGCG
Nkx2.2	CACGCAGGTCAAGATCTG	TGCCCGCCTGGAAGGTGGCG
NMDA-2D	GTTCGCTGGAGGACCTGAG	CGCGTCATACCTCGGACT
NT3	GATCCAGGCGGATATCTTGA	AGCGTCTCTGTTGCCGTAGT
Olig1	GCCCCACCAAGTACCTGTCTC	GGGACCAGATGCGGGAAC
Olig2	CACAGGAGGGACTGTGTCCT	GGTGCTGGAGGAAGATGACT
PPARγ	CACAATGCCATCAGGTTTGG	GCTGGTCGATATCACTGGAGATG
Smad1	CCGCCTGCTTACCTGCCTCCTGAA	GAACGCTTCGCCCACACGGTTGT
Smad4	GATAGCGTCTGTGTGAACC	GTACTGGTGGCATTAGACTC

Sox9	CGTGGACATCGGTGAACTGA	GGCAAGTATTGGTCAAACTC
STAT1	AGGTCCGTCAGCAGCTTAAA	CGATCGGATAACAACTGCTT
STAT3	GCGAAAGGAAACACCTATG	GTGGACATTAACCAGGTTGA
TGF-β-1	GACGGAATACAGGGCTTTCG	CCTCGACGTTTGGGACTGAT
VEGF	ACGAAGCGCAAGAAATCCC	TTAACTCAAGCTGCCTCGCC

Table 2.1. Primers used in qRT-PCR reactions. Details given include sequences of forward (sense) and reverse (antisense) primers.

2.12 Purchase of reagents and chemicals

All reagents and chemicals were purchased from Sigma-Aldrich (www.sigmaaldrich.com), unless otherwise stated in the main body of text.

Chapter 3

ESTABLISHMENT OF MSC CULTURES AND ASSESSMENT OF THEIR FUNCTIONAL BEHAVIOUR WHEN GROWN IN TWO AND THREE DIMENSIONAL POLYSTYRENE SUBSTRATES

3.1 Introduction

Traditionally, cell culture is performed in the two-dimensional environment (2D) of plastic tissue culture flasks and petri dishes. Although culturing cells using such methodology has increased our knowledge of biological processes and resulted in many important medical breakthroughs, culturing cells in tissue culture plastic makes an important assumption – that cells cultured in this artificial 2D environment behave in an analogous manner to which they would when functioning as part of a living tissue *in vivo*. It is now generally accepted that this is not the case as many different factors need to be taken into consideration when trying to artificially mimic the *in vivo* environment of a cell. Such factors include cellular geometry and arrangement, exposure to extracellular matrix components and cell-cell interactions, amongst others. Therefore, in spite of the obvious advantages of culturing cells in 2D in terms of convenience and high levels of standardisation, this methodology is fundamentally flawed in that the 2D plastic environment is far removed from the complex environment in which a cell exists *in vivo*. Factors such as extracellular matrix and cell-cell interactions all interplay to provide biochemical and mechanical cues that influence cellular behaviour such as proliferation and differentiation (Bissel *et al.*, 2002; Kleinman *et al.*, 2003; Pampaloni *et al.*, 2007).

Reproducing the exact *in vivo* environment for cell growth within the lab is an extremely difficult and virtually impossible task, though efforts can be made to mimic certain elements of the *in vivo* environment e.g. coating flasks with extracellular matrix proteins such as laminin to provide an environment more permissive to the culture of neural cell types (Carbonetto and Cochard, 1987). Although a useful compromise, coating cellware with individual (or even combinations of) extracellular matrix proteins is not an ideal practise as extracellular matrix does not simply consist of a basic mixture of proteins; rather, it is a specific yet dynamic combination of proteins, arranged purposely and present at precise concentrations. It is this delicate balance between arrangement and concentration that results in the execution of specialised responses in cells *in vivo* (Adams and Watt, 1993). Unsurprisingly, simple changes in extracellular matrix architecture and component composition can radically alter the functional behaviour of a cell.

Because of the highly unnatural environment provided by conventional tissue culture plastic, there is a high demand for alternative systems in which to culture cells *in vitro* that would provide an environment more representative of that in which they exist *in vivo* (Elsdale and Bard, 1972). Many studies have demonstrated that culturing cells on 3D scaffolds dramatically alters their behaviour compared to conventional 2D cultures and this has been shown in a number of different cellular systems (Beningo *et al.*, 2004; Witte and Kao, 2005). Malignant breast cells undergo dramatic changes in gene expression when cultured on 3D compared to 2D (Weaver *et al.*, 1997; Wang *et al.*, 1998). Fibroblasts also demonstrate altered behaviour when cultured on 3D in terms of increased rates of proliferation and migration compared to control cultures (Cukierman *et al.*, 2001). Perhaps most exciting of all are the numerous reports that embryonal carcinoma (EC) stem cells cultured on 3D demonstrate enhanced functionality compared to their 2D equivalents (Levenberg *et al.*, 2003; Hayman *et al.*, 2004; Bokhari *et al.*, 2007a; Bokhari *et al.*, 2007b).

Although the advantages of culturing cells in 3D compared to 2D are generally well-acknowledged and well-accepted, routine 3D culture is far from commonplace in the lab due to a lack of standardisation (Pampaloni, 2007; Bradley *et al.*, 2009). That said, efforts have been and continue to be made to produce robust 3D *in vitro* cell culture models, as these could lead to more accurate and sensitive drug screening assays, significantly reducing the need for animal testing, and would also be invaluable in the fields of tumour biology, epithelial biology, gene expression studies and developmental studies in particular (Bissell *et al.*, 1982; O'Brien *et al.*, 2002; Friedl, 2004; Kunz-Schughart *et al.*, 2004; Marx and Sandig, 2006; Bradley *et al.*, 2009).

Some of the earliest successful examples of 3D cell culture involved culturing cells on biodegradable polymers such as poly(glycolic acid) and poly(lactic acid) (Mikos *et al.*, 1993; Bokhari *et al.*, 2007). Biodegradable polymers have been shown to successfully promote proliferation, migration and differentiation of various neural cell types, providing great potential for repair of the damaged nervous system (Tabesh *et al.*, 2009). In such instances, the polymer would need to provide a suitable substratum with the crucial functional property of promoting axonal growth and encouraging reconstruction of neural circuitry. Biodegradable polymers have also received much attention for their potential use in the production of vascular structures *in vitro*, for

application such as bypass operations (Campbell and Campbell, 2007; Ko *et al.*, 2007). Several studies also report the suitability of such polymers for delivery of cellular material to the damaged retina (Lu *et al.*, 2001; Klassen, 2007). Likewise, their potential for use in bone and cartilage tissue engineering has been discussed at length (Temenoff and Mikos, 2000; Koch and Gorti, 2002; Liu and Ma, 2004). Although most interest in biodegradable polymers is tailored towards their potential use in cellular therapies and tissue engineering strategies, chitosan-based products have been used for controlled drug delivery (Prabaharan, 2008). The physical properties that make biodegradable polymers ideal for use in cellular therapy, however, also make them unsuitable for everyday use in the lab as they are subject to degradation following prolonged storage (Bokhari *et al.*, 2007).

More recent examples of 3D cell culture involve culturing cells on structures known as hydrogels. As the name would imply, hydrogels are hydrated polymers, in that they consist of a 3D network of polymer chains that spread out to form a gel due to the fact that the spaces between the chains are filled with water. In some cases, hydrogels can contain up to 99% water. This property of being able to retain water whilst maintaining their physical integrity and mechanical properties means that hydrogels are used for applications such as contact lenses. However, they are becoming more frequently used in cell culture applications, and can be made from a wide variety of materials such as agarose, collagen, fibrin, gelatin and laminin (Bokhari *et al.*, 2007; Bradley *et al.*, 2009). Collagen-based gels are perhaps the most commonly used to date and have been used quite extensively in the culture of neural cell types. Ganglia can be maintained in collagen-based gels for up to 4 weeks *in vitro* (Blackshaw *et al.*, 1997). Fibrin-based gels have also been reported to promote nerve regeneration due to their neurite-promoting properties (Sakiyama *et al.*, 1999). Agarose gels with laminin covalently attached have also been reported to promote axonal extension and neuronal regeneration (Yu *et al.*, 1999). Although hydrogels are used extensively in the study of neural cell types, they are also ideal for the study of other cellular systems; for example, in cartilage grafts, as articular cartilage is a water-laden tissue (Fisher *et al.*, 2004; Park *et al.*, 2005). Similarly to biodegradable polymers, there is also evidence to suggest that hydrogels may be suitable for the release of biomolecules (Lin and Anseth, 2009).

Although biodegradable polymers and hydrogels are the most commonly used substrates for the 3D culture of cells *in vitro*, they are by no means the only technologies being employed in the lab for 3D culture. For example, sponges have been examined for their potential as suitable growth substrates for MSCs *in vitro* (Takahashi *et al.*, 2005). Sponges possess a similar structural morphology to gels but differ in that they possess larger pores and as such have a far more open structure to permit infiltration of larger cell types. This structure can be modified appropriately to influence cellular behaviour. The use of microcarriers is another method for culturing cells in 3D, though this methodology is very infrequent compared to those described above. Microcarriers are extremely small spheres of an approximate diameter of 500 µm which can be coated with extracellular matrix molecules to permit attachment of cells. The main advantage of this technology is that these spheres provide an enormous surface area, allowing the culture of a large number of cells in a small volume (Bradley *et al.*, 2009). Although infrequently used in an academic environment, the main advantage of this technology is found in industry where they are commonly used for mass bioproduction.

Reinnervate Ltd manufactures a novel, polystyrene-based scaffold, with the aim of providing an easily-handled material that can be used as an everyday tool in the lab for 3D cell culture. This scaffold is branded Alvetex^{TM} and demonstrates several advantages over the above technologies. For example, it is manufactured under robust and highly reproducible conditions, unlike sponges and gels which are subject to variability in their formation and can undergo structural alterations with long-term storage. Another major advantage of the scaffold is that it is produced under animal-free conditions as cells generally attach to the scaffold without the need for coating with extracellular matrix molecules, though coating can be performed when required for the culture of particularly fastidious cell types such as neural cells. The chemistry behind the production of the scaffold will be considered briefly.

The scaffold chemistry has been published extensively (Barbetta *et al.*, 2000; Cameron and Barbetta, 2000; Krajnc *et al.*, 2002). Scaffolds are commonly referred to as poly(HIPE)s as they are manufactured by polymerisation in high internal phase emulsions (HIPEs). A key property of HIPEs is that they consist of a heterogeneous liquid-liquid mixture possessing a large volume of internal (droplet) phase (Cameron

and Sherington, 1996; Bokhari *et al*., 2007). Phase droplets are deformed into polyhedra when the volume fraction exceeds 0.74 and these polyhedra are separated by thin layers of continuous (non-droplet) phase. In order for polymerisation to occur, two key criteria must be satisfied: firstly, at least one phase of the emulsion must contain a monameric species; secondly, a cross-linker must be present in the continuous, non-droplet phase. Only when both criteria are satisfied can polymerisation occur. Accumulation of monomer units occurs around the dispersed, deformed phase droplets, creating polyhedral-like voids in the resulting material (Cameron and Sherington, 1996; Cameron, 2005). These voids are connected to surrounding voids by films of continuous phase known as 'interconnects' (Cameron *et al.*, 2006). The resulting monolith can be sectioned into wafer thin slices of 120µm thickness, providing a robust material than can be easily used in cell culture. Physical parameters such as void size can be easily controlled using this methodology, which is advantageous as it allows scaffolds to be tailored specifically to meet the needs of the particular cells being cultured. This technology has been exemplified for use in routine cell culture using a number of different cellular systems, including the HepG2 (liver carcinoma) and TERA2.cl.SP12 (embryonal carcinoma) cell lines (Hayman and Przyborski, 2004; Hayman *et al.*, 2005; Bokhari *et al.*, 2007a; Bokhari *et al.*, 2007b).

The present study provides the first data regarding the culture of MSCs on Reinnervate's novel 3D scaffold. MSCs are extremely exciting candidates for use in cellular therapies and tissue engineering for a number of reasons, as discussed in Section 1.4.1 . Because of the advantages of using MSCs in a clinical setting compared to other stem cell types e.g. embryonic stem cells, there is a huge interest in MSC research at present. However, before the use of MSCs in the clinic becomes more commonplace, it is crucial to gain a more comprehensive understanding of the complex molecular and cellular mechanism(s) by which MSCs commit to a given fate and undergo differentiation to produce mature, fully-functional derivatives. Much of our present knowledge has been gained from studies performed on the highly unnatural, 2D environment of tissue culture plastic. A better understanding of such complex processes therefore requires 3D cell culture systems that more accurate mimic the niche of MSCs *in vivo*. In the present study, the ability of Reinnervate's scaffold to support the viable culture of MSCs *in vitro* and support their functional ability to undergo osteogenic and adipogenic differentiation is examined. Results obtained

suggest that the scaffold may provide a more realistic and physiologically-relevant system in which to study the processes of osteogenic and adipogenic differentiation in a manner more pertinent to that which occurs *in vivo*.

Hypothesis

MSCs cultured on Alvetex™ will demonstrate enhanced cellular viability and functionality in terms of their potential to undergo osteogenic and adipogenic differentiation, compared to MSCs cultured on tissue culture plastic.

Aims of chapter

To isolate MSCs from the bone marrow of adult rats and produce primary *in vitro* cultures. Examine how the behaviour of MSCs is influenced following culture on Alvetex™ compared to the conventional 2D environment of tissue culture plastic.

Objectives

1. Establish early passage cultures of MSCs from rat bone marrow.

2. Verify the MSC-like phenotype of isolated cells in terms of cell surface and intracellular marker expression and potential to differentiate into mesodermal derivatives

3. Assess the behaviour of MSCs cultured in 3D using Alvetex™ compared to conventional 2D cultures in terms of cell viability, osteogenic and adipogenic differentiation

3.2 Materials and methods

3.2.1 Osteogenic differentiation of MSCs and von Kossa staining

The osteogenic potential of MSCs was determined using the following differentiation protocol. Cells were seeded at a density of $3x10^3$ cells/cm^2 into 6 well plates, in CCM, and allowed to reach 70-80% confluency. When the desired degree of confluency was reached, osteogenic differentiation was induced in MSCs by switching their culture medium from CCM to osteogenic medium (DMEM supplemented with 10% FCS, dexamethasone (100nM), ascorbic acid 2-phosphate (50μM) and β-glycerophosphate (10nM)). Cells were maintained in this osteogenic medium for 3 weeks, with media changes every 3-4 days. After 3 weeks, cells were washed in PBS and fixed in 4% PFA for 30 minutes at room temperature. In order to assess osteogenic differentiation and the formation of calcified bone nodules, von Kossa staining was performed. Briefly, cells were incubated in 5% silver nitrate solution for 10 minutes in the dark, washed thoroughly with distilled water, and exposed to bright light for 1 hour. Following this, cells were again washed with distilled water and counterstained with haematoxylin for 5 minutes.

3.2.2 Adipogenic differentiation of MSCs and oil red O staining

The adipogenic potential of MSCs was determined using the following differentiation protocol. Cells were seeded at a density of $2x10^4$ cells/cm^2 into 12 well plates, in CCM, and allowed to reach 100% confluency. When the desired degree of confluency was reached, adipogenic differentiation was induced in MSCs by switching their culture medium from CCM to adipogenic induction medium (DMEM supplemented with 10% FCS, dexamethasone (1μM), indomethacin (0.2mM), insulin (10μg/ml) and 3-isobutyl-1-methylxanthine (0.5mM)). After 3 days, adipogenic induction medium was replaced by adipogenic maintenance medium (DMEM supplemented with 10% FCS and insulin (10μg/ml)) for 2 days. This induction/maintenance cycle was repeated for 3 weeks. After 3 weeks, cells were washed in PBS and fixed in 4% PFA for 30 minutes at room temperature. In order to assess adipogenic differentiation and the formation of neutral lipid droplets, oil red O staining was performed as follows. Briefly, cells were incubated in 0.3% oil red O (in isopropanol) for 30 minutes at room temperature and washed thoroughly with distilled water. Following this, cells were

counterstained with haematoxylin for 5 minutes, and blued (3% ammonia solution in water) for 30 seconds.

3.2.3 Growth curve of MSCs and estimation of doubling time

To assess the growth rate and doubling time of MSCs cultured under standard conditions (CCM), a growth curve was constructed by seeding MSCs at a wide range of densities into a standard 12-well plate and periodically determining the number of viable cells in culture. Five different densities were used – 1000, 2000, 3000, 5000 and 10,000 cells/well – and this was designated as T=0. The number of viable cells in culture was determined every 24 hours periodically for up to 120 hours, using the MTS proliferation assay, as previously detailed in Section 2.8. All assays were performed in triplicate, and the means calculated and plotted ± SEM.

3.2.4 Preparation of Alvetex™

Solid, highly porous 3D Alvetex™ monoliths were prepared and fabricated as detailed in the literature (Bokhari *et al.*, 2007a; Bokhari *et al.*, 2007b). The resulting monolith was sectioned to a thickness of 120μm, using a vibratome, to produce wafer thin discs of scaffold suitable for use in cell culture. These scaffold discs were inserted into the wells of 6-well plates, and pre-wetted and sterilised in 70% ethanol for one hour at room temperature, followed by UV treatment (5 minutes). Following this, discs were subsequently washed in PBS x 3 before used for cell culture.

3.2.5 Preparation of samples for scanning electron microscopy (SEM)

To prepare biological samples for surface analysis using scanning electron microscopy (SEM), cells cultured on the 3D scaffold were fixed in 2% PFA and 2.5% glutaraldehyde in 0.1M Sorenson's phosphate buffer (pH 7.4) for 1 hour at room temperature. Following fixation, samples were rinsed in 0.1M phosphate buffer and subsequently post-fixed in 1% OsO_4 solution for 1 hour at room temperature, followed by sample dehydration by bringing the samples through progressive ethanol washes as follows: 50%, 70%, 95% and 100% ethanol (4 x 5 minutes in each condition). Dry scaffold samples were then sectioned into smaller pieces of approximately 25mm^2 in area, mounted onto specimen holders (stubs) using an epoxy resin adhesive and dried from CO_2 at 38^{o}C at 1200 psi. The final preparation step involved low vacuum sputter coating with a 7nm layer of chromium (to prevent the accumulation of static electric

charge on the sample during electron irradiation), after which samples were ready for visualisation using a Hitachi S5200 scanning electron microscope.

3.2.6 Histology preparation for MSCs cultured under osteogenic and adipogenic conditions on Alvetex™

MSCs were seeded onto the scaffold at a density of 1 x 10^6 cells/scaffold and cultured under osteogenic and adipogenic conditions for 21 days, after which samples were prepared for histology analysis. To assess osteogenic differentiation, samples were washed in PBS x 1 and fixed directly in the 3D scaffold in 4% PFA overnight at 4^{o}C. Following fixation, sample dehydration was achieved by bringing samples through progressive ethanol washes – 50%, 70%, 95% and 100% ethanol (4 x 5 minutes in each condition) – followed by embedment in paraffin and sectioning to produce 7μm sections ready for staining. Von Kossa and Masson's trichrome staining was performed to demonstrate the formation of calcified bone nodules and the deposition of extracellular collagen, respectively. Specific details of von Kossa staining are described in Section 3.2.1. For Masson's trichrome staining, sections were first counterstained with haematoxylin for 5 minutes, followed by sequential staining with solutions A (10 minutes), B (5 minutes) and C (10 minutes). Solution A (plasma stain) consisted of 1% glacial acetic acid, 0.5% acid fuchsin and 0.5% xylidine ponceau in distilled water; solution B consisted of 1% phosphomolybdic acid in distilled water; and solution C (fibre stain) consisted of 2% glacial acetic acid and 2% light green SF yellowish in distilled water. To assess adipogenic differentiation, oil red O staining was performed to demonstrate the accumulation of neutral lipid droplets within the cell cytoplasm. MSCs were washed in PBS x 1 and trypsinsed out of the 3D scaffold, resuspended in CCM to inactivate the trypsin, cytospun onto non-coated glass slides at 800rpm for 5 minutes and fixed in 4% PFA overnight at 4^{o}C. Following fixation, oil red O staining was performed as described in Section 3.2.2.

3.2.7 Determination of alkaline phosphatise (ALP) activity and osteocalcin levels

Alkaline phosphatase (ALP) activity was determined using a commercially-available, highly sensitive colorimetric assay kit (Sigma, N7653), following the manufacturer's instructions. The kit exploits the use of *p*-nitrophenyl phosphate (pNNP) as a substrate for ALP, where ALP catalyses the hydrolysis of pNNP to *p*-nitrophenol. This

hydrolysis reaction yields a yellow soluble end-product, which can be measured spectrophotometrically at 405nm. Absorbance at 405nm is therefore directly proportional to ALP activity. The methodology was adapted from the manufacturer's protocols and what had previously been published in the literature (Wang *et al.*, 2007). Briefly, MSCs were cultured under osteogenic conditions for 5, 7, 14 and 21 days on tissue culture plastic (2D) vs. scaffold (3D), and ALP activity determined at each of these time points. Cells were washed in ice-cold PBS x 2 and protein extracts made by homogenising the samples in 2ml lysis buffer (0.2% IGEPAL, 10mM Tris-HCl and 1mM MgCl2 at pH 7.5). Lysates were centrifuged at 12,000 rpm for 10 minutes at 4^{o}C and the supernatant removed and subsequently assayed for ALP activity. 50µl sample supernatant and 50µl pNNP (1mg/ml) were mixed together and incubated on a bench shaker for 30 minutes in the dark. Absorbances at 405m were determined immediately following this incubation. A standard curve was constructed using absorbance values determined for known concentrations of *p*-nitrophenol, allowing conversion of our sample absorbance values into protein concentrations. ALP activity was subsequently normalised to total protein content (determined using Bradford assay) and expressed as nmol/hour/mg protein.

Levels of osteocalcin secreted into the culture media were determined using a commercially-available, rat-specific sandwich ELISA kit, following the manufacturer's instructions (BT-490 kit, BTI, Stoughton, MA). The methodology was as follows. MSCs were cultured under osteogenic conditions for 14, 21 and 35 days on tissue culture plastic (2D) vs. scaffold (3D), and osteocalcin secretion into the culture media determined at each of these time points. The microtiter plate provided by the manufacturers was pre-coated with a monoclonal antibody specific for the N-terminal region of rat osteocalcin. 100µl sample media was added to each well and the plates incubated at 4^{o}C for 24 hours. Following this, sample media was aspirated off and each well washed in PBS x 3. 100µl of a second antibody was then added to each well – a goat polyclonal antibody specific for the C-terminal region of rat osteocalcin – and plates incubated for 1 hour at 37^{o}C. Each well was washed in PBS x 3, and 100µl of a donkey anti-goat IgG peroxidase was subsequently added to each well, and plates incubated for 1 hour at room temperature. Following a further three washes in PBS, 100µl of substrate mix (consisting of one volume of TMB solution: one volume of hydrogen peroxide) was added to each well and plates incubated in the dark for 30

minutes at room temperature. 100µl stop solution was added to each well to stop the reaction, and absorbance at 450nm measured immediately. Concentration of osteocalcin in the culture media was directly proportional to absorbance. A standard curve was constructed using absorbance values determined for known concentrations of osteocalcin, allowing conversion of our sample absorbance values into actual concentrations. The standard curve covered a range of concentrations from 0.25-20ng/ml. Osteocalcin levels were subsequently normalised to total DNA content and expressed as ng/ml/µg DNA.

3.2.8 Oil red O quantification

Quantification of intracellular lipid accumulation was achieved by using a colorimetric assay based on elution of oil red O. MSCs were cultured under adipogenic conditions for 7 and 21 days on tissue culture plastic (2D) vs. scaffold (3D), and oil red O staining performed as previously described. Following staining with oil red O, cells were subsequently destained in 100% isopropanol for 30 minutes at room temperature, and the absorbance of the subsequent solution measured at 500nm using a Nanodrop spectrophotometer. Concentration of oil red O stain in the solution was directly proportional to absorbance at 500nm. Negative controls were ran in parallel, consisting of MSCs cultured on 2D and 3D under standard culture conditions i.e. non-adipogenic conditions. Each assay was performed in triplicate, and the means calculated and subsequently expressed as a percentage above control levels.

3.2.9 Nile red staining and flow cytometric analysis

Further quantification of intracellular lipid accumulation was achieved by staining cells with the lipophilic Nile red fluorescent dye, followed by flow cytometric analysis, using a methodology adapted from Bäckesjö *et al.*, 2006. MSCs were cultured under adipogenic conditions for 21 days on tissue culture plastic (2D) vs. scaffold (3D). Following this 21 day differentiation period, cells were washed in PBS x 1, trypsinised, resuspended in CCM to inactive the trypsin and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and the cell pellets resuspended in 4% PFA and incubated at 4^{o}C overnight. Following overnight fixation, cells were stained with Nile red (10µg/ml) for 45 minutes at room temperature, then analysed using a FACSCalibur flow cytometer (BD Biosciences). Fluorescent emission was detected in the FL1 band-pass filter, and the fraction of cells exhibiting positivity for Nile red

fluorescence was determined for MSCs cultured under adipogenic conditions on 2D and 3D, in terms of the percentage of positive cells and mean fluorescence intensity (MFI). Ten thousand events per sample were determined.

3.2.10 Cyclic AMP (cAMP) enzyme immunoassay

MSCs were cultured under adipogenic conditions on 2D and 3D for 7 and 21 days, and the cyclic AMP (cAMP) levels determined at both time points using a commercially available cAMP EIA kit (Cayman Chemical, Ann Arbor, MI), following the manufacturer's protocol. Cells were trypsinised and centrifuged at 2000 rpm for 5 minutes to form a pellet, which was subsequently dissolved in 1N NaOH and protein content measured using the Biorad protein assay. cAMP levels determined by the EIA were normalised to total protein levels, as determined by the above Biorad protein assay. The basis of the EIA assay is the competition between free cAMP and a cAMP tracer (cAMP-acetylcholinesterase (AChE) conjugate) for a limited number of rabbit antibody binding sites specific for cAMP. Throughout the assay the concentration of cAMP tracer remains constant, whereas the levels of free cAMP vary depending on the levels of cAMP present in the samples being tested, therefore the levels of cAMP tracer which bind to the rabbit antibody binding site is inversely proportional to the levels of cAMP in the sample. The subsequent mixture of rabbit antibody-cAMP and rabbit antibody-cAMP tracer complexes bind to a mouse anti-rabbit antibody which is pre-attached to an ELISA plate. In order to develop the plate, Ellman's reagent, which contains the substrate to the cAMP tracer, must be added. The product of this reaction is yellow in colour, and the intensity of this colour is proportional the amount of cAMP tracer in the well, which in turn is inversely proportional to the amount of free cAMP in the well. Intensity of product absorption was determined by measuring absorbance at 415nm, and these absorbance values were used in subsequent downstream calculations in order to determine the exact levels of cAMP in the samples.

3.3 Results

3.3.1 Stromal cells isolated from the bone marrow of postnatal rats demonstrate morphology in keeping with an MSC origin

MSCs were isolated from the femurs and tibiae of Wistar rats, aged between 6-8 months, using the methodology described in Section 2.1. Plastic adherence was used as the main criterion to allow selection of MSCs from the various other cell types that reside within the bone marrow, particularly the haematopoietic population. Whilst efforts continue to be made to exploit marker expression in order to produce a more homogeneous starting population, there is little standardisation between laboratories. As such, plastic adherence still remains the most commonly used method for MSC selection by far, despite the obvious limitations of the heterogeneous nature of the starting material.

Figure 3.1 shows MSCs at various stages during the isolation and expansion process. Following the plating of whole bone marrow aspirates into T75 cm^2 flasks, non-adherent cells (haematopoietic) were removed after 48 hours, leaving behind an adherent (stromal/MSC) population (Figure 3.1A). Even at this early stage, morphological heterogeneity within the adherent population was evident. Cells were small in size, largely retractile and spindle-shaped. Contaminating haematopoietic cells, mainly erythrocytes, were readily observable at this stage of the isolation process even after several PBS washes/media changes, and remained as phase bright, highly refractile cells in suspension. Some contamination of MSC cultures with haematopoietic cells is inevitable, especially during the initial isolation procedure and early passage cultures. These contaminants are removed from culture via continual PBS washes, media changes and by passaging, with the end result being a heterogeneous mixture of stromal cells/MSCs at various stages of development. Within 2-3 days, the small, adherent, spindle-shaped cells began to divide rapidly and flatten out to form larger, flatter cells with a morphology more in keeping with that described for an MSC population (Figure 3.1B). The arrowheads in Figure 3.1B show actively dividing cells, which demonstrate a very spherical morphology as they undergo cytokinesis. Following division, the cells increased in size and spread out, acquiring a more stromal, fibroblastic-like morphology. Cells continued to proliferate and form colonies, which grew very rapidly and eventually merged together to form a

monolayer culture (Figure 3.1C). When a culture reached 70-100% confluency (Figure 3.1D), typically taking between 7-14 days following initial plating of the bone marrow aspirate, we described this culture as Passage 0 (P0). A small degree of haematopoietic contamination (approximately 1-2%) was still evident in this confluent P0 culture. To passage confluent MSC cultures, the methodology described in Section 2.1 was followed. The resulting cultures were designated as Passage 1 (P1) and Figure 3.1E shows MSCs 24 hours post-passage. Cells were larger in size and demonstrated a more fibroblastic-like morphology than their initially-isolated counterparts (Figure 3.1A). These cultures typically reached confluency between 7-10 days (Figure 3.1F).

3.3.2 Flow cytometric and immunocytochemical analysis of marker expression in bone marrow stromal cells verify the isolation of a true MSC population

As described previously in Section 3.3.1 stromal cells isolated from rat postnatal bone marrow aspirates via plastic adherence display a morphological phenotype very characteristic of an MSC population. Although useful in many settings, morphological data alone is limited and insufficient to confirm with any certainty the isolation of a true MSC population. In order to verify that the cells isolated and subsequently used in all downstream studies demonstrated behaviour indicative of an MSC origin, two approaches were adopted. Firstly, expression of a wide range of generic cell surface and intracellular markers were examined to produce a detailed expression profile for the isolated cells. Secondly, the potential of the isolated cells to differentiate into mature mesodermal derivatives such as bone and fat was examined, and this will be considered in the proceeding section.

Cell surface marker expression was assessed via live cell staining with antibodies directed against cell surface antigens, followed by flow cytometric analysis. Staining with *P3X* antibody served as a suitable negative control, allowing the flow cytometer parameters to be set to distinguish between specific fluorescence and non-specific fluorescence resulting from loosely-bound antibody/autofluorescence. Negative controls appear as clear traces in Figure 3.2. Confirming the isolation of a true MSC population is hindered by the lack of any definitive markers that are expressed purely in MSCs.

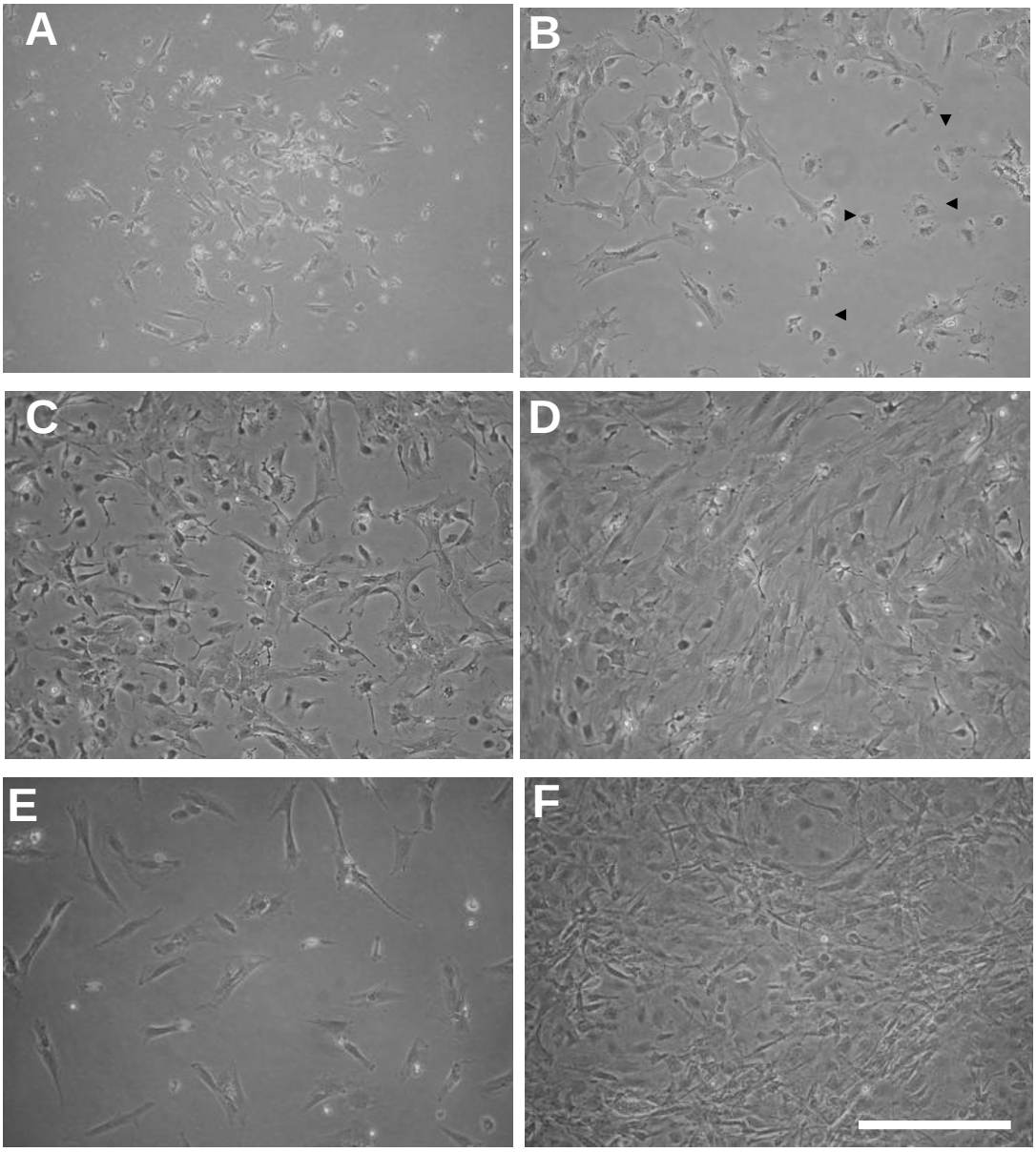

Figure 3.1. Isolation of MSCs from the bone marrow of postnatal rat. A-F shows MSCs at various stages during the isolation procedure. **A:** 48 hours post isolation. MSCs adhere to tissue culture plastic, whereas contaminating haematopoietic cells remain in suspension as highly refractile, phase bright cells; **B:** Adherent cells begin to divide and flatten out, adopting a more fibroblastic-like morphology. Arrowheads indicate actively-dividing cells; **C:** Culture at 7 days, where colonies are expanding rapidly and beginning to merge to form a monolayer; **D**: A confluent P0 culture prior to passaging; **E:** Cell morphology of a passage 1 (P1) culture, 24 hours after passaging; **F:** A confluent P1 culture. Scale bar = 100µm.

Therefore it is necessary to examine the expression of a range of markers, none of whose expression is exclusive to MSCs. Representative flow cytometric traces for a wide range of cell surface markers are shown in Figure 3.2. CD34 and CD45 are both significantly expressed on the haematopoietic cell types present within the same bone marrow niche as MSCs. Contamination of MSC cultures with haematopoietic cell

types can be problematic, particularly during the initial isolation steps and in early passage cultures. The representative traces shown in Figure 3.2A are from passage 2 (P2) cultures, the lowest passage used in any study throughout this thesis. As the traces clearly show, the cells are largely negative for CD34 and CD45 expression (black peaks), confirming that the cultures used throughout this thesis were not contaminated/ very minimally contaminated by any haematopoietic cell types. Expression of a wide range of positive markers were also assessed via flow cytometry, including CD9, CD13, CD15, CD44, CD58, CD71, CD90, CD105 and CD138 (Figure 3.2B). The isolated cells demonstrated positive expression for each of these markers when assessed via flow cytometry, although the traces do indicate that the markers are expressed at varying intensities. Some markers show a degree of overlap with the *P3X* negative control e.g. CD13 and CD58, whereas other markers show little, if any, overlap with the *P3X* negative control e.g. CD90 and CD105. Isolated cells were also examined for expression of neural markers including nestin, Tuj-1, GFAP and GalC, and were negative for expression in all instances (data not shown).

In addition to flow cytometric analysis of cell surface marker expression, immunocytochemistry was employed to examine the expression of a range of intracellular cytoskeletal and extracellular matrix markers indicative of an MSC phenotype. Cells demonstrated intense staining for the mesodermal markers α-smooth muscle actin, vimentin, fibronectin and β-actin, which are largely associated with mesenchymal stem cell types (Figure 3.3). Immunocytochemistry was also performed for the neural markers nestin, Tuj-1, GFAP and GalC, and the isolated cells were negative in all instances (data not shown), confirming the observations made via flow cytometry.

Whilst expression of these cell surface and intracellular markers give little information with regards the function of the isolated cells, the expression profile observed is indicative of an MSC origin. Results from both flow cytometric and immunocytochemistry analyses are summarised in Table 3.1.

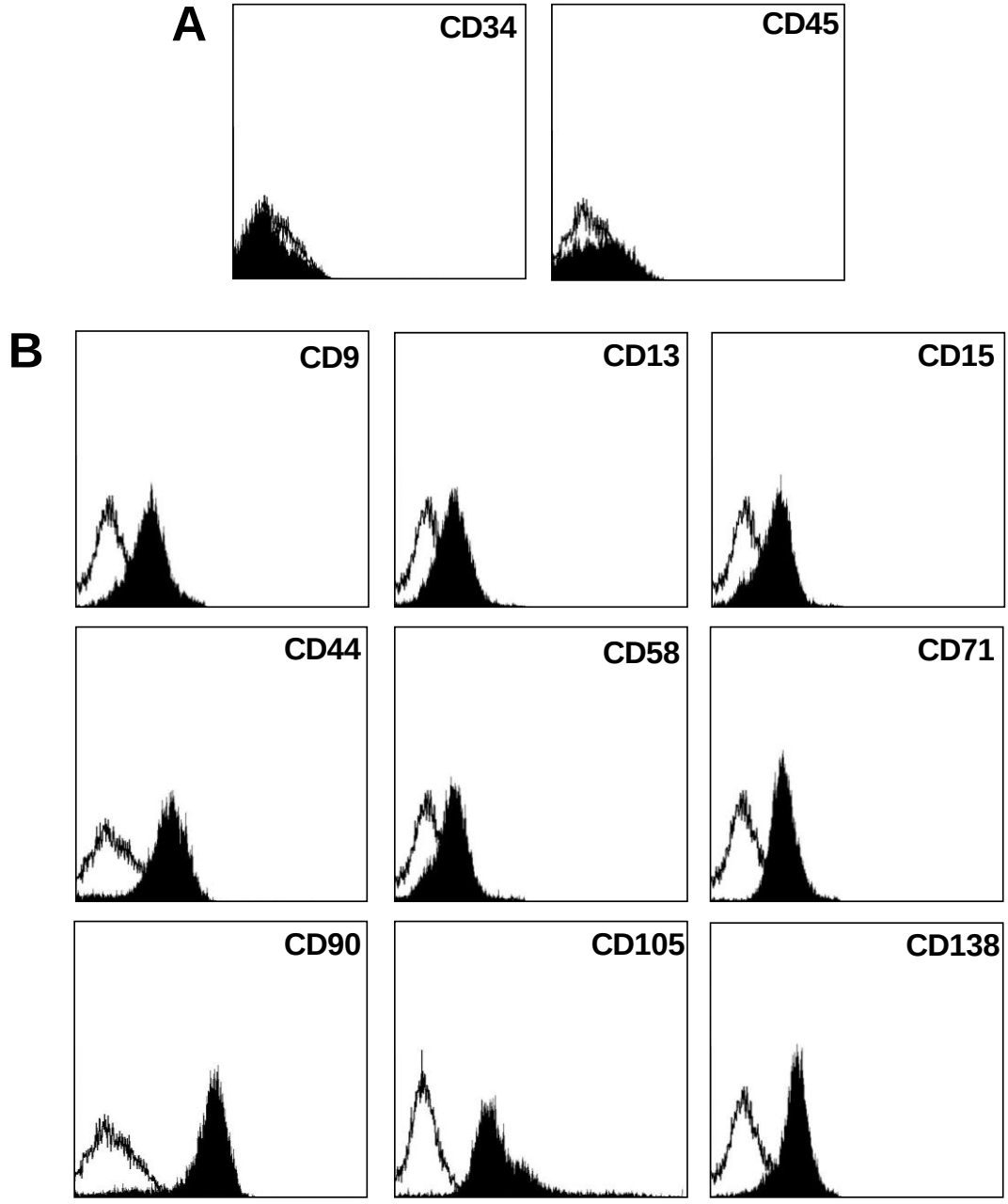

Figure 3.2. Flow cytometric analysis of cell surface marker expression in MSCs isolated from the bone marrow of postnatal rat. Representative traces showing typical cell surface marker expression profiles for haematopoietic (A) and non-haematopoietic (B) markers. Clear traces correspond to the *P3X* negative control and black traces correspond to specific positive fluorescence. MSCs are largely negative for haematopoietic marker expression, suggesting any haematopoietic contamination to be minimal, and positive for more specific MSC marker expression.

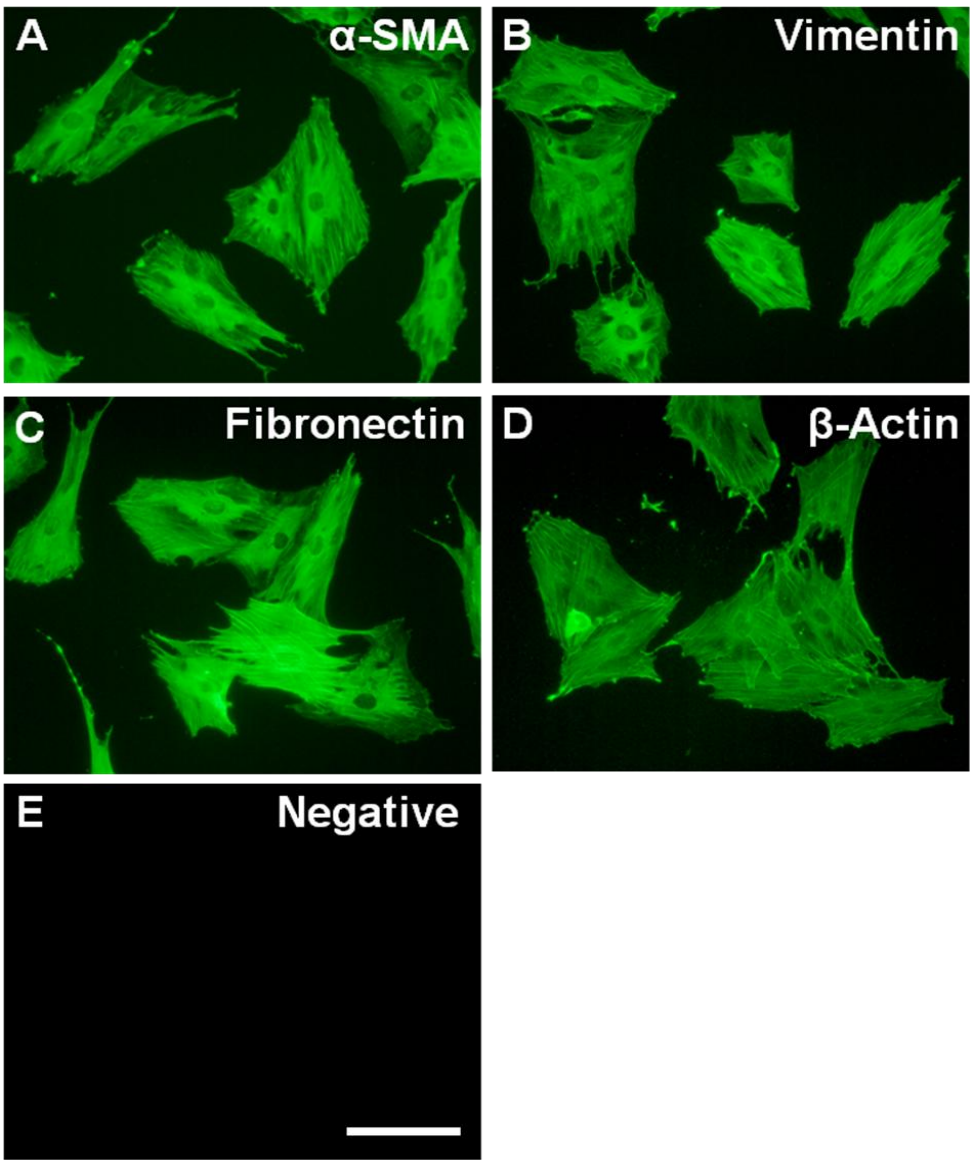

Figure 3.3. Immunocytochemical analysis of intracellular marker expression in MSCs isolated from the bone marrow of postnatal rats. Representative images showing expression of a range of intracellular antigens known to be present in immature MSCs: **A**, α-SMA; **B**, vimentin; **C**, fibronectin; **D**, β-actin. Negative control, consisting of staining with secondary antibody only (i.e. no primary antibody), is also shown (**E**). Scale bar = 100μm.

Antigen	**Reactivity**
CD9	+
CD13	+
CD15	+
CD34	–
CD44	+
CD45	–
CD58	+
CD71	+
CD90	+
CD105	+
CD138	+
α-SMA	+
Vimentin	+
Fibronectin	+
β-Actin	+

Table 3.1. Summary of marker expression in MSCs. A combined summary of cell surface and intracellular marker expression in MSCs isolated from the bone marrow of postnatal rat, as determined using flow cytometry (cell surface) and immunocytochemistry (intracellular).

3.3.3 Bone marrow stromal cells demonstrate multilineage potential owing to their ability to differentiate into multiple mesodermal cell types such as bone and fat

The previous section clearly demonstrates that stromal cells isolated from the bone marrow of postnatal rat display a marker expression profile highly indicative of an MSC origin. A second standard to authenticate the MSC-like behaviour of these cells was to assess their potential to differentiate into mature mesodermal derivatives such as bone and fat, a crucial property of MSCs. As the definition of a stem cell is a functional one, the demonstration that these cells have the potential to differentiate into more mature mesodermal derivatives is even more crucial in confirming the isolation of a true MSC population than marker expression alone.

MSCs were induced to undergo osteogenesis and adipogenesis using the differentiation protocols described in Sections 3.2.1 and 3.2.2. MSCs were cultured under osteogenic conditions for a period of two weeks, after which the differentiative state of the cultures was assessed using the von Kossa histological stain to identify the formation of calcified bone nodules on the surface of the cellware. Bone nodules were readily detectable at time points as early as one week, though such nodules were small in size and very infrequent throughout the culture (data not shown). After culturing cells under osteogenic conditions for two weeks however, the majority of the cellware showed a significant level of calcification and large bone nodules were evident (Figure 3.4B). Negative controls were run in parallel to differentiated cultures, consisting of cells grown under standard culture conditions (unsupplemented CCM). Von Kossa staining in such cultures showed little evidence of calcification or bone nodule formation (Figure 3.4A).

MSCs were cultured under adipogenic conditions for a period of three weeks, after which the differentiative state of the cultures was assessed using the oil red O histological stain to identify the presence of neutral lipid droplets within the cytoplasm of the differentiated cells. Lipid droplets were readily detectable at time points as early as one week, though these droplets were small in size and very few in number (data not shown). After culturing cells under adipogenic conditions for three weeks however, large neutral lipid droplets were readily detectable in the cytoplasm of a significant proportion of all cells (approximately one-third), suggesting that cells were

differentiating into more mature pre-adipocytes and adipocytes (Figure 3.4D). Lipid droplets were not detectable in negative controls (Figure 3.4C).

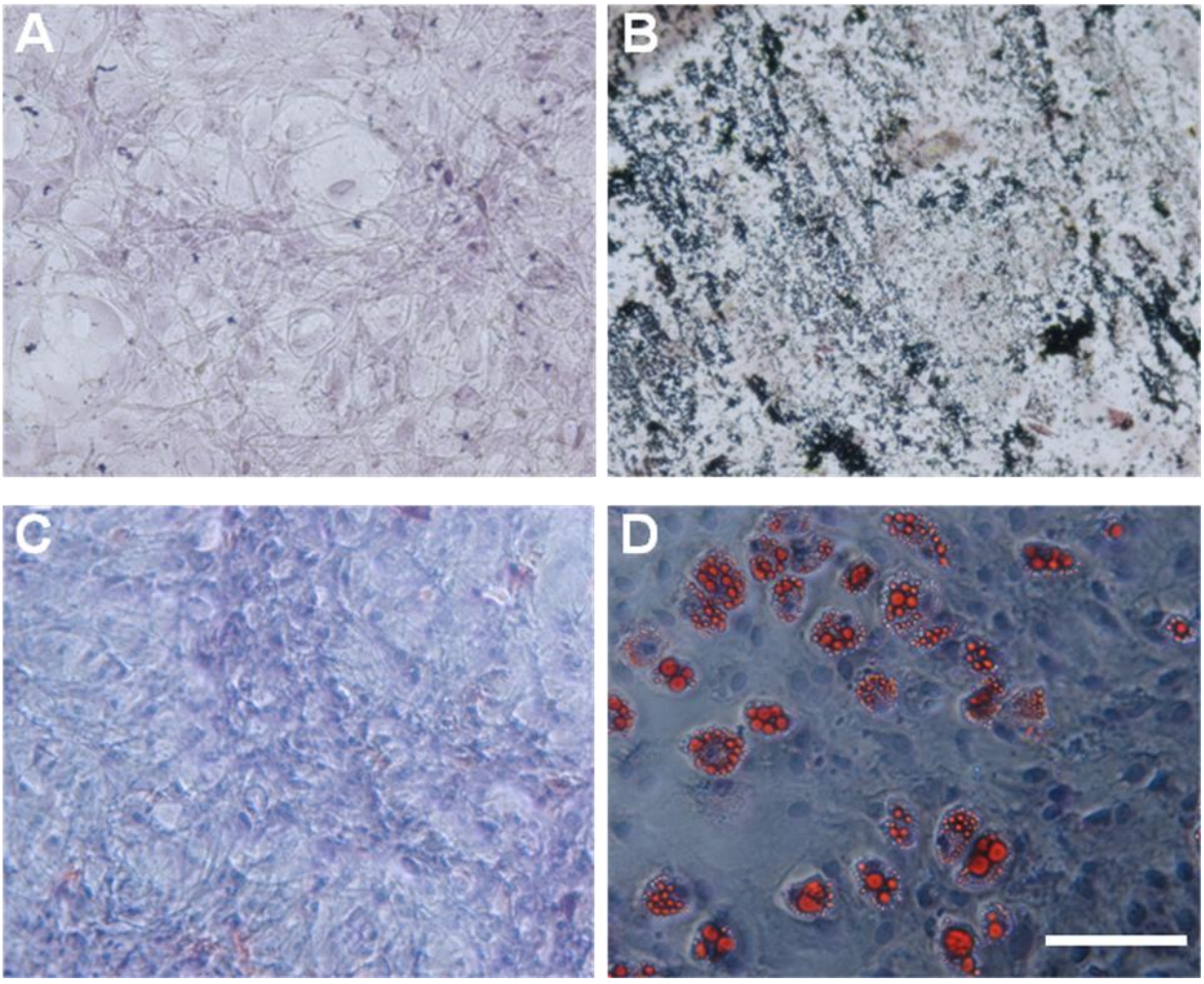

Figure 3.4. Histological analysis of osteogenic and adipogenic differentiation in MSCs. MSCs were cultured under osteogenic conditions for a period of 2 weeks and processed for von Kossa staining to allow visualisation of bone nodule formation and the deposition of a calcified matrix. **A** corresponds to the negative control and **B** corresponds to MSCs cultured under osteogenic conditions. Mineralisation is clearly evident in **B**. MSCs were also cultured under adipogenic conditions for a period of 3 weeks and processed for oil red O staining to allow visualisation of intracellular lipid droplets. **C** corresponds to negative control and **D** corresponds to MSCs cultured under adipogenic conditions. Accumulation of lipid droplets in **D** is clearly evident. Scale bar = 100µm.

Taking into account the observations in Sections 3.3.2 and 3.3.3, stromal cells isolated from rat postnatal bone marrow using the selection criteria of plastic adherence demonstrate both a marker expression profile and potential for differentiation that is indicative of an MSC origin. Such data allow the confident conclusion that the cells isolated and used for subsequent downstream studies in this thesis are MSCs, and will be referred to as such for the remainder of this thesis.

3.3.4 MSCs cultured under standard culture conditions demonstrate density-dependent growth kinetics and a doubling time of approximately 72 hours

A growth curve was constructed by seeding MSCs at a range of densities – 1000, 2000, 3000, 5000 and 10,000 cells/well – into a standard 12-well plate and determining the number of viable cells in culture using the MTS assay every 24 hours for a period of 120 hours (5 days). Data for each timepoint was performed in at least triplicate and the means calculated and plotted as a line graph, as shown in Figure 3.5. MSC doubling time worked out to be approximately 72 hours, though differences were evident depending on the initial seeding densities of the cells. Doubling time was lower for MSCs seeded at higher densities and higher for MSCs seeded at lower densities. This trend is in keeping with what has been previously published (Guillot *et al.*, 2007). Doubling times of MSCs reported in the literature can vary quite significantly depending on the tissue from which the cells were isolated, age of donor etc. Doubling times as quick as one or two days have been reported in some instances, whereas other studies report doubling times as long as 111 hours (Kadiyala *et al.*, 1997; Conget and Minguell, 1999; Suva *et al.*, 2004; Guillot *et al.*, 2007; van der Bogt *et al.*, 2009; Zhang *et al.*, 2009).

3.3.5 MSCs cultured on Alvetex™ demonstrate enhanced ability in vitro to undergo osteogenic and adipogenic differentiation

The two-dimensional (2D) environment in which cells are typically grown in culture is in no way representative of their highly complex three-dimensional (3D) niche *in vivo*. Because of this fundamental limitation of routine tissue culture, there has been much emphasis placed on the development and fabrication of 3D natural and synthetic structures/scaffolds on which to culture cells *in vitro*, with the aim of producing a growth environment more characteristic of that in which cells exist *in vivo*. Such products would be of huge benefit in a research setting, providing more accurate models to study complex cellular processes such as differentiation.

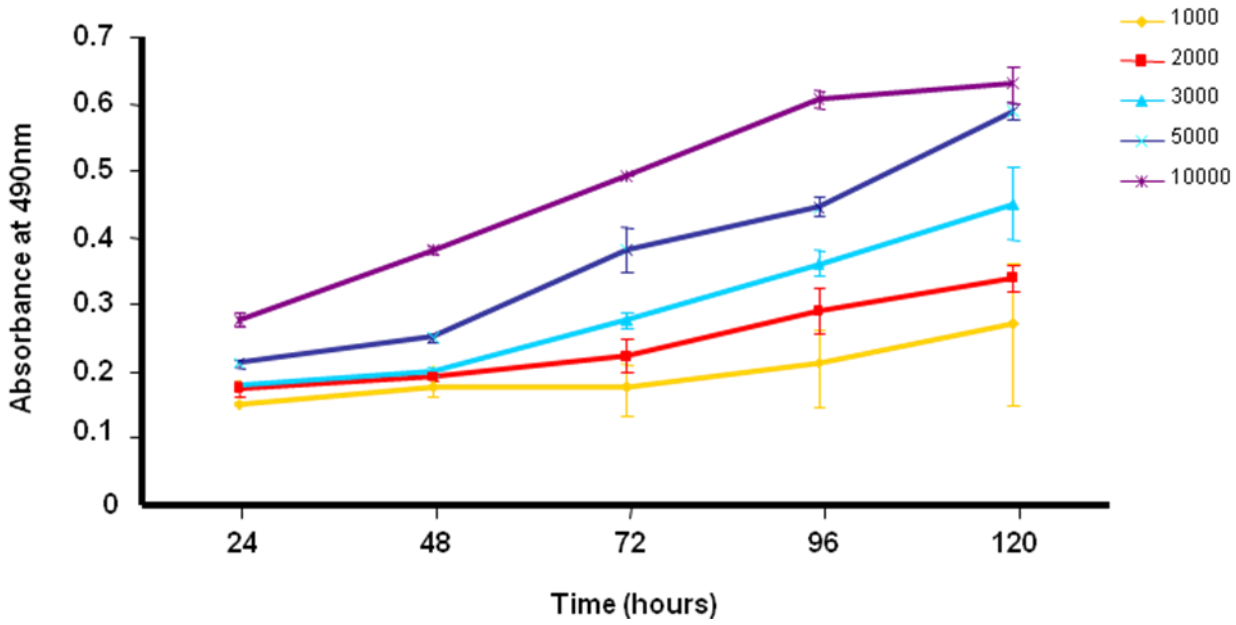

Figure 3.5. A curve showing the growth kinetics of MSCs cultured under standard culture conditions. Number of viable cells in culture was determined every 24 hours for up to 120 hours using the MTS assay. Kinetics of MSC growth were density-dependent.

Reinnervate, the spin-out company from Durham University funding this research, produce a novel 3D poly-HIPE scaffold (Alvetex^{TM}) on which to culture cells *in vitro*, and this technology has been exemplified using many different biological systems, including HepG2 (human liver carcinoma) and TERA2.sp.12 (human embryonal carcinoma) cell lines (Bokhari et al., 2007a; Bokhari et al., 2007b; Hayman et al., 2004; Hayman et al., 2005). The performance of MSCs cultured on this scaffold compared to routine 2D cultures is addressed in the remainder of this chapter, in terms of the capacity of the scaffold to permit the attachment and viable growth/expansion of MSCs, and also in terms of the potential of attached MSCs to differentiate along osteogenic and adipogenic lineages. MSCs cultured on the scaffold were compared to MSCs cultured on the 2D environment of standard cellware.

3.3.6 MSCs cultured on Alvetex^{TM} display excellent morphology and scaffold coverage but poor scaffold penetration

Scanning electron microscopy (SEM) was used to examine the surface topography of MSCs cultured on the scaffold under standard culture conditions (CCM) for 21 days (Figure 3.6). This allowed us to address several key questions, including:

a) Does the scaffold surface permit the attachment of MSCs?
b) Does the scaffold surface support the viable growth and expansion of MSCs?
c) Do MSCs demonstrate characteristic morphology when cultured on the scaffold?
d) Do MSCs demonstrate extensive scaffold coverage following 21 days culture?
e) Do MSCs penetrate throughout the scaffold to grow in multiple levels?

Figure 3.6 contains several SEM micrographs of MSCs cultured on the scaffold under standard culture conditions (CCM) for 21 days, at various magnifications and angles. All five micrographs (Figure 3.6A-E) clearly demonstrate that the scaffold not only permits the attachment of MSCs, but maintains their viability and supports their growth to such an extent that the entire surface of the scaffold is covered by MSCs following 21 days in culture. Figure 3.6A is an excellent example to highlight this, as the surface of the entire sample is covered by MSCs. Tears in the sample are resultant from the many handling steps involved in sample preparation for SEM and are in no way indicative of any patches of non-cellular attachment on the scaffold. MSCs interact with one another to form many close associations, packing tightly together and exhibiting a characteristic 'swirly' pattern that was observed on all samples. This distinctive pattern can be seen at a higher magnification in Figure 3.6B. At this level of detail, the surface of the scaffold cannot be observed because the cells are so densely packed that they are completely covering the top surface of the scaffold. At higher magnification, however, it becomes possible to see more detail at the individual cellular level (Figure 3.6C). The surface of the scaffold can now be observed, though it is still difficult to visualise in any detail because the cells are very densely packed following 21 days in culture. Cells attached to the scaffold appear viable and healthy at the morphological level, demonstrating a typically large, flat, fibroblastic morphology that is highly characteristic of an MSC phenotype. Such is the availability of space on the scaffold that the MSCs spread out to their maximum capacity and interact with neighbouring MSCs within the 3D environment of the scaffold by extending cellular projections across voids. MSCs are also fed from above and below. Such interactions are likely to be more representative of the cell-cell interactions that occur within the niche of the bone marrow *in vivo* compared to those interactions that occur in standard 2D *in vitro* culture. In terms of scaffold penetration, MSCs do not appear to be readily growing throughout all available levels of the scaffold (Figure 3.6D and 3.6E). These

micrographs do demonstrate a small degree of penetration as some MSCs do appear to be growing within the middle layers of the scaffold, though their appearance is sporadic and at low frequency. A sheet of healthy MSCs growing on the top surface of the scaffold is apparent despite the low levels of penetration (Figure 3.6D and 3.6E).

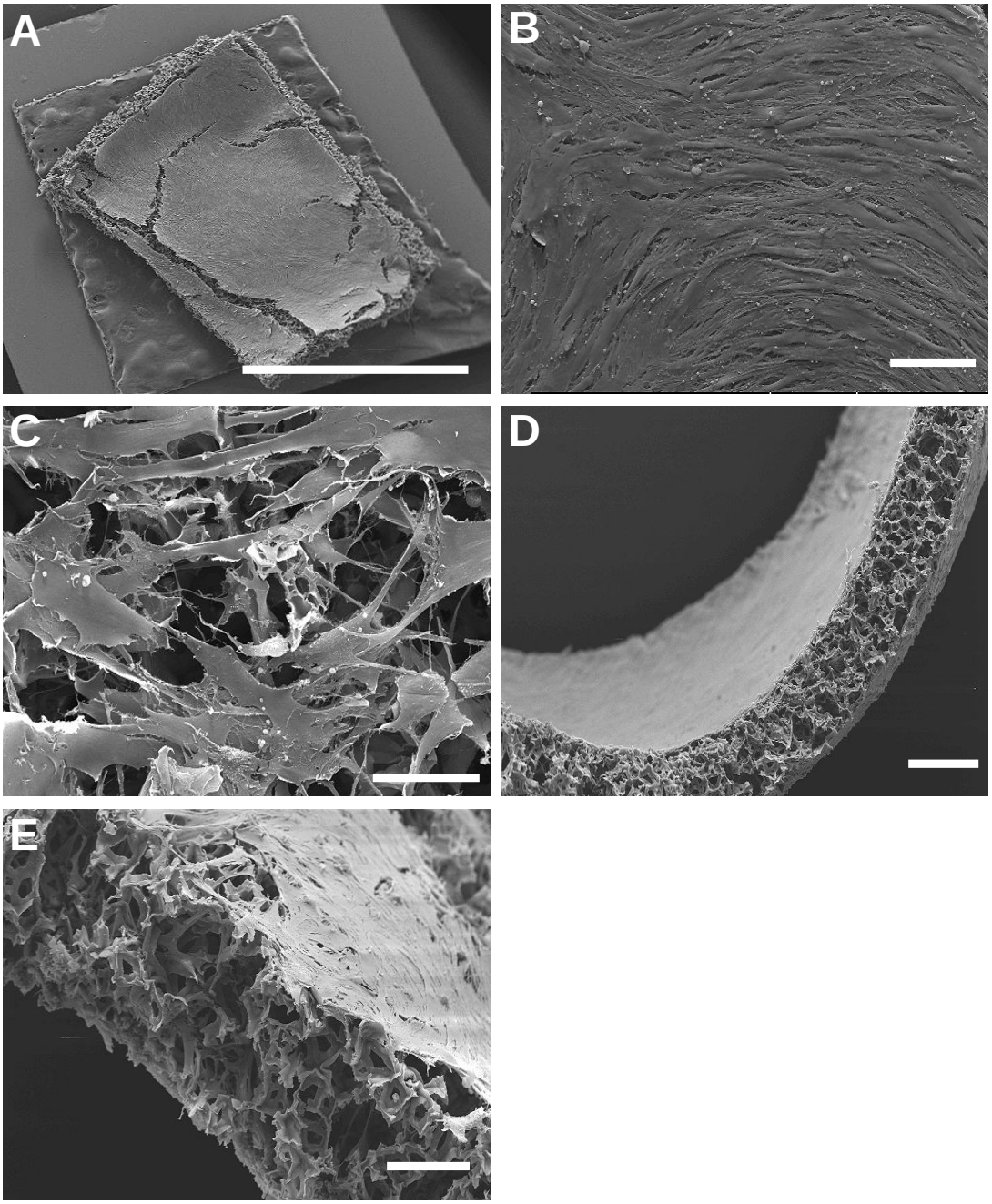

Figure 3.6. SEM micrographs of MSCs cultured under standard conditions on Alvetex™ for 21 days at various magnifications and angles. Scale bars are as follows: **A:** 2mm; **B:** 100µm; **C:** 50µm; **D:** 200µm; **E:** 100µm.

3.3.7 MSCs cultured on Alvetex™ show enhanced viability compared to their 2D counterparts

SEM analysis clearly shows that the scaffold surface supports the attachment of MSCs and encourages their growth, resulting in complete coverage of the scaffold surface following 21 days in culture. Although morphological evidence suggests that MSCs adhered to the scaffold surface are healthy and viable, this data on its own is of limited use, especially due to its non-quantitative nature, as the aim of this section is to determine whether MSCs cultured on the scaffold show enhanced viability and cellular function compared to their culture on standard 2D cellware. The MTS assay provides an alternative, quantitative approach to assess viability of MSCs cultured on 2D vs. 3D, and the methodology for this assay is described in detail in Section 2.8. MSCs were seeded at equivalent densities into standard 6-well plates for 2D culture and onto the scaffold for 3D culture as described in Section 3.2.6 and this was designated as Day 0. The number of viable cells was subsequently determined at days 7, 14 and 21 for both conditions and the MTS absorbance values at 490nm are illustrated in Figure 3.7. Viable cells were successfully cultured on both 2D and 3D at each of the timepoints examined with both substrates clearly supporting the proliferation of MSCs, as the MTS absorbance values increased with time for both 2D and 3D cultures. However, at all of the timepoints examined, the number of viable cells was greater in 3D cultures compared to their equivalent 2D cultures, and this difference was highly significant at the statistical level ($p<0.001$).

3.3.8 MSCs cultured under osteogenic conditions on 2D (tissue culture plastic) and 3D (Alvetex™) growth substrates demonstrate the acquirement of a mature osteoblastic phenotype as determined histologically using von Kossa and Masson's trichrome staining

MSCs were cultured under osteogenic conditions on tissue culture plastic (2D) and scaffold (3D) for a period of 21 days, as detailed in Section 3.2.6. In order to assess the phenotype of MSCs following this culture, histological techniques were employed to examine bone nodule formation and the deposition of a mineralised matrix, two crucial events in osteogenesis. Two histological methods were used in this section – von Kossa and Masson's trichrome staining – which allow the identification of extracellular calcium and collagen deposits respectively. Visualisation at the cellular

level was achieved by counterstaining cells with the nuclear stain haematoxylin (staining nuclei a bright purple colour).

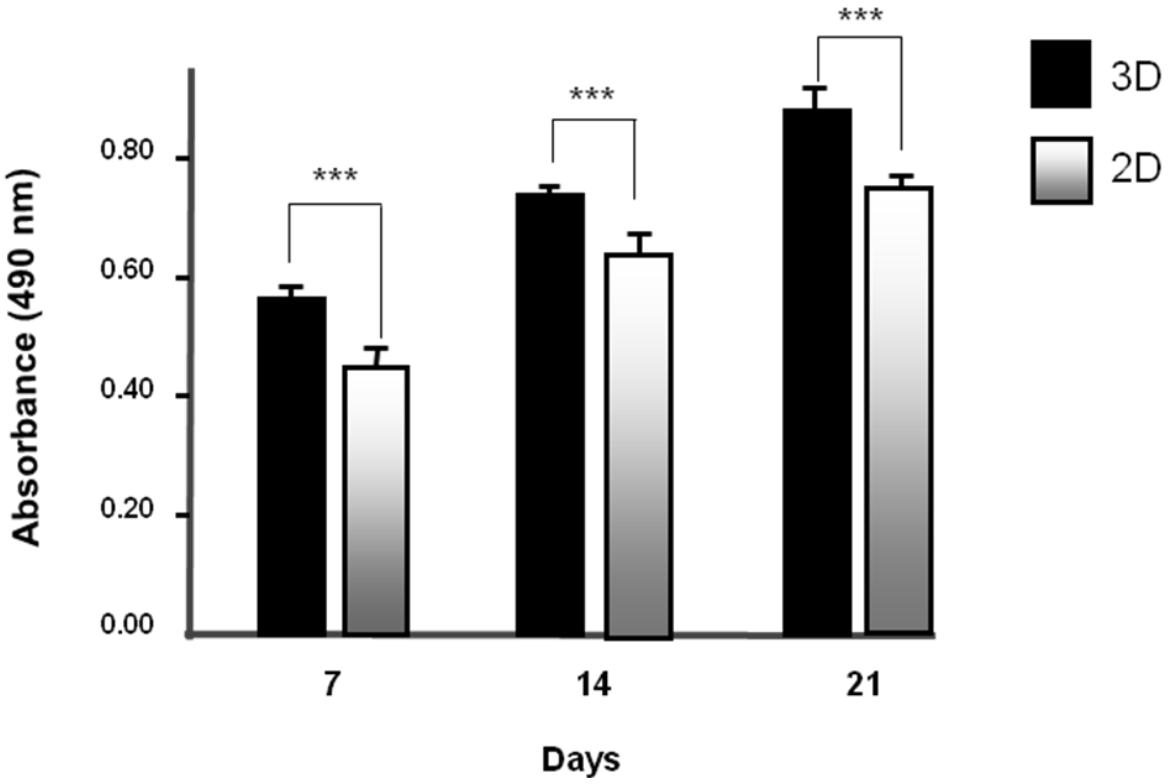

Figure 3.7. MTS absorbance values for MSCs cultured on Alvetex™ and tissue culture plastic under standard conditions for 7, 14 and 21 days. MSCs were simultaneously cultured on the scaffold (3D) and tissue culture plastic (2D) for up to 21 days under standard conditions, and the MTS assay used to determine the number of viable cells in culture at days 7, 14 and 21. Each bar represents the mean (n=3)+SEM, *** $p<0.001$.

Figure 3.8 shows representative staining for MSCs cultured under osteogenic conditions on both 2D (Figure 3.8A and 3.8C) and 3D (Figure 3.8B and 3.8D) substrates. Von Kossa staining (Figure XA and XB) is the most commonly used technique to assess osteoblastic behaviour, staining calcified bone nodules an intense black colour (as indicated by arrowheads in Figures 3.8A and 3.8B). Although it is widely accepted that von Kossa staining identifies calcium, the stain itself is not actually specific for the calcium ion. Rather, it demonstrates the presence of the anion (carbonate or phosphate ions) rather than the cation (calcium ion). Carbonate and phosphate ions displace silver ions from the silver nitrate solution used in von Kossa

staining, which then appear as densely black areas. As carbonate and phosphate ions are virtually always tightly associated with calcium ions, this technique is considered to demonstrate the presence of calcium. Bone nodules formation and mineralisation were apparent for MSCs cultured on both 2D (Figure 3.8A) and 3D (Figure 3.8B) substrates, confirming that MSCs demonstrate a potential to differentiate into mature osteoblastic derivatives on both substrates.

Another key element of osteogenesis is the production and secretion of collagen in the extracellular matrix (ECM) by cells displaying an osteoblastic phenotype. This process of collagen synthesis and deposition is a pre-requisite for bone nodule formation, therefore the presence of collagen should precede the presence of calcium in cells actively undergoing osteogenesis. Masson's trichrome staining (Figure 3.8C and 3.8D) is another commonly used histological technique and can be used to stain various structures, including muscle (red), amongst others. From an osteoblastic perspective, Masson's trichrome staining is used to identify collagen deposition in the ECM by cells displaying an osteoblastic phenotype. Masson's trichrome stains collagen an intense green colour (Figure 3.8C and 3.8D). The presence of collagenous proteins were readily detectable for MSCs cultured on both 2D (Figure 3.8C) and 3D (Figure 3.8D) substrates, providing further evidence that MSCs are able to differentiate into mature osteoblastic derivatives on both 2D and 3D substrates. Negative controls for both histological stains described above were performed using MSCs that had not been induced to undergo osteogenic differentiation, but instead were cultured (simultaneously) under standard culture conditions (CCM). These controls did not demonstrate any evidence of calcium or collagen deposition (data not shown).

Due to the qualitative nature of histological analysis, coupled with the widely varying methods of staining 2D samples compared to 3D samples i.e. directly in the cellware for 2D vs. paraffin embedding and sectioning for 3D, it would be an inaccurate comparison to make any conclusions regarding the ability of MSCs to undergo osteogenesis on 2D vs. 3D from these data alone. The following sections will describe quantitative, functional assays that were performed as a more balanced approach to compare the osteogenic potential of MSCs cultured on 2D vs. 3D.

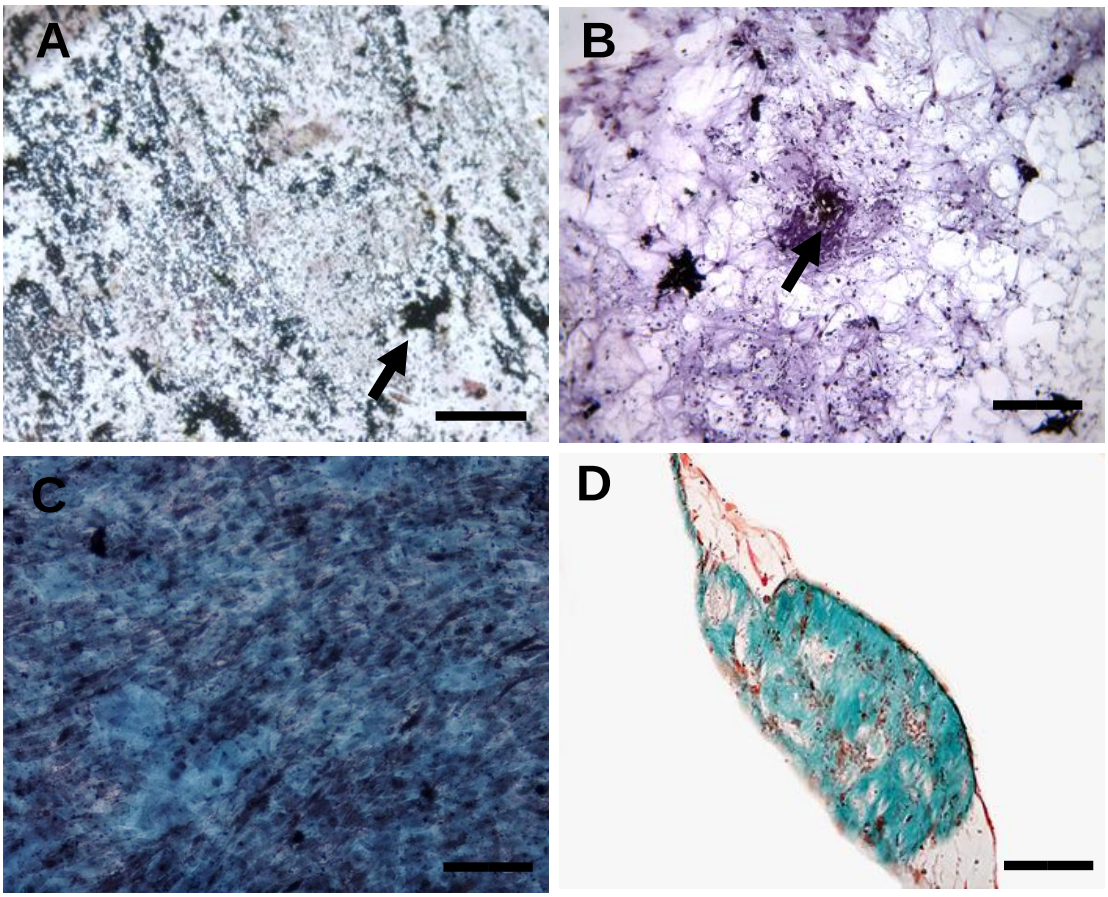

Figure 3.8. Histological analysis of osteogenic differentiation in MSCs cultured on 2D and 3D. Von Kossa (**A**, **B**) and Masson's trichrome (**C**, **D**) staining was performed on MSCs cultured under osteogenic conditions for 21 days on 2D (**A**, **C**) and 3D (**B**, **D**) to demonstrate calcium and collagen deposition respectively. Intensely stained black areas represent calcium deposition and bone nodule formation is indicated by the arrowhead. Green staining represents collagen deposition. Scale bars: **A**, 100µm; **B**, 200µm; **C**, 100µm; **D**, 200µm.

3.3.9 MSCs cultured under osteogenic conditions on Alvetex™ demonstrate enhanced osteoblastic functional behaviour including increased alkaline phosphatase activity and increased secretion of osteocalcin compared to their 2D counterparts

Alkaline phosphatase (ALP) activity is one of the most commonly used markers of osteogenic differentiation/behaviour (Lian and Stein, 1992). In this section, ALP activity was determined for MSCs cultured under osteogenic conditions on tissue culture plastic (2D) and scaffold (3D) at days 5, 7, 14 and 21 post-induction of differentiation, as described in Section 3.2.7. Results are illustrated in Figure 3.9. ALP activity was detectable on both 2D and 3D substrates as early as day 5, albeit at low levels, indicating that even at this early timepoint MSCs were beginning to acquire functional characteristics more indicative of an osteoblastic phenotype rather than a

stem/progenitor cell phenotype. However, it was not possible to ascertain whether low levels of ALP activity were attributable to only a small percentage of cells undergoing differentiation at this point, or whether a large percentage of cells were undergoing differentiation but were still relatively immature at this point. With increasing time, ALP activity increased substantially for MSCs cultured on both 2D and 3D substrates as they became more committed to an osteogenic differentiation program and began to express a more mature functional osteoblastic phenotype. Maximum activity was attained at 14 days for both 2D and 3D cultures. Thereafter, ALP activity began to decrease, as determined at the 21 day timepoint, though the level of ALP activity at 21 days was still relatively high and comparable with that observed at 7 days. This trend was comparable between MSCs cultured on both substrates, though at each of the timepoints examined ALP activity was significantly enhanced when MSCs were cultured on 3D compared to 2D, suggesting that MSCs cultured on 3D were acquiring a more mature osteoblastic phenotype than their 2D counterparts. These differences were highly significant at the statistical level for days 5, 7 and 14 ($p<0.001$).

A further marker of osteogenic differentiation is osteocalcin, a late marker of osteogenesis whose production and secretion is highly indicative of the expression of a mature osteoblastic phenotype (Lian and Stein, 1992; Lian and Stein, 1993). In this section, osteocalcin production and secretion was determined for MSCs cultured under osteogenic conditions on 2D and 3D, as described in Section 3.2.7, as an indicator of more mature osteoblastic behaviour. As it is a late marker of osteogenesis, levels of osteocalcin were determined at the later timepoints of 14, 21 and 35 days post-induction of differentiation. Results are illustrated in Figure 3.10. Osteocalcin was readily detectable in the culture media of MSCs cultured on both 2D and 3D substrates at each of the timepoints assessed, including the earliest timepoint of 14 days, indicating that cells were highly committed to an osteogenic differentiation pathway and were beginning to display functional behaviour indicative of a more mature osteoblastic phenotype. Levels of osteocalcin in the culture media increased with time in both conditions, with maximum levels observed after 35 days post-induction of differentiation, suggesting that cells were still differentiating and becoming more osteoblast-like even at this late timepoint. Although a clear increase in osteocalcin levels were observed for MSCs cultured on both 2D and 3D, levels were significantly higher for MSCs cultured on 3D compared to their 2D counterparts at all of the

timepoints assessed ($p<0.001$). These data further support for the conclusions made from the ALP data above that MSCs cultured on the scaffold display enhanced functional behaviour indicative of the acquirement of a more mature osteoblastic phenotype compared to equivalent 2D cultures.

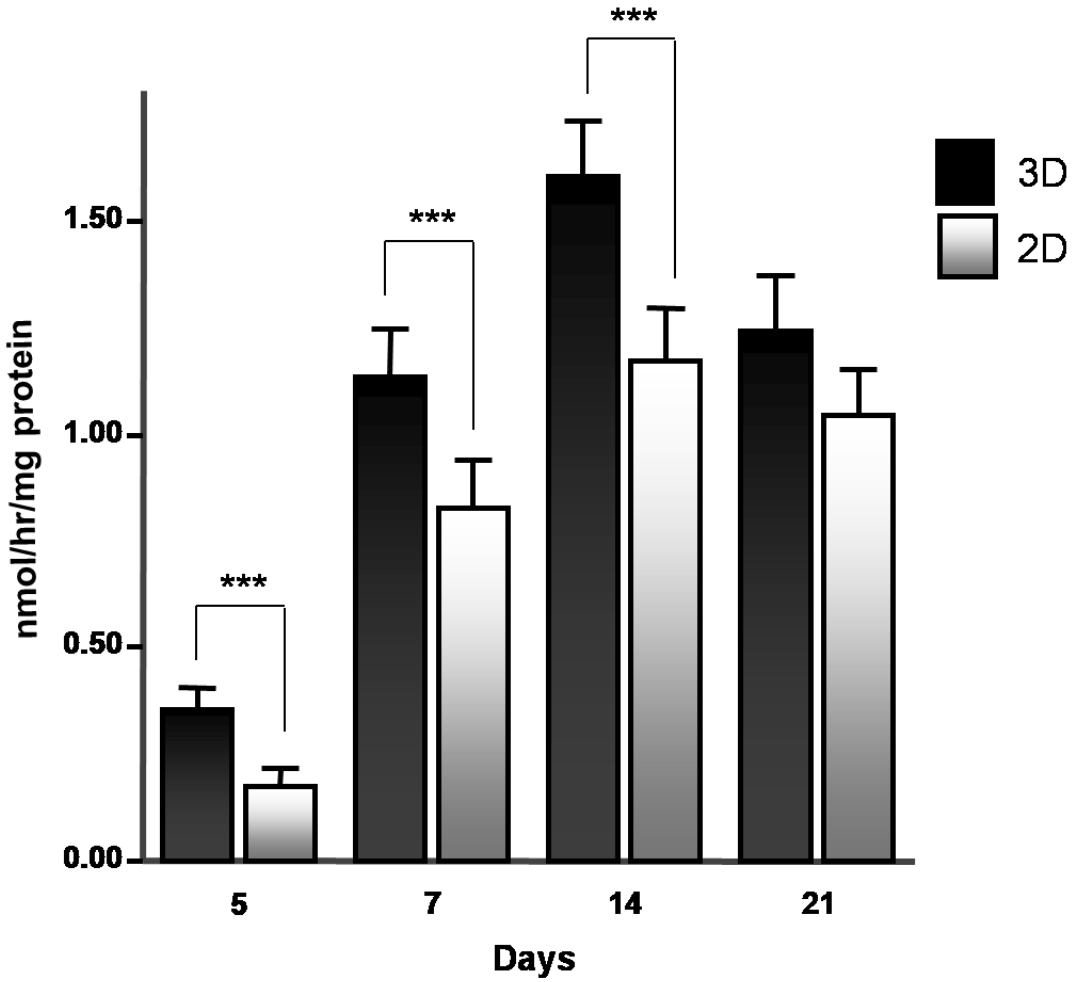

Figure 3.9. ALP activity in MSCs cultured on Alvetex™ and tissue culture plastic under osteogenic conditions for up to 21 days. MSCs were simultaneously cultured on the scaffold (3D) and tissue culture plastic (2D) for up to 21 days under osteogenic conditions and ALP activity determined at days 5, 7, 14 and 21. Each bar represents the mean (n=3)+SEM, *** $p<0.001$.

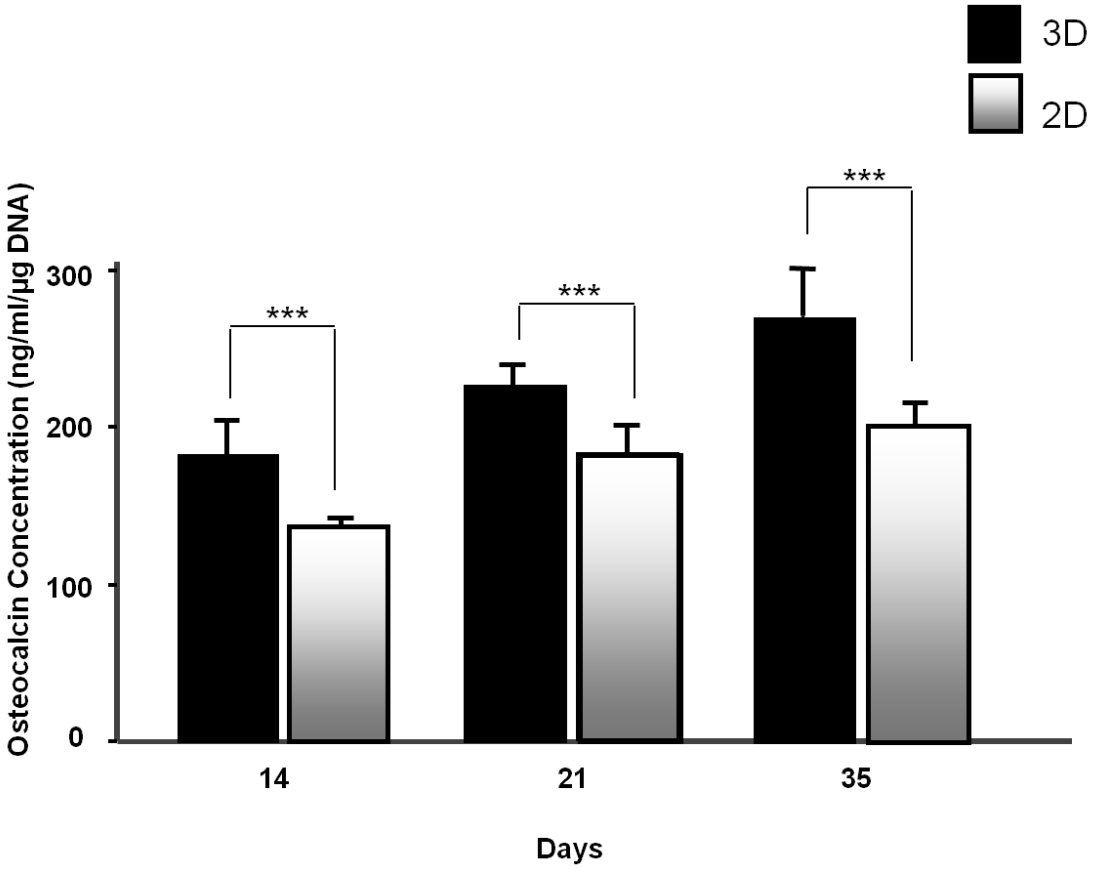

Figure 3.10. Osteocalcin secretion by MSCs cultured under osteogenic conditions on Alvetex™ and tissue culture plastic for up to 35 days. MSCs were simultaneously cultured on the scaffold (3D) and tissue culture plastic (2D) for up to 35 days under osteogenic conditions and levels of osteocalcin secretion into the culture media determined at days 14, 21 and 35. Each bar represents the mean (n=3)+SEM, *** $p<0.001$.

3.3.10 MSCs cultured under adipogenic conditions on Alvetex™ demonstrate enhanced accumulation of intracellular lipid droplets compared to their 2D counterparts as determined histologically using oil red O staining and by using an oil red O elution method

MSCs were cultured under adipogenic conditions on tissue culture plastic (2D) and scaffold (3D) for a period of 21 days, as detailed in Section 3.2.6. To assess the phenotype of MSCs following this culture period, oil red O staining was performed to allow identification of neutral lipid droplets within the cell cytoplasm and allow a measure of adipogenesis. Visualisation at the cellular level was achieved by counterstaining cells with haematoxylin followed by 'blueing' in 3% ammonia solution, as described previously in Section 3.2.6. Due to technical difficulties encountered when performing oil red O staining on paraffin-embedded sections (solvent extraction of lipoid material during paraffin processing), a cytospin method

was adopted whereby cells were trypsinised out of the scaffold and cytospun onto non-coated glass slides as described in Section 3.2.6. This method of oil red O staining is advantageous over staining frozen sections (a viable alternative) because it allows visualisation of stained cells at an individual level, allowing direct comparisons to be made between cells cultured on 2D and 3D. Figure 3.11 shows representative oil red O staining for MSCs cultured under adipogenic conditions for 21 days on both 2D (Figure 3.11C) and 3D (Figure 3.11D) substrates. Intracellular lipid accumulation was apparent for MSCs cultured under both conditions, confirming that MSCs demonstrate a potential to differentiate into mature adipogenic derivatives on both substrates. Negative controls using MSCs that had not been induced to undergo adipogenic differentiation, but instead were cultured (simultaneously) under standard culture conditions (CCM), did not demonstrate any evidence of lipid droplet accumulation within the cell cytoplasm (Figures 3.11A and 3.11B). At this morphological level, MSCs that had been cultured under adipogenic conditions on 3D (Figure 3.11D) contained substantially more lipid droplets per cell than their 2D counterparts (Figure 3.11C). In order to quantify these observations and allow a statistical comparison between MSCs cultured on 2D vs. 3D, an oil red O elution technique was performed as described in Section 3.2.8. Results are illustrated in Figure 3.12. At both 7 and 21 days, MSCs cultured on the scaffold demonstrated significantly higher levels of oil red O compared to their 2D counterparts, validating the morphological conclusions above and confirming that MSCs cultured on the scaffold underwent enhanced adipogenic differentiation and displayed a more mature adipogenic phenotype compared to cells cultured on standard tissue culture plastic. Differences in oil red O elution were highly statistically significant at both time points ($p<0.001$).

3.3.11 Culture of MSCs under adipogenic conditions on Alvetex™ does not cause an increase in the percentage of cells undergoing adipogenic differentiation but does result in the demonstration of a more mature adipogenic phenotype compared to 2D counterparts, as determined by Nile red staining followed by flow cytometric analysis

MSCs were cultured under adipogenic conditions on tissue culture plastic (2D) and scaffold (3D) for a period of 21 days. Oil red O is by far the most commonly used stain to demonstrate the presence of intracellular lipid droplets within differentiated cells.

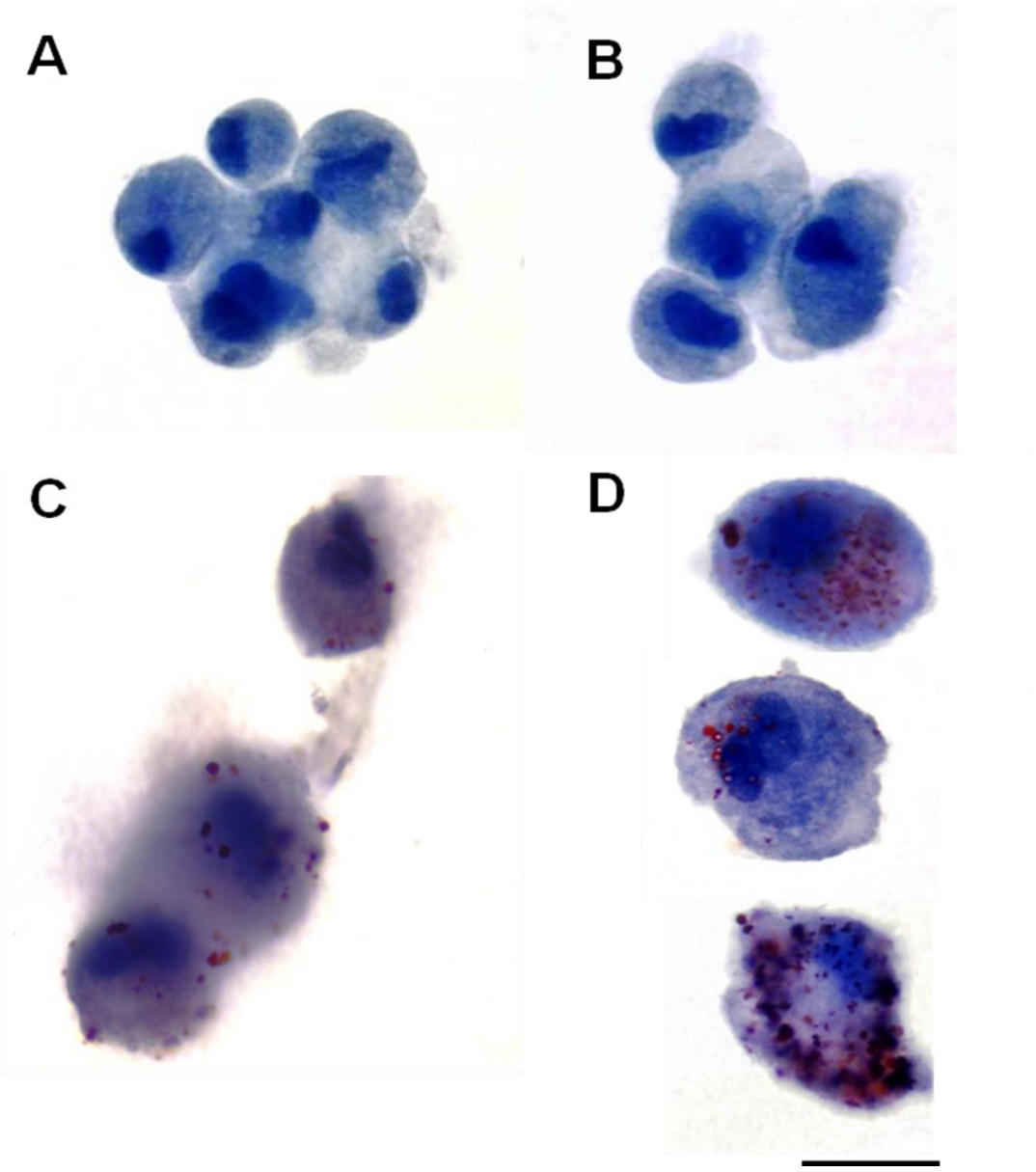

Figure 3.11. Oil red O staining in MSCs cultured under adipogenic conditions on tissue culture plastic (2D) and Alvetex™ (3D) for 21 days, using the cytospin method. Negative controls for both conditions (**A**, **B**) showed no evidence of intracellular lipid accumulation. MSCs cultured under adipogenic conditions on 2D (**C**) demonstrated some evidence of intracellular lipid accumulation and MSCs cultured on 3D demonstrated a significant degree of intracellular lipid accumulation (**D**).

An alternative lipophilic stain is Nile red, which is advantageous over oil red O due to its fluorescent properties. Lipid-bound Nile red emits fluorescence at a wavelength of approximately 525nm, allowing analysis of adipogenesis using techniques such as immunofluorescence and flow cytometry.

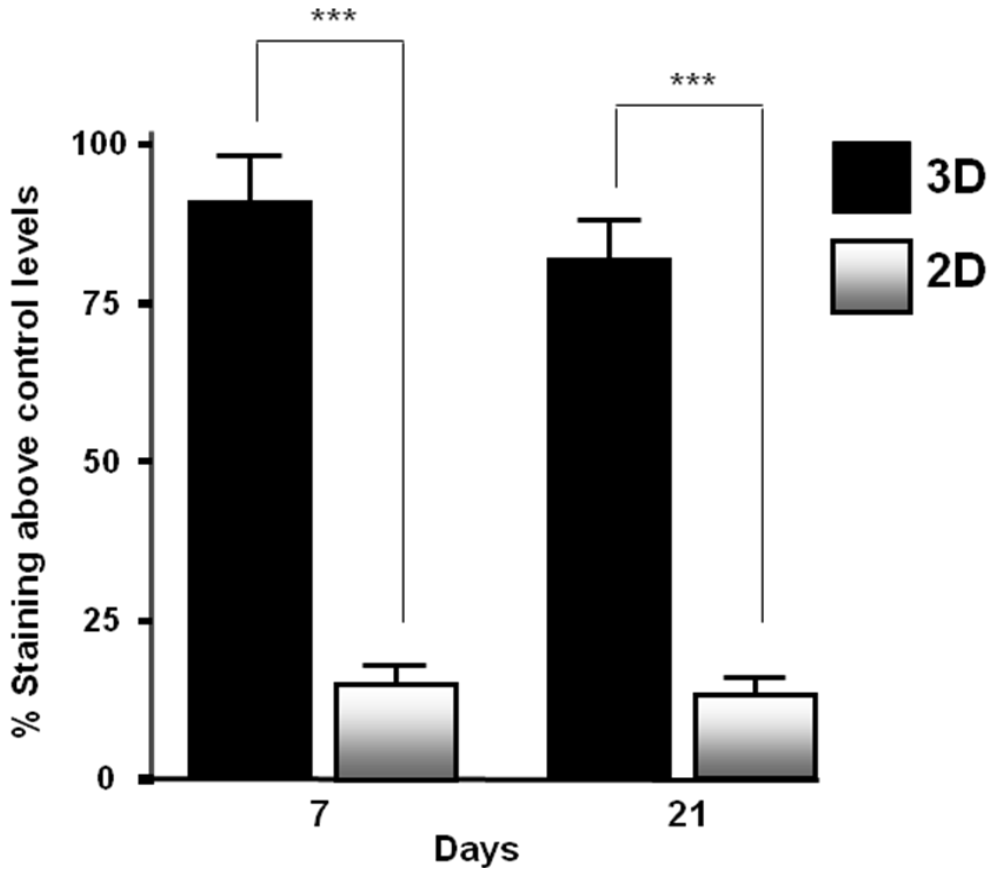

Figure 3.12. Quantification of oil red O staining in MSCs cultured under adipogenic conditions on tissue culture plastic (2D) and Alvetex™ (3D) using an elution method. MSCs were simultaneously cultured on the scaffold (3D) and tissue culture plastic (2D) for up to 14 days under adipogenic conditions and levels of oil red O staining determined at days 7 and 14. Each bar represents the mean (n=3)+SEM, *** $p<0.001$.

Here, MSCs cultured under adipogenic conditions on 2D and 3D for 21 days were stained with Nile red followed by flow cytometric analysis, in terms of the percentage of positive cells for Nile red staining and mean fluorescence intensity (MFI) of staining, allowing a distinction between cells that were weakly positive and cells that were strongly positive for Nile red. Figure 3.13 shows representative flow cytometric traces for negative control (Figure 3.13A) and MSCs cultured under adipogenic conditions on both 2D (Figure 3.13B) and 3D (Figure 3.13C) substrates. The negative control consisted of undifferentiated MSCs cultured under standard culture conditions, to determine levels of background/non-specific fluorescence and determine the threshold against which positive and negative events would be measured (Figure *3.13A*). Any events to the left of this threshold (R1) were considered negative for Nile red staining and, as such, indicative of an undifferentiated phenotype. Conversely, any

events to the right of this threshold (R2) were considered positive for Nile red staining and, as such, indicative of an adipogenic phenotype. Collated results are shown graphically in terms of percentage of cells displaying positivity for Nile red staining (Figure 3.13D) and in terms of MFI (Figure 3.13E).

Positive events were detected for MSCs cultured under adipogenic conditions on both 2D (Figure 3.13B) and 3D (Figure 3.13C) substrates, though there was no statistical difference in the number of positive cells between these conditions, approximately 30% each (Figure 3.13D). This demonstrates that around one-third of MSCs cultured under adipogenic conditions were actively undergoing differentiation to form more mature adipogenic derivatives and this was the case whether they were cultured on 2D or 3D. However, such is the sensitivity of flow cytometry that even weak fluorescence will be detected and reported as a positive event, therefore this analysis alone is insufficient to distinguish between cells that are only weakly positive and those that are strongly positive i.e. cells that have a low level of intracellular lipid accumulation compared to those with a high level of intracellular lipid accumulation. Determination of MFI allows an analysis of fluorescence intensity. MSCs cultured on the scaffold demonstrate a significantly higher MFI than their 2D counterparts (Figure 3.13E), validating the oil red O elution results above (Section 3.3.10) and providing further evidence to suggest that MSCs cultured on the scaffold display an enhanced ability to undergo adipogenic differentiation. These differences were significant at the statistical level ($p<0.01$). This difference in MFI can also be observed on the raw flow cytometric traces, as the positive events for MSCs cultured on 3D (Figure 3.13C) are shifted more towards the right than the corresponding 2D events (Figure 3.13B), which is indicative of a higher intensity of fluorescence. This data alone is insufficient to allow conclusion as to whether this increase in Nile red intensity is a consequence of an increase in the number of droplets per cell or an increase in the size of droplets per cell for MSCs cultured on 3D compared to 2D. However, the oil red O staining in Figure 3.11 clearly shows that MSCs cultured on the scaffold demonstrate a greater number of lipid droplets per individual cell compared to their 2D counterparts, suggesting that the increase in MFI of Nile red is likely to be attributable to a greater number of lipid droplets per cell rather than the presence of larger droplets.

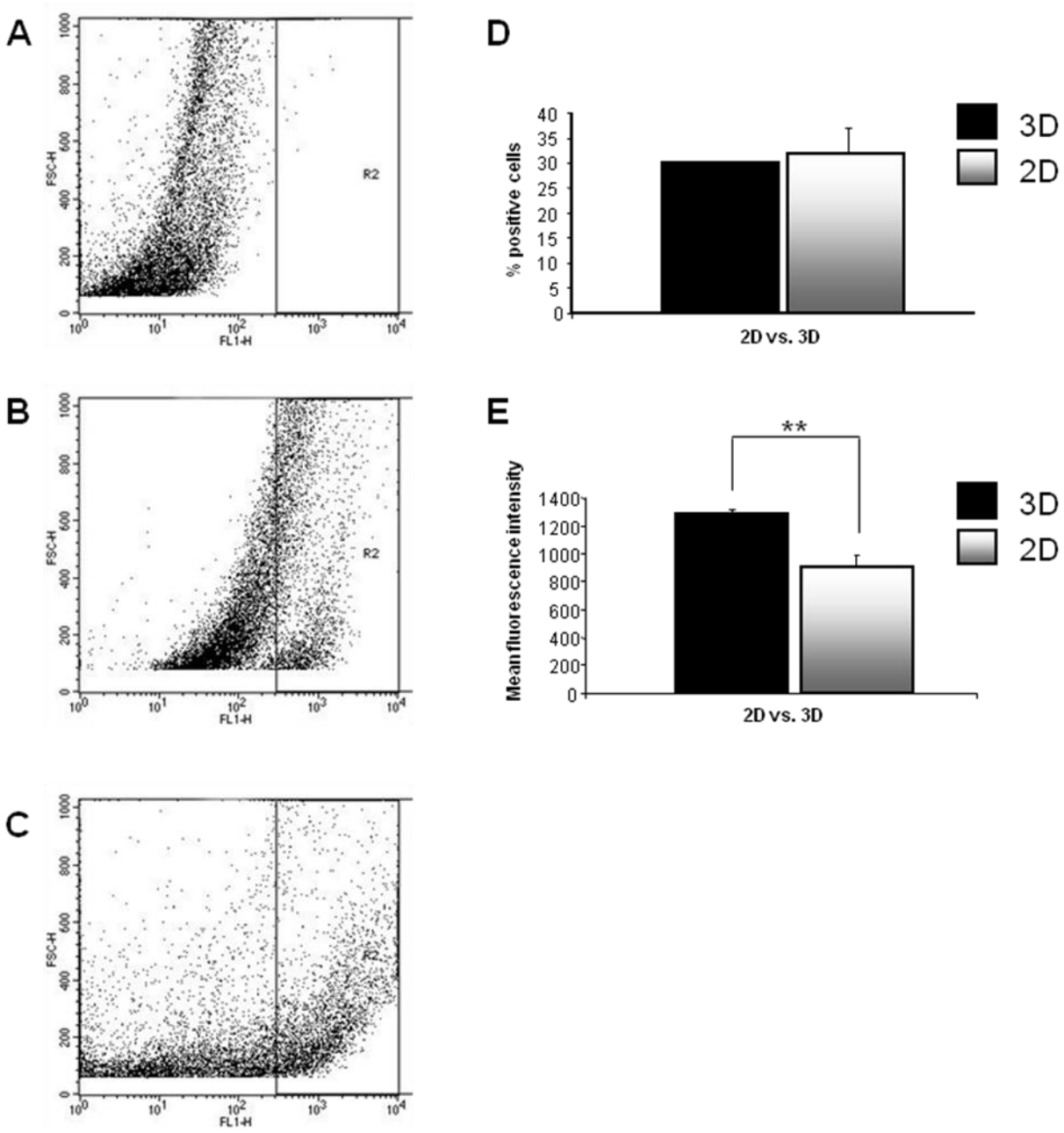

Figure 3.13. **Flow cytometric analysis of Nile red staining in MSCs cultured under adipogenic conditions on Alvetex™ (3D) and tissue culture plastic (2D) for 21 days.** Representative flow cytometric traces are shown for MSCs stained with Nile red following 21 days culture under adipogenic conditions: **A**, negative control; **B**, MSCs cultured on 2D; **C**, MSCs cultured on 3D. Results from three independent studies were combined and collated to give an indication of the cells demonstrating positivity for Nile red staining (**D**) and mean fluorescence intensity (MFI) of staining (**E**). Each bar represents the mean (n=3)+SEM, ** $p<0.01$.

3.3.12 MSCs cultured under adipogenic conditions on Alvetex™ demonstrate a significantly higher level of metabolic activity compared to MSCs cultured on standard tissue culture plastic as indicated by increased levels of cyclic adenosine monophosphate (cAMP)

To assess the functional phenotype of MSCs differentiated along an osteogenic lineage on 2D vs. 3D, functional assays were performed to determine ALP activity and levels of osteocalcin secreted into the culture media (Section 3.3.9). Unfortunately, there are a lack of reliable markers and biochemical assays to assess functional adipogenic behaviour, which is typically measured using non-functional methods such as oil red O (Section 3.3.10) and Nile red (Section 3.3.11) staining. In order to gain an appreciation of how functionally active cells were when cultured under adipogenic conditions on the scaffold (3D) compared to plastic (2D), levels of the signalling molecule cyclic adenosine monophosphate (cAMP) were determined using a commercially-available enzyme immunoassay (EIA) kit as detailed in Section 3.2.10. cAMP plays a fundamental role in adipogenesis, predominantly through its activation of protein kinase A (PKA), which in turn activates a number of transcription factors including cAMP-responsive element binding protein (CREB), a key transcription factor involved in expression of multiple genes crucial for adipogenic differentiation (Kurten *et al.*, 1988; Reusch *et al.*, 2000; Petersen *et al.*, 2008; Yang *et al.*, 2008). In this section, cAMP levels were determined for MSCs cultured under adipogenic conditions on tissue culture plastic (2D) and scaffold (3D) at days 7 and 21 post-induction of differentiation, as described in Section 3.2.10. Results are illustrated in Figure 3.14. cAMP was readily detectable in samples from both growth substrates at both timepoints, indicating that cells were actively undergoing metabolic processes requiring cAMP-mediated signalling events. Although cAMP is a crucial mediator of adipogenesis, it is a universal second signalling molecule and as such is involved in a wide range of cellular processes besides adipogenic differentiation. It is therefore not possible to conclude with any certainty that levels of cAMP determined in the above assay were exclusively attributable to adipogenic differentiation, though increased levels of adipogenesis would certainly contribute to increased levels of cAMP. What can be concluded with confidence from these data is that MSCs cultured on Alvetex™ were significantly more metabolically active than their 2D equivalents, especially at the 7 day timepoint. At day 7, the level of cAMP was significantly greater in cells cultured on 3D compared to 2D, indicating that cells cultured on the scaffold were

more metabolically active than their 2D counterparts and it is probable that this higher metabolic activity was a direct consequence of an enhanced rate of adipogenesis. Levels of cAMP in 2D cultures were much lower, suggesting that these cells were undergoing adipogenesis much less efficiently or at a much lower rate of occurrence. At day 21, levels of cAMP increased in cells cultured on 2D but decreased in cells cultured on 3D compared to levels observed at the 7 day timepoint. Levels of cAMP were equivalent for both cultures at this timepoint. The increase in cAMP level for MSCs cultured on 2D is likely to be attributable to a greater degree of adipogenic differentiation occurring at this timepoint compared to the relatively early timepoint of 7 days. For MSCs cultured on 3D, however, maximum levels of cAMP were observed at 7 days. Because the 3D structure of the scaffold appears to functionally enhance MSC performance during standard culture, a maximum cAMP level at day 7 suggests that cells cultured on the scaffold committed to differentiation earlier than their 2D counterparts due to a greater efficiency at undergoing the necessary changes in protein complement required to undergo adipogenesis. In crude terms, MSCs cultured on 2D may 'lag' behind those cultured on 3D, in terms of committing to the differentiation process and acquiring a more mature adipogenic phenotype. The difference between cAMP levels in MSCs cultured on 2D and 3D were significant at the statistical level for the 7 day time point ($p<0.001$) but not the 21 day timepoint.

3.3.13 MSCs cultured under adipogenic conditions on Alvetex™ demonstrate enhanced expression of adipocyte-related genes compared to MSCs cultured on 2D

As mentioned in the previous section, there are a lack of reliable markers and biochemical assays to assess functional adipogenic behaviour. To circumvent this and gain a more mechanistic insight into how MSCs were behaving when cultured under adipogenic conditions on the scaffold (3D) compared to plastic (2D), expression of a number of adipocyte-related genes were examined, at various timepoints up to 14 days differentiation, using qRT-PCR. Specific genes were selected, based on current knowledge within the literature, to cover both the early and late stages of the adipogenic differentiation program. Expression of adiphilin, an early marker of adipogenesis, and PPARγ and adipsin, later markers of adipogenesis, were determined and normalised to GAPDH levels as detailed in Section 2.11.2.

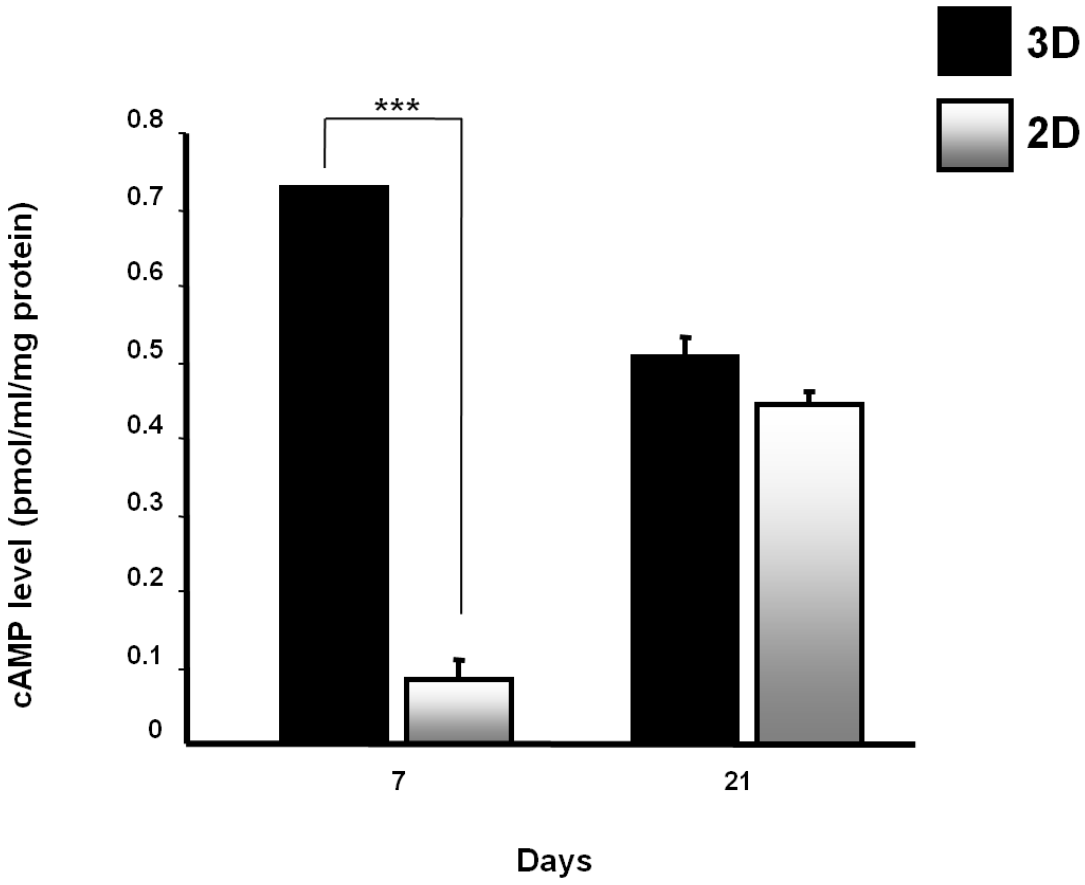

Figure 3.14. Intracellular cAMP levels in MSCs cultured under adipogenic conditions on Alvetex™ (3D) and tissue culture plastic (2D). Intracellular cAMP levels were determined in MSCs cultured under adipogenic conditions on 2D and 3D at 7 and 21 days using a commercially available ELISA-based method. Each bar represents mean (n=2)+SEM, *** $p<0.001$.

The role(s) of all three genes in the adipogenic differentiation process have been highly documented in the literature (Chawla and Lazar, 1994; Schoonjans *et al.*, 1996; Cowherd *et al.*, 1999; Fruhbeck *et al*., 2001; Bildirici *et al.*, 2003). Normalised levels of expression for each gene are shown, as a percentage of the maximum expresser, in Figure 3.15. mRNA transcript levels of all three genes were determined in MSCs cultured on 2D and 3D prior to induction of differentiation, to ensure that there were no differences in transcript levels prior to the start of the experimental timepoints (data not shown).

Adipophilin is an early marker of adipogenesis, forming an association with minute lipid droplets formed during the initial stages of differentiation (Brasaemle *et al.*, 2007). At the 5 day timepoint, MSCs cultured on 3D demonstrated enhanced expression of adipophilin mRNA compared to their 2D counterparts, and this

difference was significant at the statistical level ($p<0.01$). Increased levels of adipophilin suggested that MSCs cultured on 3D were actively undergoing adipogenesis and were in the early stages of differentiation at this timepoint. Lower levels of expression in MSCs cultured on 2D suggested one of two things: a) MSCs cultured on 2D 'lagged behind' those cultured on 3D in terms of committing to differentiation, or b) MSCs cultured on 2D were committing to differentiation at the same time as those cultured on 3D, but were less efficient in bringing about the complex intracellular changes required for differentiation. At the 7 day timepoint, expression of adipophilin increased in MSCs cultured on both 2D and 3D, with those cultured on 2D demonstrating slightly higher levels of expression than those on 3D, though this difference was not significant at the statistical level. This pattern of expression indicated that MSCs cultured on 2D were entering the initial, adipophilin-dependent stage of adipogenesis at this timepoint, whereas MSCs cultured on 3D were further along the linear pathway of events that occurs during adipogenesis and were making the transition from adipophilin-dependency to adipophilin-independency. At the 14 day timepoint, expression of adipophilin decreased in MSCs cultured on both 2D on 3D, though expression remained significantly higher for those cultured on 2D compared to those cultured on 3D ($p<0.01$). This pattern of expression suggests that MSCs cultured on 2D are beginning to make the transition into the later, adipophilin-independent stages of adipogenesis but are still largely in the earlier, adipophilin-dependent stages of adipogenesis, whereas MSCs cultured on 3D are largely in the later stages of differentiation.

PPARγ is a member of the peroxisome proliferator-activated receptor family nuclear hormone receptors and its expression is restricted to adipose tissue. It is known to play a crucial role during adipogenesis, controlling the expression of a large number of genes required throughout the later stages of differentiation and also in lipid metabolism (Schoonjans *et al.*, 1996; Grimaldi *et al.*, 2001). At the 5 day timepoint, levels of PPARγ expression were low and equal for MSCs cultured under adipogenic conditions on both 2D and 3D. At this timepoint, MSCs were in the early stages of adipogenesis (as indicated by expression of the early marker, adipophilin) which are largely independent of PPARγ-controlled transcriptional events. At the 7 day timepoint, however, there is a massive increase in PPARγ expression in MSCs cultured on 3D, suggesting that these cells were in the mid-late stages of the differentiation

process. Levels of expression of PPARγ remained unchanged in MSCs cultured on 2D. At the 14 day timepoint, levels of PPARγ returned to a low level for MSCs cultured on 3D, to a level comparable with cells cultured on 2D. Increased expression of PPARγ in MSCs cultured on 3D is likely to be a major factor contributing to the enhanced adipogenic effect of MSCs cultured on 3D compared to 2D.

Adipsin, a serine protease produced and secreted by mature adipocytes, is a very late marker of adipogenesis and its expression correlates with the last stages of the linear adipogenic pathway (Fruhbeck *et al.*, 2001; De Gemmis *et al.*, 2006). Prior to induction of differentiation, adipsin mRNA was undetectable in MSCs cultured on both 2D and 3D (data not shown). At the 5 day timepoint, however, adipsin mRNA transcripts were readily detectable in MSCs cultured under adipogenic conditions on both 2D and 3D substrates. Expression was slightly higher in MSCs cultured on the scaffold compared to tissue culture plastic, though this difference was slight and not significant at the statistical level. At the 7 day timepoint, MSCs cultured on the scaffold demonstrated a further increase in expression of adipsin, whereas MSCs cultured on 2D actually showed a decrease in expression. An increase in expression of adipsin mRNA suggested that MSCs cultured on the scaffold were beginning to demonstrate a more mature adipogenic phenotype, especially in comparison to their 2D counterparts, as MSCs cultured on 2D demonstrated a significantly lower level of expression ($p<0.01$). At the 14 day timepoint, adipsin transcript levels had returned to low levels for MSCs cultured on 3D and remained relatively low for MSCs cultured on 2D, though levels of transcript were higher in MSCs cultured on 2D compared to 3D. This was significant at the statistical level ($p<0.01$). This expression profile is representative of what has been previously reported in the literature for MSCs cultured on 3D substrates (De Gemmis *et al.*, 2006). Lower levels of adipsin in MSCs cultured on 2D suggests that adipogenic derivatives formed by these cells do not appear to reach the levels of maturity of those formed by cells cultured on 3D.

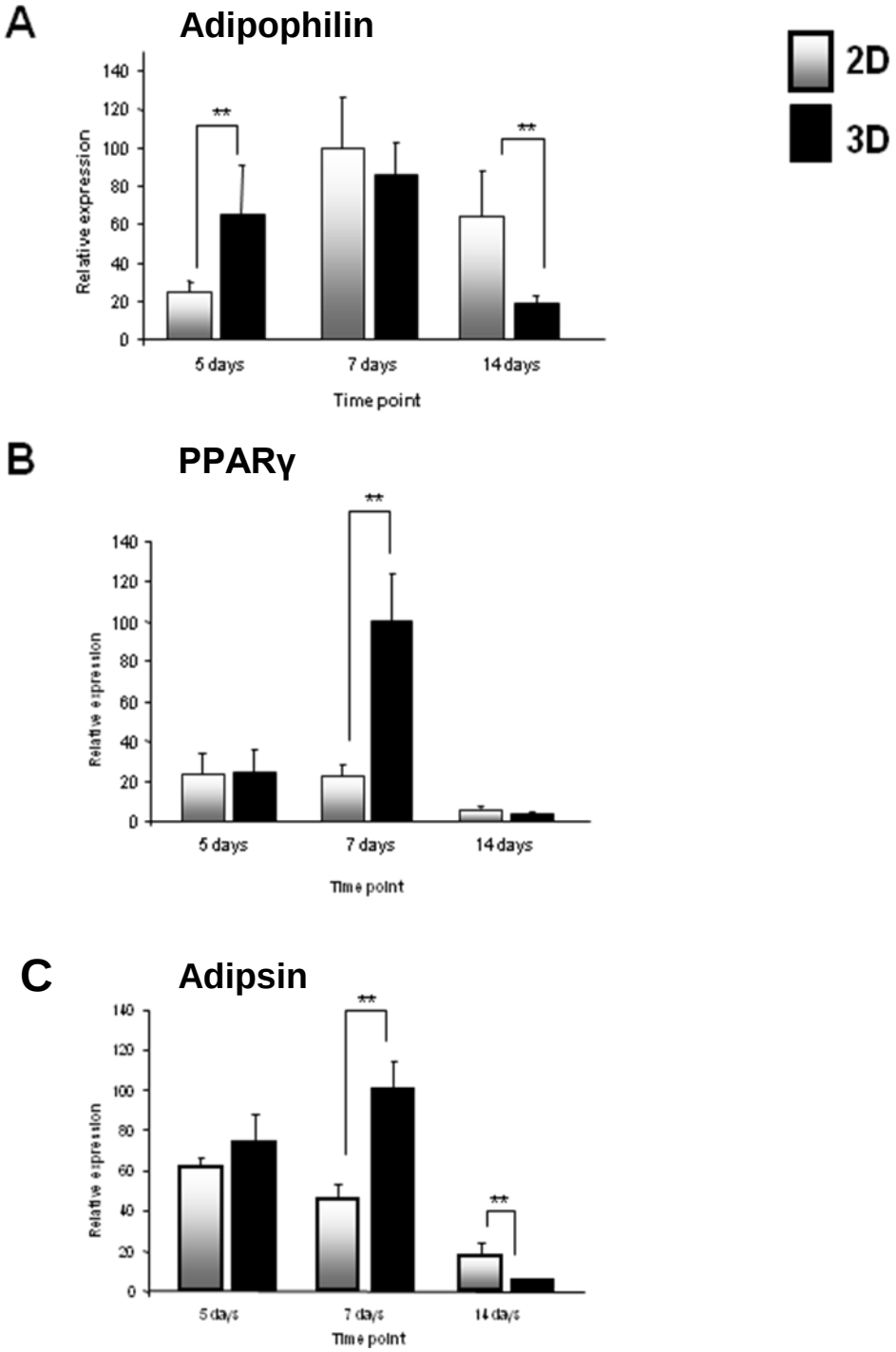

Figure 3.15. Expression of adipocyte-specific genes in MSCs cultured under adipogenic conditions on Alvetex™ (3D) and tissue culture plastic (2D). QRT-PCR comparing mRNA transcript levels of three adipocyte-specific genes – **A**, adipophilin; **B**, PPARγ; and **C**, adipsin – in MSCs cultured under adipogenic conditions for 5, 7 and 14 days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, ** $p<0.01$, ** $p<0.01$.

3.3.14 MSCs demonstrate marked differences in expression of soluble factors and cytokines when cultured under both osteogenic and adipogenic conditions on Alvetex™ compared to standard tissue culture plastic

There are many studies throughout the literature examining the effects of various three-dimensional surfaces and scaffolds on the differentiation of MSCs and other cell types, and in many cases an enhanced differentiative effect is observed. Whilst this enhanced effect can be attributed to the profound effects of 3D cellular culture compared to 2D, little work has been done at the molecular level to ascertain what basic changes (if any) are occurring in cells cultured on 3D to bring about such significant results. This study aims to address that. It is well documented that bone and fat function as autocrine and paracrine tissues *in vivo*, with the proliferation and differentiation of osteoblasts and adipocytes being controlled to a certain extent by soluble factors and cytokines secreted by cells within their niche (Baylink *et al.*, 1993; Spiegelman and Flier, 1996; Mohamed-Ali *et al.*, 1998; Pages *et al.*, 2000; Sykara and Opperman, 2003). Here, expression of a number of soluble factors and cytokines previously documented to exert a role of osteogenic and adipogenic differentiation were examined in MSCs cultured on the scaffold versus tissue culture plastic using qRT-PCR. All expressions were normalised to GAPDH as previous detailed in Section 2.11.2, with normalised levels of expression shown as a percentage of the maximum level of expression in Figure 3.16 and Figure 3.17.

Figure 3.16 shows the mRNA expression profiles of a number of soluble factors and cytokines (TGF-β1, VEGF, IGF-1, TNF-α, BMP2, BMP4 and BMP7) in MSCs cultured under osteogenic conditions for 5 and 7 days on 2D and 3D. Expression of TGF-β1, the founding member of the transforming growth factor beta superfamily of cytokines, was low in MSCs cultured on both 2D and 3D at the 5 day timepoint, but despite such low levels of expression MSCs cultured on 3D demonstrated significantly higher mRNA levels than their 2D counterparts ($p<0.05$). At the 7 day timepoint, expression of TGF-β1 increased in MSCs cultured under both conditions when compared to levels at the 5 day timepoint, with MSCs cultured on the scaffold demonstrating vastly greater expression than their 2D equivalents ($p<0.001$). VEGF, belonging to a subfamily of the platelet-derived growth factor family of cytokines, was undetectable at the mRNA level in MSCs cultured on 2D at the 5 day timepoint, though was detectable at very high levels in MSCs cultured on 3D ($p<0.01$). At the 7

day timepoint, however, VEGF was detectable at high levels in MSCs cultured on both 2D and 3D, with MSCs cultured on 3D demonstrating slightly higher levels of expression, though this was not significant at the statistical level. IGF-1, a polypeptide hormone so called due to its similarity in structure to insulin, was undetectable in MSCs cultured under both conditions at the 5 day timepoint and remained undetectable at the 7 day timepoint for MSCs cultured on tissue culture plastic. MSCs cultured on the scaffold, however, demonstrated a significant upregulation in IGF-1 at this timepoint ($p<0.05$). TNF-α, belong to the TNF superfamily of cytokines, was virtually undetectable at the 5 day timepoint in MSCs cultured under both conditions, with MSCs cultured on tissue culture plastic lacking expression completely and MSCs cultured on the scaffold demonstrating only very slight expression. Expression remained low in MSCs cultured on the scaffold at the 7 day timepoint, though interestingly underwent a massive upregulation in MSCs cultured on tissue culture plastic ($p<0.05$). Bone morphogenetic proteins are perhaps the most well-characterised of all growth factors and cytokines known to influence osteogenesis and BMP2, BMP4 and BMP7 were examined in this study. The pattern of expression of BMP2 mirrored that of TGF-β1, in that MSCs cultured on the scaffold demonstrated significantly higher levels of expression than their 2D counterparts at the both the 5 day ($p<0.001$) and 7 day ($p<0.05$) timepoints. BMP4 was undetectable in MSCs cultured on 2D at both the 5 and 7 day timepoints, but was detectable at high levels in MSCs cultured on 3D at both the timepoints examined ($p<0.01$). BMP7 mRNA transcripts were not detectable in MSCs cultured under either conditions at the 5 day timepoint, though were detectable at the 7 day timepoint, with MSCs cultured on 2D demonstrating vastly higher levels of expression than their 3D counterparts. This difference, however, was not significant at the statistical level.

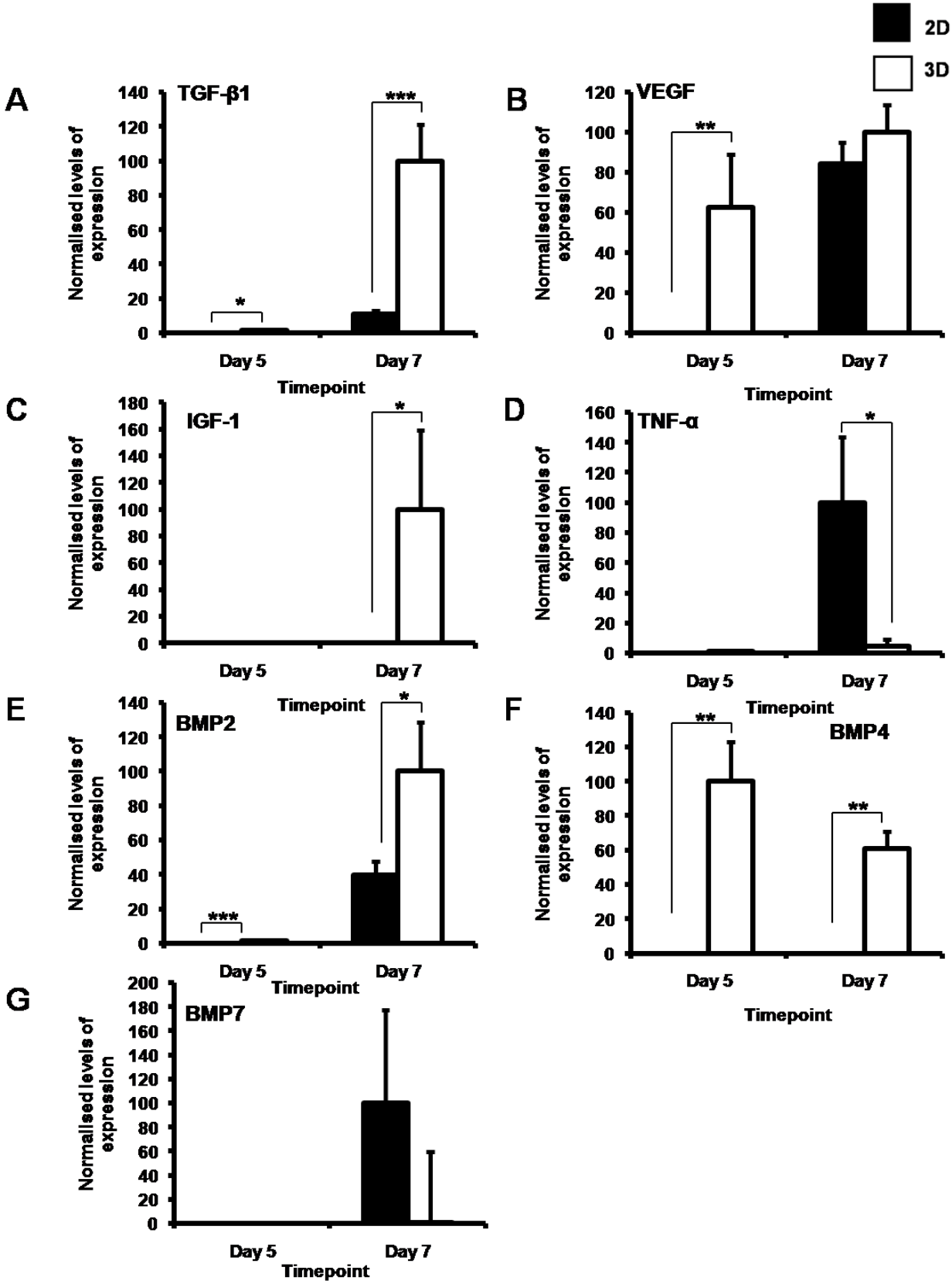

Figure 3.16. Expression of several soluble factors, as determined by qRT-PCR, in MSCs cultured under osteogenic conditions on Alvetex™ (3D) and tissue culture plastic (2D). A: TGF-β1; **B:** VEGF; **C:** IGF-1; **D:** TNF-α; **E:** BMP2; **F:** BMP4; **G:** BMP7. Each bar represents the mean (n=3)+SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3.17 shows the mRNA expression profiles of a number of soluble factors and cytokines (IGF-1, TGF-β1, TNF-α, BMP2, BMP4 and BMP7) in MSCs cultured under adipogenic conditions for 5 and 7 days on 2D and 3D. There is a certain amount of redundancy and crosstalk between different soluble factor/cytokine-induced signalling pathways and, as such, many of the soluble factors reported to be involved in osteogenesis are similarly reported to play important roles in adipogenesis also (Zehentner *et al.*, 2000; zur Nieden *et al.*, 2004; Holly *et al.*, 2006; Levi *et al.*, 2010). IGF-1 mRNA transcripts were detectable in MSCs cultured on both 2D and 3D substrates at both 5 and 7 days and at both timepoints MSCs cultured on 3D demonstrated higher levels of expression than their 2D counterparts. However, these differences were not significant at the statistical level ($p>0.05$). TGF-β1 was expressed at extremely low levels at the 5 day timepoint in MSCs cultured on both 2D and 3D and remained at such low levels at the 7 day timepoint in MSCs cultured on 3D. MSCs cultured on 2D, however, demonstrated a significant upregulation in TGF-β1 expression at the 7 day timepoint ($p<0.01$). The expression profile of TNF-α demonstrated a remarkable similarity to the expression profile observed for IGF-1, in that TNF-α mRNA transcripts were detectable in MSCs cultured on both 2D and 3D substrates at both 5 and 7 days and at both timepoints MSCs cultured on 3D demonstrated higher levels of expression than their 2D counterparts. No statistically significant difference was observed ($p>0.05$). BMP2 was virtually undetectable in MSCs at the 5 day timepoint regardless of their growth substrate and remained at similarly low levels for MSCs cultured on 2D at the 7 day timepoint also. MSCs cultured on the scaffold, however, demonstrated a substantial increase in expression at the 7 day timepoint, but this was not significant at the statistical level ($p>0.05$). BMP4 was detectable at higher levels in MSCs cultured on the scaffold compared to tissue culture plastic at both timepoints examined and these differences were statistically significant for both the 5 day ($p<0.01$) and the 7 day ($p<0.05$) timepoint. At the 5 day timepoint, BMP7 was not detectable in MSCs cultured on either growth substrate and remained undetectable for MSCs cultured on the scaffold even at the 7 day timepoint. However, MSCs cultured on tissue culture plastic underwent a massive upregulation in BMP7 expression between 5 and 7 days, and this difference was highly significant at the statistical level ($p<0.01$).

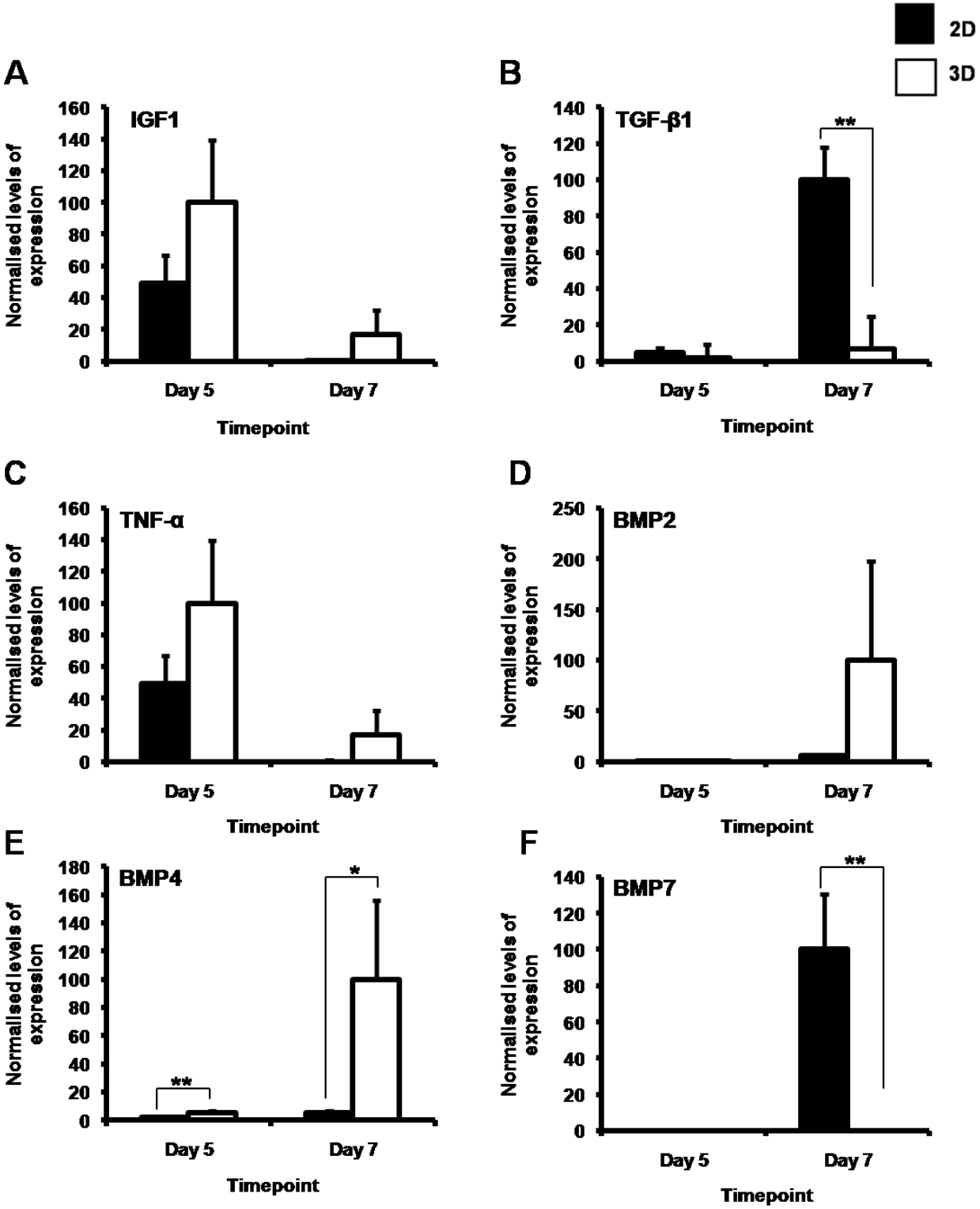

Figure 3.17. Expression of several soluble factors, as determined by qRT-PCR, in MSCs cultured under adipogenic conditions on Alvetex™ (3D) and tissue culture plastic (2D). A: IGF-1; **B:** TGF-β1; **C:** TNF-α; **D:** BMP2; **E:** BMP4; **F:** BMP7. Each bar represents the mean (n=3)+SEM, * $p<0.05$, ** $p<0.01$.

3.4 Discussion

Establishment of primary MSC cultures, marker expression profiles and multilineage differentiation potential

In this study it was confirmed that stromal cells isolated from bone marrow, using plastic adherence as the main selection method, displayed a morphological phenotype, marker expression profile and differentiation potential that were highly characteristic of an MSC population. This method of MSC isolation is the traditional and most commonly used method to date, whereby whole bone marrow aspirates are plated out into tissue culture plastic for 48 hours, allowing sufficient time for MSCs to attach to the cellware (Luria *et al.*, 1971). Haematopoietic cells do not attach to the cellware and remain as highly refractile cells in suspension, which are removed by continual media changes and passaging. Although plastic adherence remains the most commonly used approach for isolating MSCs from bone marrow, it is disadvantageous in that resulting cultures are highly heterogeneous, consisting of a mixture of cell types all at various stages of development. Variations on this protocol include centrifuging whole bone marrow aspirates over a Percoll gradient, allowing separation of the various cell types within the bone marrow according to density, and selecting only the nucleated fraction for culture. This again, however, has the disadvantage of non-specificity and heterogeneity (Dazzi *et al*., 2006). A major aim of research at present is to generate new protocols that result in the isolation of a more homogeneous starting population; this will ideally involve exploitation of cell surface marker expression to allow positive selection of a population expressing a specific stem cell marker using methods such as FACS and immunomagnetic sorting. Such methods have been in practice for many years regarding the isolation of haematopoietic stem cells (Spangrude *et al.*, 1988; Morrison and Weissman, 1994; Yin *et al.*, 1997) and similar methods of isolation are becoming commonplace in the isolation of stem cells from various other tissues such as embryonal carcinomas and even prostate (Przyborski, 2001; Richardson *et al.*, 2004). However, this situation is more complicated in MSC biology due to the lack of any cell surface markers whose expression is exclusive to MSCs. As such, expression of a range of cell surface markers was determined in this study to confirm the isolation of a true MSC population, including CD9, CD13, CD15, CD44, CD58, CD71, CD90 CD105, CD138 and also the haematopoietic markers CD34 and CD45. This is by no means an exhaustive list of MSC markers. Notable absentees from this list include

CD29, CD31, CD54, CD63, CD73, CD106, CD117, CD140b, CD166, Stro-1 and Sca-1 (Gronthos *et al.*, 1944; Pittenger *et al.*, 1999; Bobis *et al.*, 2006; Jackson *et al.*, 2007). Likewise, CD34 and CD45 are not the only markers of haematopoietic cell types, with absentees including CD11b, CD14, CD31, CD33 and CD133 (Pittenger *et al.*, 1999; Bobis *et al.*, 2006). Because of a lack of consensus as to which markers are more indicative of an MSC phenotype than others, a common problem occurs in that different groups examine different panels of markers in order to assess the phenotype of putative MSCs. A lack of an exclusive marker of MSCs also means it is difficult to isolate a homogeneous starting population by methods such as those described above. Several efforts have been published in recent years to find an accurate combination of cell surface markers that allows for the selection of putative MSCs from their more differentiated derivatives. For example, Pittenger and colleagues report that co-expression of CD105 and CD73 is sufficient to allow isolation of MSC from bone marrow, whereas a different study suggests that CD166 and CD105 are more important antigenic determinants of MSC phenotype (Pittenger *et al.*, 1999; Alsalemah *et al.*, 2004). Stro-1 antibody has also been reported to select for a pure population of cells demonstrating MSC characteristics (Simmons *et al.*, 1999; Dennis *et al.*, 2002; Gronthos *et al.*, 2003).

As the definition of a stem cell is a functional one, it is perhaps more important to demonstrate that stromal cells isolated from the bone marrow display the potential to differentiate into a range of mature mesodermal cell types, a fundamental property of MSCs. In this study, it was demonstrated that stromal cells displayed at least bipotentiality in that they demonstrated the ability to differentiate into bone and fat, using well-established protocols (Pittenger *et al.*, 1999). This suggests the isolation of a true MSC population, rather than the isolation of pre-osteoblasts and/or pre-adipocytes that were already committed to differentiate into bone and fat lineages respectively.

Exemplification of Alvetex™

As discussed in the introduction to this Chapter, routine cell culture performed on tissue culture plastic (2D) is fundamentally flawed in that it forces cells to adopt highly unnatural conformations/morphologies that are in no way representative of their *in vivo* growth environments (3D), where they develop highly complex interactions with

neighbouring cells and extracellular matrix within their niche. As such, there is a demand for 3D culture systems for *in vitro* use. Reinnervate produces Alvetex™, a novel 3D polystyrene scaffold that can be used in the routine culture of cells *in vitro*, with the aim of providing more accurate analyses of complex cellular phenomena such as cellular differentiation. This technology has previously been exemplified using two main cell types: the HepG2 (liver carcinoma) and TERA2.cl.SP12 (embryonal carcinoma) cell lines (Hayman and Przyborski, 2004; Hayman *et al.*, 2005; Bokhari *et al.*, 2007a; Bokhari *et al.*, 2007b). In each case, cells cultured on the scaffold demonstrated enhanced viability and functional activity compared to their counterparts that were cultured on the 2D environment of tissue culture plastic. HepG2 cells cultured on the scaffold displayed excellent morphology, higher levels of viability (determined via MTT assay) and enhanced functional activity (determined by production of albumin, a commonly-used marker of liver-specific function/metabolic activity) compared to their 2D counterparts (Bokhari *et al.*, 2007a). In a further paper by the same authors it was demonstrated that HepG2 cells cultured on the scaffold displayed a greater resistance against the cytotoxic drug methotrexate (MTX) compared to those cultured on 2D, suggesting that the scaffold may provide a useful and more physiologically-relevant system in which to perform pharmaco-toxicological testing (Bokhari *et al.*, 2007b). Hayman and colleagues also examined the suitability of the scaffold to permit the viable attachment and growth of TERA2.cl.SP12-derived neurons (Hayman and Przyborski, 2004; Hayman *et al.*, 2005). The untreated scaffold did not support the attachment of viable neurons. However, upon coating the scaffold surface with the biologically active molecules poly-D-lysine and laminin, a large number of viable neurons attached to the scaffold surface (Hayman *et al.*, 2005). Neurons cultured on coated scaffolds also demonstrated the formation of more extensive and complex neural networks compared to their 2D counterparts, and such complex 3D networks are likely to be more indicative of those which form *in vivo* (Hayman *et al.*, 2004). Expression of neural markers in neurons cultured on 2D and 3D were also examined in this study. Neurons cultured on 2D demonstrated positive expression of markers associated with mature, post-mitotic neuronal phenotypes such as NeuroD and MAP2ab, but lacked expression of markers associated with the developing nervous system such as nestin, MAP2c and GAP43. Markers associated with developing neurons, however, were detectable in MSCs cultured on the scaffold, strongly suggesting that neural networks were still expanding and neurite outgrowth

was still occurring in neurons cultured on this system. Expression of mature neuronal markers were also readily detectable in these cells.

Morphology and viability of MSCs cultured on Alvetex™

In the present study, it has been demonstrated for the first time that the novel 3D polystyrene scaffold manufactured by Reinnervate is biocompatible to support the attachment and growth of viable MSCs *in vitro*. SEM analysis revealed that the untreated scaffold surface supported the attachment and viable growth of MSCs, in keeping with previous studies performed on the HepG2 cell line (Bokhari *et al.*, 2007a). Although Hayman and colleagues reported that it was first necessary to coat the scaffold surface with the biologically active molecules poly-D-lysine and laminin to permit attachment and growth of TERA2.cl.SP12-derived neurons, this necessity can be attributable to the very particular growth substrate requirements of neural cell types *in vitro* (Hayman *et al.*, 2005). This is also the case for neural cells cultured on tissue culture plastic. SEM analysis of MSCs cultured on the scaffold also revealed that these cells displayed excellent morphology – a characteristically large, fibroblastic morphology – with cells spreading out to their maximum capacity over the scaffold surface to form a sheet of healthy cells. Cell viability was determined quantitatively for MSCs cultured on 2D and 3D at a range of timepoints up to and including 21 days, using the MTS assay. The results from the assay allowed two main conclusions to be drawn: a) MSCs were proliferating over the 21 day time course of the experiment when cultured on both 2D and 3D substrates, and b) at each of the timepoints examined, there was a significantly higher number of viable cells on 3D compared to 2D ($p<0.001$). It is anticipated that the observed increase in cell viability is a result of the scaffold enabling MSCs to grow in a manner more pertinent to that in which they grow within their bone marrow niche, allowing them to form cell-cell interactions that are more representative of events that occur *in vivo*. Culturing MSCs on the scaffold is therefore likely to provide a more accurate and physiologically-relevant system in which to study MSC biology. Several efforts have already been made to fabricate 3D scaffolds suitable for *in vitro* culture of MSCs, with many studies reporting that 3D culture promotes proliferation and increases viability of MSCs. Such examples include nanofibrous type I collagen scaffolds, hyaluronan-based scaffolds, chitosan-gelatin-chondroitin-based scaffolds, gelatin/PLLA (poly($_{L}$-lactic acid))-based scaffolds and poly(lactic-*co*-glycolic acid) (PLGA)-based scaffolds (Shih *et al.*, 2006; Machado *et*

al., 2007; Jin *et al.*, 2008; Mattii *et al.*, 2008; Pasquinelli *et al.*, 2008). However, not all 3D scaffolds increase viability and promote proliferation of MSCs *in vitro*. For example, MSCs cultured on a photocrosslinked oligo[(polyethylene glycol) fumarate] (OPF) hydrogel did not show any significant proliferation over a period of 14 days (Dadsetan *et al.*, 2008). In another study, culturing MSCs on a nano-hydroxyapatite/polyamide (m-HA/PA) scaffold did promote proliferation over a 7 day time period, however, there was no statistically significant difference between the number of viable cells cultured on the scaffold compared to routine 2D cultures (Wang *et al.*, 2007). The present study clearly demonstrates that the polystyrene scaffold manufactured by ReInnervate not only promotes proliferation of MSCs over time, but increases viability beyond the level observed on tissue culture plastic.

Osteogenic differentiation of MSCs

In addition to cell morphology and viability analysis, this study also addressed the pivotal issue of whether MSCs cultured on the scaffold: a) retained their potential to differentiate into the mesodermal derivatives bone and fat, and b) whether this differentiative potential was enhanced following 3D culture compared to conventional 2D culture. Mineralisation and deposition of a collagenous matrix were determined using von Kossa and Masson's trichrome staining respectively. MSCs cultured on 2D had previously demonstrated the ability to differentiate into osteoblastic derivatives and histological analysis revealed that MSCs cultured on the scaffold retained this ability to differentiate. However, histological analysis alone is insufficient to allow comparisons to be made regarding the functionality of MSCs cultured on 2D vs. 3D due to its intrinsically qualitative nature and also the widely varying methodologies adopted for staining 2D vs. 3D cultures.

Alkaline phosphatase and osteocalcin

Fortunately, a number of functional assays are commercially available to assess osteoblastic behaviour. Alkaline phosphatase (ALP) activity is perhaps the most commonly used marker of osteogenic differentiation (Lian and Stein, 1992). ALP is an ectoenzyme produced by osteoblasts that is involved in the degradation of inorganic pyrophosphate to produce a sufficient location concentration of phosphate or inorganic pyrophosphate for matrix mineralisation to occur (Marom *et al.*, 2005). The pattern of ALP activity observed in this study – an increase in activity up to a maximum at 14

days followed by a subsequent decrease at 21 days – is highly characteristic of what has previously been reported (Owen *et al*., 1990; Datta *et al*., 2006; Hosseinkhani *et al*., 2006; Donzelli *et al*., 2007; Na *et al*., 2007). Maximum ALP activity at 14 days is associated with the end of the proliferative stage and the subsequent decrease in activity is associated with advanced matrix mineralisation and the acquisition of a more mature osteoblastic phenotype. Although this trend was comparable between MSCs cultured on 2D and 3D, ALP activity was significantly enhanced in MSCs cultured on 3D compared to 2D at each the timepoints assessed, suggesting that these cells were displaying enhanced osteoblastic functional behaviour.

A second commonly used marker of osteogenic differentiation is osteocalcin. Osteocalcin is a much later marker of osteogenesis than ALP that is produced and secreted by mature osteoblasts and contributes to the ground substance of bone extracellular matrix (Lian and Stein, 1992; Lian and Stein, 1993; Buranasinsup *et al.*, 2006). This ground substance, in combination with extracellular collagen, makes up bone extracellular matrix (osteoid). Osteocalcin was readily detectable in the media of MSCs cultured under osteogenic conditions on both 2D and 3D substrates at each of the timepoints assessed, with maximum levels observed at the 35 day timepoint. This is highly indicative of the expression of a mature osteoblastic phenotype. As for ALP activity, the trend in osteocalcin secretion was comparable between MSCs cultured on 2D and 3D, though osteocalcin levels were significant greater in the media of MSCs cultured on 3D compared to 2D. These data further suggest that MSCs cultured on 3D displayed enhanced functional behaviour in keeping with the expression of a mature osteoblastic phenotype.

Combined, these results suggest that the scaffold provides a superior environment in which to culture MSCs and support their differentiation into osteoblastic derivatives. Other scaffolds which support and enhance osteogenic differentiation of MSCs include OPF hydrogels, n-HA/PA scaffolds, collagen sponges (Gingistat1), collagen-based scaffolds and gels, silk-based scaffolds, peptide-amphiphile (PA)-based scaffolds, osteopontin-derived peptide (ODP)-based hydrogels, coralline-based scaffolds, poly-l-caprolactone (PCL)-based scaffolds and gelatin-based sponges (Meinel *et al.*, 2004; Shin *et al.*, 2004; Takahashi *et al.*, 2004; Abramovitch-Gottlib *et al.*, 2005; Li *et al.*,

2005; Yoneno *et al.*, 2005; Hosseinkhani *et al.*, 2006; Donzelli *et al.*, 2007; Wang *et al.*, 2007; Dadsetan *et al.*, 2008).

Adipogenic differentiation of MSCs

The above results clearly demonstrate that culturing MSCs within the 3D environment of the scaffold not only supports but also enhances osteogenic differentiation of MSCs. As these cells previously demonstrated bipotentiality in their ability to differentiate into both osteogenic and adipogenic lineages in 2D culture, the next aim of this study was to investigate whether MSCs cultured on the scaffold retained their potential to undergo adipogenic differentiation and, if so, whether this potential was enhanced following 3D culture compared to conventional 2D culture. The formation and accumulation of neutral lipid droplets within the cell cytoplasm was examined using oil red O and Nile red staining, two synergistic methods for examining lipid formation.

Oil red O and Nile red staining

Adipogenic differentiation of MSCs cultured under conventional 2D conditions had previously been demonstrated and histological analysis using oil red O staining revealed that MSCs cultured on the scaffold retained this differentiative ability. However, whereas histological analysis was unsuitable to allow direct comparisons to be made between osteoblastic derivatives formed on 2D and 3D, the case is somewhat different here to due to varying methodology. The cytospin method of staining required differentiated cells to be trypsinised out of the scaffold and spun onto non-coated glass slides prior to oil red O staining – this was advantageous as it permitted analysis at the individual cellular level and, as such, allowed direct comparisons to be made between MSCs cultured on 2D vs. 3D. Morphological observations in terms of the number of lipid droplets per cell demonstrated that MSCs cultured on 3D contained more droplets within their cytoplasm than their 2D counterparts, an observation further supported by oil red O elution studies. A more sophisticated method to examine intracellular lipid accumulation involved staining cells with the Nile red fluorescent dye, followed by flow cytometric analysis. These studies revealed that approximately one-third of MSCs actively underwent adipogenic differentiation regardless of their culture substrate, though MSCs cultured on the scaffold demonstrated a higher intensity of Nile red staining compared to their 2D counterparts. This strongly suggests that culturing MSCs on the scaffold did not result in an increase in the number of cells

undergoing differentiation, but rather caused those cells that did undergo differentiation to do so in a more efficient manner, resulting in these cells acquiring a more mature adipogenic phenotype than their 2D counterparts.

However, analysis of lipid accumulation within differentiated cells only gives information with regards the final phenotype of the cells and gives little, if any, information regarding the functionality of differentiated cells. Whereas osteogenic differentiation can be assessed *in vitro* using a range of commercially-available functional assays such as determination of ALP activity and osteocalcin levels as has been performed in the present study, the study of adipogenesis is somewhat more complex due to the lack of functional biochemical assays available to assess adipogenic behaviour. In the absence of such biochemical assays, adipogenic behaviour is usually measured using non-functional methods, typically via the assessment of lipid droplet formation using lipophilic stains such as oil red O and Nile red. Although such methods are of some use, this information is limited when wanting to compare how cells are behaving when cultured on 3D compared to 2D.

Cyclic AMP (cAMP) activity

In order to gain a more functional insight into how MSCs were behaving when cultured on the scaffold compared to tissue culture plastic, cyclic AMP (cAMP) levels were determined using a commercially-available enzyme immunoassay kit. cAMP response element binding protein (CREB) is a central transcription factor in adipogenesis and plays a crucial role in initiation and during the early stages of adipogenesis, a role which has been documented extensively throughout the literature (Reusch *et al.*, 2000; Petersen *et al.*, 2008). It is constitutively expressed in 3T3-L1 fibroblasts prior to induction of differentiation and is upregulated in preadipocytes and even mature adipocytes following their treatment with conventional adipogenic culture media containing cAMP-inducing agents such as dexamethasone and IBMX (Reusch *et al.*, 2000). Convincing evidence for the key role of CREB in adipogenesis also comes from observations that adipogenesis is impaired in a number of CREB-deficient models (Zhang *et al.*, 2004; Fox *et al.*, 2006). From a more mechanistic perspective, CREB binds to putative cAMP response elements (CREs) present within the promoters of several adipocyte-specific genes, such as PPARγ and LPL, and can manipulate adipogenesis via its transcriptional regulation of these key adipogenic components

(Hansen *et al.*, 2001; Madsen *et al.*, 2003; Tzameli *et al.*, 2004; Yang *et al.*, 2008). CREB has also been suggested to play a role as a survival factor of adipocytes, controlling the expression of pro- and anti-apoptotic genes (Reusch and Klemm, 2002). However, an interesting concern is raised by Reusch and colleagues in their study regarding the paradoxical role of cAMP signalling both adipogenesis and lipolysis, two conflicting processes (Reusch *et al.*, 2000). Although they report that cAMP demonstrates a key role in potentiating adipogenesis consistent with previous observations in the literature, other studies have reports that chemically-induced stimulation of the cAMP signalling cascade results in increased lipid breakdown (MacDougald and Lane, 1995; Collins *et al.*, 1997; Danforth and Himms-Hagen, 1997; Yarwood *et al.*, 1998; Weyer *et al.*, 1999; Reusch *et al.*, 2000). There are several possible reasons regarding these opposing observations. For example, each of these studies has used different experimental models and cAMP is likely to have different roles depending on the cellular system in which it is studied. It is also likely to be dependent upon the differentiated state of the cell, as cAMP signalling in undifferentiated cells has been reported to exert a different effect to cAMP signalling in more mature adipocytes (Reusch *et al.*, 2000; Petersen *et al.*, 2008). As a universal signalling molecule, cAMP has many different downstream effectors besides CREB and as such it is highly probable that a CREB-independent mechanism could cause these different observations. In fact, even within the process of adipogenesis cAMP can act via protein kinase A (PKA)-dependent and independent mechanisms (Martini *et al.*, 2009). Although cAMP is a universal second messenger molecule and has crucial roles in many different processes and functions outside adipogenesis, it was still considered a useful exercise to determine cAMP levels in MSCs cultured on 2D vs. 3D in order to give an idea as to how the cells were performing at a metabolic level. At the earlier timepoint of 7 days, cAMP levels were significantly higher for MSCs cultured on 3D compared to 2D, which is in keeping with previous reports of cAMP playing a crucial role during initiation and the early stages of adipogenesis. Low levels of cAMP in MSCs cultured on 2D at this timepoint suggest that few cells are undergoing adipogenesis at present or are doing so at a very low level. At the later timepoint of 21 days, cAMP levels decrease in MSCs cultured on 3D and increase in MSCs cultured on 2D, to comparable levels for the two conditions. This suggests that MSCs cultured on 3D are exiting the early, cAMP-dependent stages of adipogenesis whereas MSCs cultured on 2D are further behind the differentiation process and are

still in the earlier, cAMP-dependent stages. This strongly suggests that MSCs cultured on the scaffold are more efficient at undergoing adipogenic differentiation, a property that can be attributable, at least in part, to increased cAMP-signalling events.

Expression of adipocyte-related genes

Data regarding levels of cAMP in MSCs cultured on 2D and 3D are useful in that they allow an appreciation as to how cells are functioning at a metabolic level. Although elevated cAMP in MSCs cultured on the scaffold compared to tissue culture plastic strongly suggests that these cells were undergoing enhanced adipogenesis, it must be remembered that cAMP is not a specific marker of adipogenesis. A lack of specific functional assays is a major limiting factor in adipogenesis research. In order to gain a more mechanistic insight into how MSCs were behaving when cultured under adipogenic conditions on both 2D and 3D, expression of several adipocyte-related genes were examined using qRT-PCR – adipophilin, PPARγ and adipsin – the roles of which have been reported extensively throughout the literature. Adipogenesis is known to occur in four distinct stages – preconfluent proliferation, confluence growth arrest, hormonal induction and permanent growth arrest (terminal differentiation) – and these genes have been selected to cover a range of these stages (Cowherd *et al.*, 1999). Adipophilin associates with minute droplets formed during the early stages of adipogenesis, suggesting a role for adipophilin in the nucleation of lipid droplets (Brasaemle *et al.*, 1997). Therefore, adipophilin can be considered an early marker of adipogenesis. As differentiation proceeds and lipid droplets increase in size, adipophilin is gradually replaced by another protein, perilipin, to such an extent that mature adipocytes show virtually exclusive perilipin expression and little (if any) expression of adipophilin. PPARγ belongs to the peroxisome proliferator-activated receptor family of nuclear hormone receptors and, although there are three known families of receptor – α, β and γ – it is PPARγ whose expression is restricted to adipose tissue. Expression of PPARγ is increased rapidly after hormonal induction during stage 3 and it plays a key role during adipogenesis, controlling the expression of a number of crucial genes required during the differentiation process (De Gemmis *et al.*, 2006). Adipsin is a serine protease secreted by mature adipocytes and, as such, is a very late marker of adipogenesis whose maximum expression occurs in stage 4 (Fruhbeck *et al.*, 2001; De Gemmis *et al.*, 2006). Consequently, production and secretion of adipsin is largely dependent on differentiation occurring. MSCs cultured

on the 3D environment of the scaffold demonstrated enhanced expression of these genes compared to those cultured on 2D tissue culture plastic, keeping an expression profile expected during adipogenesis. Levels of expression in MSCs cultured on 2D tended to be lower than those in cultured on 3D and in some cases even failed to express (as was the case for PPARγ in particular and also adipsin). A lack of expression of adipocyte-related genes in cells cultured on 2D following appropriate stimulation with adipogenic factors has previously been reported (Hwang *et al.*, 1997; Kang *et al.*, 2007). Such observations are not unique to the study of adipogenesis. It is now commonly acknowledged that cells cultured in 3D demonstrate markedly different gene expression profiles than those cultured in 2D (Pampaloni *et al.*, 2007). For example, melanoma cells cultured on 3D compared to 2D demonstrate an upregulation of genes whose expression is reported to be upregulated in cancerous tissue (Ghosh *et al.*, 2005). This further demonstrates the limitations of culturing cells in a 2D environment and further stresses the need for more accurate models in which to study cell differentiation *in vitro*.

Expression of soluble factors and cytokines

Combined, results from this study clearly demonstrate that MSCs cultured on the scaffold display enhanced functionality in their ability to undergo osteogenic and adipogenic differentiation when compared to MSCs cultured on tissue culture plastic. However, whilst such observations are not totally unsurprising, with many studies reporting enhanced cellular viability and functionality following culture on 3D surfaces and scaffolds, the large majority of studies end their argument there and do not proceed to examine the molecular changes that occur in cells cultured on 3D to bring about such improvements. Experiments in this study aimed to address this. *In vivo*, osteogenic and adipogenic differentiation are influenced by autocrine and paracrine mechanisms, whereby cells within their niche secrete a range of soluble factors and cytokines that are able to influence proliferation and differentiation (Baylink *et al.*, 1993; Spiegelman and Flier, 1996; Mohamed-Ali *et al.*, 1998; Pages *et al.*, 2000; Sykara and Opperman, 2003). Here, it is proposed that MSCs cultured on the scaffold demonstrate enhanced differentiation via an autocrine mechanism, with cells cultured on the scaffold upregulating a number of positive soluble factors and simultaneously decreasing expression of a number of inhibitory factors. Specific soluble factors to examine were selected based on literature searches and expression

was examined at the mRNA level using qRT-PCR. Although mRNA levels are not always indicative of actual protein levels and give no information as to whether the protein is actually secreted or not, it is advantageous in that it provides a rapid and straightforward technology in which to screen many factors. Changes at the transcriptional level are useful in themselves as indicators of cellular activity.

MSCs cultured under osteogenic conditions on 2D and 3D demonstrated markedly different expression profiles of a number of key osteogenic factors at the mRNA level. TGF-β1, VEGF, IGF1, BMP2 and BMP4 were all upregulated in MSCs cultured on the scaffold compared to their equivalent cultures on tissue culture plastic, and these observations were significant at the statistical level. The osteogenic roles of these factors have all been highly documented in the literature. TGF-β1 is reported to be highly expressed in cells demonstrating an osteoblastic phenotype, with addition of free TGF-β1 to culture media resulting in further enhancement of osteogenic marker expression, such as ALP and osteocalcin (Long *et al.*, 1995; Eingartner *et al.*, 1999; Gosain *et al.*, 2000; Weiss *et al.*, 2002; Simionescu *et al.*, 2005). It is also highly expressed during distraction osteogenesis, a surgical procedure designed to lengthen long bones (Yates *et al.*, 2002). VEGF has also been reported to be a valuable soluble factor in the process of osteogenesis – however, as the majority of VEGF studies have been performed using *in vivo* models, such positive effects on osteogenesis have been attributed in main to increased angiogenesis, resulting in enhanced blood supply to osteoblastic tissue (Peng *et al.*, 2002; Weiss *et al.*, 2005; Casap *et al.*, 2008). Despite this, there has been some evidence to suggest that VEGF can function as a positive mediator of osteogenesis via a non-angiogenic mechanism and, indeed, VEGF has been reported to be produced and secreted during osteogenesis itself, being a key process in the promotion of bone nodule formation and mineralisation (Mayer *et al.*, 2005; Mandu-Hrit *et al.*, 2008). Although better known for its role in adipogenesis, IGF1 may also function as an osteogenic factor as it is highly expressed during distraction osteogenesis and is also reported to enhance cellular viability, which could be one mechanism to explain why cells cultured on the scaffold demonstrate enhanced viability compared to equivalent 2D cultures (Yates *et al.*, 2002; Pountos *et al.*, 2007; Jeong *et al.*, 2008). Exogenous application of IGF1 is also reported to exert a positive influence on osteoblastic activity of cells during distraction osteogenesis (Stewart *et al.*, 1999). The best characterised of all the soluble factors that influence osteogenesis

are, as their name suggests, the BMP family. MSCs cultured on the scaffold demonstrated a significant upregulation in expression of BMP2 compared to their 2D counterparts and BMP4 expression was found exclusively in MSCs cultured on the scaffold. BMP2 is a fundamental regulator of osteogenesis and has even been used in the clinic in the treatment of osteogenic defects, though high doses were required in order to observe any positive effects (Schwarz *et al.*, 2008). Further support for this comes from studies in which inhibition of BMP2 expression prevented bone nodule formation and matrix mineralisation (Hassan *et al.*, 2006; Bais *et al.*, 2009). Cheng and colleagues even go as far as claiming that BMP2 was the most potent inducer of osteogenesis out of 14 BMPs examined in their study (Cheng *et al.*, 2003). BMP4 is also an effective inducer of osteogenic differentiation, albeit not as efficacious as its family member, BMP2 (Li *et al.*, 1998; Yates *et al.*, 2002). MSCs cultured on 2D demonstrated significantly reduced levels of the above soluble factors at the mRNA level, providing compelling evidence to suggest that an autocrine mechanism was responsible, at least in part, for the enhanced differentiative effect observed when MSCs were cultured on the scaffold.

However, MSCs cultured on 2D did not show reduced expression of all the soluble factors examined. TNF-α and BMP7 were expressed at greater levels in MSCs cultured on 2D compared to 3D, although this observation was only significant at the statistical level for TNF-α ($p<0.05$). Interestingly, BMP7 is reported to play a role in the commitment and differentiation of MSCs to an osteoblastic-like phenotype, though the function of BMP7 in osteogenesis has been studied less extensively than its counterparts, BMP2 and BMP4 (Hassan *et al.*, 2006). Conversely, TNF-α has not been reported to exert a positive effect on osteogenesis, with several studies suggesting that TNF-α inhibits osteogenesis *in vitro*, an effect that can be reversed by blocking the TNF-α signalling cascade (Tomomatsu *et al.*, 2009). TNF-α has even been reported to induce osteoclastogenesis, though this effect is usually seen in combination with interleukin molecules such as IL-1β (Wang *et al.*, 2005). These results combined strongly suggest that increased expression of osteogenic-promoting soluble factors concomitant with a decrease in expression of the osteoclastic-promoting TNF-α in MSCs cultured on the scaffold result in enhanced expression of an osteoblastic phenotype in MSCs cultured on the scaffold compared to 2D counterparts. It is likely

that this phenomenon is a result of a combinatorial effect rather than just one individual factor or a couple of factors inducing this effect.

MSCs cultured under adipogenic conditions on the scaffold and tissue culture plastic also demonstrated markedly different expression profiles of a number of adipogenic factors at the mRNA level. IGF1, TNF-α, BMP2, BMP4 were all upregulated in MSCs cultured on the scaffold compared to their equivalent cultures on tissue culture plastic. However, although these differences in expression are quite apparent graphically, only BMP4 was upregulated at a statistically significant level. The adipogenic roles of these factors have all been highly documented in the literature. IGF1 is a well-characterised, potent inducer of adipogenesis and is significantly upregulated in cells undergoing adipogenic differentiation, playing a crucial role in the proliferation stage of adipogenesis (stage 1) (Wright and Hausman, 1995; Rajkumar *et al.*, 1999). It is such a potent inducer of differentiation that IGF1 is reported to be able to substitute for insulin in the traditional adipogenic cocktail and has also been suggested to function in an autocrine/paracrine fashion when in its free form (Smith *et al.*, 1988; Hausman, 1989; Blake and Clarke, 1990; Schmidt *et al.*, 1990; Tchouklova *et al.*, 2009). TNF-α, interestingly, is reported to be an anti-adipogenic factor, with one particular study demonstrating that treating mature adipocytes with TNF-α resulted in a loss of intracellular lipid material (Torti *et al.*, 1989). The effect was so striking that the cells appeared to have undergone a dedifferentiation process and resembled their undifferentiated precursors. Although their name may suggest that their biological role is exclusive to promoting osteogenic differentiation, BMPs are also known to be important determinants of adipogenic differentiation. BMP2 has frequently been reported to stimulate adipogenesis as well as osteogenesis (Zehentner *et al.*, 2000; zur Nieden *et al.*, 2004). However, an adipogenic role of BMP2 is somewhat controversial, with some studies reporting BMP2 to be inhibitory to adipogenic differentiation, whilst another study reports that BMP2 strongly stimulates adipogenic in the presence of other adipogenic-inducing factors but is incapable of doing so on its own (Gimble *et al.*, 1995; Gori *et al.*, 1999; Sottile and Seuwen, 2000). A dual-role of BMP2 in osteogenesis and adipogenesis has been proposed to occur as a result of BMP2 acting through different receptor units for each process (Wang *et al.*, 1993; Asahina *et al.*, 1996; Chen *et al.*, 1998; Kang *et al.*, 2009). The role of BMP4 in adipogenesis is much more straightforward. There is compelling evidence to suggest that BMP4 is a key

factor in adipogenesis, as BMP4-producing cells convert into adipocytes at high frequency and efficiency (Tang *et al.*, 2004; Bowers *et al.*, 2006; Taha *et al.*, 2006; Bowers and Lane, 2007; Kang *et al.*, 2009). As MSCs cultured on the scaffold demonstrate upregulation of a number of positive regulators (albeit only BMP4 at a statistically significant level), a similar argument holds here that, as for osteogenesis, an autocrine mechanism was responsible, at least partially, for the enhanced effect on differentiation observed when MSCs were cultured in 3D.

However, MSCs cultured on 2D showed enhanced expression of TGF-β1 and BMP7 compared to their 3D counterparts, and these differences were significant at the statistical level in both instances ($p<0.01$). TGF-β1 is a potent inhibitor of adipogenesis (Serrero and Mills, 1991; Bortel *et al.*, 1994; Choy *et al.*, 2000; Choy and Derynck, 2003; Turner *et al.*, 2008). BMP7, displaying a similar expression profile to TGF-β1, is reported to be involved in adipogenesis (Choy *et al.*, 2000; Neumann *et al.*, 2007; Kang *et al.*, 2009). However, it is reported that BMP7 promotes differentiation of cells into brown adipocytes as opposed to white adipocytes, as is the case for all other members of the BMP family (Tseng *et al.*, 2008). The decrease in expression in TGF-β1 is likely to be a contributing factor to the increased adipogenic effect in MSCs cultured on the scaffold.

Overall summary

MSCs cultured in 3D using Alvetex^{TM}, a novel, polystyrene scaffold, demonstrate enhanced viability and functionality in their ability to undergo osteogenic and adipogenic differentiation. Current tissue culture practise consists, on the main, of culturing cells in 2D plastic flasks, an environment which is far removed from the complexities of their niche *in vivo*. Considerations of cell shape and morphology, as well as interactions with other cells, is an aspect of cell biology that is often overlooked despite the critical role of such factors in influencing cell behaviour. For example, osteogenic and adipogenic differentiation of MSCs has been reported to be significantly controlled by cell shape and cytoskeletal tension (Sordella *et al.*, 2002; McBeath *et al.*, 2004; Jakkaraju *et al.*, 2005; Meyers *et al.*, 2005). As such, current knowledge obtained from studying osteogenic and adipogenic differentiation in a 2D environment may not be entirely accurate and there is an urgent need for scaffolds that can be used for routine *in vitro* culture which allow cells to adopt conformations and

form interactions that are more representative of those that occur *in vivo*. Although culturing cells in such scaffolds often results in increased cellular viability and functionality, little work has been done at present to determine what molecular changes, if any, are occurring in cells following this 3D culture. This study addresses that issue, providing evidence to suggest that MSCs cultured on the scaffold undergo enhanced differentiation, at least in part, as a result of increased autocrine behaviour.

Chapter 4

MSCs FUNCTION AS PARACRINE MEDIATORS OF NEURAL DIFFERENTIATION *VIA* THE RELEASE OF TROPHIC FACTORS AND CYTOKINES

4.1 Introduction

For many years it was largely believed that the adult mammalian brain only had a very limited capacity for regeneration following injury. Neurogenesis – the process by which new neurons are generated in the nervous system – was thought to be restricted to the developing brain in keeping with Cajal's central dogma of 'no new nerve cells after birth' (Ramón y Cajal, 1928; Ehninger and Kempermann, 2008). However, Cajal's views are now known to be somewhat inaccurate, with a study being published as early as 1965 providing evidence to suggest that neurogenesis is a constitutively-occurring process in the hippocampus of postnatal rats (Altman and Das, 1965). Although this was a pivotal study at the time, very little progress was made in terms of understanding this phenomenon until the development of more sophisticated techniques in the 1990s resulted in significant advances (von Bohlen and Halbach, 2007). Currently, it is widely accepted that neurogenesis occurs in two distinct regions of the adult brain – the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Eriksson *et al.*, 1998; Gould *et al.*, 1999; Garcia *et al.*, 2004; Maslov *et al.*, 2004; Ehninger and Kempermann, 2008). In both instances, neurogenesis and the development of new neurons has been attributed directly to the neuronal differentiation of neural stem and progenitor cells residing in nearby niches. For example, neural stem and progenitor cells located in the SVZ enter a rostral migratory stream and eventually differentiate into neurons in the olfactory bulb, whereas neural stem and progenitor cells located in the SGZ undergo proliferation and migration to the granule cell layer where they eventually differentiate into hippocampal granule cells (Altman and Das, 1965; Stanfield and Trice, 1988; Goldman, 1995; Kuhn *et al.*, 1996). Interestingly, although neural stem and progenitor cells from the SGZ and SVZ occupy different niches *in vivo* i.e. the hippocampus and lateral ventricles of the adult brain respectively, they appear to demonstrate a degree of functional interchangeability in that hippocampal progenitors demonstrate the potential to migrate to the olfactory bulb and differentiate into olfactory bulb neurons following grafting into the rostral migratory stream (Suhonen *et al.*, 1996). Such a key observation suggests that neural stem and progenitor cells isolated from the SGZ and SVZ are either a) extremely closely related to one another phenotypically and are able to respond in a similar manner to the same environmental cues or b) possess a greater plasticity than previously anticipated.

Although the vast majority of original research articles and review articles state that the SGZ and SVZ are the only regions of the adult brain retaining the capacity for neurogenesis, several studies have been published reporting that neurogenesis can occur in many other regions of the adult brain, including the amygdala, neocortex, striatum, subcallosal zone and substantia nigra (Gould *et al.*, 1996; Bernier *et al.*, 2002; Zhao *et al.*, 2003; Van Kampen *et al.*, 2004; Bedard *et al.*, 2006; Seri *et al.*, 2006). That said, rates of neurogenesis observed in these areas of the adult brain are substantially lower than those rates observed in the SGZ and SVZ (von Bohlen and Halbach, 2007).

The existence of neural stem and progenitor cells throughout the SGZ and SVZ of the developed adult mammalian brain is supported by several fundamental experimental observations. Primary *in vitro* cultures of phenotypically-immature cells isolated from the adult mammalian brain demonstrated a high capacity for proliferation in the presence of mitogens such as EGF and FGF and underwent differentiation to express a more mature neural phenotype following removal of these growth factors from the culture media (Reynolds and Weiss, 1992; Kukekov *et al.*, 1999). Retroviral tracing studies also demonstrated that a specific subpopulation of cells within the SGZ and SVZ always remained within the proliferative hotspots of these structures and cell cycle analysis allowed this cellular subpopulation to be further divided into one of two categories: a slowly dividing population (approximately 15 days) and a rapidly dividing population (<15 hours) (Morshead *et al.*, 1994; Tropepe *et al.*, 1997; Morshead *et al.*, 1998; Doetsch *et al.*, 1999). A slow rate of cell division is akin to a stem cell phenotype whilst a rapid rate of cell division is more akin to a progenitor phenotype, therefore by extension the slowly dividing cells were considered to belong to the neural stem cell population and the rapidly dividing cells were considered to belong to the neural progenitor cell population. The most crucial observation of all, however, was that after administration of cytarabine (Ara-C) to target and eliminate the rapidly dividing population, the rapidly dividing population was regenerated following subsequent removal of Ara-C from the culture media (Doetsch *et al.*, 1999). This observation offers substantial support for the asymmetric division model discussed by Gage in his recent review (2000), whereby neural stem cells in the brain are largely quiescent in nature and undergo asymmetric division to produce two phenotypically and functionally distinct daughter cells – a stem cell phenotypically

identical to the mother cell (self-renewal) and a more lineage-restricted but rapidly proliferating progenitor cell (Michalopoulos and DeFrances, 1997; Zhu and Watt; 1999; Gage, 2000). Although progenitor cells are rapidly proliferating they are only able to divide for a limited number of cycles after which they undergo terminal differentiation to produce more mature, fully-functioning neural cell types. Therefore, in Doetsch's study described above, the rapidly dividing population is regenerated by the slowly dividing population as a consequence of asymmetrical cell division.

Significant progress has been made over the last decade or so in terms of increased knowledge and understanding of neural stem cell biology within the adult mammalian brain. Intensity surrounding such research continues to escalate for a number of reasons. Neural stem and progenitor cells can be propagated as *in vitro* cultures, providing a crucial research tool with which to study complex biological phenomena such as neural differentiation and development. Perhaps more interestingly, however, is the very realistic possibility that neural stem and progenitor cells within the adult brain may be exploited as potential targets of regenerative therapy in the treatment of various neurological and neurodegenerative disorders (Reynolds and Weiss, 1992; Richards *et al.*, 1992; Johansson *et al.*, 1999). Such a distinct possibility is exciting in a system that, for many years, was considered incapable of regeneration following neurological injury/deficit.

As modern medical advances have resulted in an ever-ageing population, there is an increasing demand for new, more effective therapies against age-related neurological and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, amongst many others (Ormerod *et al.*, 2008). Such disorders are notoriously difficult to treat effectively due to the significantly varying pathologies between disorders and even between individuals with the same disorder and, as such, current treatment strategies are largely inadequate. An example is the treatment of Parkinson's disease. The causative mechanism of Parkinson's disease is substantial damage/death of dopaminergic neurons within the substantia nigra pars compacta region of the brain (Chiocco *et al.*, 2007). Current clinical intervention involves administration of the neurotransmitter dopamine to replace that lost as a consequence of dopaminergic neuron damage/death. However, although administration of dopamine results in a transient amelioration of clinical symptoms, it fails to halt/slow the

progression of the disease. Therefore, if the complex mechanism(s) by which neural stem and progenitor cells differentiate into dopaminergic neurons could be elucidated, then the distinct possibility arises whereby either a) neural stem/progenitor cells are induced to undergo dopaminergic differentiation *in vitro* followed by subsequent engraftment into the damaged region of the brain or b) endogenous neural stem/progenitor cells are promoted to undergo dopaminergic differentiation *in vivo*.

A more comprehensive understanding of the complex cellular and molecular mechanisms of neural differentiation and a greater appreciation of the niche-specific signals governing stem cell behaviour *in vivo* are required if promotion of endogenous repair is to become a viable clinical option (Emsley *et al.*, 2005). The neural stem cell niche is composed many different structural and functional components such as neurons, astrocytes, oligodendrocytes, microglia, immune cells, vasculature and extracellular matrix, with each of these components interacting extensively in order to influence the behaviour of neural stem cells (Mercier *et al.*, 2002). Astrocyte- and vasculature-derived factors are thought to be particularly important in maintenance/regulation of neural stem cell function (Palmer *et al.*, 2000; Jin *et al.*, 2002; Song *et al.*, 2002; Wurmser *et al.*, 2004; Shapiro *et al.*, 2005; Barkho *et al.*, 2006; Plumpe *et al.*, 2006; Ehninger and Kempermann, 2008).

In their review, Ormerod and colleagues propose three potential mechanisms by which stem cell transplantation may be used as a regenerative therapy for neurological and neurodegenerative disorders:

1. Stem cells could act as delivery vehicles for soluble factors and cytokines to modulate biological activity of endogenous cell types

2. Stem cells could undergo differentiation into mature neural cell types to directly replace the cells lost/damaged as a consequence of disease

3. Stem cells could be differentiated into neural cell types *in vitro* followed by transplantation into a network to aid construction of neural circuitry

It is the first mechanism that is of principal interest to this thesis. MSCs have been previously shown to be able to significantly influence cellular differentiation via the expression of soluble factors and cytokines (Chapter 3). Similarly, the inaccessibility of neural stem and progenitor cells within the adult brain concomitant with the considerable technical challenges of culturing neural cells *in vitro* means that alternative stem cell sources are highly desirable from a clinical perspective. Many studies (Chapter 1) have previously shown that MSC transplantation in numerous models of neurological and neurodegenerative disorders results in beneficial effects and functional recovery to a certain degree. The mechanism behind such beneficial effects, though still controversial to an extent, is largely considered to be the result of a paracrine relationship between transplanted MSCs and host neural stem cells in that MSCs release a plethora of soluble factors and cytokines that direct the proliferation/migration/differentiation of endogenous neural stem cells. MSCs have been reported to synthesise and secrete a wide range of neurotrophins and neurotrophic factors both *in vitro* and *in vivo* and cellular-mediated delivery of such factors rather than exogenous administration may be advantageous as neurotrophins are large peptides that diffuse poorly through the blood brain barrier (Ebadi *et al.*, 1997). An increased understanding of the paracrine relationship between MSCs and neural stem/progenitor cells would aid in the development of strategies to promote endogenous repair following neurological damage in the adult brain.

The present study investigated the paracrine function(s) of MSCs in terms of their effect(s) on neural development and plasticity using an *in vitro* conditioned media (CM) model. Studies in Chapter 3 clearly demonstrate that MSCs behave differently when cultured in 3D compared to 2D. An alternative, scaffold-free method of 3D cell culture involves encouraging cells to form large, multicellular aggregates in order to promote structural interactions and re-establishment of mutual contacts between cells that are more representative of those that occur *in vivo* (Pampaloni *et al.*, 2007). MSCs cultured as large, multicellular aggregates share many of the phenotypic properties of neural stem cell-derived neurospheres, and it has been demonstrated that MSCs cultured as 3D aggregates alter their secretory profile accordingly (Hermann *et al.*, 2004; Croft and Przyborski, 2009). In the present study, MSCs cultured as 3D aggregates were allowed to condition media for 3 days, after which CM was collected and used in the downstream culture of neural stem cells. Neural stem and progenitor

cells isolated from both adult and fetal brains have been propagated as genetically stable cell lines, making them extremely useful and a highly convenient tool for use in an *in vitro* setting (Palmer *et al.*, 1997; Svendsen *et al.*, 1998; Carpenter *et al.*, 1999; Vescovi *et al.*, 1999). The cell line used in the studies performed in this thesis was the HCN-nitGFP adult hippocampal progenitor cell (AHPC) line, originally propagated by and a generous gift from Fred Gage (Salk Institute). Although primary cultures are often advantageous and considered to be a more physiologically accurate system in which to perform studies, the AHPC cell line is beneficial in that it has been extensively characterised, highly used in our lab and can be cultured as a monolayer, which is much less technically demanding than culturing primary adult neural stem/progenitor cells as neurospheres. Similarly, isolation of neural stem/progenitor cells from primary sources is extremely technically challenging e.g. ensuring dissection of the same region of brain each time, a lack of standardisation means that culture conditions employed vary significantly between different laboratories and the strain of rodent is rarely controlled for (Gage, 2000). The influence of MSC aggregate-derived soluble factors on AHPC fate was investigated at both the cellular and molecular level in this study.

Hypothesis

MSCs cultured as large, multicellular 3D aggregates secrete a specific complement of soluble factors and cytokines that influence the cell fate decisions of neural stem and progenitor cells.

Aims of chapter

To culture MSCs as multicellular spherical aggregates and examine the effect of soluble factor(s) secrete by such aggregates in terms of their ability to influence the cell fate decisions of the AHPC cell line.

Objectives

1. Culture MSCs as multicellular 3D aggregates and examine the phenotype of such aggregates in terms of morphology and marker expression

2. Examine the soluble factor(s) produced by aggregates by allowing them to condition media for 3 days and using this CM in the subsequent culture of AHPCs

3. Examine the cellular mechanism(s) by which soluble factor(s) in CM were bringing about their effect

4. Examine the molecular mechanism(s) by which soluble factor(s) in CM were bringing about their effect

4.2 Materials and methods

4.2.1 Formation of 3D cellular aggregates by MSCs

MSCs were induced to form 3D cellular aggregates using a previously published protocol (Hermann et al., 2004; Hermann et al., 2006a). Briefly, MSCs were maintained and expanded as monolayer cultures under standard conditions, as described in Section 2.1. When confluency was reached, cells were detached from the cellware by washing in PBS x 1, followed by incubation with 0.25% trypsin and 0.1% EDTA for 5-10 minutes at 37^{o}C. Trypsin was inactivated by the addition of complete culture medium (CCM) to the tissue culture flask. Cells were centrifuged at 250 x *g* for 5 minutes, the supernatant removed, and cells resuspended in 1ml of aggregate induction medium (AIM: DMEM:F12, supplemented with N2 supplement (1x), L-glutamine (12μM), penicillin (100 U/ml), streptomycin (100 μg/ml), heparin (sodium salt, 40ng/ml), EGF (10ng/ml) and FGF (10ng/ml). Cells were seeded into normal cellware at a density of 1-2 x 10^5 cells/cm^2 and incubated at 37^{o}C, 5% CO_2 and in a low O_2 (5%) environment. Aggregate formation on the surface of the tissue culture plastic was apparent within 1-3 days, and with continued culture aggregates enlarged in size and eventually lifted off the cellware surface (within approximately 5-14 days) to exist as free-floating 3D aggregates in the media. For routine culture of aggregates, growth factors (EGF and FGF) were replenished twice a week, and medium was changed once a week.

4.2.2 Production and processing of conditioned media by 3D MSC aggregates

As described in Section 4.2.1, MSCs can be induced to form cellular aggregates when cultured under appropriately-defined conditions, namely the removal of serum and addition of growth factors (EGF and FGF) to the culture medium. In order to examine the soluble factors and/or cytokines produced and secreted by these aggregates, and determine the effect(s) (if any) on the cell fate decisions of neural stem/progenitor cells, a conditioned media approach was adopted. MSCs were seeded out for aggregate formation into T25s as described in Section 4.2.1 (each T25 contained 8ml media) and allowed to condition the medium for 3 days, with day 0 starting from the initial seeding of the MSCs from standard culture conditions to aggregate-inducing conditions. After 3 days, the conditioned medium was removed from the flask,

centrifuged at 2000rpm for 5 minutes to remove cell debris, supernatant removed and filtered using a 0.22μm pore filter and stored at -20^{o}C until required.

4.2.3 Adult hippocampal progenitor cell (AHPC) culture with conditioned medium (CM)

AHPCs were maintained and expanded as monolayer cultures under standard conditions, as described in Section 2.2. When a confluency of 70-80% was reached, cells were detached from the cellware by quickly rinsing with 0.25% trypsin and 0.1% EDTA for 1-2 minutes at 37^{o}C and subsequently seeded out a density of 10,000 cells/cm^{2} into cellware coated with poly-L-ornithine (10μg/ml) and laminin (5μg/ml), in N2 medium, and allowed 24 hours to settle (designated day –1). Following this 24 hour settling period (day 0), medium was removed and replaced with CM or appropriate control medium (UC: unconditioned medium; PC: proliferation control) and AHPCs cultured for a further 7 days. Media were replenished on the 4th day. UC medium consisted of DMEM:F12, N2 supplement (1x), L-glutamine, (12μM), penicillin (100 U/ml), streptomycin (100 μg/ml) in the absence of the growth factors EGF and FGF, as mass spectrometric analysis of CM revealed that these growth factors were depleted during the 3 day conditioning process (data not shown).

4.2.4 Analysis of cell proliferation and cell death using flow cytometry

To assess cell proliferation in AHPC cultures at days 4 and 7, 10μM 5-bromo-2-deoxyuridine (BrdU) was added to the culture medium for a period of 24 hours prior to these timepoints i.e. at days 3 and 6. Following this, cells were then washed in PBS x 1, fixed in 2% PFA overnight at 4^{o}C and processed for dual staining as follows. Cells were spun down at 2000rpm for 5 minutes and the supernatant removed. Pellets were resuspended gently in 1ml permeabilising solution (0.2% Triton-X in PBS) and incubated at 37^{o}C for 30 minutes. Then, 1ml FACS buffer (2% NGS and 0.2% Tween in PBS) was added, the cells spun down at 2000rpm for 5 minutes, the supernatant removed and the pellet gently resuspended in 2M HCl and incubated for 30 minutes at room temperature, to unravel DNA to form single strands. Following this, cells were spun down at 2000rpm for 5 minutes, the supernatant removed and cells washed in 0.5M borate buffer (pH 8.5) for 10 minutes at room temperature, followed by additional washes in FACS buffer x 3. Cells were then dual-stained with primary

antibodies in the following combinations: anti-Tuj1/anti-BrdU, anti-GFAP/anti-BrdU and anti-GalC/anti-BrdU overnight at 4^{o}C. Primary antibodies for Tuj1, GFAP and GalC were used at dilutions previously detailed, and the primary antibody for BrdU was used at a dilution of 1:40. Following this incubation, cells were then washed in FACS buffer x 3 and stained with appropriate fluorochrome-conjugated secondary antibodies for 30 minutes at room temperature. Tuj1 was detected with an APC-labelled anti-rabbit secondary antibody (1:300), GFAP and GalC were detected with an APC-labelled anti-mouse secondary antibody (1:600) and BrdU was detected with a PE-labelled secondary antibody (1:600). Following this incubation, cells were then washed in FACS buffer x 3 and subsequently processed for flow cytometric analysis. PE-fluorescence was detected in the FL2 band pass filter and APC-fluorescence detected in the FL4 band pass filter, and the fraction of cells exhibiting positive staining for both BrdU and each of the neural markers Tuj1, GFAP and GalC was determined. 10,000 events were determined for each condition.

To assess cell death in AHPC cultures at days 4 and 7, 50μg/ml propidium iodide (PI) was added to the culture medium for a period of 10 minutes in the dark. Following this, cells were then washed in PBS x 1, fixed in 2% PFA overnight at 4^{o}C and processed for staining as follows. (Note – it was crucial to get cells floating in the media as well as adherent cells to assess cell death). Cells were spun down at 2000rpm for 5 minutes and the supernatant removed. Pellets were resuspended gently in 1ml permeabilising solution (0.2% Triton-X in PBS) and incubated at 37^{o}C for 30 minutes. Then, 1ml FACS buffer (2% NGS and 0.2% Tween in PBS) was added, the cells spun down at 2000rpm for 5 minutes and the supernatant removed. Cells were then stained with primary antibodies directed against Tuj1, GFAP and GalC overnight at 4^{o}C at the dilutions previously described. Following this incubation, cells were then washed in FACS buffer x 3 and stained with appropriate fluorochrome-conjugated secondary antibodies for 30 minutes at room temperature. Tuj1 was detected with an APC-labelled anti-rabbit secondary antibody, and GFAP and GalC were detected with an APC-labelled anti-mouse secondary antibody, at the dilutions previously described. Following this incubation, cells were then washed in FACS buffer x 3 and subsequently processed for flow cytometric analysis. PI-fluorescence was detected in the FL2 band pass filter and APC-fluorescence detected in the FL4 band pass filter, and the fraction of cells exhibiting positive staining for both PI and each of the neural

markers Tuj1, GFAP and GalC was determined. 10,000 events were determined for each condition.

4.2.5 Analysis of apoptosis in GFAP$^+$ and GalC$^+$ AHPC subpopulations using flow cytometry

To assess apoptosis in GFAP$^+$ and GalC$^+$ subpopulations of AHPCs at days 4 and 7, cells were washed in PBS x 1, fixed in 2% PFA overnight at 4^{o}C and processed for staining as follows. (Note – it was crucial to get cells floating in the media as well as adherent cells to assess cell death). Cells were spun down at 2000rpm for 5 minutes and the supernatant removed. Pellets were resuspended gently in 1ml permeabilising solution (0.2% Triton-X in PBS) and incubated at 37^{o}C for 30 minutes. Then, 1ml FACS buffer (2% NGS and 0.2% Tween in PBS) was added, the cells spun down at 2000rpm for 5 minutes and the supernatant removed. Cells were then dual-stained with primary antibodies in the following combinations: anti-GFAP/anti-caspase 3 and anti-GalC/anti-caspase 3, and incubated overnight at 4^{o}C. Primary antibodies for GFAP and GalC were used at dilutions previously detailed, and the primary antibody for caspase 3 was used at a dilution of 1:200. Following this incubation, cells were then washed in FACS buffer x 3 and stained with appropriate fluorochrome-conjugated secondary antibodies for 30 minutes at room temperature. GFAP and GalC were detected with an APC-labelled anti-mouse secondary antibody and caspase 3 was detected with a PE-labelled anti-rabbit secondary antibody, at the dilutions previously detailed. Following this incubation, cells were then washed in FACS buffer x 3 and subsequently processed for flow cytometric analysis. PE-fluorescence was detected in the FL2 band pass filter and APC-fluorescence detected in the FL4 band pass filter, and the fraction of cells exhibiting positive staining for both caspase 3 and each of the neural markers, GFAP and GalC, was determined. 10,000 events were determined for each condition.

4.2.6 Analysis of early- and late-apoptosis in 33B cells using flow cytometry

To assess apoptosis in 33B cells following culture with CM media and appropriate controls (UC) for 4 and 7 days, cells were incubated with 50μg/ml propidium iodide (PI) for a period of 10 minutes in the dark. Following this, cells were then washed in PBS x 1, fixed in 2% PFA overnight at 4^{o}C and processed for staining as follows. (Note – it was crucial to get cells floating in the media as well as adherent cells to assess cell death). Cells were spun down at 2000rpm for 5 minutes and the supernatant

removed. Pellets were resuspended gently in 1ml permeabilising solution (0.2% Triton-X in PBS) and incubated at 37^{o}C for 30 minutes. Then, 1ml FACS buffer (2% NGS and 0.2% Tween in PBS) was added, the cells spun down at 2000rpm for 5 minutes and the supernatant removed. Cells were than stained with an anti-caspase 3 primary antibody (1:200) overnight at 4^{o}C. Following this incubation, cells were then washed in FACS buffer x 3 and stained with an APC-labeled anti-rabbit secondary antibody (1:300) for 30 minutes at room temperature. Following this incubation, cells were then washed in FACS buffer x 3 and subsequently processed for flow cytometric analysis. PI-fluorescence was detected in the FL2 band pass filter and APC-fluorescence detected in the FL4 band pass filter, and the fraction of cells exhibiting positive staining for caspase 3 alone and caspase-3/PI-dual staining was determined. 10,000 events were determined for each condition.

4.3 Results

4.3.1 MSCs can be induced to form cellular aggregates when cultured under serum-free conditions in the presence of mitogens, EGF and FGF

The principal aims of the studies described in Chapter 3 were to investigate the effect(s) of culturing MSCs in the 3D environment of the novel polystyrene scaffold, Alvetex™. These studies clearly demonstrated that MSCs cultured in 3D displayed markedly different behavioural traits, in terms of cellular viability and potential to undergo osteogenic and adipogenic differentiation, compared to their equivalent cultures on the 2D environment of tissue culture plastic. As such, culturing cells in 3D is advantageous in that it provides a more realistic and physiologically-relevant system in which to study complex biological phenomena such as differentiation.

An alternative method of culturing cells in 3D involves encouraging them to form cellular aggregates. The formation of such aggregates *in vitro* has been reported to result in the re-establishment of mutual contacts between cells which better represent cell-cell interactions that exist *in vivo* (Pampaloni *et al.*, 2007). This method of 3D culture is advantageous in that it does not require external scaffolds, such as the polystyrene scaffold used in Chapter 3, and the methodology of inducing cells to form aggregates is relatively simple. Many techniques employed in the literature exploit the ability of cells to naturally aggregate with one another when cultured using the hanging drop technique or, similarly, when cultured in rotating vessels (Pampaloni *et al.*, 2007). However, in the present study a slightly different methodology was adopted. A number of studies have reported that MSCs can be induced to form cellular aggregates when cultured under identical conditions to those used for the propagation of neurospheres from neural stem cells (Croft and Pryzborski, 2004; Hermann *et al.*, 2004; Bossolasco *et al.*, 2005; Hermann *et al.*, 2006). It is this technique that has been employed in the present study and is described in Section 4.2.1.

Figure 4.1 shows MSCs cultured under standard and aggregate-inducing conditions. Under standard culture conditions, MSCs demonstrated a large, flat, fibroblastic morphology as previously reported in Section 3.3.1 (Figure 4.1A). However, when switched to culture conditions described for the propagation of neurospheres from neural stem cells, MSCs formed cellular aggregates that were morphologically

indistinguishable from neural stem cell-derived neurospheres (Figure 4.1B and 4.1C). Aggregate-inducing culture conditions are described in detail in Section 4.2.1 and involve seeding MSCs at a high density (1-2 x 10^5 cells/cm^2) in serum-free (N2) media in the presence of mitogens (EGF and FGF).

Within 24-72 hours, small spherical aggregations of cells were evident on the surface of the cellware (Figure 4.1B). However, not all of the seeded cells formed aggregates, with a small proportion of adherent cells forming flat morphologies as per standard culture, though the large majority of cells did form aggregates to the extent that multiple aggregates were present in each field of view, as illustrated in Figure 4.1B. With continued culture and further addition of mitogens to the culture media, cells within the aggregates continued to proliferate and aggregates continued to enlarge in size, eventually lifting off the cellware surface and forming free-floating aggregates suspended in the culture media (Figure 4.1C). This typically occurs between 5 and 14 days. Free-floating aggregates remained highly viable in suspension and with prolonged culture began to co-aggregate with each other (Figure 4.1C).

4.3.2 3D MSC aggregates demonstrate an increase in expression of neural antigens and a decrease in expression of mesenchymal antigens compared to standard 2D cultures

Culturing MSCs under aggregate-forming conditions such as those detailed in Section 4.2.1 is reported to result in the formation of spherical cellular masses that are morphologically indistinguishable from neural stem cell-derived neurospheres (Bossolasco *et al.*, 2005). Morphological images of such MSC-derived aggregates are shown in Figure 4.1. One of the key, unique properties of MSC aggregates is that they express high levels of neural antigens such as nestin, Tuj-1 and GFAP, concomitant with a decrease in expression of characteristic MSC markers such as α-SMA (Kabos *et al.*, 2002; Croft and Przyborski, 2004; Hermann *et al.*, 2004; Suzuki *et al.*, 2004; Hermann *et al.*, 2006; Kim *et al.*, 2006). In order to examine this behaviour in the present study, expression of the neural markers, nestin and GFAP, and the mesenchymal marker, α-SMA, were examined via immunocytochemical and flow cytometric methods.

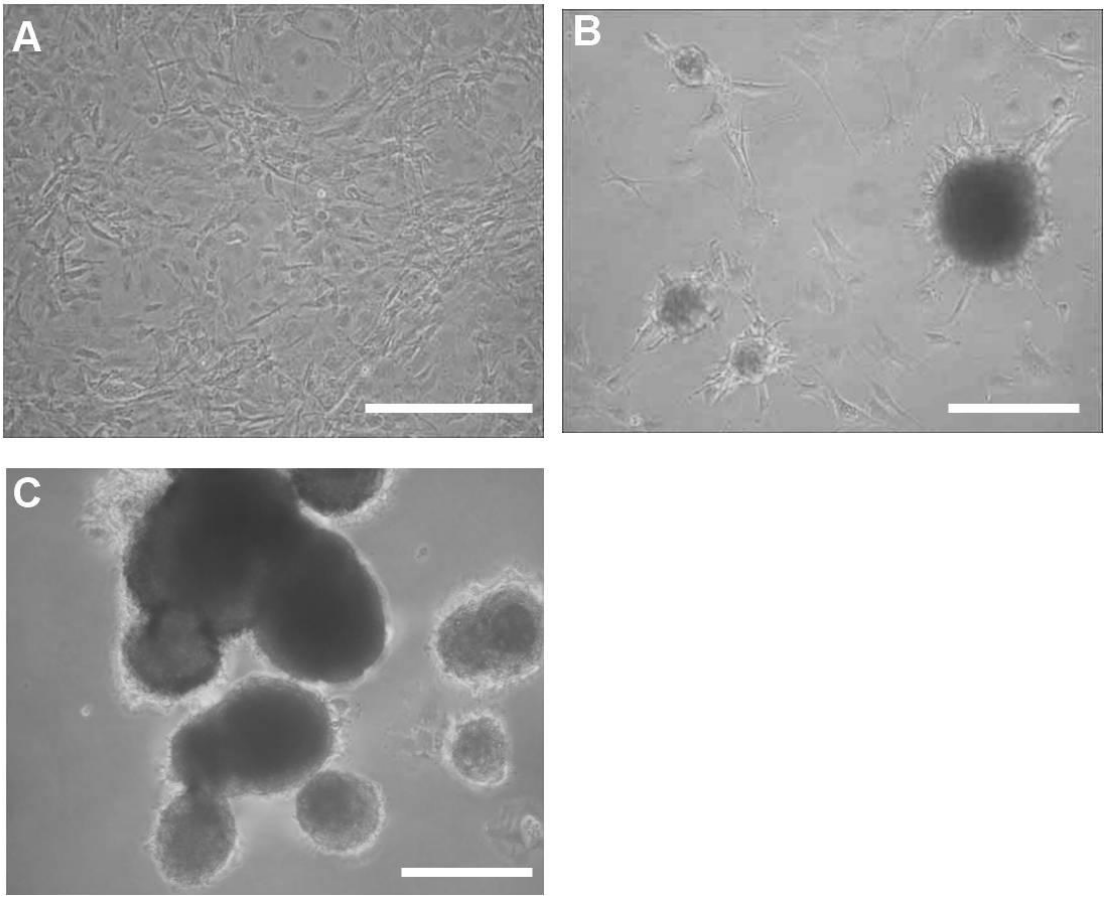

Figure 4.1. Formation of cellular aggregates by MSCs. Under defined conditions, namely serum removal and addition of mitogens to the growth media, MSCs can be induced to form large cellular aggregates. Under standard culture conditions MSCs exist as large, flat cells in a monolayer (**A**). Upon aggregate inducing conditions, they form cellular aggregates, initially on the surface of the cellware (**B**), though as these aggregates continue to grow and enlarge, they eventually lift off the cellware surface and exist in suspension. These free-floating aggregates are perfectly viable and continue to grow, eventually co-aggregating with one another (**C**). Scale bars = 100µm.

Aggregates were fixed directly as whole aggregates by incubation in 4% PFA overnight at 4^{o}C and processed for immunocytochemistry against nestin, GFAP and α-SMA as described in Section 2.7. The neural markers, nestin and GFAP, demonstrated intense levels of staining in aggregates, suggesting very high levels of expression (Figure 4.2B and 4.2D). This is consistent with previous studies. However, expression of nestin and GFAP could not be detected in MSCs cultured under standard conditions (data not shown), demonstrating that aggregates were upregulating expression of neural antigens compared to standard cultures. Expression of the mesenchymal marker, α-SMA, was also readily detectable in aggregates, though levels of staining were far less intense than those observed for neural markers (Figure 4.2F). MSCs cultured

under standard conditions demonstrated intense levels of α-SMA expression, as shown previously in Figure 3.3.

To confirm these immunocytochemical observations, expression of the previous markers in MSC-derived aggregates were also examined using flow cytometric methods (Figure 4.3). Flow cytometry circumvents potential problems associated with staining aggregates as whole structures – such as the possibility of false negative results occurring due to antibody entrapment within the aggregate rather than specific staining – as the methodology first requires aggregates to be dissociated into a single cell suspension. This was achieved enzymatically using trypsin. Once a single cell suspension had been achieved, cells were fixed in 2% PFA overnight at 4^{o}C and processed for flow cytometry as detailed in Section 2.6. This method of analysis allows more confidence as the expression data achieved is at the individual cellular level. Cells derived from MSC aggregates demonstrated positive expression of the neural markers nestin and GFAP and, to a lesser extent, α-SMA, which is consistent with the immunocytochemical observations (Figure 4.3).

4.3.3 MSC-derived aggregates secrete soluble factor(s) and cytokine(s) that influence cell fate decisions of neural stem and progenitor cells

There is an abundance of evidence in the literature to suggest that MSC-based cellular therapies may provide a means of treating neurological and neurodegenerative disorders (Section 1.4.1). Although the significant improvements in neurological function observed in such studies were undoubted, the mechanism(s) via which MSCs elicited this recovery remained (and still remain to this date) a subject of intense debate. Proposed mechanisms included: a) *'trans'*-differentiation; b) cell fusion; and c) stimulation of endogenous repair, and all three mechanisms are discussed in detail in Section 1.6.3. However, it is now becoming more commonly accepted that the main mechanism by which MSCs promote functional recovery of neurological deficits is via the release of trophic factors and cytokines that stimulate endogenous repair processes in the host (Chen *et al.*, 2001; Chen *et al.*, 2002; Chen *et al.*, 2004; Mahmood *et al.*, 2005). Such endogenous processes are likely to occur via the effect(s) of MSC-derived soluble factors on resident neural stem cells. Indeed, it has been reported that MSC transplantation results in an increase in the proliferation and migration of neural stem cells in the transplant recipient (Munoz *et al.*, 2005).

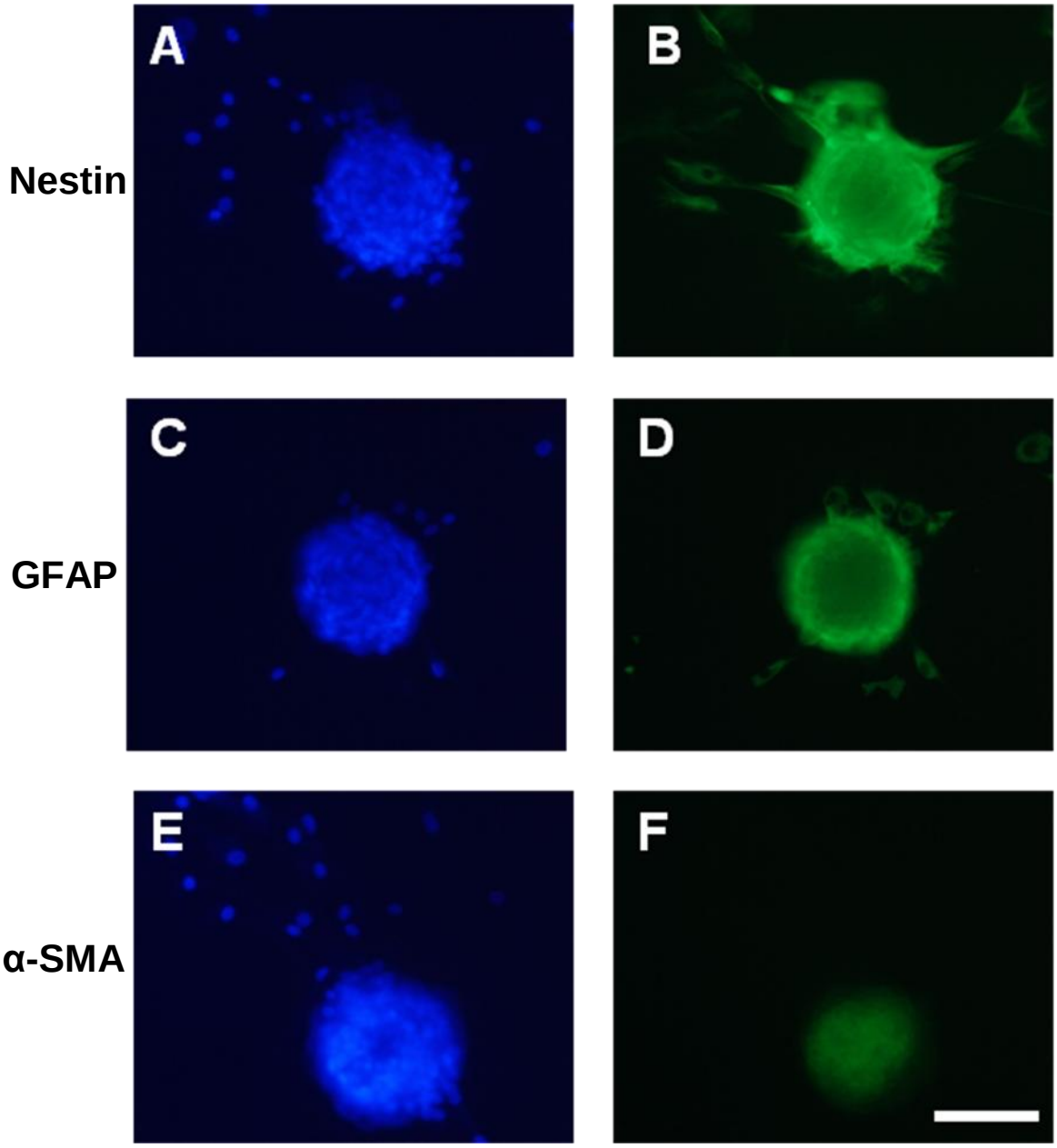

Figure 4.2. Immunocytochemical analysis of expression of neural and mesenchymal markers in MSC aggregates. Images **A**, **C** and **E** correspond to the nuclear DAPI stain, whereas images **B**, **D** and **F** correspond to nestin, GFAP and α-SMA respectively. α-SMA is expressed highly in MSCs cultured under standard conditions (Figure 3.3); however, detection of the neural antigens nestin and GFAP could not be detected (data not shown). Scale bar = 100μm.

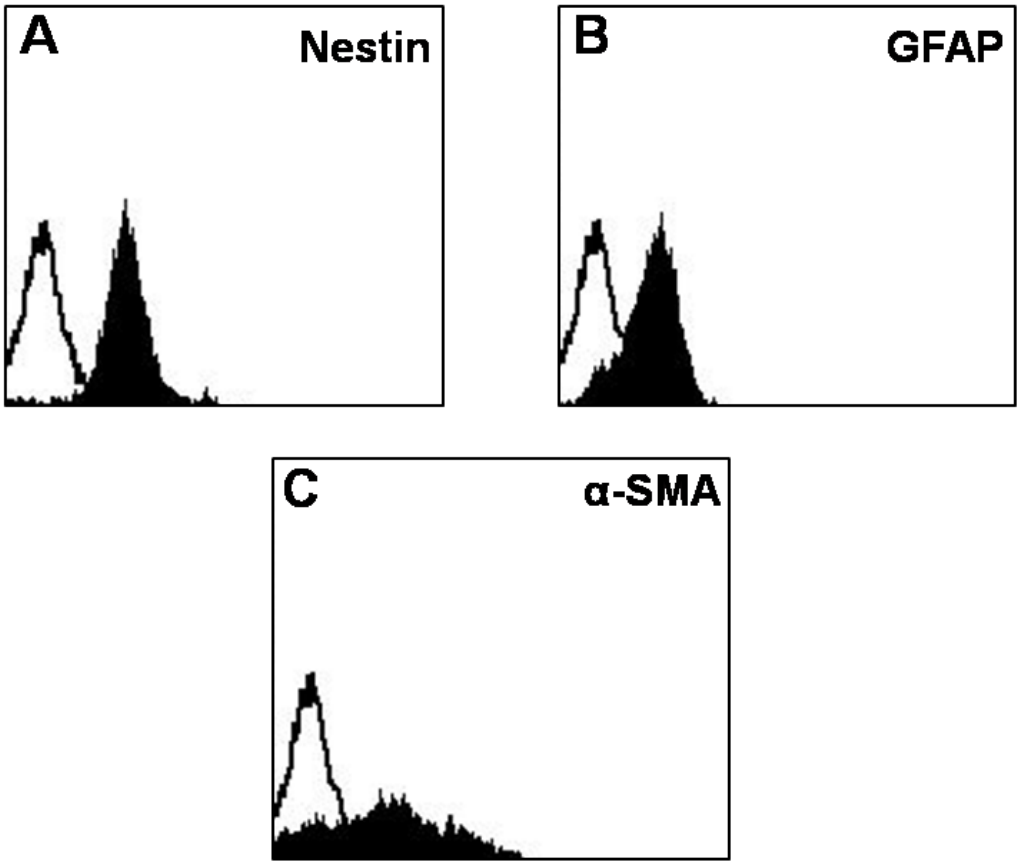

Figure 4.3. Flow cytometric analysis of expression of neural and mesenchymal markers in MSC aggregates. Representative traces showing typical marker expression profiles for nestin (**A**), GFAP (**B**) and α-SMA (**C**) in MSCs cultured as aggregates. Clear traces correspond to the *P3X* negative control and black traces correspond to specific positive fluorescence.

Previous work in our lab has demonstrated that MSC-derived aggregates are able to influence the cell fate decision of embryonic neural stem cells following co-culture *in vitro* (Croft and Przyborski, 2009). This effect had to be the result of a paracrine relationship between the two cell types as both cell types were separated by a porous membrane i.e. could only communicate with each other via the action of soluble factors and cytokines. This setup is advantageous in that it allows intercellular communication between the two cell types as would be the case *in vivo*, and MSCs have been previously reported to change their secretome depending on the specific cellular microenvironment (Chen *et al.*, 2002). However, despite this major advantage, the setup is fundamentally flawed in terms of identifying the soluble factors present in the media as it would be extremely difficult to determine the cellular origin of soluble factors present. Even if cells from different species were used in such a study, such is the cross-reactivity of proteins from different species in experiments such as ELISAs that very little useful data would be achieved.

A simpler approach would involve setting up a condition media (CM) assay, in which MSC-derived aggregates are allowed condition media for a specified time, after which the media removed and used to culture neural stem/progenitor cells. This allows that any cellular effect observed can be attributable to aggregate-derived soluble factors and, as such, would allow easier identification of the active component(s) present in the media.

In the present study, an aggregate-based conditioned media (CM) system was set up using previously published protocols regarding the generation of CM from MSCs. The effect of this CM on neural development and the mechanisms by which such effect(s) occurred were evaluated using a progenitor cell line routinely used in our lab, the rat adult hippocampal progenitor cell (AHPC) line. This cell line can be easily expanded in the lab and has previously shown the ability to differentiate into all three major neural lineages; neurons, astrocytes and oligodendrocytes.

4.3.4 Aggregate-derived soluble factors induce the formation of a glial morphology in AHPCs following 7 days culture

MSCs were seeded out for aggregate formation and allowed to condition media for 3 days, as described in Section 4.2.1. Following this, media was collected and prepared as detailed prior to downstream culture of AHPCs. For the remainder of this thesis, such media will be referred to as CM (conditioned media). As detailed in Section 4.2.3, AHPCs were seeded onto cellware coated with PLO and laminin at a density of 10,000 cells/cm^2 in their normal proliferative media and were allowed 24 hours to settle (designated day –1). Following this 24 hour period (day 0), media was removed and replaced with CM or appropriate control media (UC: unconditioned control i.e. media in which no cells had been cultured; PC: proliferation control i.e. standard AHPC culture medium) and AHPCs cultured for a further 7 days. Media was replenished on the 4th day. After 7 days, AHPCs were analysed for any obvious changes in cellular morphology (Figure 4.4). PC AHPCs were largely phase dark and very densely packed following 7 days culture (Figure 4.4A). Cell bodies were very rounded with no obvious evidence of processes or cytoplasmic extensions forming, suggesting that they were largely undifferentiated. This is in complete contrast to AHPCs cultured under UC and CM conditions, where the large majority of cells demonstrated phase-bright soma in contrast to the dark-phase soma observed in PC. In

addition, AHPCs cultured under UC conditions possessed numerous short processes, which again was in complete contrast to PC. Although possessing numerous processes per soma, UC cells still appeared largely undifferentiated, as these processes were very short in length and did not demonstrate any obvious characteristics of neuronal or glial morphologies (Figure 4.4B).

AHPCs cultured with CM, however, possessed multiple processes per soma that did acquire morphologies very characteristic of a more mature neural phenotype (Figure 4.4C). The presence of extensive multipolar soma suggested that AHPCs were producing glia under these conditions as opposed to neurons, whose morphologies would be typically bipolar with processes terminating in growth cones. Morphological evidence suggested that AHPCs were forming astrocytes as opposed to oligodendrocytes when cultured with CM, as they possessed a highly characteristic stellate, star-shaped morphology as opposed to a more intricate oligodendrocytic morphology consisting of extensive secondary and tertiary branching.

4.3.5 Culturing AHPCs in CM for 7 days results in expression of the structural astrocytic marker, GFAP, as determined by immunocytochemistry and flow cytometric analysis

Although morphological analysis is useful, it is limited in terms of what conclusions can be drawn from it because it cannot be used solely to confirm the phenotype of a specific cell type. In order to gain more information regarding the phenotype of AHPCs cultured in CM and appropriate controls for 7 days, expression of a range of neural markers were examined using immunocytochemistry and flow cytometry. Markers specific for neuroprogenitors and the three major neural cell types were used – nestin (neuroprogenitor marker), Tuj-1 (neuronal marker), GFAP (astrocytic marker) and GalC (oligodendrocytic marker).

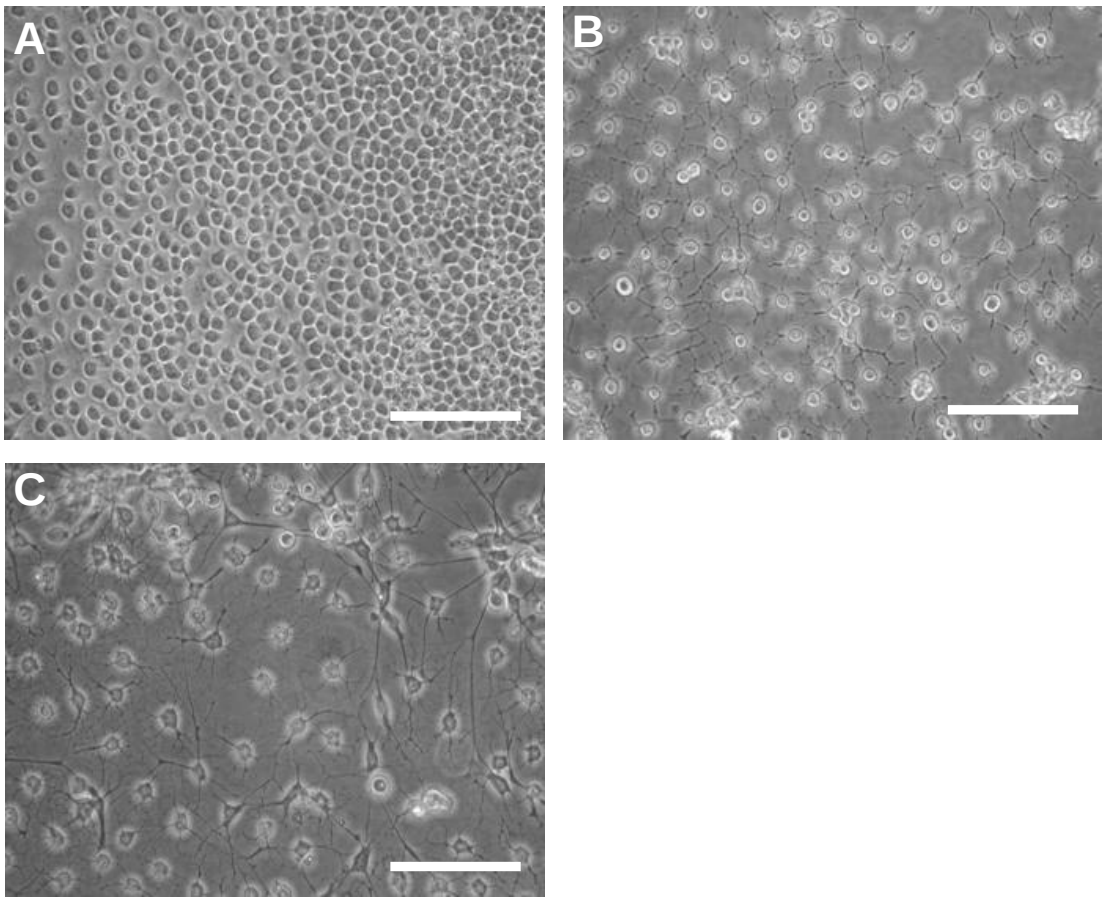

Figure 4.4. Morphology of AHPCs after 7 days culture with conditioned media and appropriate controls. A: AHPCs cultured under their normal proliferative conditions; **B**: AHPCs cultured with unconditioned media; **C**: AHPCs cultured with conditioned media. Cells in **A** and **B** appear largely undifferentiated, whereas cells in **C** appear to have adopted a glial morphology, having multipolar cell bodies with numerous processes, as opposed to neuronal cell types, which are typically bipolar. Scale bar = 100μm.

AHPCs were seeded out for marker analysis experiments as described in Section 4.2.3 and cultured in CM and appropriate controls (PC and UC) for 7 days. Following 7 days culture, AHPCs were fixed in 4% PFA for 30 minutes at room temperature and processed for immunocytochemistry directed against nestin, Tuj-1, GFAP and GalC as described in Section 2.7. AHPCs cultured under all three conditions – PC, UC and CM – demonstrated intense staining for the neuroprogenitor marker, nestin (Figures 4.5A, 4.5B and 4.5C). Patterns of staining corresponded with the cellular morphologies described in Section 4.3.4. Conversely, expression of the neuronal marker, Tuj-1, was undetectable in AHPCs cultured under all three conditions (Figures 4.5D, 4.5E and 4.5F). In terms of GalC expression, the oligodendrocytic marker, AHPCs cultured under UC and CM conditions demonstrated very weak, diffuse staining that was present in the large majority of cells per field of view (Figures 4.5K and 4.5L). GalC

was undetectable in AHPCs cultured under normal proliferative conditions (Figure 4.5J). However, the most striking result from these experiments was in terms of GFAP expression, the astrocytic marker. GFAP was undetectable in AHPCs cultured under PC and UC conditions (Figures 4.5G and 4.5H). AHPCs cultured with CM for 7 days, on the other hand, demonstrated a significant increase in expression of GFAP such that the large majority of cells per field of view stained intensely for GFAP (Figure 4.5I). The specificity of GFAP expression to CM cultures strongly suggested the presence of some unknown soluble factor(s) present in CM that induced expression of GFAP and hence an astrocytic phenotype in AHPCs. These marker analyses were in keeping with previous morphological observations which suggested that culturing AHPCs with CM caused them to adopt an astrocytic morphology (Figure 4.5C). Patterns of GFAP staining corresponded with this astrocytic morphology and can be seen at higher magnification in Figure 4.6.

Analysis of cell marker expression using immunocytochemical methods is useful in that it allows a visualisation of cellular morphology and determination of protein location at the subcellular level. However, one major limitation of this technique is that it lacks the sensitivity of more sophisticated techniques such as flow cytometry. Flow cytometry allows even the slightest of changes in protein expression to be detected and similarly allows an accurate measurement of protein intensity to be made (MFI). Therefore, using these techniques simultaneously yields the most comprehensive results.

AHPCs were seeded out as described above and cultured in CM and appropriate controls (PC and UC) for 7 days. Following 7 days culture, AHPCs were fixed in 2% PFA at 4^{o}C overnight and processed for flow cytometry against nestin, Tuj-1, GFAP and GalC as described in Section 2.6. Data in terms of the percentage of cells expressing marker and MFI of marker expression are shown graphically in Figure 4.7 in terms of raw data (Figures 4.7A and 4.7B) and as a fold-increase above PC (Figures 4.7C and 4.7D). As AHPCs cultured under normal, proliferative conditions (PC) should remain largely undifferentiated, expression levels in PC samples should reflect endogenous levels of expression in undifferentiated AHPCs. Expressing UC and CM data as a fold-increase or fold-decrease compared to PC essentially normalises against

basal levels of expression and allows more useful comparisons to be made between UC and CM samples.

In terms of the percentage of cells expressing each neural marker, there was very little difference between each of the samples (Figure 4.7A and 4.7C). Any differences in expression that were apparent – such as the increase in the percentage of cells expressing GFAP and GalC and the decrease in percentage of cells expressing nestin and Tuj-1 – were very slight and not significant at the statistical level. Such is the sensitivity of flow cytometry that cells expressing extremely low levels of antigen will be detected as positive events, even when the level is so low it is unlikely to be biologically relevant. That is why it is necessary to examine percentage of cells expressing marker simultaneously with MFI of marker expression, as it allows discrimination between cells that are weakly and strongly positive for expression. In terms of MFI of nestin, Tuj-1 and GalC expression, there was very little observable difference between UC and CM samples (Figures 4.7B and 4.7D). Any slight differences were not significant at the statistical level. However, in terms of MFI of GFAP expression, AHPCs cultured with CM for 7 days demonstrated a dramatic increase in marker intensity, a greater than 20-fold increase compared to PC and UC (Figures 4.7B and 4.7D). This observation was highly significant at the statistical level ($p<0.001$). This observation strongly supports the immunocytochemical data (Figure 4.5).

4.3.6 AHPCs cultured with CM for 7 days exit the cell cycle, providing further evidence that soluble factors present within CM induce differentiation in AHPCs

There is a delicate balance between cell proliferation and cell differentiation that must be maintained *in vivo* for proper growth to occur and prevent disorders such as cancers. The process of cell proliferation is strictly controlled during the differentiation of stem cells, with differentiation of stem cells frequently occurring silmultaneously with exit from the cell cycle. Indeed, exit from the cell cycle has been reported to precede the differentiation of neural progenitors towards astrocytes (Galderisi *et al.*, 2003). Therefore, if the formation of an astrocytic morphology and increase in GFAP expression in AHPCs following culture in CM for 7 days is indicative of true astrocytic differentiation, it would be expected that AHPCs undergo exit from the cell cycle. This was examined in the present study. AHPCs were seeded out as previously

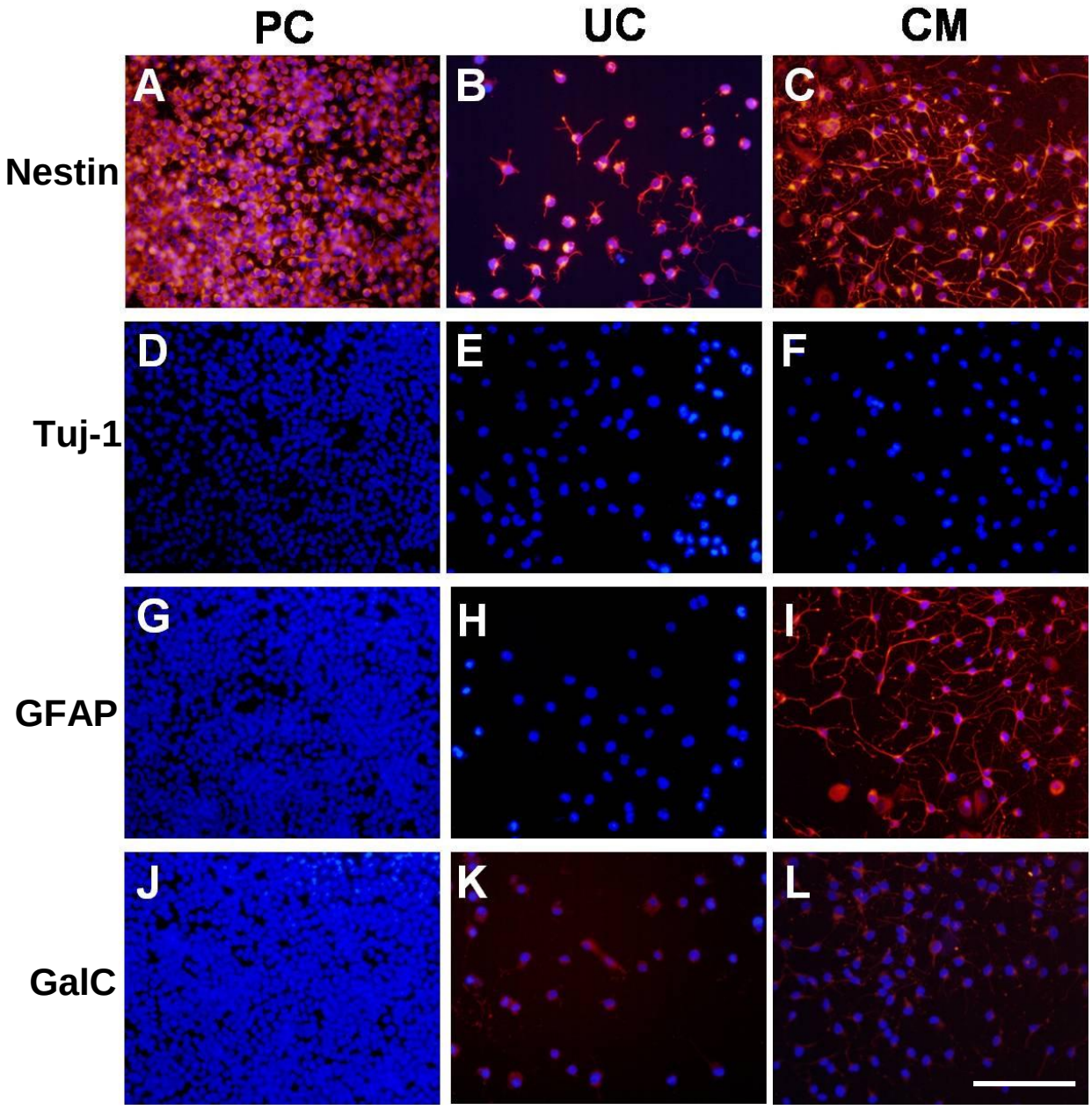

Figure 4.5. Expression of neural markers in AHPCs following 7 days culture with conditioned media and appropriate controls. A, B and **C:** expression of nestin; **D, E** and **F:** expression of Tuj-1; **G, H** and **I:** expression of GFAP; **J, K** and **L:** expression of GalC. Treatment of AHPCs with conditioned media results in a significant upregulation in GFAP expression, suggesting that MSC-derived aggregates secrete a complement of soluble factors/cytokines that induce AHPCs to undergo gliogenesis. Scale bar = 100μm.

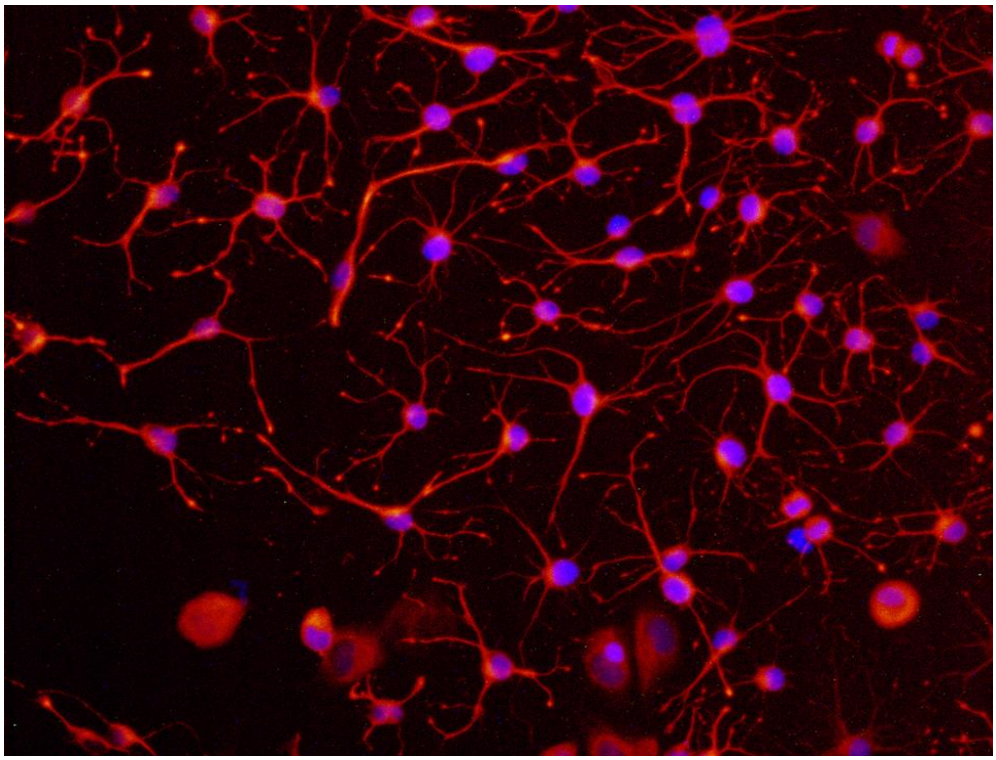

Figure 4.6. Morphology of GFAP$^+$ AHPCs following culture with conditioned media for 7 days. Treatment of AHPCs with conditioned media for 7 days results in a significant upregulation of GFAP expression, suggesting that MSC-derived aggregates secrete a complement of soluble factors/cytokines that induce AHPCs to undergo gliogenesis. Analysis of GFAP$^+$ AHPCs at the morphological level suggests that these cells are adopting a predominantly glial morphology, having multipolar cell bodies with numerous processes, as opposed to neuronal cell types, which are typically bipolar. Morphological evidence alone suggested AHPCs were forming astrocytes as opposed to oligodendrocytes, possessing a characteristic stellate, star-shaped morphology as opposed to a more intricate oligodendrocytic morphology consisting of extensive secondary and tertiary branching. Magnification is X40.

detailed in Section 4.2.3, switched to CM and appropriate control conditions (PC and UC) and the number of viable cells in culture determined at the 1, 3 and 7 day timepoints using the MTS proliferation assay. At both the 1 and 3 day timepoints, there was little observable difference regarding the number of viable cells in culture between control (PC and UC) and CM conditions (Figure 4.8). At the 7 day timepoint, however, there was a huge difference between the number of AHPCs in PC cultures compared to UC and CM cultures – the presence of FGF2 as a component of the PC media stimulated the proliferation of AHPCs. Despite this marked difference in viable cell number between PC and the two other conditions, there was no observable difference between viable cell number in UC vs. CM cultures at the 7 day timepoint. Absorbance values at 490nm for UC and CM samples were approximately one-third the absorbance values of PC samples (Figure 4.8). These data clearly suggest that AHPCs cultured under UC and CM conditions were not proliferating and, by extension, were exiting

the cell cycle. The lack of FGF2 in the UC media explains why little proliferation of AHPCs was observed over the 7 day time course of the experiment compared to PC. An equivalent number of cells were observed in CM samples, strongly suggesting that soluble factors present in CM did not exert any proliferative effects.

4.3.7 In addition to upregulation of structural astrocytic markers such as GFAP, AHPCs cultured with CM for 7 days also demonstrate a marked upregulation in the expression of functional biochemical markers indicative of an astrocytic phenotype

Previous data in this study has demonstrated that aggregate-derived soluble factors present in CM induce AHPCs to acquire an astrocytic morphology concomitant with a significant upregulation in expression of the structural astrocytic marker, GFAP. Combined, these data strongly suggest that AHPCs are being induced to undergo astrocytic differentiation. In terms of assessing cell differentiation, however, it is perhaps functional behaviour rather than simple marker analysis that provides more convincing evidence of true differentiation. Such functional assays were performed in Chapter 3 to assess osteogenic (ALP activity and osteocalcin secretion) and adipogenic differentiation (cAMP activity).

In terms of neuronal differentiation, functional assessment of differentiation would typically be measured in terms of electrical activity such as the generation of action potentials (Jurga *et al.*, 2009). However, assessing the functional activity of astrocytes is somewhat more complex, as commonly reported functions of astrocytes include supporting neuronal growth by providing nutrients, influencing neuronal behaviour by releasing neurotrophic soluble factors and cytokines, maintenance of a proper ion balance in the extracellular space *in vivo* and forming the blood-brain barrier, amongst many others (Kimelberg, 1983; Nimmerjahn, 2009). Unsurprisingly, these are all functions that are extremely difficult to assess *in vitro*. Therefore, the most commonly used method of assessing functional astrocytic behaviour *in vitro* is by examining expression of various functional biochemical receptors. Here, expression of four major biochemical receptors indicative of an astrocytic phenotype were examined at the mRNA level for AHPCs cultured with CM and appropriate controls for 7 days. These were AMPA receptor 3 (AMPA3), GABA-A, NMDA receptor 3 (NMDA-2D) and 5-HT 1A, all of which have been reported as functional biochemical markers of astrocytic behaviour (Kamnasaran *et al.*, 2008).

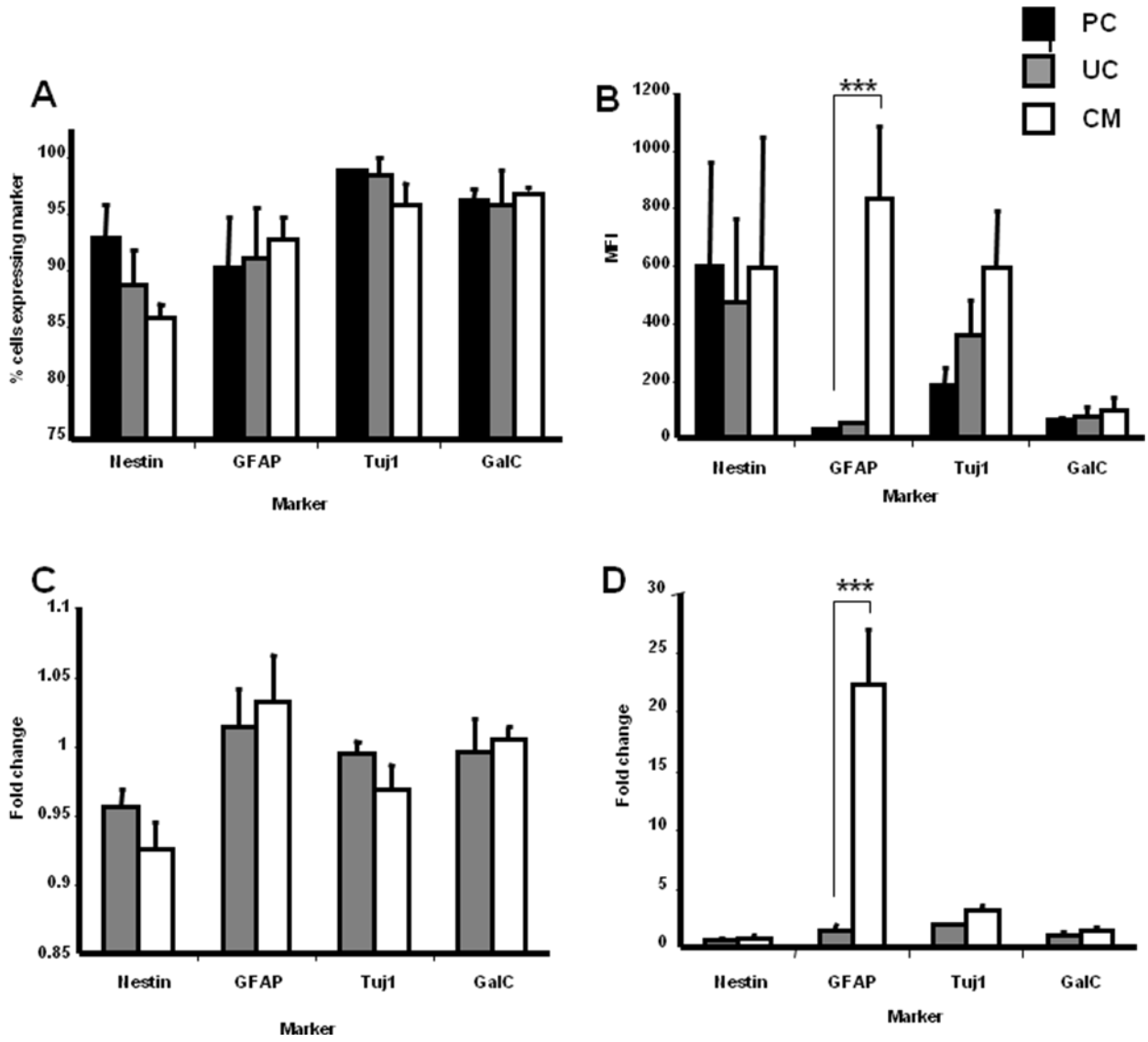

Figure 4.7. Flow cytometric analysis of marker expression in AHPCs treated with conditioned media and controls. **A**: % cells expressing marker; **B**: mean fluorescence intensity of marker expression; **C**: fold change in the % cells expressing marker (compared to proliferation control); **D**: fold change in mean fluorescence intensity of marker expression (compared to proliferation control). Treatment of AHPCs with conditioned media results in ~25-fold increase in the intensity of GFAP expression, suggesting that MSC-derived aggregates secrete a complement of soluble factors/cytokines that cause AHPCs to undergo gliogenesis; in particular, astrogenesis at the expense of oligodendrogenesis. Each bar represents the mean (n=3)+SEM, *** $p<0.001$.

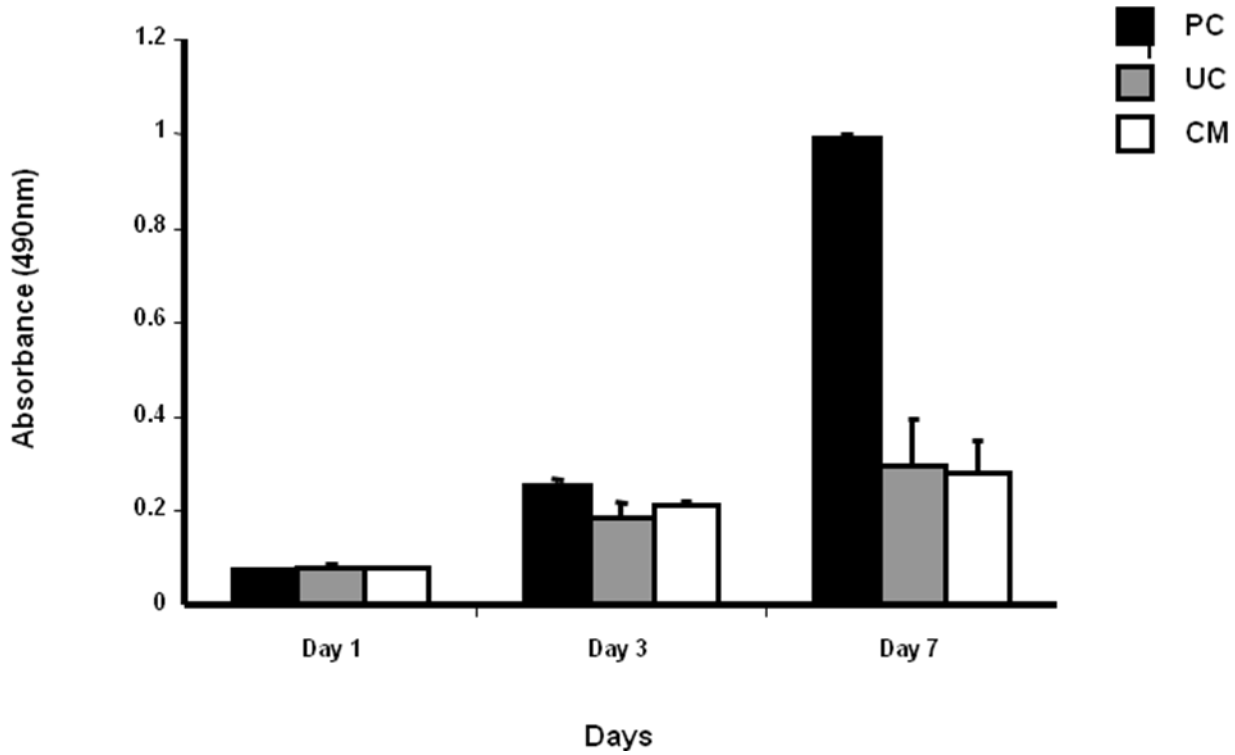

Figure 4.8. Determination of the number of viable AHPCs in culture following treatment with conditioned media and appropriate controls for 1, 3 and 7 days post-induction of differentiation. AHPCs were seeded out and cultured in conditioned media and appropriate controls (PC and UC) and the number of viable cells in culture determined using the colorimetric MTS assay. Each bar represents the mean (n=3)+SEM.

Following 7 days culture with CM and appropriate controls (PC and UC), RNA was isolated from AHPCs using the TRIreagent (Section 2.9), reverse transcribed to cDNA (Section 2.10) and processed for qRT-PCR (Section 2.11) as detailed in the specified sections. Expression profiles are shown in Figure 4.9. Each of the four receptors were significantly upregulated in AHPCs cultured with CM compared to both controls, validating previous observations from this study and providing further evidence to suggest that soluble factors present in CM induced an astrocytic phenotype in AHPCs. AMPA3 was the only receptor that could be readily detected in AHPCs cultured under all three conditions (Figure 4.9A). PC and UC samples expressed AMPA3 at low and comparable levels to each other, whereas CM samples demonstrated a highly significant upregulation in expression, an observation that was highly significant at the statistical level ($p<0.001$). The remaining three receptors – GABA-A (Figure 4.9B), NMDA-2D (Figure 4.9C) and 5-HT 1A (Figure 4.9D) – demonstrated very similar expression profiles. All three receptors were expressed at extremely low levels in PC

samples and were undetectable in UC samples. However, significant upregulation was observed following culture with CM for 7 days, observations that were significant at the statistical level for all three receptors ($p<0.01$ for GABA-A and 5-HT-1A and $p<0.001$ for NMDA-2D).

These data (representative of astrocytic function) combined with the previous structural marker data strongly suggest that MSC-derived aggregates secrete a soluble factor/combination of soluble factors that induce astrogenesis in AHPCs.

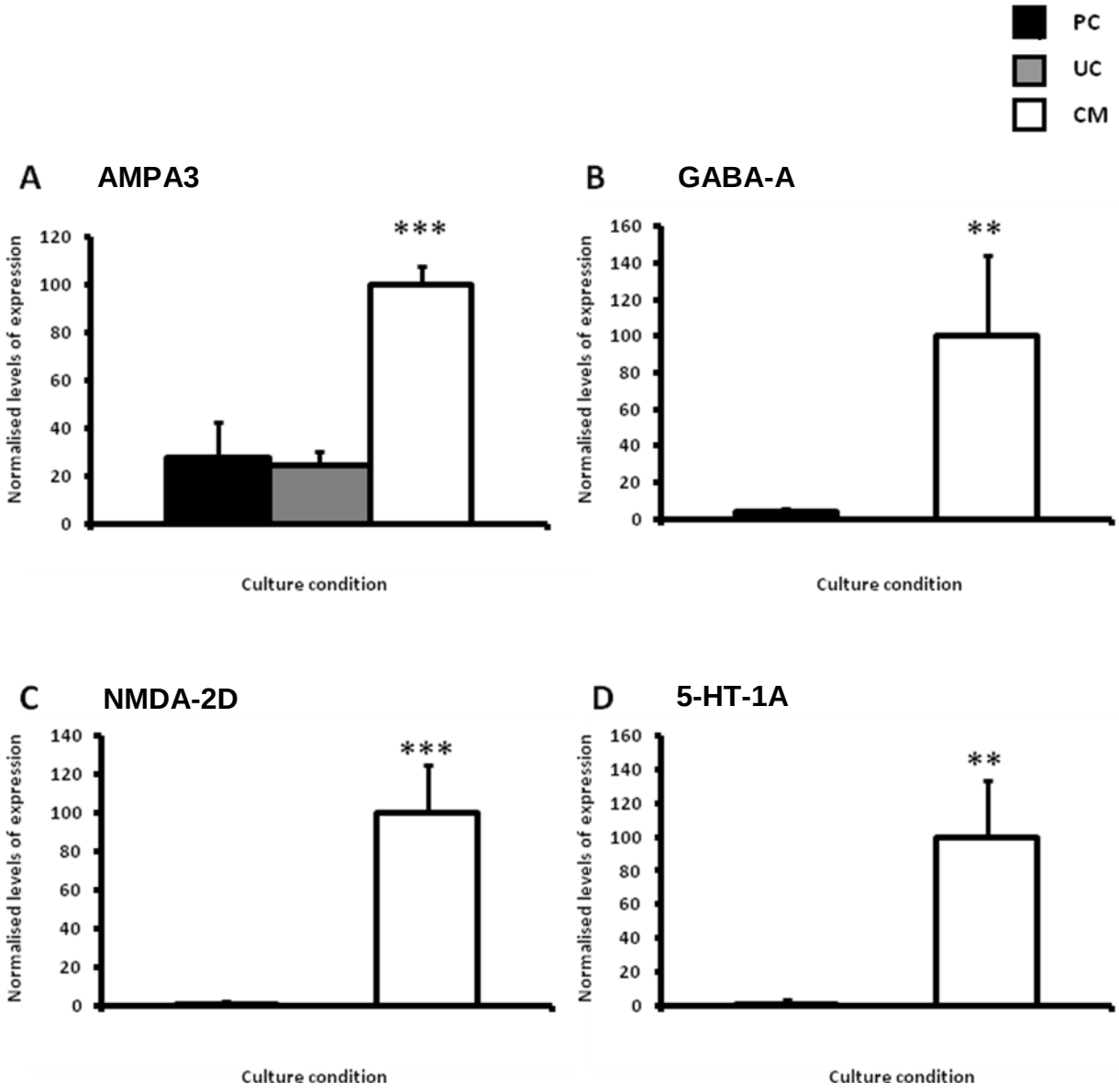

Figure 4.9. Expression of functional biochemical markers indicative of an astrocytic phenotype in AHPCs cultured with conditioned media and appropriate controls for 7 days. QRT-PCR analysis was performed to compare mRNA transcript levels of four genes encoding functional biochemical markers indicative of astrocytic behaviour – **A:** AMPA3; **B:** GABA-A; **C:** NMDA-2D and **D:** 5-HT-1A – in AHPCs cultured in conditioned media and appropriate controls for 7 days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, ** $p<0.01$.

4.3.8 The astrocytic fate of AHPCs is specifically the result of the action(s) of 3D aggregate-derived soluble factors and cannot be reproduced following culture of AHPCs in media conditioned by MSCs as 2D adherent monolayers

Results in this study so far have demonstrated that MSC-derived 3D aggregates secrete active component(s) into their culture media that exert an astrogenic effect on AHPCs following 7 days culture. However, these experiments do not answer the critical question of whether this astrogenic effect is a specific consequence of the action(s) of aggregate-derived soluble factors or is a consequence of the action(s) of more generally-secreted MSC-derived soluble factors. To address this key question, MSCs cultured as standard, adherent 2D monolayer cultures were allowed to condition media for 3 days (the same timeframe as for aggregate-derived CM), after which media was subsequently collected and processed as detailed in Section 4.2.2. AHPCs were seeded out under identical conditions to those used to assess the effect of aggregate-derived CM (Section 4.2.3) and cultured in this monolayer-conditioned media (MONO-CM) for 7 days, after which cell phenotype was assessed in terms of morphology and expression of neural markers (Figures 4.10, 4.11 and 4.12).

4.3.9 AHPCs cultured with MONO-CM demonstrate no apparent changes in morphology compared to equivalent control cultures following a 7 day culture period

As for the above studies, AHPCs were seeded onto cellware coated with PLO and laminin at a density of 10,000 cells/cm^2 in their normal proliferative media and allowed 24 hours to settle (designated day –1). Following this 24 hour period (day 0), medium was removed and replaced with MONO-CM or appropriate control media (UC: unconditioned media; PC: proliferation control) and AHPCs cultured for a further 7 days. Medium was replenished on the 4th day. After 7 days, AHPCs were analysed for any obvious changes in cellular morphology (Figure 4.10). As observed previously in Section 4.3.4, PC AHPCs were largely phase dark and very densely packed following 7 days culture (Figure 4.10A). Cell bodies were very rounded with no obvious evidence of processes or cytoplasmic extensions forming, suggesting that they were largely undifferentiated. Also in keeping with previous observations, AHPCs cultured under UC conditions were largely phase bright and possessed numerous short processes (Figure 4.10B). Although possessing numerous processes per soma, UC cells still appeared largely undifferentiated, as these processes were very

short in length and did not demonstrate any obvious characteristics of neuronal or glial morphologies. However, whereas AHPCs cultured with aggregate-derived CM demonstrated obvious differences in morphology compared to equivalent UC control cultures (Figure 4.4), AHPCs cultured with MONO-CM demonstrated no apparent differences in morphology compared to UC controls (Figure 4.10C). Culturing AHPCs with MONO-CM did not induce the formation of the stellate, star-shaped morphology that was observed following culture with aggregate-derived CM. Instead, cells possessed numerous short processes that were more indicative of an immature state as opposed to a more mature neural phenotype (Figure 4.10C). These morphological data combined suggest that soluble factors secreted by adherent 2D monolayer MSCs (MONO-CM) do not induce differentiation of AHPCs, whereas soluble factors secreted by MSC-derived 3D aggregates induce astrocytic differentiation of AHPCs following 7 days culture

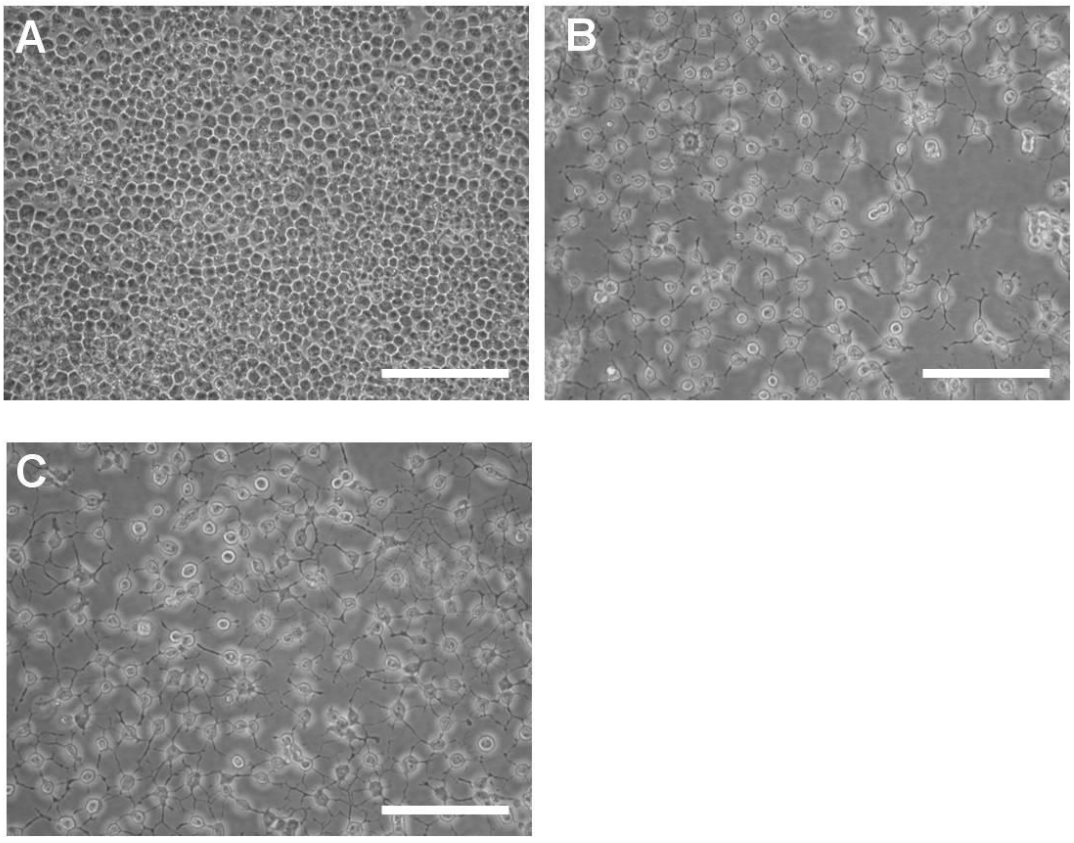

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Figure 4.10. Morphology of AHPCs following 7 days culture with MONO-CM and appropriate controls. A: AHPCs cultured under their normal proliferative conditions; **B:** AHPCs cultured with unconditioned media; **C:** AHPCs cultured with MONO-CM. AHPCs cultured under both control conditions (**A** and **B**) and with MONO-CM (**C**) appeared largely undifferentiated, though AHPCs cultured with MONO-CM did possess numerous short process; however, these were not representative of the stellate, star-shaped morphologies acquired following culture with MSC aggregate-derived CM. Scale bar = 100μm.

4.3.10 AHPCs cultured with MONO-CM for 7 days demonstrate expression of the neuroprogenitor marker, nestin, but not of the more mature neural markers, Tuj-1, GFAP and GalC

In order to gain more information regarding the phenotype of AHPCs cultured in MONO-CM and appropriate controls for 7 days, expression of a range of neural markers were examined using immunocytochemistry and flow cytometry. Markers specific for neuroprogenitors and the three major neural cell types were examined – nestin (neuroprogenitor marker), Tuj-1 (neuronal marker), GFAP (astrocytic marker) and GalC (oligodendrocytic marker). AHPCs were seeded out for these experiments and processed for marker analysis as described in Section 4.2.3. Immunocytochemical images are shown in Figure 4.11 and flow cytometric data in Figure 4.12.

AHPCs cultured under all three conditions – PC, UC and CM – demonstrated intense staining for the neuroprogenitor marker, nestin (Figures 4.11A, 4.11B and 4.11C). Patterns of staining corresponded with the cellular morphologies described in Section 4.3.9. Conversely, expression of the three other neural markers – Tuj-1, GFAP and GalC – were undetectable in AHPCs cultured under all three conditions (Figure 4.11D–L). For PC and UC samples, these observations are consistent with those previously observed (Figure 4.5). For MONO-CM samples, however, these observations differed from those previously observed following culture with aggregate-derived CM, which induced a significant increase in expression of GFAP such that the large majority of cells per field of view stained intensely for GFAP (Figure 4.5I). No such staining for GFAP was observed following culture under identical conditions with MONO-CM (Figure 4.11I). These marker analyses were in keeping with morphological observations which suggested that AHPCs cultured with MONO-CM retained an immature phenotype and did not acquire a more mature neural morphology.

Expression of neural markers in AHPCs cultured with MONO-CM and appropriate controls were assessed using flow cytometry in terms of percentage of cells expressing marker and MFI of marker expression. Cells were processed as described previously in Section 2.6 and results shown in Figure 4.12 in terms of raw data (Figures 4.12A and 4.12B) and as a fold-increase above PC (Figures 4.12C and 4.12D). In terms of the percentage of cells expressing each neural marker, there was very little difference

between each of the samples (Figures 4.12A and 4.12C). Any differences in expression that were apparent – such as a slight tendency for the percentage of cells expressing nestin, GFAP and GalC to increase and the percentage of cells expressing Tuj-1 to decrease – were very slight and not significant at the statistical level. These slight trends were similar to those previously observed following culture with aggregate-derived CM and controls (Figure 4.7). In terms of MFI of nestin, Tuj-1 and GalC expression, there was very little observable difference between UC and MONO-CM samples (Figures 4.12B and 4.12D). Any slight differences were not significant at the statistical level. However, in terms of MFI of GFAP expression, AHPCs cultured with MONO-CM for 7 days demonstrated an almost 2.5-fold increase compared to UC control cultures (Figures 4.12B and 4.12D). This trend was also observed following culture of AHPCs with aggregate-derived CM for 7 days, though this effect was much more dramatic, a greater than 20-fold increase (Figure 4.7D). The observation following culture with MONO-CM was not significant at the statistical level, whereas it was highly significant ($p<0.001$) following culture with aggregate-derived CM. These data combined strongly support morphological observations that AHPCs cultured with MONO-CM retain their immature phenotype following 7 days culture, whereas AHPCs cultured with aggregate-derived CM are induced to undergo astrocytic differentiation. Although there is a slight tendency for expression of the astrocytic marker GFAP to increase following culture with MONO-CM this effect is not statistically significant and as such it is highly feasible that MSC-derived aggregates secrete additional component(s) into their media that are responsible for enhanced astrocytic differentiation.

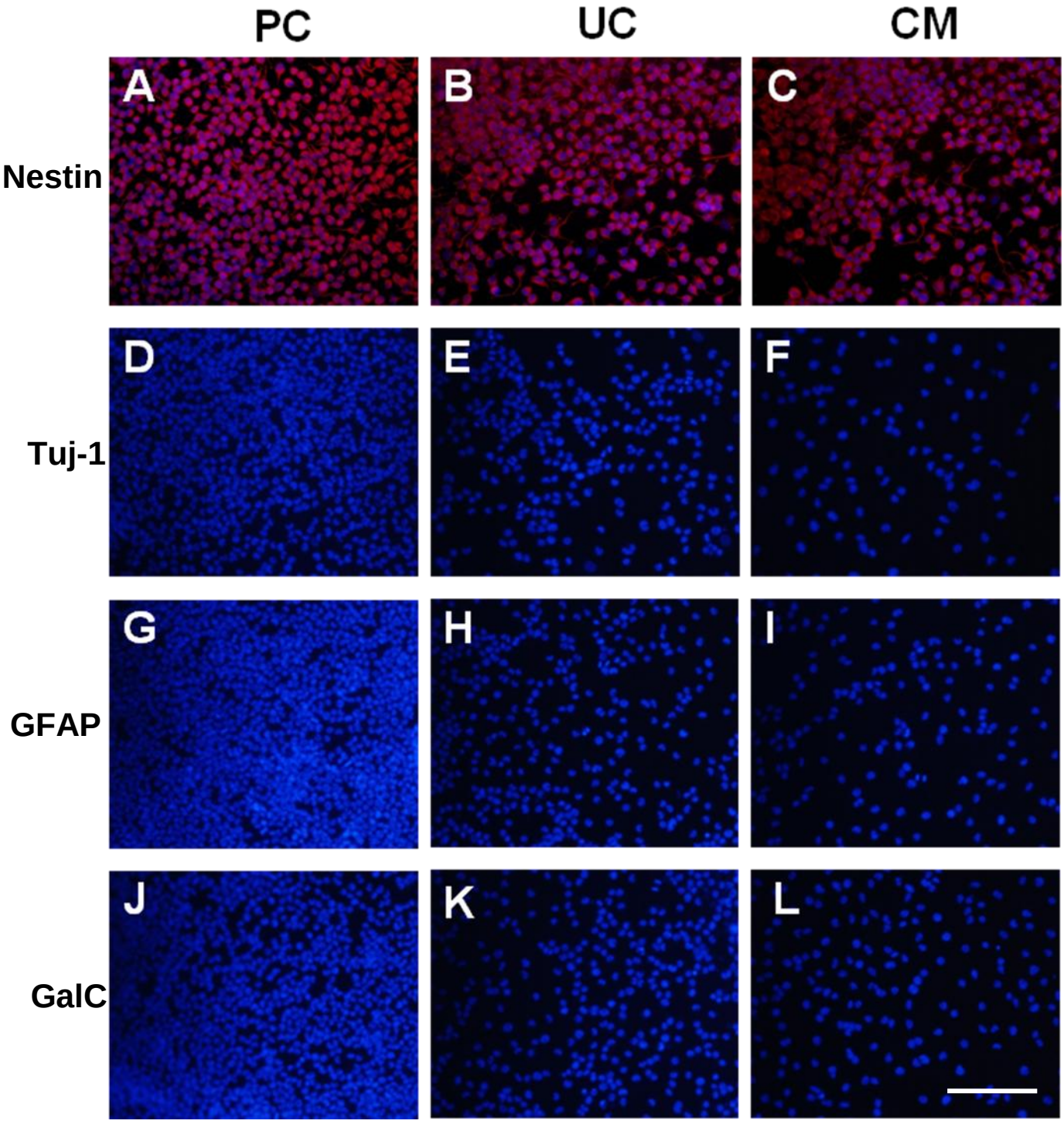


Figure 4.11. Expression of neural markers in AHPCs following 7 days culture with MONO-CM and appropriate controls. A, B and **C:** expression of nestin; **D, E** and **F:** expression of Tuj-1; **G, H** and **I:** expression of GFAP; **J, K** and **L:** expression of GalC. MONO-CM did not induce the significant upregulation in GFAP expression observed following culture with MSC aggregate-derived CM. Expression of the mature neural markers Tuj-1, GFAP and GalC could not be detected in AHPCs cultured in either MONO-CM or appropriate controls. Scale bar = 100μm.

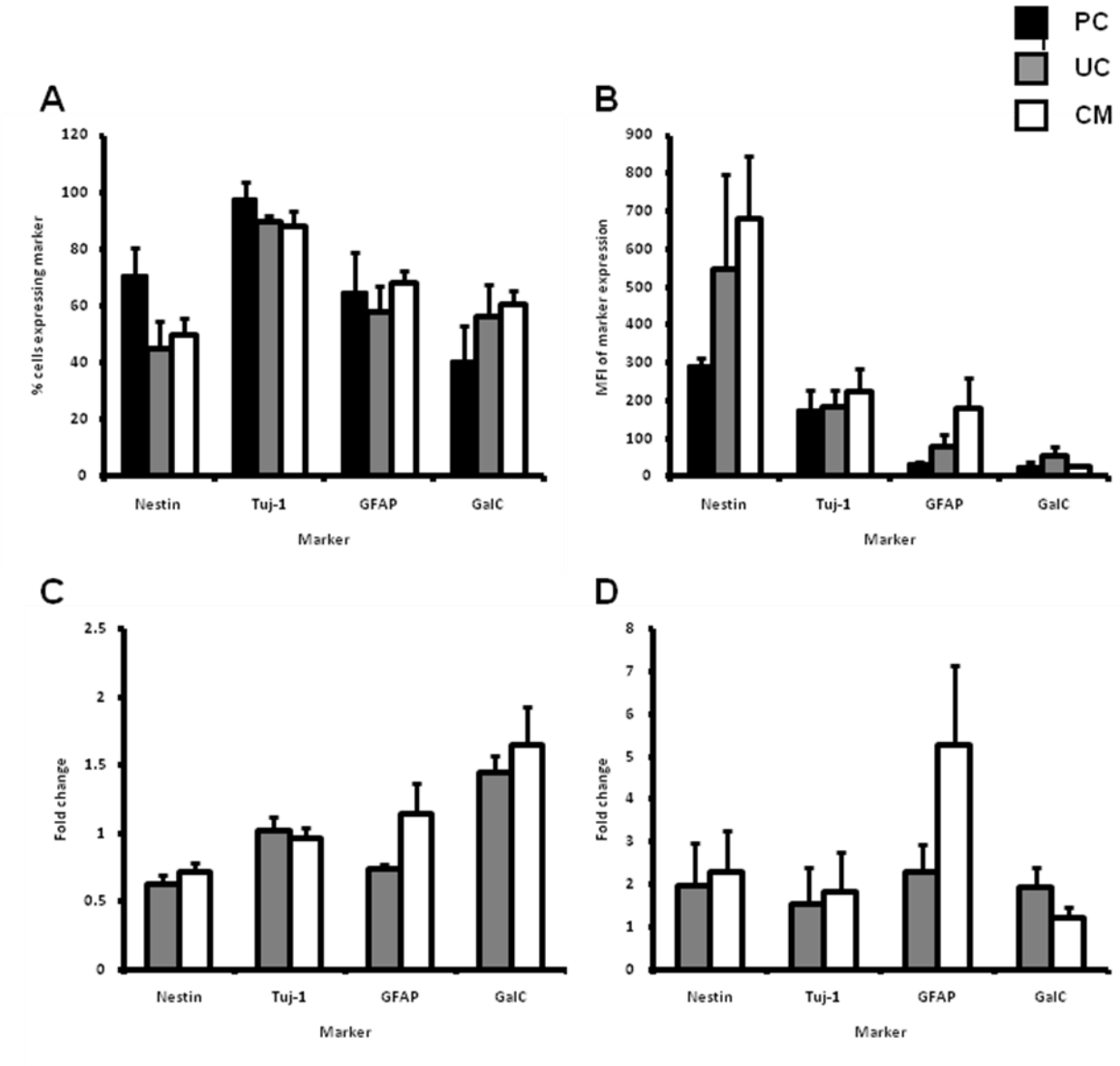


Figure 4.12. Flow cytometric analysis of marker expression in AHPCs treated with MONO-CM and controls. A: % cells expressing marker; **B:** mean fluorescence intensity of marker expression; **C:** fold change in the % cells expressing marker (compared to proliferation control); **D:** fold change in mean fluorescence intensity of marker expression (compared to proliferation control). In terms of the percentage of cell expressing each neural marker, there was very little difference between each of the samples. Similarly, in terms of intensity of marker expression, there was very little difference between each of the samples. Treatment of AHPCs with MONO-CM results in a ~2.5-fold increase in the intensity of GFAP expression, though this observation was quite variable and not statistically significant. Each bar represents the mean (n=3) + SEM

4.3.11 Oxygen tension is a crucial parameter in the culture of MSCs as cellular aggregates

Cellular behaviour in standard *in vitro* cultures has been reported to be radically altered upon exposure to varying oxygen concentrations. Under standard culture conditions, cells are exposed to atmospheric levels of oxygen, which is approximately 20%. However, this oxygen tension far exceeds the levels of oxygen which cells are exposed to *in vivo*, which is typically closer to 3-5% depending on the specific cell population and tissue in question (Ivanovic, 2009). In the bone marrow, physiological oxygen concentration has been reported as 5% (Zscharnack *et al.*, 2009). As such, studies with cells cultured under atmospheric oxygen levels may be somewhat misleading and it may therefore be more accurate to study cells cultured under lower oxygen levels that are closer to physiologically-relevant concentrations. Evidence suggests that oxygen tension can dramatically affect many aspects of cellular behaviour and MSCs cultured under low oxygen tension have demonstrated enhanced rates of proliferation and mitogenic activity, reduced cell death and enhanced survival, reduced senescence, enhanced lifespan and enhanced levels of differentiation (Ren *et al.*, 2006; Krinner *et al.*, 2009; Zscharnack *et al.*, 2009). Similar properties have been observed in other cell types, including neural stem cells (Clarke and van der Kooy, 2009).

The importance of oxygen tension as a parameter in cell culture is particularly significant in the culture of cellular aggregates and neurospheres, where control of gaseous exchange and diffusion of soluble factors has been reported to be a crucial aspect contributing to the longevity and viability of aggregates (Griffith and Swarz, 2006). MSCs induced to form aggregates in these studies using the protocol described in Section 4.2.1 were cultured under lower oxygen levels (5%) in keeping with previously published protocols (Croft and Przyborski, 2004; Hermann et al., 2004; Bossolasco et al., 2005; Hermann et al., 2006). In order to ascertain how crucial a role oxygen tension played in the culture of MSC-derived aggregates, MSCs were seeded out for aggregate formation as described in Section 4.2.1 but maintained under atmospheric oxygen levels as opposed to lower, physiological levels of oxygen. Behaviour of aggregates was assessed in terms of morphology, marker expression, rate of proliferation and also in terms of their secretome to see if the astrocytic effect of aggregate-derived CM on AHPCs could be reproduced.

4.3.12 MSCs can be induced to form cellular aggregates when cultured under atmospheric oxygen conditions

MSCs were seeded out for aggregate formation as detailed previously (Section 4.2.1) and maintained under atmospheric oxygen concentrations (20%) as opposed to physiological oxygen concentrations (5%). Figure 4.13 shows MSCs cultured in aggregate-inducing conditions under atmospheric oxygen concentrations after 72 hours. Under such conditions, MSCs formed cellular aggregates that were morphologically indistinguishable from aggregates formed under low oxygen tension (Figure 4.13). Small, spherical aggregations of cells formed on the surface of the cellware and, as previously described for aggregates cultured under low oxygen tensions, not all of the seeded cells formed aggregates with a small proportion of adherent cells forming flat morphologies as per standard culture (Figure 4.13).

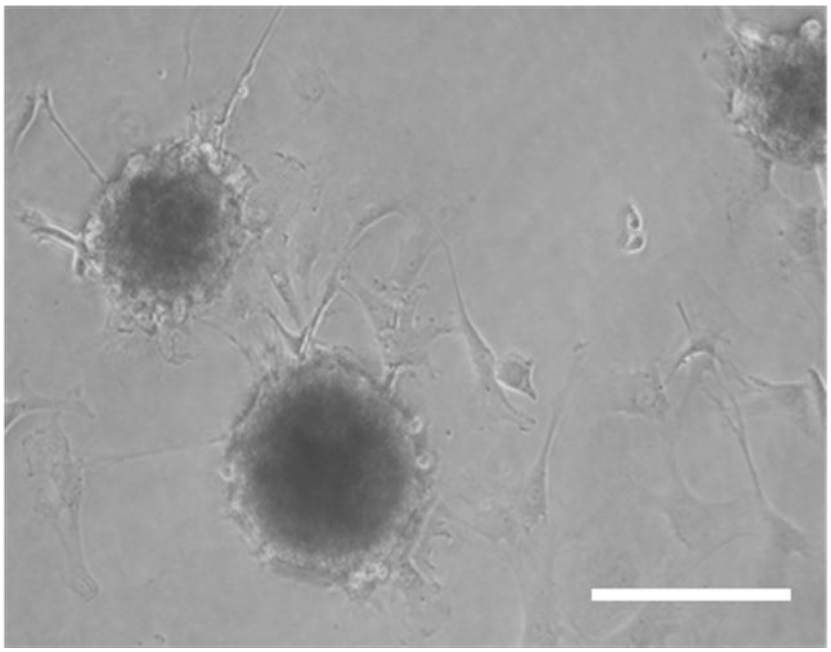


Figure 4.13. Formation of large, multicellular aggregates by MSCs cultured under atmospheric oxygen tension for 72 hours. MSCs were seeded out for aggregate formation and maintained under atmospheric oxygen tension (20%) as opposed to physiological oxygen tension (5%). Under such conditions, MSCs formed cellular aggregates that were morphologically indistinguishable from aggregates formed under physiological oxygen tension. Therefore, oxygen tension itself appears not to be a critical parameter in the formation of large multicellular aggregates from MSCs. Scale bar = 100μm.

4.3.13 MSC aggregates cultured under atmospheric oxygen conditions demonstrate a similar marker expression profile to MSC aggregates cultured under physiological oxygen conditions in terms of nestin, GFAP and α-SMA expression

Previous studies in this thesis have shown that MSCs cultured as aggregates under low, physiological oxygen levels demonstrated intense expression of the neural markers, nestin and GFAP, concomitant with weak expression of the mesenchymal marker, α-SMA (Section 4.3.2). These observations were in keeping with previously published data (Kabos *et al.*, 2002; Croft and Przyborski, 2004; Hermann *et al.*, 2004; Suzuki *et al.*, 2004; Hermann *et al.*, 2006; Kim *et al.*, 2006). In order to ascertain whether MSCs cultured as aggregates under atmospheric oxygen levels retained this marker expression profile, aggregates were fixed and processed for marker analysis using immunocytochemistry and flow cytometry as previously described (Sections 2.6 and 2.7).

Aggregates were fixed in 4% PFA and processed for immunocytochemistry against nestin, GFAP and α-SMA as previously detailed in Section 2.6. As observed for MSCs cultured as aggregates under low oxygen tension (Section 4.3.2) the neural markers, nestin and GFAP, demonstrated intense levels of staining in aggregates cultured under high oxygen tension, indicative of very high levels of expression (Figure 4.14B and 4.14D respectively). Similarly, expression of the mesenchymal marker, α-SMA, was readily detectable in aggregates cultured under high oxygen tension, though levels of staining were very weak and diffuse, indicative of only weak expression (Figure 4.14F). This is also in keeping with observations from aggregates cultured under low oxygen tension (Figure 4.2). Expression of the previous markers were also examined using flow cytometric methods, as previously detailed in Section 4.3.2. Cells derived from MSC aggregates under atmospheric oxygen tensions demonstrated positive expression of the neural markers nestin and GFAP and, to a lesser extent, α-SMA, which is consistent with the immunocytochemical observations (Figure 4.14).

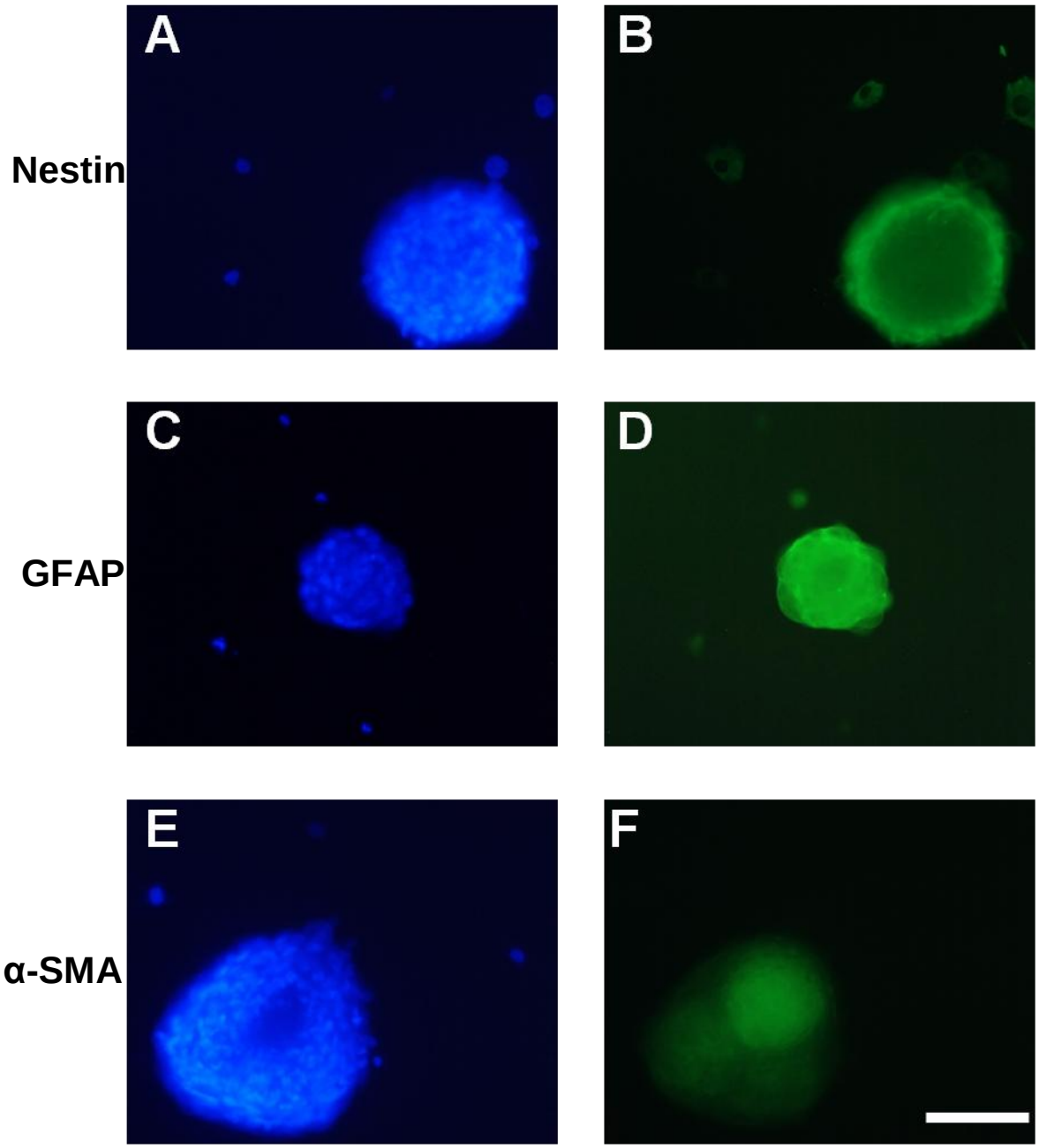


Figure 4.14. Immunocytochemical analysis of expression of neural and mesenchymal markers in MSC aggregates cultured under atmospheric oxygen tension (20%) as opposed to physiological oxygen tension (5%). Images **A**, **C** and **E** correspond to the nuclear DAPI stain, whereas **B**, **D** and **F** correspond to nestin, GFAP and α-SMA respectively. Expression profiles of markers in MSC aggregates cultured under atmospheric oxygen tension is highly comparable to the expression profiles observed following culture of MSC aggregates under physiological oxygen tension. Scale bar = 100μm.

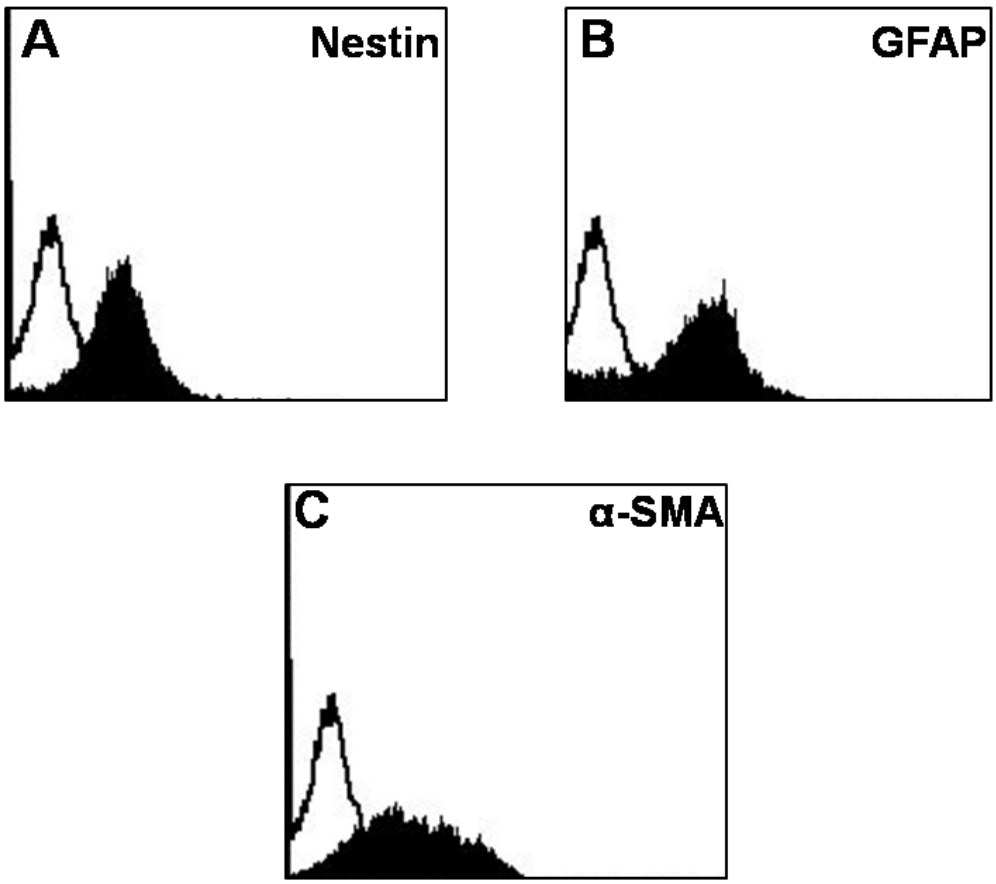


Figure 4.15. Flow cytometric analysis of expression of neural and mesenchymal markers in MSC aggregates cultured under atmospheric oxygen tension (20%) as opposed to physiological oxygen tension (5%). Representative traces showing typical marker expression profiles for nestin (**A**), GFAP (**B**) and α-SMA (**C**) in MSCs cultured as aggregates. Clear traces correspond to the *P3X* negative control and black traces correspond to specific positive fluorescence. Expression profiles of markers in MSC aggregates cultured under atmospheric oxygen tension is highly comparable to the expression profiles observed following culture of MSC aggregates under physiological oxygen tension (Figure 4.3).

4.3.14 MSC aggregates cultured under physiological oxygen conditions contain a greater number of viable cells compared to those cultured under atmospheric oxygen conditions

One of the most crucial aspects of cellular behaviour that oxygen concentration is known to dramatically affect is mitogenic activity and proliferation, and this has been reported in a range of different cell types in culture (Held and Sönnichsen, 1984; Falanga and Kirsner, 1993; Studer *et al.*, 2000; Krinner *et al.*, 2009). This study aimed to address the question of how crucial a role, if any, oxygen concentration played in the proliferation of MSCs cultured as aggregates *in vitro*.

MSCs were seeded out for aggregate formation as previously described in Section 4.2.1 and were maintained under low (5%) and high (20%) oxygen tensions for 3 days. After this period, the number of viable cells in each culture was determined quantitatively using the MTS proliferation assay, methodology for which is detailed in Section 2.8. The MTS proliferation assay is an accepted method for determining rates of proliferation in cellular spheroids and aggregates, and has been used in previously published studies (Qihao et al, 2007). Absorbance values at 490nm are directly proportional to the number of viable cells in culture and are shown in Figure 4.16.

MSCs cultured as aggregates under low oxygen tension demonstrated an absorbance at 490nm approximately two-fold higher than MSCs cultured as aggregates under high oxygen tension (Figure 4.16). This result was highly significant at the statistical level ($p<0.001$). These MTS data demonstrate that MSCs cultured as aggregates under low oxygen tension contain a significantly greater number of viable cells compared to their counterparts cultured under high oxygen tension. This is presumably a consequence of enhanced cellular proliferation following culture under low oxygen tension, though alternative mechanisms cannot be discounted e.g. reduced apoptosis, enhanced survival and reduced senescence, as all of these phenomena have been attributed to MSCs cultured under lowered oxygen states (Ren *et al.*, 2006; Krinner *et al.*, 2009; Zscharnack *et al.*, 2009).

4.3.15 Aggregates cultured under atmospheric oxygen conditions exert a similar but diminished effect on cell fate decisions of AHPCs following 7 days culture in CM compared to aggregates cultured under physiological oxygen conditions

The final and perhaps most crucial aim of the studies investigating the effects of oxygen concentration on the behaviour of MSC aggregates in culture was to determine whether varying oxygen concentration had any effect on the secretory activity of aggregates. There are a number of studies reporting that oxygen concentration is a crucial parameter influencing secretory behaviour of a range of cell types (Koos, 1986; Papas *et al.*, 1996; Zhao *et al.*, 2009). In order to ascertain whether varying oxygen concentration influenced the secretome of MSC aggregates, media conditioned by aggregates under high oxygen tension was assessed to see if the astrocytic effect observed following the culture of AHPCs with media conditioned by aggregates under low oxygen tension (Section 4.3.5) could be reproduced.

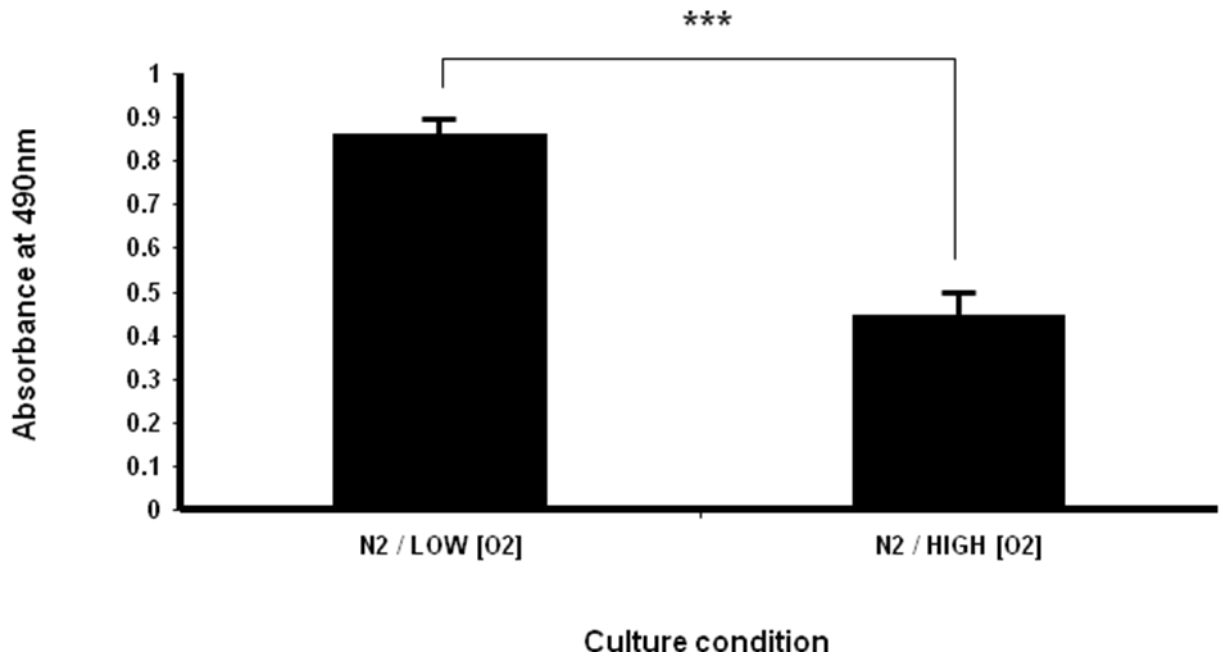


Figure 4.16. Determination of the viable number of MSCs in aggregates cultured under physiological (low) and atmospheric (high) oxygen tensions for 3 days. MSCs were seeded out for aggregate formation and cultured under physiological (low) and atmospheric (high) oxygen tensions for three days and the number of viable cells in culture determined using the colorimetric MTS assay. Each bar represents the mean (n=3)+SEM, *** $p<0.001$.

MSCs were seeded out for aggregate formation under low (5%) and high (20%) oxygen tensions and allowed to condition media for 3 days, after which the media was subsequently collected and prepared as described previously (Section 4.2.2). AHPCs were seeded out onto cellware coated with PLO and laminin at a density of 10,000 cells/cm^2 in their normal proliferative media and were allowed 24 hours to settle (designated day –1). Following this 24 hour period (day 0), media was removed and replaced with CM produced under low (CM/lo) or high (CM/hi) oxygen tensions and AHPCs cultured for a further 7 days. Media was replenished on the 4th day. After 7 days, AHPCs were processed for flow cytometry as described previously (Section 2.6) and analysed for expression of the neural markers nestin, Tuj-1, GFAP and GalC using flow cytometry (Figure 4.17).

In terms of the percentage of cells expressing each neural marker, there was no statistically significant difference between AHPCs cultured with CM/lo or CM/hi for any of the markers examined (Figure 4.17A). Both media samples resulted in an

increase in the percentage of AHPCs expressing GFAP compared to PC control, though the percentage was slightly higher in AHPCs cultured with CM/lo compared to CM/hi. In terms of MFI of marker expression, there was little observable difference in intensity of nestin, Tuj-1 and GalC expression in AHPCs cultured with CM/lo and CM/hi (Figure 4.17B). Differences in intensity of GFAP expression, however, were observed. Culturing AHPCs with CM/lo and CM/hi resulted in a substantial increase in intensity of GFAP expression compared to PC control in both instances, which strongly suggested that aggregates cultured under both low and high oxygen tensions secreted soluble factor(s) that induced astrogenesis in AHPCs following 7 days culture. However, AHPCs cultured with CM/lo demonstrated an MFI more than 2-fold greater than that observed following culture with CM/hi, a value of approximately 1100 for CM/lo compared to a value of 400 for CM/hi. This difference was significant at the statistical level ($p<0.05$). These flow cytometry data strongly suggest that MSC aggregates secrete astrocytic-promoting soluble factor(s) regardless of the oxygen tension in which they are cultured, though aggregates cultured under low oxygen exert a more significant astrocytic effect than aggregates cultured under high oxygen in terms of MFI of GFAP expression. A greater intensity of GFAP expression is likely to be indicative of a more mature astrocytic phenotype being expressed.

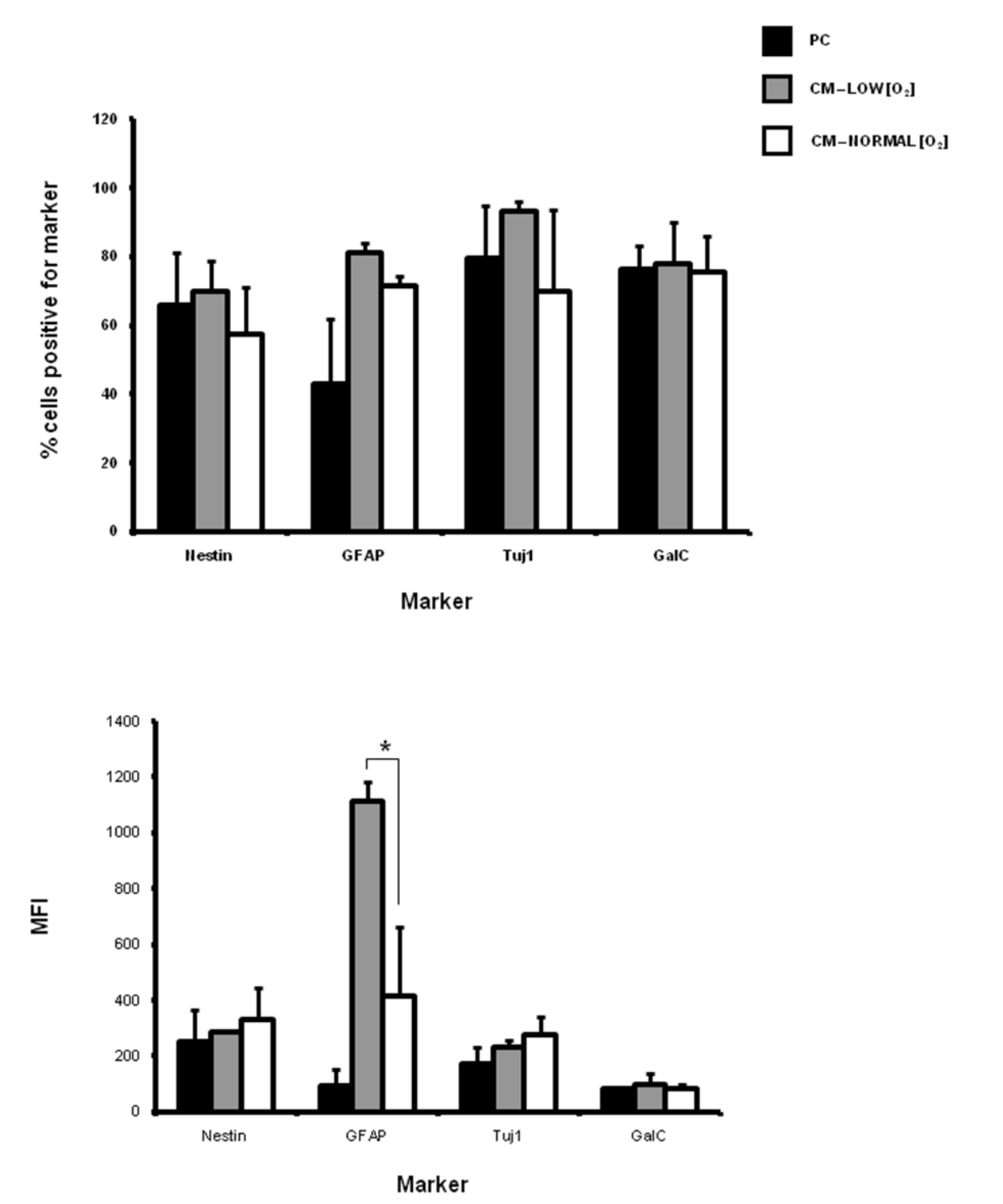


Figure 4.17. Flow cytometric analysis of marker expression in AHPCs treated with media conditioned by aggregates cultured under physiological (CM/lo) and atmospheric (CM/hi) oxygen tensions. A: % cells expressing marker; **B**: mean fluorescence intensity of marker expression. In terms of the percentage of cells expressing each neural marker, there was no statistically significant difference between AHPCs cultured with CM/lo or CM/hi for any of the markers examined (**A**). In terms of MFI of marker expression, there was little observable difference in intensity of nestin, Tuj-1 and GalC expression in AHPCs cultured with CM/lo and CM/hi (**B**). However, culturing AHPCs with CM/lo and CM/hi resulted in a substantial increase in intensity of GFAP expression compared to PC control in both instances, though AHPCs cultured with CM/lo demonstrated an MFI more than 2-fold greater than that observed following culture with CM/hi. Each bar represents the mean (n=3)+SEM, * $p<0.05$.

4.3.16 The astrocytic-promoting effect of CM acts in a dose-dependent manner

Previous studies in this Chapter have demonstrated that media conditioned by MSC aggregates contains soluble factor(s) that influence the cell fate decisions of AHPCs, such that following 7 days culture AHPCs exit the cell cycle and commit to an astrocytic differentiation program. This has been determined by examining changes in cellular morphology and examining expression of both structural and functional markers of astrocytic differentiation. Oxygen concentration has also been demonstrated to play a crucial role in the efficacy of this process.

The next aims of this study were to examine the effect of MSC aggregate-derived CM on AHPCs at a more mechanistic level. One of the first experiments performed was a titration experiment in order to determine whether the astrocytic-promoting effect of CM occurred in a dose-dependent manner. Concentration of soluble factors and cytokines is perhaps the most crucial parameter to consider in terms of their biological activity, as soluble factors often have different efficacies at different concentrations and can even have completely different effects in the same cellular system when present at different concentrations.

AHPCs were seeded out for these titration experiments under identical conditions to those previously described i.e. in their normal proliferative media, onto cellware coated with PLO and laminin, at a density of 10,000 cells/cm^2 (Section 4.2.3). Following a 'settling' period of 24 hours, media was removed and replaced with CM of different dilution factors – undiluted, 1:2, 1:4, 1:8, 1:16 and negative control (PC) – and AHPCs cultured for a further 7 days, with media being replenished on the 4th day. Following 7 days culture, the differentiative state of AHPCs at each dose was determined via flow cytometric analysis of neural marker expression, in terms of percentage of cells expressing marker and MFI of marker expression, as detailed in Section 2.6.

The percentage of cells expressing each marker is shown in Figure 4.18a for nestin (Figure 4.18a-A), Tuj-1 (Figure 4.18a-B), GFAP (Figure 4.18a-C) and GalC (Figure 4.18a-D). CM of varying dilutions did not appear to have any significant effects on the percentage of cells expressing each marker following 7 days culture. In particular, the percentage of cells expressing Tuj-1 and GalC remained constant throughout the

titration experiment, with approximately 80% of cells expressing both Tuj-1 and GalC at all dilutions of CM examined, including negative (PC) control (Figure 4.18-B and 4.18-D). Expression of nestin appeared more sporadic throughout the titration but, despite these slight fluctuations, the percentage of cells expressing nestin remained at the same approximate level (around 50-60%). The percentage of cells expressing GFAP following culture with CM for 7 days remained constant up to a dilution factor of 1:8, with approximately 80% of cells demonstrating positivity for GFAP expression. Following further dilution however (a dilution factor of 1:16 and negative (PC) control) the percentage of cells expressing GFAP showed a tendency to decrease, with approximately 50-60% of cells expressing GFAP under these conditions.

Such uniformity in percentage of AHPCs expressing neural markers following culture in CM of varying dilutions is not particularly unexpected, as previous studies in this Chapter have shown that AHPCs demonstrate positivity for neural marker expression under standard culture conditions (Figure 4.7). It was only when MFI of marker expression was examined that significant differences in cell phenotype were revealed. Figure 4.18b shows MFI of nestin (Figure 4.18b-A), Tuj-1 (Figure 4.18b-B), GFAP (Figure 4.18b-C) and GalC (Figure 4.18b-D) expression following culture with CM of varying dilutions for 7 days. Intensity of nestin expression decreased in a dose-dependent manner with increasing dilution of CM, with AHPCs cultured under negative (PC) control conditions demonstrating the weakest level of expression (Figure 4.18b-A). Tuj-1 was expressed at a relatively constant level through the titration, though there was a very slight tendency for intensity of expression to decrease from undiluted CM up to a dilution factor of 1:4 (Figure 4.18b-B). Intensity of GalC expression was somewhat more sporadic throughout the titration experiment, with MFI values fluctuating with increasing dilution factor but not adhering to any real trend (Figure 4.18b-D). In terms of intensity of GFAP expression, which is perhaps the most interesting given previous observations (Figure 4.7), there was a clear dose-dependent relationship between MFI values and dilution factor (Figure 4.18b-C). Intensity of GFAP expression decreased in a dose-dependent manner with increasing dilution of CM, such that AHPCs cultured with undiluted CM demonstrated the most intense expression of GFAP and AHPCs cultured under negative (PC) control conditions demonstrated the weakest expression. MFI values for GFAP in AHPCs cultured with

CM diluted 1:16 were on a par with negative control and it can be concluded that the effect is completely abolished at this dilution.

Combined, these titration data clearly demonstrate that the astrocytic effect of CM acts in a highly dose-dependent manner, with maximum activity observed in undiluted CM and diminishing with increasing dilution of CM.

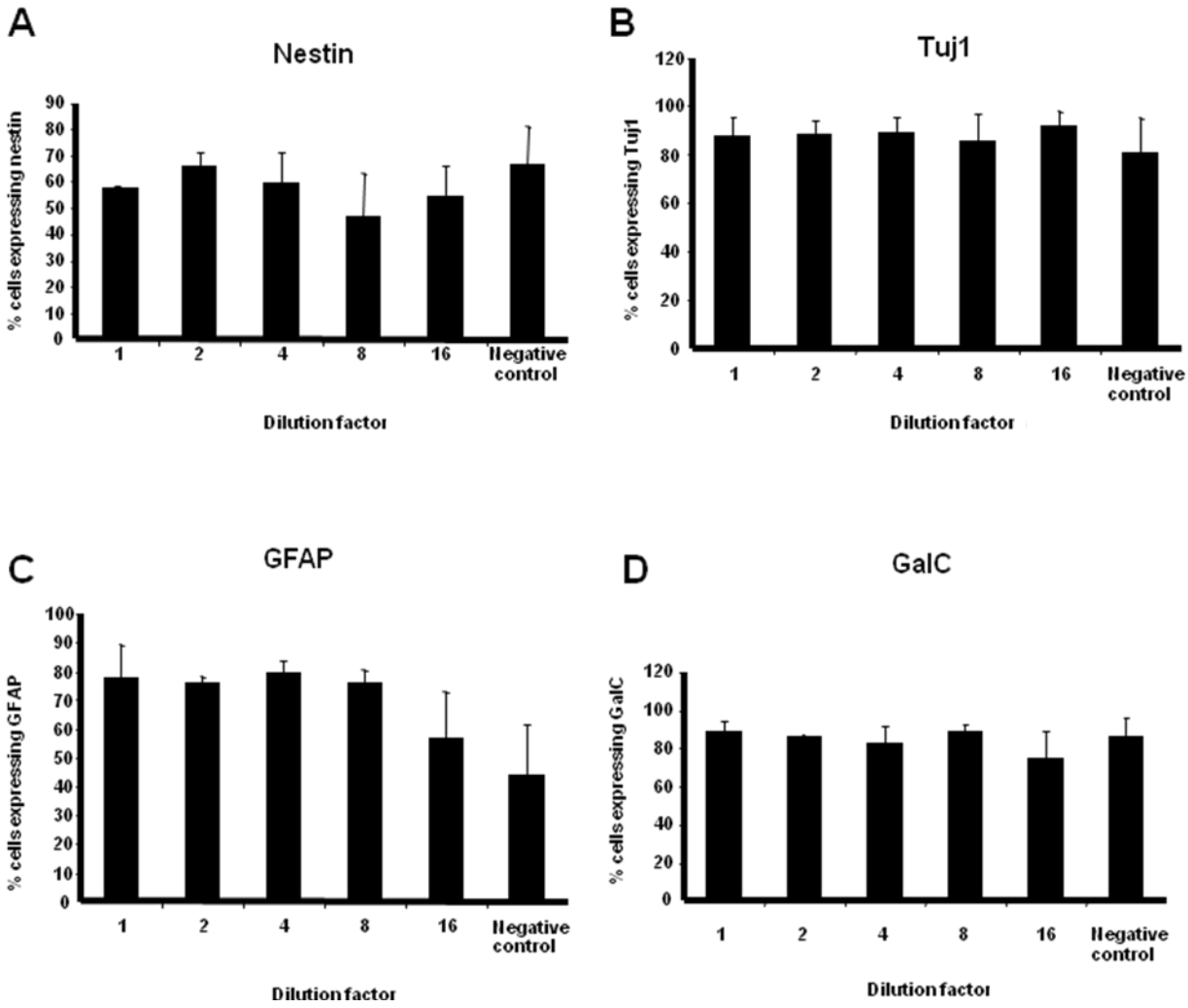


Figure 4.18a. Dose-dependency of the astrocytic-promoting effect of MSC aggregate-derived conditioned media. AHPCs were seeded out and cultured in conditioned media of varying dilution factors for 7 days and processed for flow cytometry as previously described. Expression of nestin, Tuj-1, GFAP and GalC was examined in terms of the percentage of cells expressing each marker. Each bar represents the mean (n=3)+SEM.

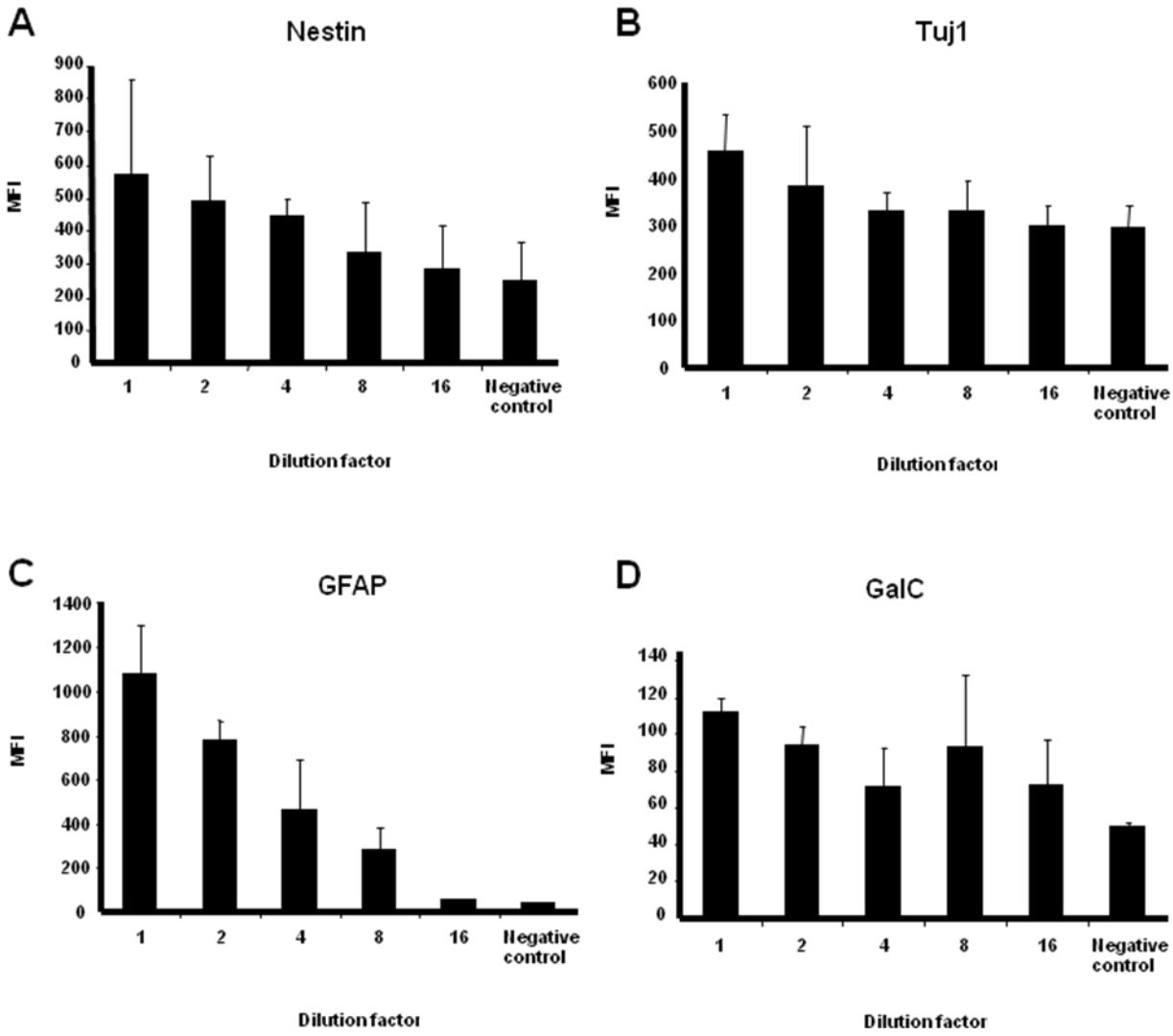


Figure 4.18b. Dose-dependency of the astrocytic-promoting effect of MSC aggregate-derived conditioned media. AHPCs were seeded out and cultured in conditioned media of varying dilution factors for 7 days and processed for flow cytometry as previously described. Expression of nestin, Tuj-1, GFAP and GalC was examined in terms of MFI of marker expression. The astrocytic-promoting effect of MSC aggregate-derived CM on AHPCs was highly dose-dependent. Each bar represents the mean (n=3)+SEM.

4.3.17 Aggregate-derived soluble factors present in CM are heat-sensitive

In a further attempt to better understand the nature of the aggregate-derived soluble factors responsible for exerting the astrogenic effect in AHPCs, the temperature-sensitivity of CM was assessed. Heat-sensitive factors in CM were inactivated by heating CM in boiling water for 10 minutes, as previously detailed in the literature (Rivera *et al.*, 2006). The aim of this experiment was to ascertain whether inactivating heat-sensitive factors in CM would abolish the astrocytic effect previously observed in AHPCs following 7 days culture.

AHPCs were seeded out for these experiments and maintained as described for all the other studies in this thesis, detailed in Section 4.2.3. In these particular experiments, AHPCs were cultured under PC conditions (negative control) or in either untreated CM (positive control) or heat-inactivated CM for 7 days, with media being replenished on the 4th day. Following 7 days culture, the differentiative state of AHPCs cultured under each condition was determined via flow cytometric analysis of neural marker expression, in terms of percentage of cells expressing marker and MFI of marker expression, as detailed in Section 2.6.

In terms of the percentage of cells expressing each marker, there appeared to be no obvious differences between AHPCs cultured with untreated CM and heat-inactivated CM (Figure 4.19A). This uniformity of neural marker expression in AHPCs has been consistent throughout studies in this thesis and it has therefore been analysis of MFI of marker expression that has been more pertinent in highlighting differences in cellular phenotype between different samples. When intensity of neural marker expression was compared between AHPCs cultured with untreated CM and heat-inactivated CM, there were no obvious differences in terms of nestin, Tuj-1 and GalC expression (Figure 4.19B). For nestin and GalC expression, MFI values were comparable to control (PC) values. MFI of Tuj-1 expression, however, increased slightly following culture with both untreated CM and heat-inactivated CM compared to PC control, though this increase was less than 2-fold and there was no statistically significant difference between the two CM samples. In terms of intensity of GFAP expression, AHPCs cultured with untreated CM and heat-inactivated CM both demonstrated a dramatic increase in MFI values compared to PC control (Figure 4.19B). This strongly suggested that AHPCs cultured with both media types were undergoing astrogenesis and adopting a more mature astrocytic phenotype. However, the intensity of GFAP expression in AHPCs cultured with untreated CM was substantially greater than that observed following culture with heat-inactivated CM, an MFI value of 700 compared to 400 respectively. This difference was significant at the statistical level ($p<0.05$). Although the intensity of GFAP expression in AHPCs cultured with heat-inactivated CM was lower than that observed following culture with untreated CM, the increase in GFAP expression observed was still a real result and significant compared to PC control. Therefore, these data demonstrate that heat-inactivation of CM only partially abolishes the astrocytic effect observed in AHPCs following 7 days culture, suggesting

that heat inactivation may change the proportion of active factor(s) in CM responsible for this effect.

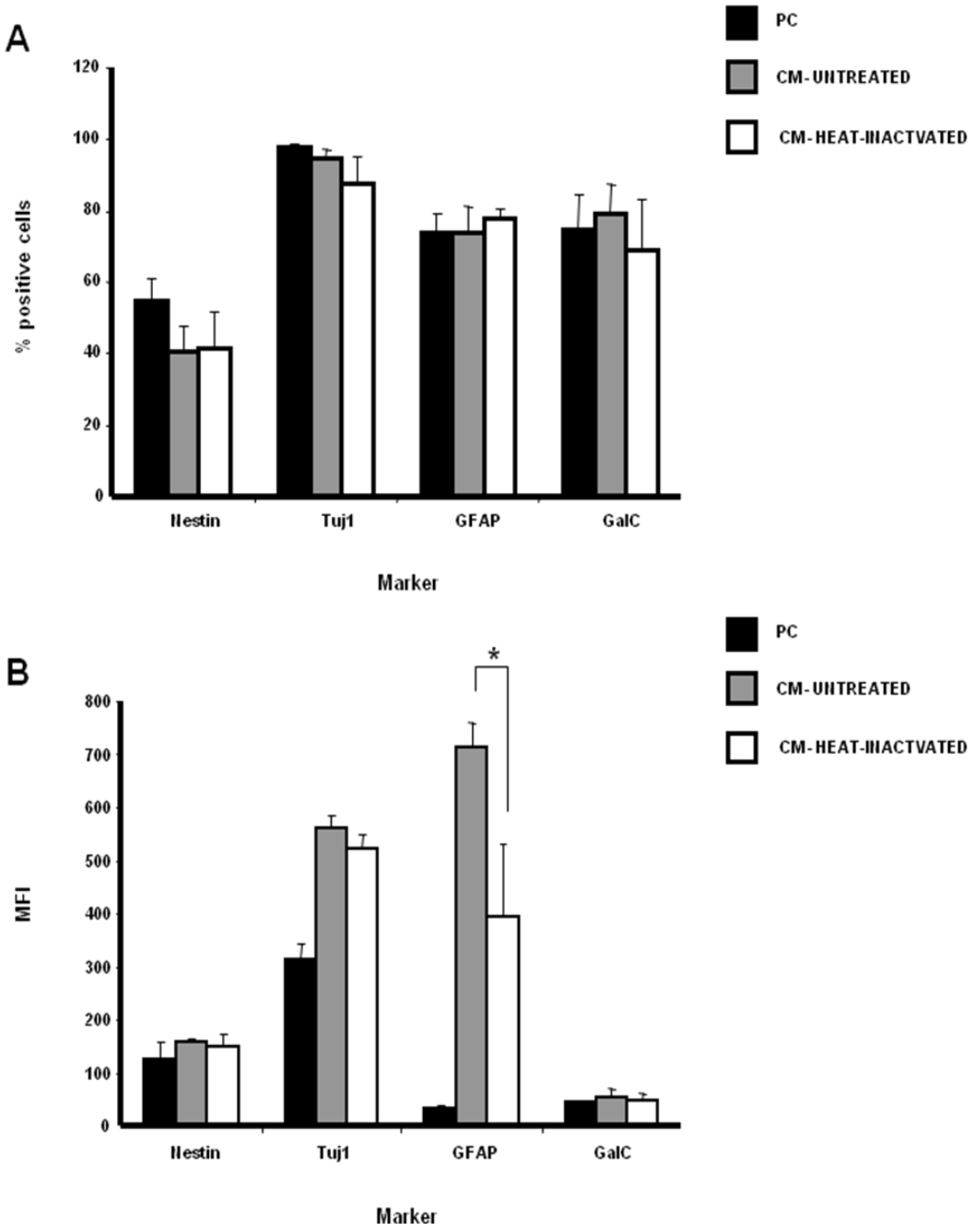


Figure 4.19. Heat-sensitivity of the astrocytic-promoting effect of MSC aggregate-derived conditioned media. AHPCs were seeded out and cultured in untreated conditioned media and heat-inactivated conditioned media (heated in boiling water for 10 minutes) for 7 days and processed for flow cytometry as previously described. Expression of nestin, Tuj-1, GFAP and GalC was examined in terms of the percentage of cells expressing each marker (**A**) and MFI of marker expression (**B**). The astrocytic-promoting effect of MSC aggregate-derived CM on AHPCs was significantly heat-sensitive. Each bar represents the mean (n=3)+SEM, * $p<0.05$.

4.3.18 Aggregate-derived soluble factors instruct AHPCs to adopt a glial cell fate, more specifically, an astrocytic fate at the expense of an oligodendrocytic fate

The next series of experiments in this study aimed to address the fundamental issue of whether soluble factors present in CM exerted an astrocytic effect in AHPCs via an instructive and/or selective mechanism. Acting via an instructive mechanism would suggest that the active components in CM acted on uncommitted AHPCs and induced them specifically to commit to and undergo astrocytic differentiation. This is in contrast to a selective mechanism, which would suggest that the active components in CM provided culture conditions that encouraged the growth/survival of AHPCs already committed to the astrocytic lineages and/or encouraged the death of AHPCs committed to other neural lineages e.g. neuronal or oligodendrocytic.

Two main studies were performed in order to determine the mechanism by which the active components in CM were exerting an astrocytic effect in AHPCs.

a) Temporal effects of CM on neural marker expression in AHPCs were examined.

b) Cell proliferation and cell death in specific subpopulations of AHPCs cultured with CM was examined.

4.3.19 AHPCs cultured with CM demonstrate a temporal increase in GFAP expression

AHPCs were seeded out for these experiments and maintained as described for all the other studies in this thesis, detailed in Section 4.2.3. In these particular experiments, AHPCs were cultured in CM or appropriate control (UC) and marker expression analysed at days 0, 4 and 7 via flow cytometric analysis in terms of percentage of cells expressing marker and MFI of marker expression, as detailed in Section 2.6. The day of cell seeding was designated day –1. Results are shown in Figure 4.20.

The temporal expression profiles in terms of the percentage of cells demonstrating positivity for marker expression when cultured in CM or appropriate control (UC) are shown in Figure 4.20 for Tuj-1 (Figure 4.20A), GFAP (Figure 4.20C) and GalC

(Figure 4.20E). Culturing AHPCs with CM did not appear to significantly affect the kinetics of Tuj-1 and GalC expression over time (Figures 4.20A and 4.20E). There was a slight trend for the percentage of cells expressing both markers to increase over the 7 day time course of the experiment, but this trend was apparent in both UC and CM cultures. In terms of the raw percentages, there was little observable difference between the two cultures. The kinetics of GFAP expression, however, did differ significantly between UC and CM cultures (Figure 4.20C). Culture with CM resulted in a significant time-dependent increase in the percentage of AHPCs expressing GFAP up to the 4 day timepoint; thereafter, no further increase was observed, with AHPCs examined at the 7 day timepoint demonstrating a similar level of expression to those examined at the 4 day timepoint. This temporal profile was in complete contrast to that observed in AHPCs cultured under UC control conditions, where the percentage of $GFAP^+$ cells showed a slight tendency to decrease up to the 4 day timepoint, followed by a subsequent increase between days 4 and 7 (Figure 4.20C).

The temporal expression profiles in terms of MFI of marker expression in AHPCs cultured in CM or appropriate control (UC) are shown in Figure 4.20 for Tuj-1 (Figure 4.20B), GFAP (Figure 4.20D) and GalC (Figure 4.20F). As was the case for the percentage of cells expressing Tuj-1 and GalC, culturing AHPCs with CM did not appear to significant affect the kinetics of Tuj-1 and GalC intensity over time (Figures 4.20B and 4.20F). Although there was a slight trend for the intensity of both markers to increase over the 7 day time course of the experiment, this trend was apparent in both UC and CM cultures. In terms of raw MFI values, intensity of expression was generally higher in AHPCs cultured with CM compared to UC controls, but such was the level of variation between independent repeats that the error bars at each timepoint overlapped extensively (Figures 4.20B and 4.20F). The kinetics of GFAP expression in AHPCs cultured under both conditions were more striking, such that AHPCs cultured in CM demonstrated a significant time-dependent increase over the 7 day time course of this experiment (Figure 4.20D). This temporal profile was in complete contrast to that observed in AHPCs cultured under UC control conditions, where intensity of expression remained extremely low and uniform throughout the 7 day time course (Figure 4.20D).

Combined, these data strongly suggest that soluble factors in CM induce AHPCs to differentiate into astrocytes at the expense of neurons and oligodendrocytes.

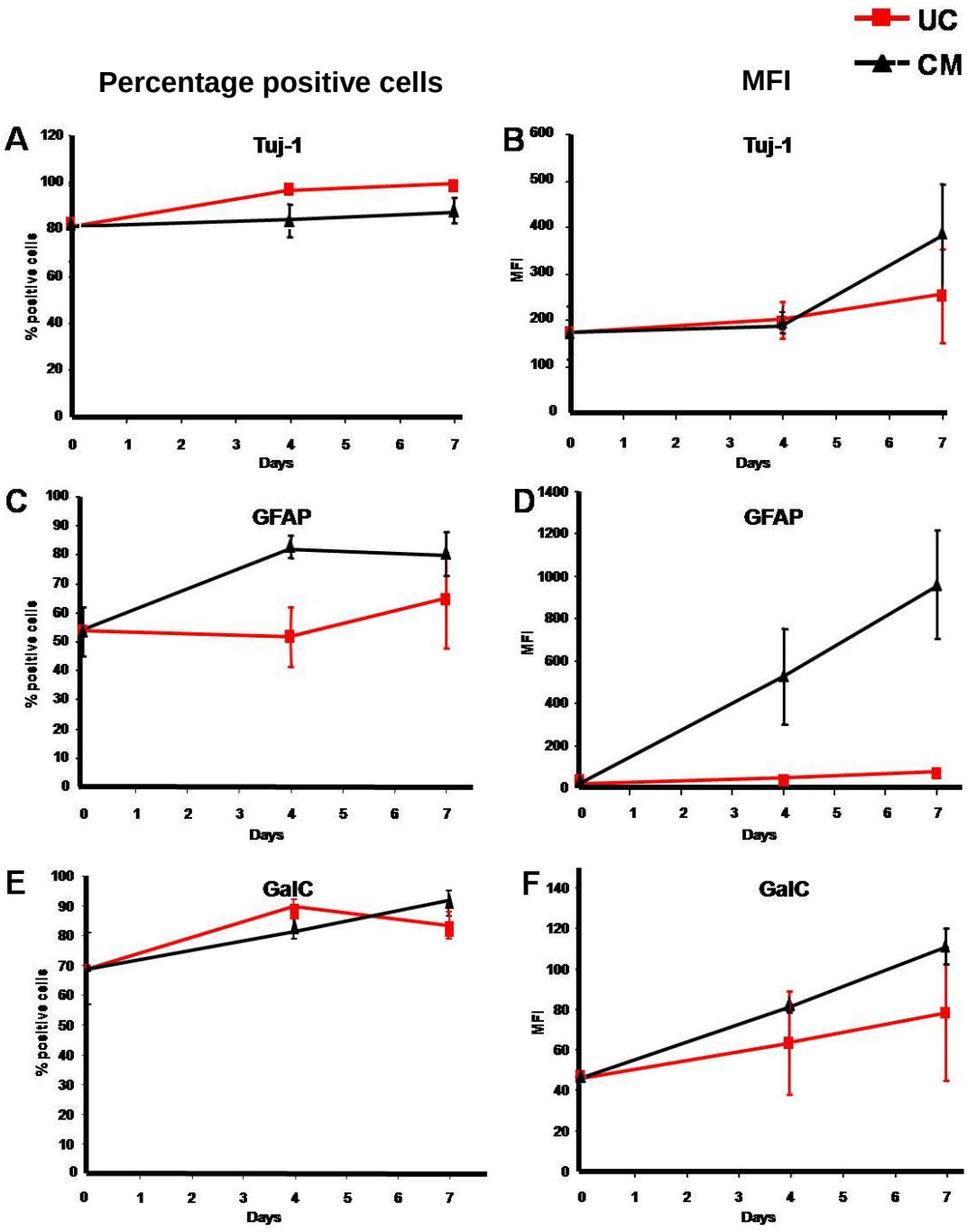


Figure 4.20. Temporal expression of neural markers in AHPCs cultured in MSC aggregate-derived conditioned media for up to and including 7 days. AHPCs were cultured in CM and appropriate control conditions (UC) and processed for flow cytometry at 4 and 7 days, as previously described. Expression of Tuj-1, GFAP and GalC was examined in terms of the percentage of cells expressing each marker (**A, C** and **E**) and MFI of marker expression (**B, D** and **F**). A significant temporal increase in the percentage of cells expressing GFAP (**C**) and similarly in the MFI of GFAP expression (**D**) was observed compared to UC controls. Each data point represents the mean (n=3)±SEM.

4.3.20 The astrocytic effect of CM in AHPCs occurs independently of effects on cell proliferation but cell death of oligodendrocytic precursors may play an important role

The previous study examining the temporal expression of neural markers in AHPCs cultured with CM and UC control suggested that AHPCs committed to and underwent astrocytic differentiation over the 7 day time course of this experiment. In order to ascertain whether a selective mechanism may also play an important role, cell proliferation and cell death were examined in specific subpopulations of AHPCs cultured with CM and UC control. Cell proliferation was assessed using bromodeoxyuridine (BrdU)-incorporation and cell death was assessed using propidium iodide (PI)-incorporation into AHPCs cultured with CM and UC control for 4 and 7 days. These cells were then processed for dual-staining with antibodies directed against the above molecules and the neural markers, either Tuj-1, GFAP or GalC, followed by flow cytometric analysis as described in detail in Section 4.2.4. The percentage of cells demonstrating dual-positivity for BrdU/PI and Tuj-1/GFAP/GalC was determined and results are shown in Figure 4.21.

Figure 4.21 demonstrates the rate of proliferation in specific subpopulations of AHPCs following culture in CM and UC control for 4 (Figure 4.21A) and 7 (Figure 4.21B) days. Subpopulations of AHPCs were defined as Tuj-1^+, $GFAP^+$ and $GalC^+$. In terms of proliferation rate, no significant difference was observed between UC and CM in either the Tuj-1^+, $GFAP^+$ or $GalC^+$ subpopulations, at both timepoints assessed (Figures 4.21A and 4.21B). These results strongly suggest that soluble factors in CM do not influence the rate of proliferation of any AHPC subpopulations.

Figure 4.21 also demonstrates the rate of cell death in specific subpopulations of AHPCs following culture in CM and UC control for 4 (Figure 4.21C) and 7 (Figure 4.21D) days. The most interesting trends emerged in the $GFAP^+$ and $GalC^+$ subpopulations following culture in CM compared to UC control. At both the 4 and 7 day timepoints, culturing AHPCs with CM resulted in a decrease in the percentage of $GFAP^+$ cells undergoing cell death compared to UC control cultures, which demonstrated higher levels of cell death. Although this trend was apparent at both timepoints assessed, these observations were not significant at the statistical level.

The $GalC^+$ subpopulation exhibited the complete opposite trend to the $GFAP^+$ subpopulation. At both the 4 and 7 day timepoints, AHPCs cultured with CM demonstrated significantly higher levels of cell death compared to UC control cultures, which demonstrated lower levels of cell death. Although this trend was apparent at both timepoints assessed, this observation was only statistically significant at the 7 day timepoint ($p<0.05$).

Combined, these flow cytometric data suggest that the astrocytic effect observed in AHPCs following culture in CM occurs independently of effects on cell proliferation within specific AHPC subpopulations. Therefore, selection of a $GFAP^+$ astrocytic-committed precursor is unlikely to be occurring in this system, suggesting an instructive mechanism as opposed to a selective mechanism. However, an important mechanism by which soluble factors in CM could be exerting their effect is via their influences on cell death in specific AHPC subpopulations. AHPCs cultured in CM demonstrated lower rates of cell death in the $GFAP^+$ subpopulation compared to controls (though not statistically significant) and higher levels of cell death in the $GalC^+$ subpopulation compared to controls ($p<0.05$). Therefore, although results from these studies suggest that astrogenesis occurs via an instructive mechanism in AHPCs, it is also highly probable that increased cell death in the $GalC^+$ oligodendrocytic subpopulation may play an important role, resulting in astrogenesis at the expense of oligodendrogenesis.

4.3.21 Increased rates of cell death in the $GalC^+$ AHPC subpopulation following culture with CM are a direct result of apoptosis rather than necrosis

In the previous section, cell death was assessed in specific subpopulations of AHPCs following culture with UC and CM for 4 and 7 days, using PI-incorporation as an indicator of cell death. AHPCs cultured in CM demonstrated lower rates of cell death in the $GFAP^+$ subpopulation compared to UC control (a statistically insignificant result) and, perhaps more interestingly, higher rates of cell death in the $GalC^+$ subpopulation compared to UC control ($p<0.05$). Although PI incorporation into dead cells (it is membrane impermeant and as such is excluded from viable cells) is perhaps the most commonly used method of assessing cell death, it has one major disadvantage in that it is unable to distinguish between cells whose membranes have been mechanically disrupted due to necrosis (cell death due to external factors) or those due

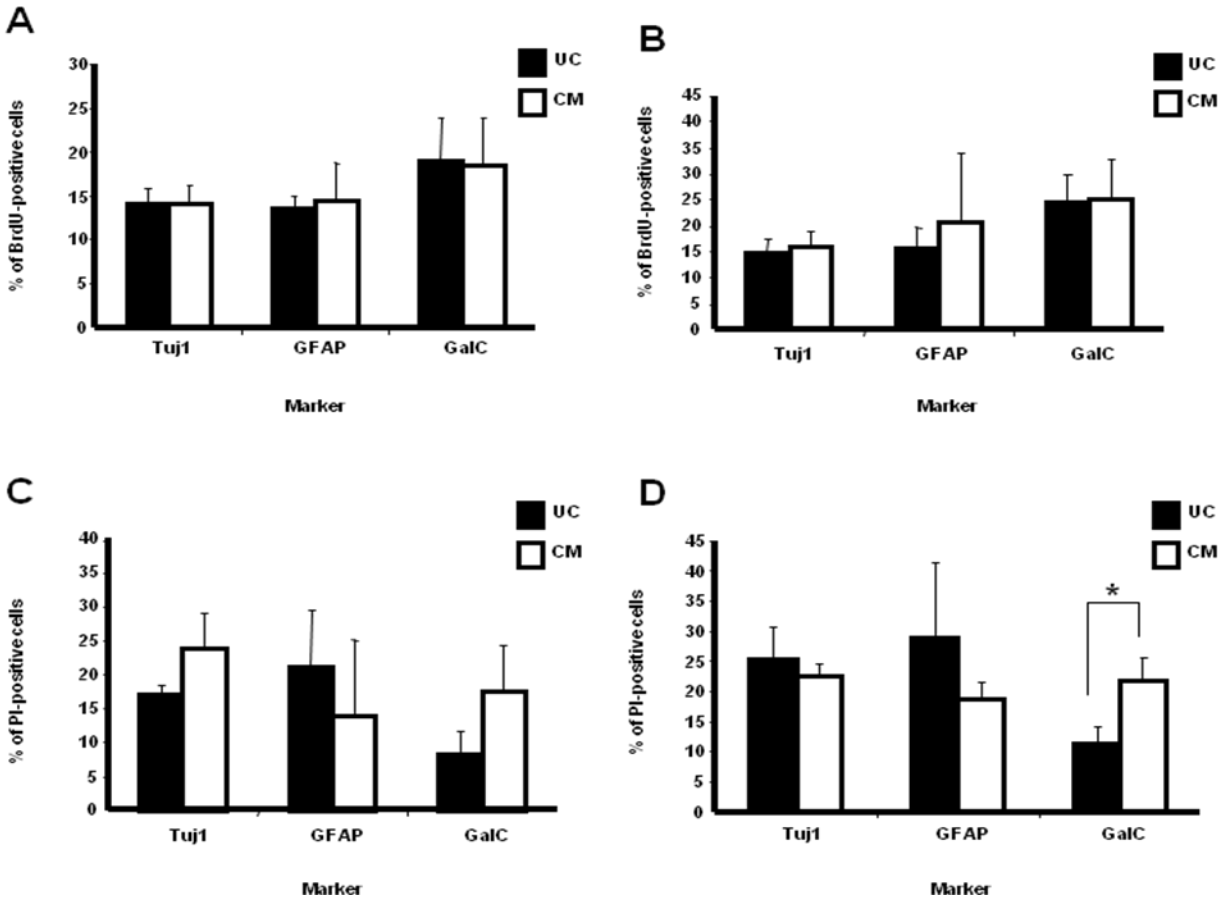


Figure 4.21. Flow cytometric analysis of rates of cell proliferation and cell death in specific subpopulations of AHPCs following culture in CM and UC control conditions for 4 and 7 days. AHPCs were seeded out and cultured in CM and appropriate control conditions (UC) and processed for flow cytometry at 4 and 7 days as previously described. Cell proliferation was assessed at 4 (**A**) and 7 (**B**) days following addition of 10μM BrdU to the culture media 24 hours prior to processing i.e. at days 3 and 6 respectively. Dual-staining was then performed, followed by flow cytometric analysis, to determine rates of proliferation in specific subpopulations of the AHPC cultures. There were no significant differences in rates of proliferation between UC and CM cultures. Cell death was assessed at 4 (**C**) and 7 (**D**) days following addition of 50μg/ml propidium iodide (PI) to the culture media 10 minutes prior to processing. Dual-staining was then performed, followed by flow cytometric analysis, to determine rates of cell death in specific subpopulations of the AHPC cultures. A significant increase in PI-incorporation into the $GalC^+$ subpopulation was observed. Each bar represents the mean (n=3)+SEM, * $p<0.05$.

to apoptosis (cell death due to internal factors; 'programmed' cell death). Therefore, it is not possible to ascertain with any certainty whether the increased rates of cell death observed in the $GalC^+$ subpopulation in the study above are a consequence of necrosis or apoptosis.

The next series of experiments aimed to determine the mechanisms of cell death in the $GFAP^+$ and $GalC^+$ AHPC subpopulations following culture with UC and CM, in terms of apoptosis vs. necrosis. Molecular events that occur during apoptosis are largely mediated by a family of cysteine proteases called caspases, of which caspase-3 is a central mediator in the apoptotic pathway (Porter and Jänicke, 1999; Fox and Aubert, 2008; Mazumder *et al.*, 2008). Activation of caspase-3 occurs during the early stages of apoptosis via the proteolytic cleavage (adjacent to Asp175) of the inactive proenzyme. Detection of cleaved caspase-3 in cells can therefore be used as a marker of apoptosis.

AHPCs were seeded out for these experiments as for all previous studies in this thesis (Section 4.2.3) and cultured under UC and CM conditions for 4 and 7 days. Following these periods, AHPCs were fixed in 2% PFA and processed for dual-staining with antibodies directed against cleaved caspase-3 and either GFAP or GalC, followed by flow cytometric analysis, as described in detail in Section 4.2.5. The percentage of cells demonstrating dual-positivity for cleaved caspase-3 and GFAP/GalC were determined and results are shown in Figure 4.22.

Although the reduced rates of cell death in the $GFAP^+$ subpopulation were not significant at the statistical level (Figure 4.21), rates of apoptosis were still determined in this subpopulation in order to ascertain whether reduced rates of cell death were mirrored in apoptosis rates. At both the 4 (Figure 4.22A) and 7 (Figure 4.22B) day timepoints, culturing AHPCs with CM resulted in a decrease in the percentage of $GFAP^+$ cells undergoing apoptosis compared to UC control cultures, which demonstrated higher levels of apoptosis. This trend was apparent at both timepoints assessed and mirrored rates of cell death determined by PI-incorporation (Figure 4.21). Similarly, these observations were not significant at the statistical level.

As observed in the previous section (Section 4.3.20), the $GalC^+$ subpopulation demonstrated the complete opposite trend to the $GFAP^+$ subpopulation. At both the 4 (Figure 4.22A) and 7 (Figure 4.22B) day timepoints, AHPCs cultured with CM demonstrated significantly higher levels of apoptosis in the $GalC^+$ subpopulation compared to UC control culture, which demonstrated lower levels of apoptosis. This trend was apparent at both timepoints assessed and mirrored rates of cell death

determined by PI-incorporation (Figure 4.21). These observations were significant at both timepoints assessed ($p<0.01$).

Data from the previous section (Section 4.3.20) suggested that soluble factors in CM could be exerting an astrocytic effect, at least in part, via their influences on cell death in the $GFAP^+$ and $GalC^+$ AHPC subpopulations. Analysis of apoptosis in these subpopulations provides further support and suggests that soluble factor(s) in CM activate apoptotic pathways in the $GalC^+$ subpopulation/ inhibit activation of apoptotic pathways in the $GFAP^+$ subpopulation. Apoptosis in the $GalC^+$ subpopulation is likely to be a key factor as to why astrogenesis is observed at the expense of oligodendrogenesis in AHPCs following culture in CM. A less significant factor is likely to be the 'protective' role observed in the $GFAP^+$ subpopulation, as indicated by decreased detection of cleaved caspase-3 following culture with CM compared to UC control.

4.3.22 Soluble factors in CM do not influence the number of viable cells in culture in the 33B oligodendroglioma cell line but demonstrate a slight tendency to induce apoptosis in this system

Combined, the above data strongly suggest that there are soluble factor(s) present in CM that result in the activation of apoptotic pathways specifically in the $GalC^+$ subpopulation of AHPCs i.e. oligodendrocytic-precursor population. One of the major causative mechanisms of cancer is aberrant cell survival resulting from a failure of proper and timely apoptosis, resulting in the development of accumulated tissue masses and tumours (Green and Evan, 2002; Kasibhatla and Tseng, 2003; Gerl and Vaux, 2005). Therefore, if this apoptotic effect can be reproduced in other oligodendrocytic systems, further analysis of the active components in CM could offer potential in the treatment of brain tumours arising from transformed oligodendrocytes i.e. oligodendrogliomas.

In order to ascertain whether this apoptotic effect was specific to AHPCs or could be reproduced in other oligodendrocytic culture systems, the effect of CM on cell viability and cell death was examined in the 33B cell line, an oligodendroglioma (more specifically, a Schwannoma) cell line derived from rat (Fields, 1977; Fields and Dammerman, 1985). These cells were a generous gift from Professor Eric Blair,

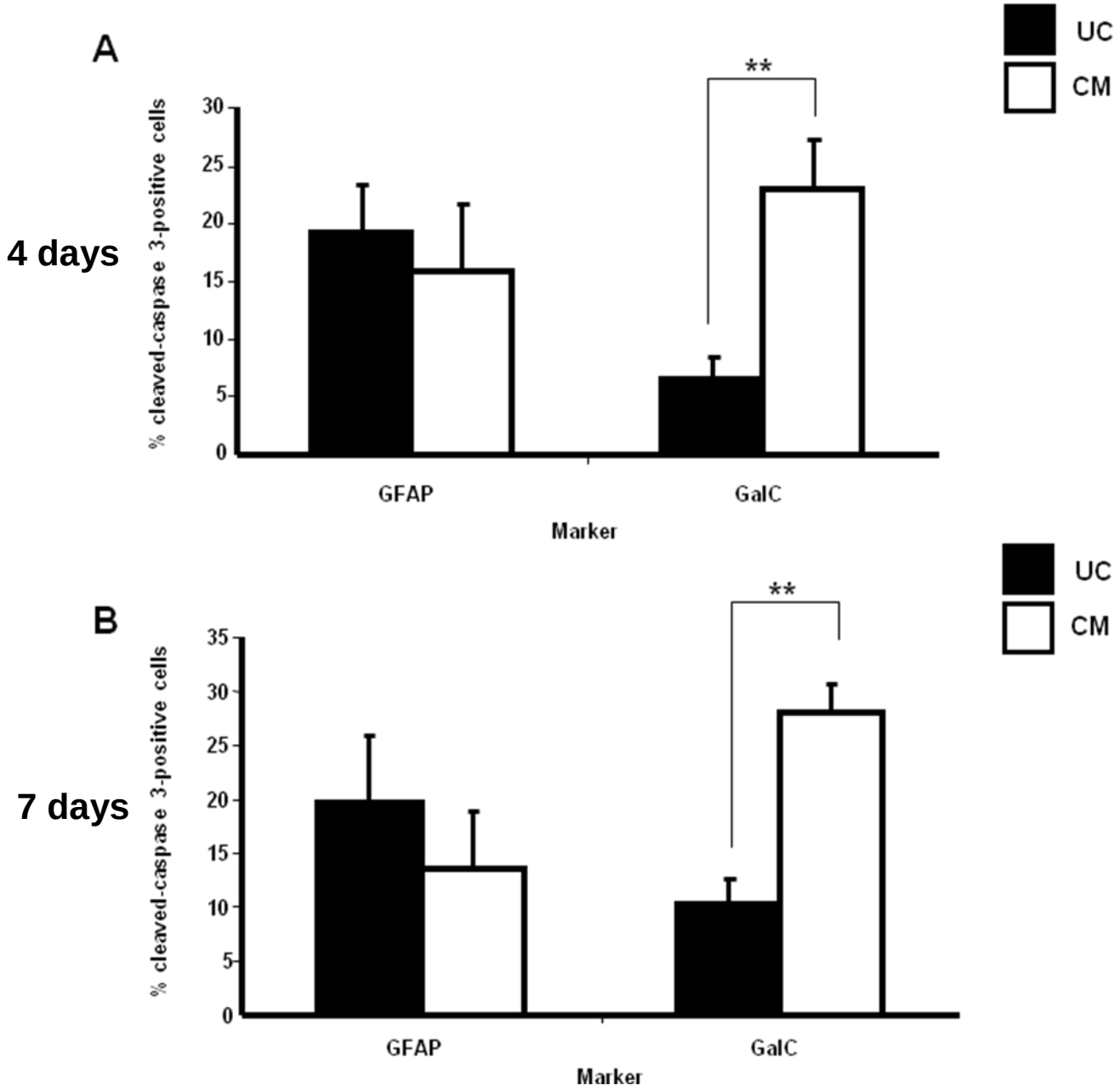


Figure 4.22. Flow cytometric analysis of apoptosis in the GFAP$^+$ and GalC$^+$ subpopulations of AHPCs following culture in CM and UC control conditions for 4 and 7 days. AHPCs were seeded out and cultured in CM and appropriate control conditions (UC) and processed for flow cytometry at 4 and 7 days as previously described. Apoptosis was assessed at 4 (**A**) and 7 (**B**) days *via* dual-staining for GFAP/GalC and cleaved caspase-3, using the latter as a direct marker of apoptosis. Flow cytometric analysis was performed to determine the percentage of cells co-expressing these markers. Rates of apoptosis mirrored rates of cell death, as previously determined by measuring PI-incorporation into AHPC subpopulations, suggesting that rates of cell death were a direct consequence of apoptosis, as opposed to necrosis. A significant increase in cleaved caspase-3 expression was observed in the GalC$^+$ AHPC subpopulation following 7 days culture, suggesting that some unidentified factor in the conditioned media was inducing apoptosis specifically in the GalC$^+$ subpopulation. Each bar represents the mean (n=3)+SEM, ** $p<0.01$.

University of Leeds, UK, and were maintained under standard conditions as described for MSCs, detailed in Section 2.3. 33B cells cultured under standard conditions demonstrated many morphological features in keeping with their neural origin, particularly demonstrating extensive cytoplasmic projections (Figure 4.23). The cancerous origin of these cells was also apparent in terms of their rapid rate of growth under standard culture conditions.

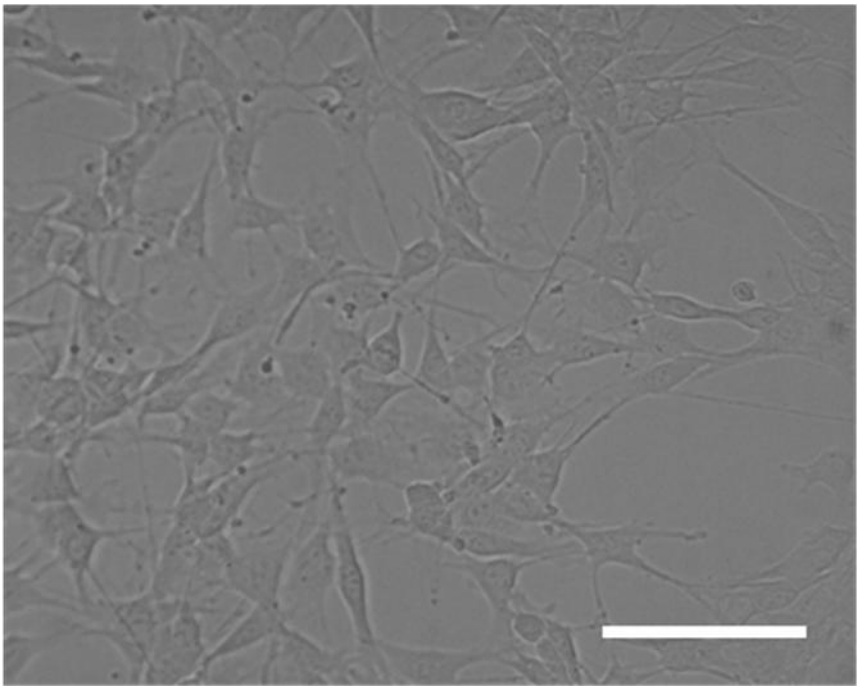


Figure 4.23. Typical morphology of the 33B cell line under standard culture conditions. The 33B cell line was originally propagated from a rat oligodendrocytoma (Schwannoma) and demonstrates morphological features in keeping with their neural origin; in particular, extensive cytoplasmic projections. Scale bar = 100μm.

4.3.23 33B cells cultured in CM do not demonstrate any apparent difference in the number of viable cells in culture compared to controls

Initially, experiments were performed in order to ascertain whether soluble factor(s) in CM affected the viability of 33B cells in culture. Viability was assessed using the MTS proliferation assay as previously described (Section 2.8). 33B cells were seeded out for these experiments under conditions mimicking those previously used for AHPC studies where possible (Section 4.2.3). Briefly, 33B cells were seeded onto non-coated cellware at a density of 10,000 cells/cm^2 in standard culture media (CCM) and allowed 24 hours to settle (designated day –1). Following this 24 hour period (day 0), media was removed and replaced with CM or appropriate control media (UC: unconditioned

media; CCM: standard culture media) and 33B cells cultured for a further 7 days. Medium was replenished on the 4th day. The number of viable cells in each culture were determined at days 1, 4 and 7 using the MTS assay as previously performed for AHPCs (Section 4.3.6) and results are shown graphically in Figure 4.24.

At each of the timepoints examined there was no observable difference in the number of viable cells in culture regardless of the culture condition employed (Figure 4.24). These data therefore suggested that soluble factor(s) in CM did not have any significant effects on the viability of 33B cells in culture when compared to control cultures. However, in terms of examining phenomena such as apoptosis and necrosis in detail, the MTS assay is perhaps not the best approach as it is crude and lacks sensitivity in comparison to more sophisticated techniques such as flow cytometry. Therefore, although the MTS data revealed no significant change in viable cell number between CM and control conditions, further analyses are required to investigate any changes at the molecular level e.g. activation of apoptotic pathways. Such experiments were performed in the proceeding section.

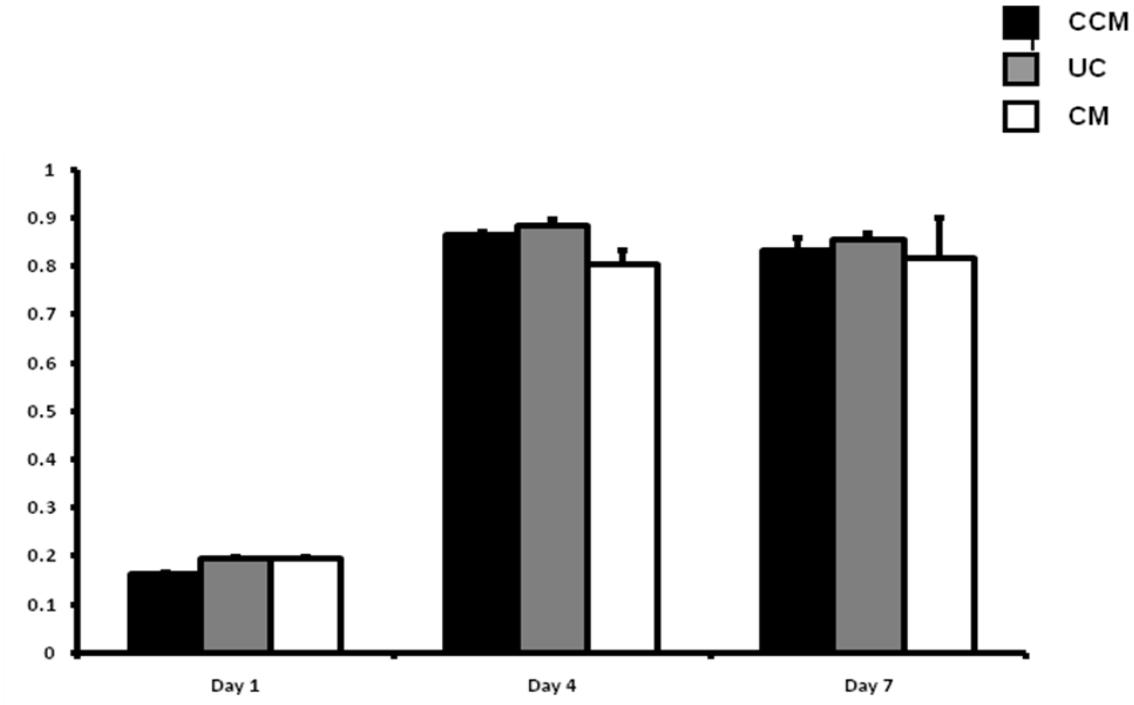


Figure 4.24. Determination of the number of viable cells in culture following treatment with conditioned media and appropriate controls for 1, 3 and 7 days. 33B cells were seeded out and cultured in conditioned media and appropriate controls (CCM and UC) and the number of viable cells in culture determined using the colorimetric MTS assay. There was no significant difference in the number of viable cells in culture between conditioned media and appropriate controls at any of the timepoints assessed. Each bar represents the mean (n=3)+SEM.

33B cells cultured in CM demonstrate a slight tendency to undergo apoptosis compared to controls

In order to gain a greater appreciation of the apoptotic/necrotic phenotype of 33B cells cultured in CM and appropriate controls, cell death was analysed in detail using flow cytometric methods as described in Section 4.2.6. Briefly, 33B cells were seeded out as described previously in Section 4.3.23 and maintained in CM and appropriate controls (UC and CCM) for 4 and 7 days. Following these culture periods, cells were dual-stained against PI and caspase-3, followed by flow cytometric analysis, as detailed in Section 4.2.6. Such analysis allows discrimination between cells at various stages of cell death, as illustrated diagrammatically in Figure 4.25.

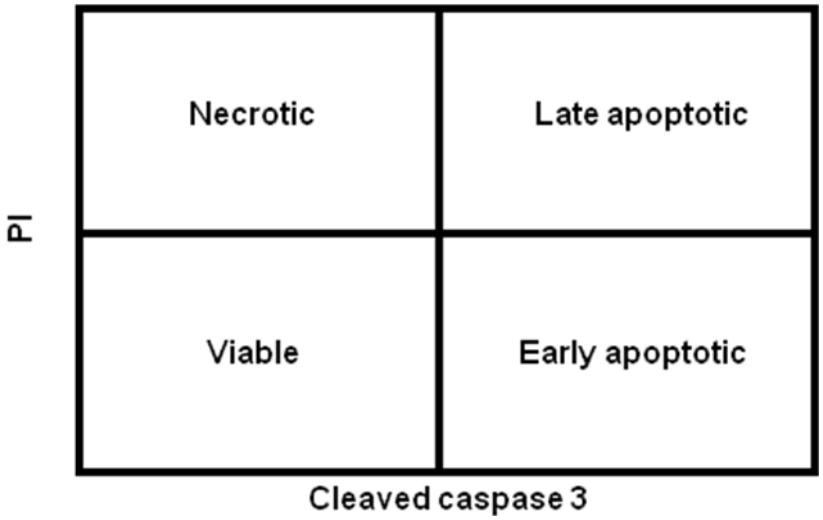


Figure 4.25. Flow cytometric analysis of cell death can be achieved by dual-labelling with propidium iodide (PI) and cleaved caspase-3, followed by flow cytometric analysis to discriminate between various stages of cell death, including early apoptosis (PI^-/caspase-3^+), late apoptosis (PI^+/caspase-3^+) and necrosis (PI^+/caspase-3^-).

- Healthy, viable cells (PI^-/caspase-3^-) Lower left quadrant
- Early apoptotic cells (PI^-/caspase-3^+) Lower right quadrant
- Late apoptotic cells (PI^+/caspase-3^+) Upper right quadrant
- Necrotic cells (PI^+/caspase-3^-) Upper left quadrant

Data from three independent repeats were collated and the results shown graphically in Figure 4.26 in terms of the percentage of early apoptotic (Figure 4.26A; PI^-/caspase-3^+) and late apoptotic (Figure 4.26B; PI^+/caspase-3^+) cells. In terms of the percentage of cells in the early stages of apoptosis, there was no obvious difference between cells cultured with CCM or UC at either of the timepoints assessed (Figure 4.26A). Following culture with CM, however, there was a tendency for the percentage of cells in the early stages of apoptosis to increase compared to control conditions and this was apparent at both the 4 and 7 day timepoints (Figure 4.26A). These observations were not significant at the statistical level. Very similar trends were observed in terms of the percentage of cells in the later stages of apoptosis (Figure 4.26B). There was no obvious difference between cells cultured with CCM or UC at either of the timepoints assessed. However, culture with CM again resulted in a tendency for the percentage of cells in the later stages of apoptosis to increase compared to control conditions, a trend that was apparent at both timepoints assessed. Similarly, these observations were not significant at the statistical level.

In summary, these data suggest that soluble factor(s) in CM induce a slight increase in rates of apoptosis in the 33B oligodendroglioma cell line compared to control cultures. Although 33B cells cultured in CM demonstrated a greater percentage of cells in both the early and later stages of apoptosis at both timepoints assessed, these observations were not significant at the statistical level. Despite the lack of statistical significance, these data still hold potential in that the trend for soluble factor(s) in CM to induce apoptosis in oligodendrocytic cell types has been reproduced here.

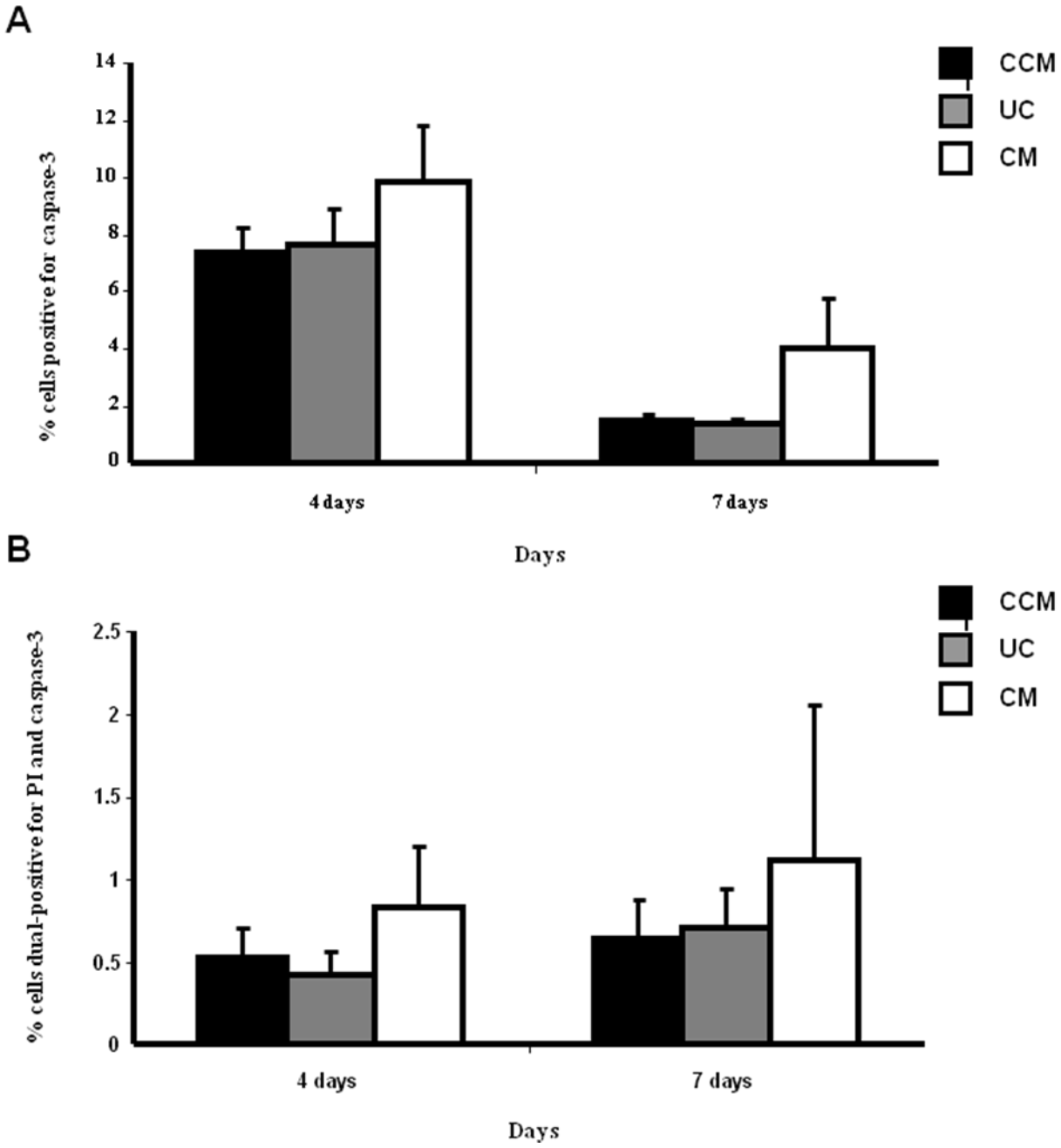


Figure 4.26. Flow cytometric determination of early-apoptotic and late-apoptotic 33B cells in culture following treatment with CM and appropriate controls (CCM and UC) for 4 and 7 days. 33B cells were seeded out and cultured in CM and appropriate controls (CCM and UC) and processed for flow cytometry at 4 and 7 days as described previously. Dual-labelling with propidium iodide (PI) and cleaved caspase-3 was performed in order to discriminate between early apoptotic (**A**; PI^-/caspase-3^+) and late-apoptotic (**B**; PI^+/caspase-3^+) cells. No statistically significant difference was observed in terms of apoptotic status between MSCs cultured with CM or under control conditions. Each bar represents the mean (n=3)+SEM.

4.3.24 Soluble factor(s) in CM induce dramatic changes in the transcriptional profiles of AHPCs in terms of patterns of expression of neural-related transcription factors

Studies in this Chapter so far have demonstrated that MSC aggregates secrete soluble factor(s) that influence the cell fate decisions of AHPCs, causing them to commit to and undergo an astrocytic differentiation program. Investigation into the mechanisms by which this effect occurs has largely been based at the cellular level thus far. Although there is evidence to suggest that activation of apoptotic pathways in $GalC^+$ oligodendrocytic precursors is likely to be a major contributing factor, temporal analysis of marker expression also suggests the involvement of an instructive mechanism, whereby soluble factor(s) in CM induce uncommitted progenitors to commit to and undergo astrocytic differentiation. In order to provide further evidence in support of an instructive mechanism, in addition to providing data at a molecular level as opposed to a cellular level, expression of transcription factors known to play key roles in neural differentiation processes were examined in AHPCs cultured with CM and UC control at days 1, 3 and 7, using qRT-PCR.

The transcription factors examined can be divided into several subcategories:

1. Astrocytic-promoting transcription factors

2. Oligodendrocytic-promoting transcription factors

3. Neuronal-promoting transcription factors

4. Neuronal-inhibiting transcription factors

Expression of a range of transcription factors belonging to each category were examined using qRT-PCR and the results shown in Figures 4.27, 4.28, 429 and 4.30. AHPCs were seeded out for these experiments as for all previous studies in this thesis (Section 4.2.3) and cultured under UC and CM conditions for 1, 3 and 7 days. Following these culture periods, total cellular RNA was isolated using the TRIreagent method (Section 2.9) followed by reverse transcription to cDNA (Section 2.10) and

finally qRT-PCR using SYBR Green chemistry (Figure 2.3). Specific primer sequences are shown in Table 2.1.

In terms of astrocytic-promoting transcription factors, expression of STAT1, STAT3, Smad1, Smad4 and Sox9 were determined at the mRNA level in AHPCs cultured with UC and CM for 1, 3 and 7 days. Results are shown in Figure 4.27. AHPCs cultured with CM demonstrated significant upregulation of each of these transcription factors compared to control cultures, albeit at various timepoints throughout the 7 day time course of this experiment. STAT1 was markedly upregulated at both the 1 ($p<0.001$) and 3 ($p<0.01$) day timepoints following culture with CM compared to UC controls, though mRNA levels dropped considerably at the 7 day timepoint to an equivalent level to UC control cultures (Figure 4.27A). Levels of STAT1 expression in control cultures remained relatively uniform throughout the 7 day time course of this experiment. STAT3 displayed a very similar expression profile, with very high levels of mRNA transcript being detected at the 1 ($p<0.001$) and 3 ($p<0.01$) day timepoints (Figure 4.27B). Interestingly, expression of STAT3 could not be detected in AHPCs cultured under UC control conditions at either of these timepoints. Transcripts were readily detectable at the 7 day timepoint for AHPCs cultured under both control and CM conditions, though expression was very low in both instances such that there was no statistically significant difference between UC and CM samples at this timepoint. Smad1 was expressed at significantly higher levels in AHPCs cultured with CM compared to UC control at the 1 day timepoint only ($p<0.05$). However, despite the statistical significance of this observation, levels of Smad1 transcript were very low in AHPCs cultured with CM and undetectable in AHPCs cultured with UC at this timepoint (Figure 4.27C). At the 3 day timepoint, expression of Smad1 was readily detectable in AHPCs cultured under both conditions, with levels of transcript being detected at virtually identical levels in each case. Interestingly, at the 7 day timepoint, expression of Smad1 increased in UC samples and decreased in CM samples, such that the difference in expression between the two samples was highly significant at the statistical level ($p<0.001$). Smad4 demonstrated an almost identical expression profile to that observed for STAT1 (Figure 4.27D), with AHPCs cultured in CM demonstrating significantly higher levels of mRNA transcript compared to controls at both the 1 ($p<0.01$) and 3 ($p<0.05$) day timepoints (Figure 4.27D). At the 7 day timepoint, expression of Smad4 decreased in AHPCs cultured with CM and increased

in AHPCs cultured with UC, such that levels of expression were comparable in AHPCs cultured under both conditions. No statistical significant was observed at the 7 day timepoint. Expression of Sox9 was unique in that it was the only transcription factor that was upregulated in AHPCs cultured with CM at all of the timepoints examined (Figure 4.27E). These observations were significant at the statistical level at each of the timepoints – days 1 ($p<0.05$), 3 ($p<0.01$) and 7 ($p<0.05$). Expression of Sox9 in UC control cultures demonstrated a similar trend to that observed for CM cultures, in that expression increased with time over the 7 day time course of the experiment. However, at each of the timepoints examined, absolute levels were lower in UC cultures compared to CM cultures. Combined, these data demonstrate that soluble factor(s) in CM cause astrogenesis, at least in part, by upregulation of a number of key transcriptional regulators of astrocytic differentiation.

However, regulation of astrogenesis at the transcriptional level is a complex, highly involved process involving extensive interaction and interplay between many positive and negative regulators of differentiation. It is now widely accepted that expression of astrocytic-promoting transcription factors, although a crucial event, is not sufficient to promote astrogenesis in uncommitted precursors – such positive regulators of astrocytic differentiation must be expressed concomitant with inhibitors of neuronal differentiation; likewise, neuronal- and oligodendrocytic-promoting transcription factors must be downregulated to permit astrogenesis (Guillemot, 2007).

In terms of oligodendrocytic-promoting transcription factors, expression of Olig1, Olig2 and Nkx2.2 were examined at the mRNA level in AHPCs cultured with UC and CM for 1, 3 and 7 days. Interestingly, Nkx2.2 was not expressed at a detectable level in AHPCs cultured under either condition at any of the timepoints examined. Olig1 and Olig2 on the other hand were readily detectable in AHPCs cultured under both conditions and results are shown in Figure 4.28. Olig1 was expressed at similar levels in AHPCs cultured with UC and CM at the earlier timepoints of 1 and 3 days (Figure 4.28*A*). Any differences in mRNA transcript levels were very slight and not significant at the statistical level. However, at the 7 day timepoint, AHPCs cultured with CM demonstrated a significant decrease in expression of Olig1 compared to AHPCs

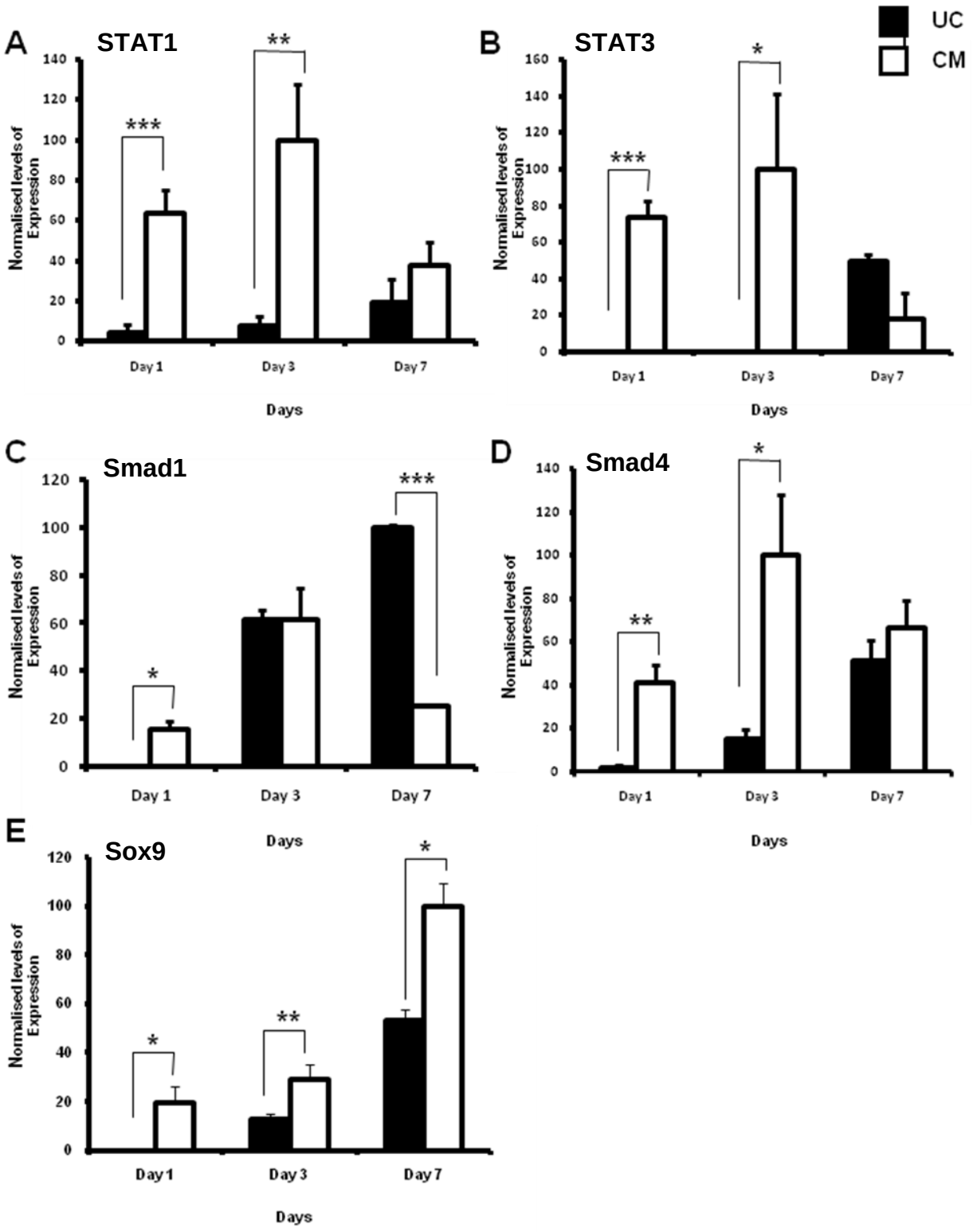


Figure 4.27. Expression of astrocytic-promoting transcription factors in AHPCs cultured with CM and UC control for 1, 3 and 7 days. QRT-PCR analysis was performed to compare mRNA transcript levels of five astrocytic-promoting transcription factors – **A:** STAT1; **B:** STAT3; **C:** Smad1; **D:** Smad4; **E:** Sox9 – in AHPCs cultured in CM and UC control for 1, 3 and 7 days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, ** $p<0.01$, *$p<0.05$.

cultured with UC, where expression of Olig1 was maintained at a similar level to that observed at the earlier timepoints. This observation was significant at the statistical level ($p<0.05$). The expression profile of Olig2 was far more intriguing (Figure 4.28*B*). This oligodendrocytic-promoting transcription factor was actually upregulated in AHPCs following culture with CM compared to UC control at the earlier timepoints of 1 and 3 days, with these observations being highly significant at the statistical level ($p<0.001$). At the 7 day timepoint, however, there was no observable difference in expression of Olig2 between AHPCs cultured under both conditions.

In terms of neuronal-promoting transcription factors, expression of the neurogenin family members Ngn1, Ngn2, Ngn3 and Mash1 were determined at the mRNA level in AHPCs cultured with UC and CM for 1, 3 and 7 days. Results are shown in Figure 4.29. In most instances, AHPCs cultured with CM demonstrated significant downregulation of these transcription factors compared to control cultures, albeit at various timepoints throughout the 7 day time course of this experiment. Ngn1 was significantly downregulated at the 1 day timepoint following culture with CM compared to UC control ($p<0.01$) (Figure 4.29A). At the later timepoints of 3 and 7 days, however, expression of Ngn1 was very low and uniform in AHPCs cultured under both conditions. Any slight differences in expression that were apparent were not significant at the statistical level. Interestingly, Ngn2 showed no differences in expression throughout the 7 day time course of this experiment, regardless of the culture condition employed (Figure 4.29B). Levels of mRNA transcript remained constant throughout the entire experiment and were detectable at comparable levels in AHPCs cultured with UC and CM. Expression of Ngn3 was undetectable in AHPCs cultured under both UC and CM at the 1 and 3 day timepoints, though became readily detectable at the 7 day timepoint (Figure 4.29C). At this timepoint, mRNA transcript levels were significantly higher in AHPCs cultured under control conditions compared to CM ($p<0.05$) (Figure 4.29C). Mash1 mRNA transcripts were readily detectable in AHPCs cultured under both UC and CM conditions at all timepoints examined (Figure 4.29D). At each of these timepoints, expression of Mash1 was greater in AHPCs cultured under control conditions compared to AHPCs cultured with CM; however, this observation was only statistically significant at the 7 day timepoint ($p<0.01$). Combined, these data demonstrate that soluble factor(s) in CM, in addition to

promoting the upregulation of astrocytic-promoting transcription factors, cause downregulation of a number of key neuronal-promoting transcription factors.

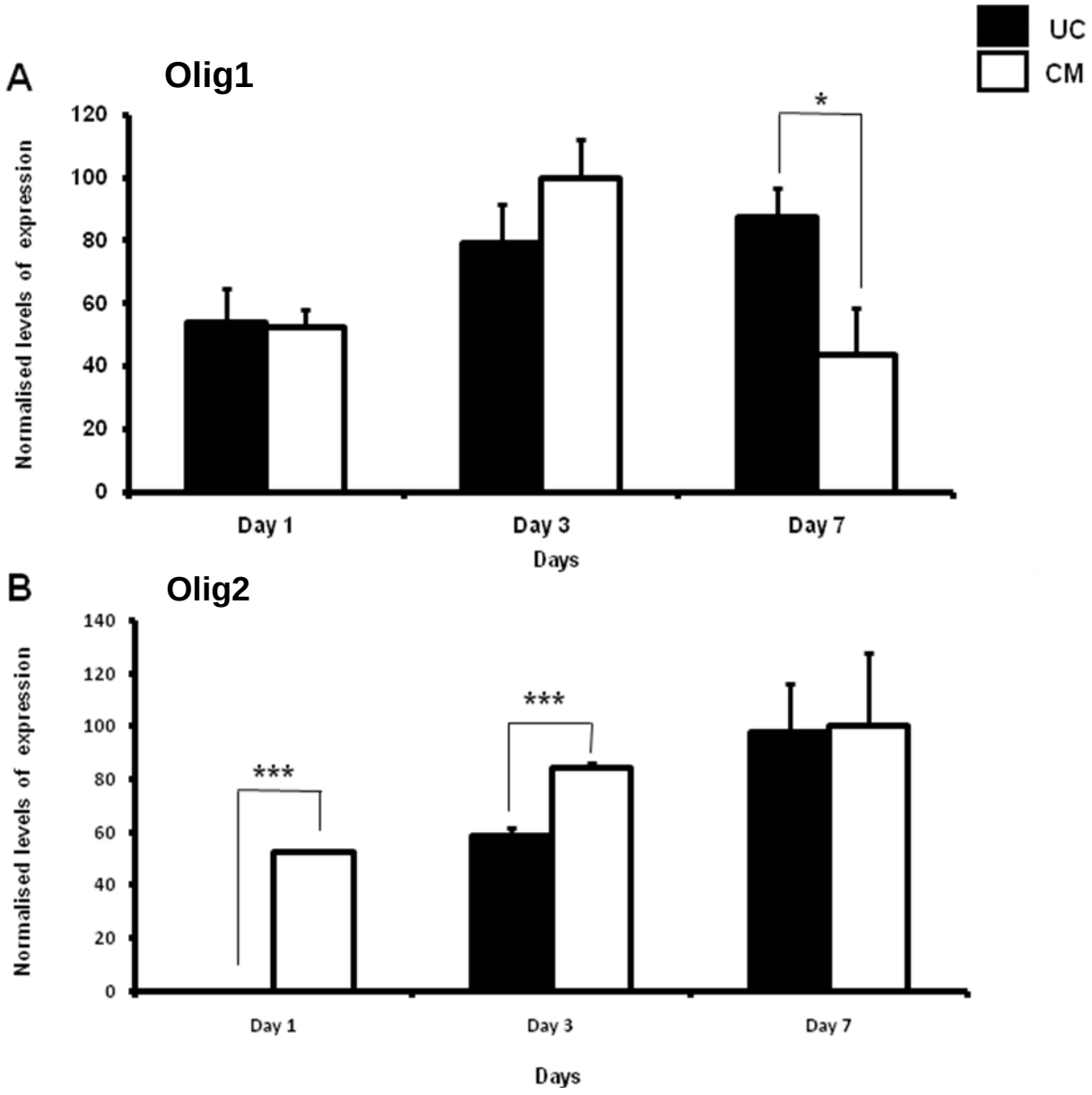


Figure 4.28. Expression of oligodendrocytic-promoting transcription factors in AHPCs cultured with CM and UC control for 1, 3 and 7 days. QRT-PCR analysis was performed to compare mRNA transcript levels of two oligodendrocytic-promoting transcription factors – **A:** Olig1 and **B:** Olig2 – in AHPCs cultured in CM and UC control for 1, 3 and 7 days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, *$p<0.05$.

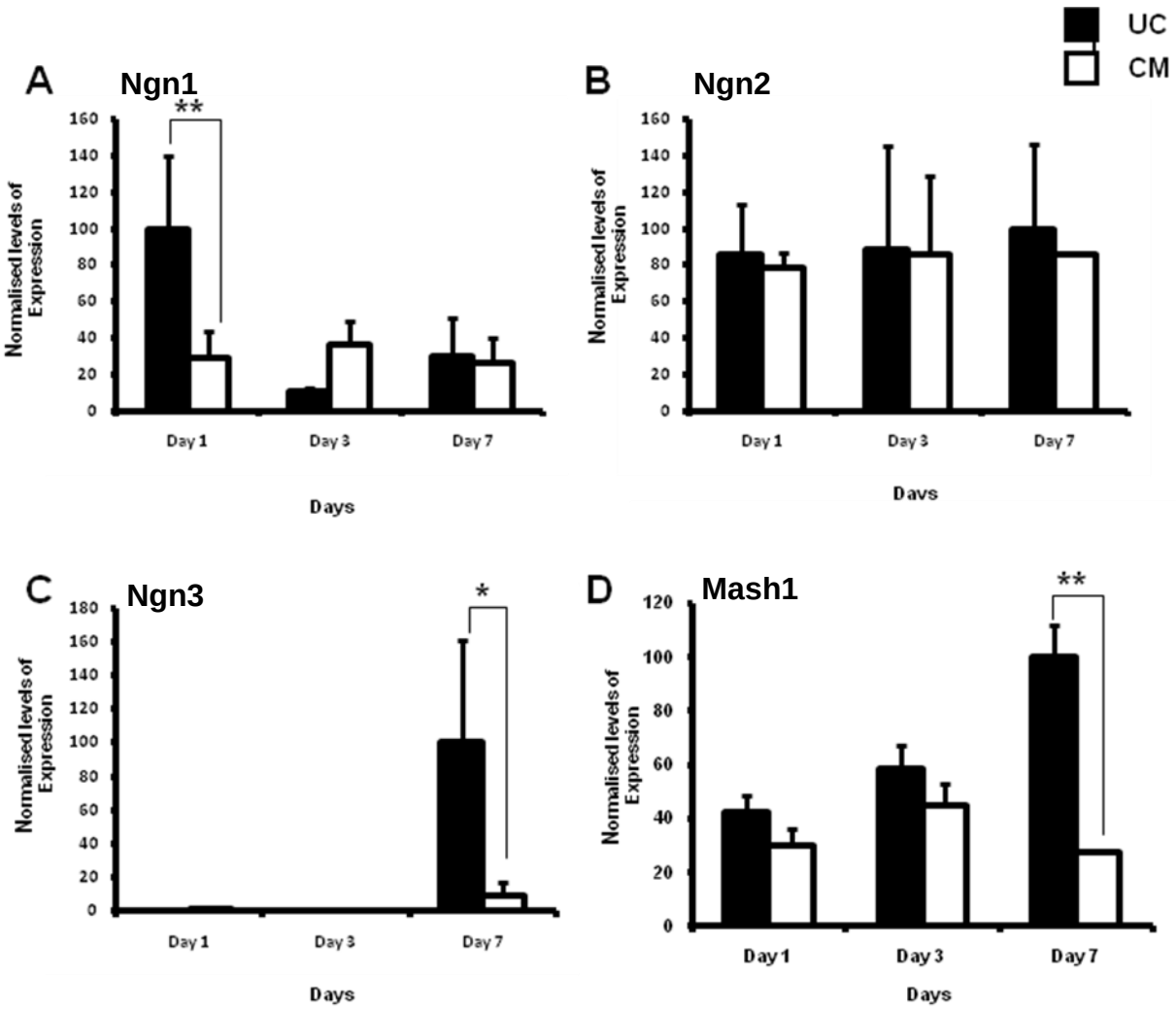


Figure 4.29. Expression of neuronal-promoting transcription factors in AHPCs cultured with CM and UC control for 1, 3 and 7 days. QRT-PCR analysis was performed to compare mRNA transcript levels of four neuronal-promoting transcription factors – **A:** Ngn1; **B:** Ngn2; **C:** Ngn3; **D:** Mash1 – in AHPCs cultured in CM and UC control for 1, 3 and 7 days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, ** $p<0.01$, *$p<0.05$.

In the final series of experiments examining the expression profiles of neural fate determinants in AHPCs, expression of a number of neuronal-inhibitory factors was determined at the mRNA level in AHPCs cultured with UC and CM for 1, 3 and 7 days. Expression of two members of the Hes protein family – Hes1 and Hes5 – along with three members of the Inhibitor of Differentiation (Id) protein family – Id1, Id2 and Id3 – were determined and results shown in Figure 4.30. In all instances, AHPCs cultured with CM demonstrated significant upregulation of these neuronal-inhibitory

factors compared to control cultures, albeit at various timepoints throughout the 7 day time course of this experiment. Hes1 was markedly upregulated in AHPCs cultured with CM compared to UC control cultures at all of the timepoints examined, though this observation was only significant at the 1 day timepoint ($p<0.05$) (Figure 4.30A). Levels of expression increased throughout the 7 day time course of the experiment such that maximum expression was observed at the 7 day timepoint. This trend was also observed in AHPCs cultured under UC control conditions, though expression was undetectable at the 1 day timepoint and remained at much lower levels at the later timepoints of 3 and 7 days compared to AHPCs cultured with CM. Similar to the trends in Hes1 expression, expression of Hes5 was also markedly upregulated in AHPCs cultured with CM compared to UC at the 1 and 3 day timepoints (Figure 4.30B). This observation was only statistically significant at the 1 day timepoint ($p<0.05$). At the 7 day timepoint, however, expression of Hes5 decreased in AHPCs cultured with CM, concomitant with increased expression in AHPCs cultured with UC, such that there was no observable difference in expression in AHPCs cultured under either condition at this timepoint. In terms of Id1 expression, AHPCs cultured with CM demonstrated a significant upregulation in mRNA transcript levels compared to controls at the 7 day timepoint only (Figure 4.30C). This observation was highly significant at the statistical level ($p<0.001$). At the earlier timepoints of 1 and 3 days, levels of Id1 transcript were extremely low in AHPCs cultured under both UC and CM conditions; however, despite such low levels of expression, AHPCs cultured under UC demonstrated significantly higher levels of expression at both of these early timepoints ($p<0.05$ at the 1 day timepoint, $p<0.001$ at the 3 day timepoint). Another member of the Id protein family – Id2 – was significantly upregulated in AHPCs cultured with CM compared to UC control at the 1 day timepoint only ($p<0.01$) (Figure 4.30D). However, this expression profile changed over the 7 day time course of the experiment such that AHPCs cultured under UC conditions demonstrated significantly higher levels of expression compared to equivalent CM cultures ($p<0.01$). The final neuronal-inhibitory factor examined in this study was Id3. At the earlier timepoints of 1 and 3 days, Id3 was expressed at significantly higher levels in AHPCs cultured with CM compared to UC (Figure 4.30E). These observations were significant at the statistical level at both the 1 ($p<0.05$) and 3 ($p<0.01$) day timepoints. However, whereas expression of Id3 underwent a massive reduction at the 7 day timepoint in AHPCs cultured with CM, expression in AHPCs cultured with UC showed the exact opposite

trend and underwent a massive upregulation in expression. Expression of Id3 in AHPCs cultured with UC at the 7 day timepoint was significant higher than expression observed in CM cultures at this timepoint ($p<0.001$).

In summary, the above data demonstrate that soluble factor(s) in CM significantly influence the behaviour of AHPCs at both the cellular and molecular level. Observations in this section of the thesis show that AHPCs cultured with CM demonstrate markedly different transcriptional profiles compared to control cultures, in terms of expression of neural fate determinants. Such differences are likely to be major contributing factors to the astrogenic effect observed in AHPCs following culture with CM for 7 days. Upregulation of astrocytic-promoting and neuronal-inhibitory factors, concomitant with downregulation of pro-neuronal and pro-oligodendrocytic factors, are crucial molecular events in the intricate and highly complex process of astrogenesis.

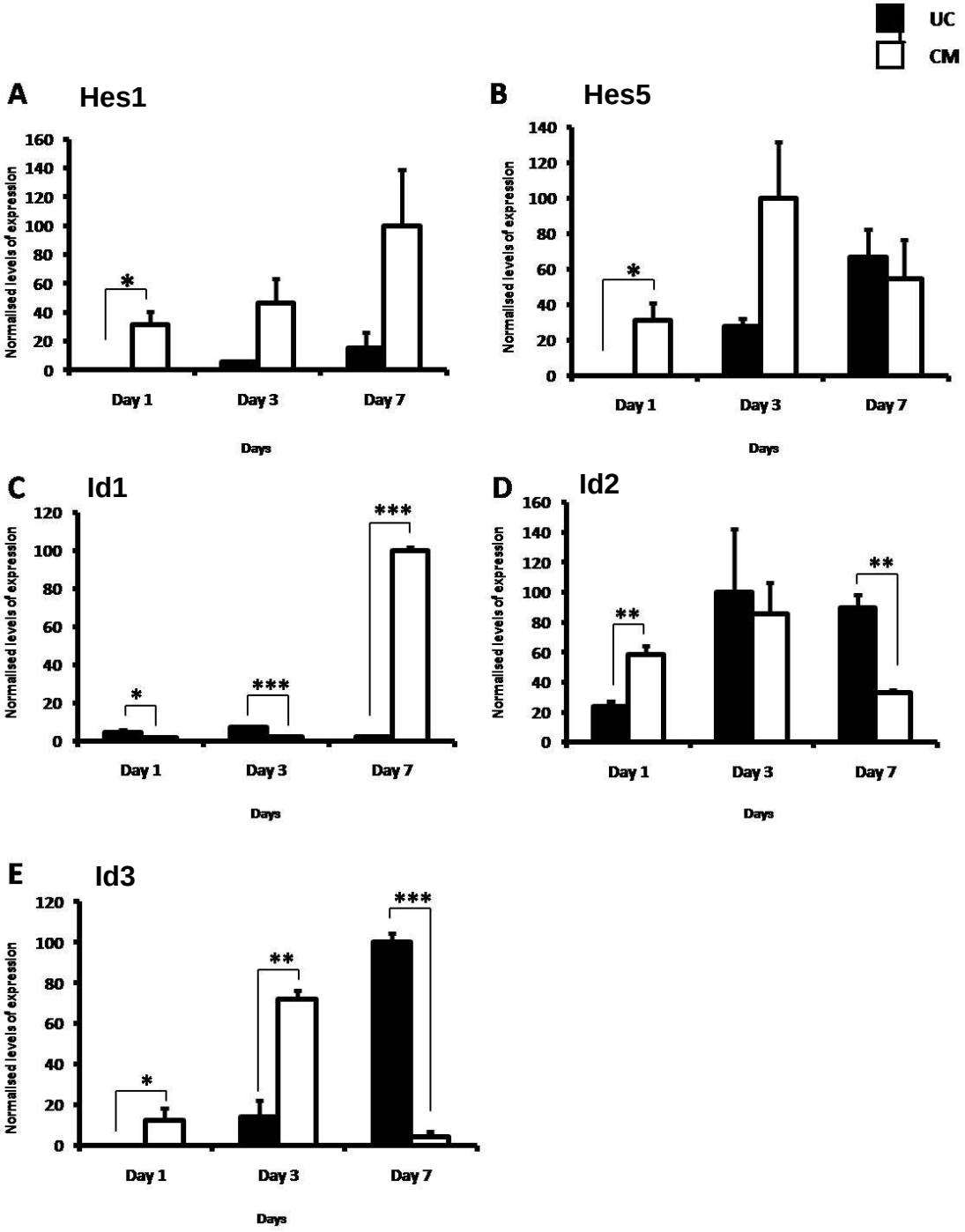


Figure 4.30. Expression of neuronal-inhibitory transcription factors in AHPCs cultured with CM and UC control for 1, 3 and 7 days. QRT-PCR analysis was performed to compare mRNA transcript levels of five neuronal-inhibitory transcription factors – **A:** Hes1; **B:** Hes5; **C:** Id1; **D:** Id2; **E:** Id3 – in AHPCs cultured in CM and UC control for 1, 3 and 7 days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

4.4 Discussion

Culture of MSCs as large, multicellular 3D aggregates

As discussed in Chapter 3, general cell culture methodology using tissue culture plastic is fundamentally flawed in that the 2D environment of tissue culture plastic forces cells to adopt highly unnatural conformations/morphologies that are in no way representative of their *in vivo* growth environments (3D), where they develop highly complex interactions with neighbouring cells and extracellular matrix within their niche. As such, there is an increasing demand for 3D culture systems for *in vitro* use. Studies in Chapter 3 investigated the behaviour of MSCs cultured on a novel polystyrene scaffold, Alvetex^{TM}, manufactured by Reinnervate. Data from these studies clearly demonstrated that MSCs cultured on the scaffold displayed markedly different behavioural traits, in terms of enhanced cellular viability and enhanced functionality in terms of their ability to undergo osteogenic and adipogenic differentiation, compared to equivalent cultures on 2D tissue culture plastic. It can therefore be argued that culturing cells on the 3D environment of the scaffold provides a more realistic and physiologically-relevant system in which to study complex biological phenomena such as differentiation.

Although findings from Chapter 3 of this thesis and many other studies in the literature demonstrate that cells cultured on 3D scaffolds behave in a manner more representative of that in which they behave *in vivo*, there are alternative methods of 3D cell culture that are advantageous in that they encourage such phenotypic behaviour but do not require the use of external scaffolds. For example, encouraging cells to form large, multicellular aggregates is a method of 3D cell culture that promotes structural interactions, such as the re-establishment of mutual contacts between cells, and functional behaviour more reflective of that observed in real tissues (Pampaloni *et al.*, 2007; Lin and Chang, 2008). To date, the large majority of studies exploiting the advantages of using cellular aggregates as 3D model systems have been in tumour biology and other oncology-related studies (Mueller-Klieser, 1987; Kunz-Schughart *et al.*, 1998; Santini and Rainaldi, 1999; Bates *et al.*, 2000; de Ridder *et al.*, 2000; Gottfried *et al.*, 2006). However, there is also increasing evidence to suggest that culturing cells as aggregates and spheroids may be useful in developmental biology, with studies reporting enhanced viability and functionality in stem and progenitor cells

cultured as aggregates compared to standard monolayer cultures (Lin and Chang, 2008). Such observations are analogous to those reported in Chapter 3 for MSCs cultured on the scaffold.

Strategies for the 3D culture of cells as large, multicellular aggregates and spheroids vary quite significantly throughout the literature. Two methods exploit the tendency of cells to naturally aggregate with each other in culture: the hanging drop technique (used extensively in the culture of embryonic stem cells to promote the formation of embryoid bodies) and culturing cells in rotating bioreactors (Pampaloni *et al.*, 2007). Both techniques have been described to promote the formation of aggregates from MSCs, though neither technique has been used extensively (Chen *et al.*, 2006; Elvenes *et al.*, 2009).

Similarities between MSC-derived aggregates and NSC-derived neurospheres

A significant number of studies have reported that MSCs can be induced to form large, multicellular, free-floating aggregates when cultured under identical conditions to those used for the propagation of neurospheres from neural stem cells (Croft and Przyborski, 2004; Hermann *et al.*, 2004; Bossolasco *et al.*, 2005; Hermann *et al.*, 2006). These culture conditions are described in Section 4.2.1, with key points of note including the absence of serum and addition of mitogens, EGF and FGF, to the culture media. Such are the phenotypic similarities between MSCs and NSCs cultured under these defined conditions that they are morphologically indistinguishable from one another. In some studies, such as that published by Suzuki and colleagues in 2004, aggregates formed by MSCs are even referred to as 'neurospheres' – however, this is somewhat misleading as neurospheres are, by definition, free-floating aggregations of cells derived from NSCs (Bez *et al.*, 2003; Campos, 2004; Suzuki *et al.*, 2004). To avoid such confusion, the terminology 'neurosphere' has been avoided throughout this thesis and MSC-derived aggregates have been referred to as 'aggregates' rather than 'neurospheres' throughout these studies.

MSCs cultured under defined, aggregate-inducing conditions in this study formed large, spherical cellular masses that were morphologically indistinguishable from those observed in previous studies (Hermann *et al.*, 2004; Bossolasco *et al.*, 2005; Croft and Przyborski, 2009). Enhanced functionality in terms of potential to undergo osteogenic,

adipogenic and cartilaginous differentiation has been reported in MSCs cultured as aggregates and such cultures have also been reported to possess a greater plasticity than previously anticipated, demonstrating a potential to '*trans*'-differentiate into hepatocyte-like cells (Qihao *et al.*, 2007; Boeuf *et al.*, 2008; Wang *et al.*, 2009). However, because MSC-derived aggregates are so phenotypically similar to NSC-derived neurospheres, the large majority of studies involving MSC-derived aggregates have been aimed at a neurological perspective.

One of the key properties of MSC aggregates that is consistently reported in studies throughout the literature is that they express very high levels of neural antigens such as nestin, Tuj-1 and GFAP concomitant with very low levels of expression of mesenchymal antigens such as α-SMA (Kabos *et al.*, 2002; Croft and Przyborski, 2004; Hermann *et al.*, 2004; Suzuki *et al.*, 2004; Hermann *et al.*, 2006; Kim *et al.*, 2006). In keeping with such previous studies, MSCs cultured as aggregates in this Chapter demonstrated enhanced expression of the neural antigens, nestin and GFAP, concomitant with reduced expression of the mesenchymal marker, α-SMA, compared to MSCs cultured under standard conditions. These expression profiles were confirmed using both immunocytochemical and flow cytometric techniques to provide information at the whole aggregate and individual cellular level respectively.

Several studies investigating the neural-like behaviour of MSCs cultured as neurosphere-like aggregates demonstrated that they lost phenotypic traits characteristic of their mesenchymal origin and gained phenotypic traits more in keeping with a neural origin. Separate studies led by Suzuki *et al* (2004) and Hermann *et al* (2004) both demonstrated that MSCs cultured as aggregates displayed the potential to differentiate into the three main neural cell types – as determined by morphological analysis and expression of mature markers indicative of the acquirement of a neuronal, astrocytic and oligodendrocytic cell fate – following plating out onto laminin-coated cellware and treatment with suitable neurogeneic factors respectively (Hermann *et al.*, 2004; Suzuki *et al.*, 2006). Other studies support such observations (Kabos *et al.*, 2002; Kim *et al.*, 2006). However, when observations in the previous chapter of this thesis are taken in account (Chapter 3), it is the study recently published by Croft and Przyborski that is perhaps the most interesting and relevant (Croft and Przyborski, 2009). In this study, it was demonstrated that MSCs cultured as aggregates were able

to influence the cell fate decisions of primary embryonic NSCs in a co-culture system. The co-culture system adopted in this study was unique in that the two cell populations in culture – MSC-derived aggregates and NSCs – were separated by a porous microfilter membrane, such that the two cell populations were only able to communicate using paracrine mechanisms i.e. via the release of trophic factors and cytokines. MSC aggregates released a combination of soluble factors that induced NSCs to commit to and undergo a neuronal differentiation program (as determined by expression of Tuj-1) whereas MSCs cultured as standard monolayers released a combination of soluble factors that induced NSCs to commit to and undergo an astrocytic differentiation program (as determined by expression of GFAP), at the expense of a neuronal one. Such evidence that MSC-derived aggregates demonstrated a paracrine relationship with NSCs is particularly interesting and relevant to previous studies in this thesis (Chapter 3) demonstrating that enhanced osteogenic and adipogenic differentiation of MSCs cultured on the scaffold was a consequence, at least in part, of enhanced autocrine activity, with MSCs cultured on the scaffold demonstrating enhanced expression of positive soluble factors concomitant with reduced expression of inhibitory factors that would aid these differentiative processes. Therefore, MSCs can be described as both autocrine and paracrine regulators of differentiation.

MSCs as paracrine mediators of neural differentiation

Following on from the study recently published by Croft and Przyborski in 2009, the major aims of this Chapter were to investigate the paracrine function(s) of MSC-derived aggregates in terms of their effect(s) on neural development and plasticity using an *in vitro* conditioned media (CM) model. A number of studies adopting a CM approach in order to investigate the effects of MSC-derived soluble factors and cytokines on the differentiative state of NSCs have already been published (Wislet-Gendebien *et al.*, 2004; Rivera *et al.*, 2006). In 2006, Rivera and colleagues reported that adult rat MSCs cultured under standard culture conditions (in CCM containing 10% serum) secreted a complement of proteins and/or other metabolic factors into their media that induced adult rat NSCs to commit to and undergo oligodendrogenic differentiation at the expense of astrocytic differentiation, as determined by increased expression of the oligodendrocytic markers, GalC and MBP, concomitant with decreased expression of the astrocytic marker, GFAP (Rivera *et al.*, 2006). Levels of

neuronal differentiation did not differ between CM and control cultures in this system, as determined by expression of the neuronal marker, Map2ab. Conversely, Wislet-Gendebien and colleagues reported that adult rat MSCs cultured under conditions that promoted the expression of the neuroprogenitor marker, nestin, secreted a complement of proteins and/or other metabolic factors into their media that induced embryonic (E16) NSCs to commit to and undergo astrocytic differentiation, as determined by expression of GFAP (Wislet-Gendebien *et al.*, 2004). Increased expression of GFAP occurred simultaneously with decreased expression of Tuj-1 and O4 (another marker of oligodendrocytic fate) and this effect was demonstrated to be, at least partially, a consequence of active BMP4 secretion.

Although the differences in observations between these studies are likely to be largely a consequence of the obvious differences in experimental setup i.e. the use of adult NSCs in the Rivera (2006) study compared to the use of embryonic NSCs in the Wislet-Gendebien (2004) study, these observations also suggest that the conditions in which MSCs are cultured and maintained considerably influence their secretory properties. This is consistent with previous studies reporting that MSCs are able to significantly vary their secretion patterns depending on specific microenvironments (Chen *et al.*, 2002). An interesting question to address in the near future will be whether MSCs should be administered to areas of neurological damage in patients under standard conditions or whether they should first be cultured under defined conditions *in vitro* in order to promote a more direct response *in vivo*.

MSC aggregate-derived soluble factors influence the cell fate decisions of AHPCs

In the present study, CM was produced from MSC-derived aggregates using methodology identical to that used in the previously-discussed studies (Wislet-Gendebien *et al.*, 2004; Rivera *et al.*, 2006). These studies reported an optimal conditioning period of 3 days in order to gain a suitable balance between allowing sufficient time for cells to secrete a concentration of soluble factors/cytokines that is likely to exert a biological effect without depleting the basal media of essential nutrients which would be deleterious to the further culture of cells. In order to assess the neurogenic properties of aggregate-derived soluble factors, CM was collected and used in the subsequent culture of a rat adult hippocampal progenitor cell (AHPC) line for 7 days. These self-renewing, multipotent cells were a generous gift from Professor

Fred Gage (Salk Institute) and have been extensively characterised (Palmer *et al.*, 1997). Routine use of this cell line in our lab confirmed the stem cell-like behaviour of these cells in terms of their ability to self-renew and differentiate into all three of the major neural cell types – neurons, astrocytes and oligodendrocytes.

Morphology and expression of neural markers

Following culture with CM for 7 days, AHPCs were induced to commit to and undergo gliogenesis, in particular, astrogenesis at the expense of oligodendrogenesis. This was determined using morphological analyses and, perhaps more convincingly, using neural marker analysis. One particularly prominent feature of the AHPC cell line used in this study, however, is that undifferentiated, proliferating cells demonstrate positivity not only for markers indicative of a neuroprogenitor phenotype, such as nestin, but also demonstrate positivity for expression of markers indicative of a more mature neural phenotype, such as NSE, Map2c and O4 (Sommer and Schachner, 1982; Black and Kurdyla, 1983; Iwanaga *et al.*, 1989; Lendahl *et al.*, 1990; Palmer *et al.*, 1997). These markers are reportedly expressed at levels of greater than 80% (Palmer *et al.*, 1997). Expression of other neural markers has also been reported in AHPCs cultured under standard, proliferative conditions, such as A2B5 and GFAP, though these levels are much lower, at levels of less than 2% (Palmer *et al.*, 1997). Expression of the neural markers GalC, MBP, NF200, Tau and NeuN were negligible (Palmer *et al.*, 1997). Combined, these marker analyses therefore raise a potential issue, as extensive co-expression of neural markers could lead to ambiguity in terms of interpretation of results. The potential for ambiguity intensifies when more sophisticated marker analyses are performed using highly sensitive techniques such as flow cytometry, as the marker expression profiles constructed in the previous studies were derived from immunocytochemical observations alone. For example, previous characterisation studies have reported that AHPCs are largely negative for GFAP as determined using immunocytochemical methods (Palmer *et al.*, 1997). Immunocytochemical observations from this study support such observations. However, when GFAP expression is examined in AHPCs maintained under identical conditions using flow cytometry, approximately 90% of cells demonstrate positivity, albeit at weak MFI. Therefore, marker expression profiles constructed using immunocytochemical analyses alone could yield false negative results due to its lack of sensitivity.

In order to assess the differentiative state of AHPCs cultured with CM, expression of the neural markers nestin, Tuj-1, GFAP and GalC were examined, using immunocytochemistry and flow cytometry, as indicators of neuroprogenitor, neuronal, astrocytic and oligodendrocytic phenotypes respectively. The most striking observation was in terms of GFAP expression – AHPCs cultured with CM demonstrated intense, highly significant levels of expression compared to AHPCs cultured under the two control conditions, which demonstrated very low, weak levels of expression. However, caution must be aired when using GFAP as an exclusive marker of astrocytic differentiation as GFAP, in addition to nestin, is widely reported to be expressed in immature neural stem and progenitor cells isolated from the hippocampus (Filippov *et al.*, 2003; Fukuda *et al.*, 2003; von Bohlen and Halbach, 2007). It has also been widely reported that, during adult neurogenesis, new neurons originate from $GFAP^+$ cells displaying astrocytic traits (Doetsch *et al.*, 1997). Therefore, it is crucial to be able to discriminate between $GFAP^+$ cells that express an immature, undifferentiated phenotype and $GFAP^+$ cells that express a more mature, astrocytic phenotype. The $GFAP^+$ cells observed in this study following culture with CM are likely to be astrocytes rather than progenitors for two reasons:

1. During adult neurogenesis, GFAP is downregulated prior to neuronal differentiation (von Bohlen and Halbach, 2007). The converse is observed in this study in that AHPCs cultured with CM increase expression of GFAP.

2. $GFAP^+$ progenitor cells demonstrate unipolar and bipolar morphologies with few cytoplasmic processes (von Bohlen and Halbach, 2007). AHPCs cultured with CM, however, demonstrate multipolar soma with extensive cytoplasmic extensions, forming stellate, star-shaped morphologies.

Another interesting observation to arise from these marker analysis studies was in terms of nestin expression – AHPCs cultured under both control and CM conditions demonstrated very high, intense levels of expression. Since nestin is traditionally

considered to be a marker of immature neural stem and progenitor cells, it could be anticipated that nestin expression would be downregulated in AHPCs actively undergoing differentiation. However, although there is a slight tendency for the percentage of cells expressing nestin to decrease following culture with CM for 7 days, this observation was not significant at the statistical level and intensity of expression (MFI) was comparable between AHPCs cultured with CM and AHPCs cultured under standard, proliferative conditions. It is now widely accepted that nestin is upregulated in astrocytes following cellular stress or pathological events e.g. cerebral ischemia, traumatic brain injury and neurotoxicity, such that nestin is considered to be an accurate marker of reactive astrocytes (Clarke *et al.*, 1994; Duggal *et al.*, 1997; Holmin *et al.*, 1997; Brook *et al.*, 1999; Sahin *et al.*, 1999; Holmin *et al.*, 2001; Yoo *et al.*, 2005). That is not to suggest that the $GFAP^+$ astrocytic-like cells formed by AHPCs following culture with CM for 7 days are necessarily experiencing cell stress events, however, as continued nestin expression has been reportedly observed in astrocytes in non-pathological states (Schmidt-Kastner and Humpel, 2002). A further study by Gu and colleagues reported that, in the mature human CNS, nestin expression was readily detectable in at least three mature neural cell types; among them, astrocytic-like cells isolated from the subependymal zone and subgranular layer of the dentate gyrus of the hippocampus (Gu *et al.*, 2002).

There is no evidence in the literature at present reporting the expression of GalC, traditionally a marker of oligodendrocytes, in astrocytic-like cells. However, immunocytochemical analysis revealed diffuse patterns of GalC staining in AHPCs cultured under both UC and CM conditions, though staining was extremely weak and of very low intensity (MFI). Previous studies using this particular AHPC cell line have reported that density arrest in the presence of FGF favoured the survival and differentiation of $GalC^+$ oligodendrocytic cell types, whereas density arrest in the absence of FGF favoured the survival and differentiation of neuronal and astrocytic cell types (Palmer *et al.*, 1997). As FGF is not a component of UC media and is depleted by aggregates during the three day conditioning period (as determined using mass spectrometry, data not shown), it would be anticipated that such conditions would facilitate neuronal and astrocytic differentiation and encumber oligodendrocytic differentiation. However, trace amounts of FGF may remain following transfer of cultures from proliferative media (containing FGF) to UC/CM media (lacking FGF),

which may account for diffuse levels of expression. Similarly, the possibility of MSC-derived aggregates secreting low levels of FGF into the CM cannot be discounted. Certainly, no oligodendrocytic morphologies are observed in AHPCs following culture with UC or CM, suggesting that weak expression of GalC may be attributable to underlying expression and not oligodendrocytic differentiation.

Combined, these marker analyses provide strong evidence to suggest that aggregate-derived soluble factors present in CM induce AHPCs to commit to and undergo astrocytic differentiation. As previously discussed, however, expression of a range of markers indicative of a more mature neural fate in undifferentiated AHPCs, concomitant with extensive co-expression of markers, can lead to ambiguity in terms of interpretation of results. Therefore, although marker analysis is extremely useful in allowing discrimination between different cell types, it is perhaps functional analysis that is more convincing in demonstrating/confirming the expression of a particular cellular phenotype.

Expression of functional astrocytic markers

Assessing the functional behaviour of $GFAP^+$ astrocytic-like cells *in vitro* is extremely challenging. Although astrocytes represent greater than 90% of the total cell population in the mammalian brain, until very recently they were considered to be passive cells whose main function was to provide structural and metabolic support to neurons (He and Sun, 2007). Their significance was considered to be largely secondary to that of neurons. This attitude has changed somewhat over recent years and an increasing number of studies have been published suggesting that the function of astrocytes is far more widespread than previously anticipated. Although the function of astrocytes still remains largely undefined to this date, they are known to perform a wide range of diverse functions throughout the CNS, including:

- Formation and maintenance of the blood-brain barrier, preventing large macromolecules entering the brain from blood plasma (Abbott, 2002).

- Balance ionic concentration in extracellular space surrounding neurons and control levels of neurotransmitters and ions, such as glutamate and dopamine,

around the synapse (Henn and Hamberger, 1971; Porter and McCarthy, 1997; Temburni and Jacob, 2001; He and Sun, 2007).

- Control many aspects of synaptogenesis, including synapse formation, synaptic function and transmission, synaptic organisation, synaptic homeostasis and synapse number (Temburni and Jacob, 2001; Ullian *et al.*, 2001; Piet *et al.*, 2004; Allen and Barres, 2005).

- Vasomodulation i.e. control of blood flow to neurons (Parri and Crunelli, 2003).

- Control of myelinating activity of oligodendrocytes (Ishibashi *et al.*, 2006).

- Immunological behaviour – astrocytes have been reported to become phagocytic scavengers following neuronal injury/death (Dong and Benveniste, 2001; He and Sun, 2007).

This is by no means an exhaustive list of all the functions of astrocytes and is merely meant as a representation of their more commonly reported functions. Taking such complex functions into account, producing an *in vitro* assay to assess the functional behaviour of astrocytes would be extremely complicated and technically demanding. Therefore, to circumvent these difficulties and gain an appreciation of how astrocytic-like cells are behaving at a functional level, the most commonly adopted method is by examining expression of a range of biochemical receptors known to be indicative of an astrocytic phenotype. Several studies have reported that astrocytes express a range of ligand-gated and voltage-dependent ion channels, allowing them to clear neurotransmitters and ions away from the synapse (Henn and Hamberger, 1971; Porter and McCarthy, 1997; Temburni and Jacob, 2001). In this study, expression of AMPA receptor 3 (AMPA3), GABA-A, NMDA receptor 3 (NMDA-2D) and 5-HT 1A were examined at the mRNA level in AHPCs cultured with CM and appropriate controls for 7 days. All four receptors have previously been reported as functional biochemical markers of astrocytic behaviour (Kamnasaran *et al.*, 2008). These receptors are just a handful of those known to contribute to astrocytic function, however, with others

including nicotinic acetylcholine (nACHR), glutamate, opioid, dopamine, glycine, serotonic, β-adrenergic and purinergic receptors (Araque *et al.*, 1999; Parpura and Haydon, 2000; Verkhratsky and Steinhauser, 2000; Sharma and Vijayaraghavan, 2001; Santello and Volterra, 2008). Each of the receptors examined were upregulated in AHPCs cultured with CM compared to controls, with each of these observations being significant at the statistical level, strongly suggesting that AHPCs cultured with CM were induced to commit to a bona fide astrocytic differentiation program, rather than induced to aberrantly express the structural astrocytic marker, GFAP.

Effect of oxygen tension on the secretory profile of MSC-derived aggregates

Observations in this Chapter have thus far demonstrated that MSC aggregate-derived soluble factors in CM induced AHPCs to express an astrocytic phenotype, as determined by positive expression of structural and functional astrocytic markers. Further studies in this Chapter went on to investigate the effect(s) of oxygen tension on fundamental aspects of aggregate behaviour and function, in particular, their ability to direct the differentiation of AHPCs via paracrine signalling. It is now becoming more generally accepted that oxygen tension is a crucially-overlooked parameter in cell culture, as oxygen tension is a critical signal for virtually all cellular processes (Csete, 2005). Routine, highly standardised methods of cell culture results in exposure of cells to atmospheric concentrations of oxygen (approximately 20%); however, cells within their natural environment *in vivo* are exposed to much lower concentrations of oxygen (approximately 2-10%, depending on tissue type, tissue arrangement etc.) (Csete, 2005; Sullivan *et al.*, 2006). Therefore, the behaviour of cells cultured under atmospheric oxygen levels *in vitro* may be far removed from their actual physiological behaviour *in vivo*. Exposure to high concentrations of oxygen can also be damaging to cells, as oxygen is toxic at higher concentrations due to the formation of reactive oxygen species, such as free radicals and peroxides (Bellomo, 1991).

Taking this into account, a significant number of studies have reported that varying oxygen tension dramatically influences the behaviour of numerous cell types *in vitro*, particularly stem cells, in terms of cellular processes such as proliferation, differentiation, migration and apoptosis (Csete, 2005). Rates of proliferation are reportedly enhanced in stem cells cultured under low oxygen compared to atmospheric oxygen tensions and this phenomenon has been observed in a range of different stem

cell types, including neural stem cells, skeletal muscle satellite cells, haematopoietic stem cells and even MSCs (Reykdal *et al.*, 1999; Morrison *et al.*, 2000; Studer *et al.*, 2000; Csete *et al.*, 2001; Lennon *et al.*, 2001). Cellular senescence, a biological process corresponding to the ageing of cells as a consequence of increased cell division, has also been reported to be delayed in cells following culture under low oxygen tension (Parrinello *et al.*, 2003). There is also much evidence to suggest that apoptosis is reduced in stem cells following culture in low oxygen compared to atmospheric oxygen tension, as fewer reactive oxygen species are generated under lower oxygen conditions (Erkkila *et al.*, 1999; Studer *et al.*, 2000; Mostafa *et al.*, 2002). Functionality of stem cells in terms of their differentiative potential has also reported to be extensively influenced by oxygen tension – for example, neural stem cells cultured under low oxygen tension demonstrated a bias to undergo dopaminergic differentiation (Studer *et al.*, 2000). Likewise, MSCs cultured under low oxygen tension demonstrated a bias to undergo myogenesis at the expense of adipogenesis (Csete *et al.*, 2001). Such unique properties of cells following culture under lower oxygen tensions compared to atmospheric oxygen tension are not exclusive to stem cells, with phenomena such as enhanced viability/proliferation/survival/functionality/ physiology and reduced senescence being reported in a range of more lineage-restricted cell types, including liver sinusoidal endothelial cells (LSECs), amniotic fluid cell cultures, fibroblasts, skin microvascular endothelial cells, mouse embryonic fibroblasts (MEFs), hepatocytes and chondrocytes (Suleiman and Steven, 1987; Zhou *et al.*, 2000; Murphy and Sambanis, 2001; Balin and Pratt, 2002; Parrinello *et al.*, 2003; Held and Sönnichsen, 2005; Martinez *et al.*, 2008).

However, several studies have also been published claiming that culturing cells under lowered oxygen tensions can actually be detrimental to cellular viability and function. For example, osteogenic differentiation of MSCs has been reported to be most efficacious at atmospheric oxygen levels, with oxygen tensions below 5% having an inhibitory effect on differentiation (Raheja *et al.*, 2009). Similarly, T cell clones reportedly demonstrated reduced proliferative capacities and lifespan when cultured under low oxygen compared to atmospheric oxygen tension (Duggan *et al.*, 2004). Perhaps most interestingly, in complete contrast to the studies with stem cells described above, both mouse and human embryonic stem cells have been reported to

demonstrate reduced rates of proliferation following cultured under low oxygen tensions compared to atmospheric tension (Kurosawa *et al.*, 2006; Chen *et al.*, 2008).

The significance of oxygen tension is of particular importance when considering the optimal culture conditions of 3D structures such as the MSC-derived aggregates used in this Chapter, as large cellular aggregates require a careful control of gaseous exchange (Griffith and Swarz, 2006). Protocols used for aggregate formation in this study clearly stated that MSCs should be seeded out under low oxygen tension rather than atmospheric oxygen tension (Hermann *et al.*, 2004; Hermann *et al.*, 2006). In order to ascertain whether the behaviour of the MSC-derived aggregates used in this study was influenced by oxygen tension, MSCs were seeded out for aggregate formation under both low (5%) and atmospheric (20%) oxygen tensions. Although aggregates cultured under both low and atmospheric oxygen tensions were indistinguishable from one another in terms of morphological appearance and expression of neural/mesenchymal markers, MSCs seeded out for aggregate formation under low oxygen tension demonstrated a significantly enhanced rate of proliferation compared to counterparts seeded out under atmospheric oxygen tensions ($p<0.001$). An enhanced rate of proliferation following culture under low oxygen tension is in keeping with the vast majority of studies described above. Furthermore, when the secretory activity of aggregates cultured under varying oxygen tensions was examined – in terms of their ability to secrete soluble factors that promote astrogenesis of AHPCs following 7 days culture – it was observed that the astrogenic effect was significantly diminished following culture with CM derived from aggregates cultured under atmospheric oxygen tension compared to low oxygen tension ($p<0.05$). One possible explanation for this observation could be the enhanced proliferation in aggregates cultured under low oxygen tension, resulting in a greater number of cells and, by extension, an increased concentration of active biological factor being secreted into CM. However, an additional possibility could be that aggregates cultured under varying oxygen tensions demonstrate different behaviour at the secretory level. This is a likely scenario, as oxygen tension has previously been reported to dramatically affect the secretory activity of a range of cell types. For example, oxygen concentration plays a pivotal role in the secretion of insulin from islet cells – insulin secretion decreases with decreasing oxygen concentration and this effect is so pronounced that in the absence of oxygen, insulin secretion cannot be detected (Aleyassine, 1970; Dionne *et*

al., 1989; Ohta *et al.*, 1990; Dionne *et al.*, 1993; Papas *et al.*, 1996). Oxygen concentration has also been reported to regulate the production and secretion of a range of angiogenic factors, with one study reporting that CM derived from granulosa cells exerted a different effect on vascular endothelial cells depending on whether they were cultured under low or atmospheric oxygen tension (Koos, 1986). In addition to protein-based soluble factors, oxygen has also been reported to influence the secretion of non-protein-based soluble factors such as steroidal hormones (Raff *et al.*, 1990). A lowered oxygen environment also results in enhanced secretion of extracellular matrix from endothelial cells (Zhao *et al.*, 2009).

Combined, these results suggest that lowered oxygen tension significantly enhanced the viability and function of MSC-derived aggregates compared to counterparts cultured under atmospheric oxygen tensions, in terms of proliferation rate and secretory activity. The mechanism(s) behind the enhanced astrogenic effect of CM produced under low oxygen is likely to be a combination of enhanced rate of proliferation (and hence an increased number of cells in culture) concomitant with altered secretory activity of aggregates cultured under lowered oxygen tension.

Temporal analysis of neural marker expression

The final aims of the study in this Chapter were to investigate the cellular and molecular mechanisms causing the observed astrocytic effect in AHPCs. When marker analysis in AHPCs was monitored temporally over the 7 day time course of the experiment, expression of GFAP increased with time both in terms of the percentage of cells positive for expression and MFI of marker expression. The percentage of $GFAP^+$ cells reached a maximum at the 4 day timepoint, suggesting that cells had committed to the process of astrogenesis by this timepoint, whilst MFI of GFAP expression increased up to a maximum at the 7 day timepoint, suggesting that committed cells were continuing to undergo astrogenesis and express a more mature astrocytic phenotype. The kinetics of GFAP expression, coupled with the observation that proliferation of the $GFAP^+$ subpopulation was not influenced following culture with CM compared to control, strongly suggested the soluble factors in CM were not positively selecting for astrocytic-committed precursors. As such, these data suggest an instructive mechanism is at play rather than a selective one.

Cell proliferation and cell death

Interestingly, however, increased rates of cell death were observed in the $GalC^+$ subpopulation of AHPCs at both the timepoints assessed following culture with CM compared to control. Although this observation was apparent at both timepoints assessed, it was only statistically significant at the 7 day timepoint ($p<0.05$). Selective cell death of the $GalC^+$ oligodendrocytic precursor subpopulation following culture with CM could be another key mechanism contributing to the astrogenic effect observed in AHPCs, resulting in astrogenesis at the expense of oligodendrogenesis. Although the vast majority of published studies report that MSC-derived CM exerts a positive influence on neural development in terms of neuroprotection and differentiation, there is also evidence to suggest that MSC-derived factors may, in some cases, be detrimental to neural cell systems. For example, MSC-CM has recently been reported to be cytotoxic and increase rates of cell death in hippocampal slice cultures (Horn *et al.*, 2009).

Apoptosis

One major limitation of the above data, however, is that PI positivity does not discriminate between necrotic and late apoptotic cells. Cells can be induced to undergo apoptosis, or programmed cell death, via a combination of intracellular and/or extracellular signals, which raises the interesting question of whether soluble factor(s) present in CM induce activation of apoptosis in the $GalC^+$ subpopulation of AHPCs or whether the increased rates of cell death observed are a consequence of necrosis. In terms of extracellular induction of apoptosis in oligodendrocytes, soluble factors including TNF-α and NGF have been reported to be potent inducers of apoptosis in this cell system (Selmaj and Raine, 1988; Casaccia *et al.*, 1996). Signalling events that occur during apoptosis are mediated by a family of cysteine proteases known as caspases, of which caspase-3 is a central mediator in the apoptotic pathway (Ellis *et al.*, 1991; Alnemri *et al.*, 1996; Gu *et al.*, 1999; Porter and Jänicke, 1999; Fox and Aubert, 2008; Mazumder *et al.*, 2008). Activity of caspases is most commonly modulated at the post-translational level, where inactive proenzymes are cleaved to produce active enzymes – activation of caspase-3 occurs during the early stages of apoptosis via the proteolytic cleavage (adjacent to Asp175) of the inactive proenzyme. Therefore, detection of cleaved caspase-3 can be considered a positive marker of apoptosis and its expression can be determined using flow cytometric methods. Rates

of apoptosis observed in the $GalC^+$ subpopulation following culture with CM and controls directly mirrored rates of cell death as determined using PI incorporation, with rates of apoptosis being significantly higher following culture with CM at both timepoints assessed ($p<0.01$). These data therefore suggest that MSC-derived aggregates secrete soluble factor(s) that induce activation of apoptotic pathways specifically in the $GalC^+$ subpopulation of AHPCs.

Analysis of cell death in the 33B oligodendrocytoma cell line

Such observations are of particular interest from an oncological perspective. One of the major causative mechanisms resulting in cancer development is uncontrolled/accelerated cellular proliferation, resulting in a massive accumulation of tissue mass i.e. tumour formation (Kasibharla and Tseng, 2003). However, an equally significant causative mechanism is a failure/dysregulation of cellular apoptosis and death, which can contribute to initiation and progression of cancer development (Lowe and Lin, 2000; Kasibharla and Tseng, 2003). Therefore, some of the most promising therapeutic strategies in the treatment of cancer are those that induce and/or increase rates of apoptosis in transformed cells (Searle *et al.*, 1975; Lowe and Lin, 2000; Gerl and Vaux, 2005). Therefore, by extension, if soluble factor(s) present in CM induce apoptosis specifically in oligodendrocytic-like ($GalC^+$) cells, then this is a promising avenue for investigation in cancers arising from transformed oligodendrocytes – oligodendrocytomas.

Oligodendrocytomas represent a small percentage of tumours of the central nervous system, accounting for approximately 10% of all gliomas i.e. tumours with their origins in glial cell types (Ney and Lassman, 2009; Vesper *et al.*, 2009). Current therapies for oligodendrocytomas are inadequate despite increasing knowledge into the aetiology of these tumours (Vesper *et al.*, 2009). Therefore, if the apoptotic effect of soluble factor(s) in CM could be exploited to induce apoptosis in transformed oligodendrocytes, this would potentially provide a novel therapy in the combat against cancers such as oligodendrocytomas. However, this apoptotic effect has only been observed in the AHPC system. In order to ascertain whether this effect could be translated to other oligodendrocytic systems, the effect of CM was examined on the 33B cell line – an oligodendrocytoma (more specifically, a Schwannoma) cell line derived from rat (Fields, 1977; Fields and Dammerman, 1985). Culture with CM did

not affect cellular viability compared to controls (using the MTS assay) and, likewise, there was no statistically significant difference in the percentage of cells detected in the early or late stages of apoptosis (using flow cytometry). Although these data were not significant at the statistical level, they still offered much promise in that CM cultures demonstrated a higher percentage of cells in the early and late stages of apoptosis. Perhaps with further optimisation a greater induction of apoptosis could be achieved in this oligodendrocytoma cell line – possible ideas include concentrating CM to produce a more potent cocktail of soluble factor(s); an increased aggregate-conditioning time; an increased 33B cell culture time; and identification of the active apoptotic factor in CM and recreation of this effect using the optimal concentration of recombinant protein.

Expression of signalling pathway components and transcription factors

Investigation into the molecular mechanisms controlling the specification of astrogenesis in AHPCs following culture with CM was performed by assessing the expression of a range of signalling pathway components and transcription factors known to play key roles in neural differentiation processes. Expression of such factors was determined at the mRNA level in AHPCs cultured with CM and UC control at days 1, 3 and 7, using qRT-PCR. The transcription factors examined can be divided into one of four categories based on the differentiative processes in which they are key components – astrocytic-promoting; neuronal-promoting; oligodendrocytic-promoting; and neuronal-inhibitory proteins. Combined, expression profiles constructed from these studies suggested that uncommitted AHPCs committed to an astrocytic differentiation program at the genetic level following culture with CM.

Expression of astrocytic-promoting transcription factors

AHPCs demonstrated significant upregulation of the five astrocytic-promoting transcription factors examined in this study – STAT1, STAT3, Smad1, Smad4 and Sox9 – and these observations were significant at the statistical level at various timepoints examined. The crucial roles these factors play in astrogenesis have been highly documented in the literature and are extensively reviewed by Guillemot (Guillemot, 2007). The two main highly conserved signalling pathways reported to play a key role in astrogenesis of uncommitted neural stem and progenitor cells are the janus kinase-signal transducer and activator of transcription (JAK-STAT) and BMP

signalling pathways (Gross *et al.*, 1996; Bonni *et al.*, 1997; Rajan and McKay, 1998; Guillemot, 2007; Nakashima *et al.*, 2001; He *et al.*, 2005; Kessaris *et al.*, 2008). Notch signalling has also been implicated in astrogenesis but has been omitted from analysis for the purpose of this study as Notch signalling requires interaction of membrane-bound cell surface receptors on cells – as the approach adopted in this study is a CM approach, it can be assumed that the mechanism at play is a result of soluble factor-mediated signalling (Gaiano *et al.*, 2000; Tanigaki *et al.*, 2001). The downstream effectors of JAK-STAT signalling are the STAT family of transcription factors, of which STAT1 and STAT3 are the most commonly implicated in astrogenesis (Bonni *et al.*, 1997; Nakashima *et al.*, 1999). In terms of the BMP signalling, the downstream effectors of this pathway are the Smad family of transcription factors, of which Smad1 and Smad4 are the most commonly implicated in astrogenesis (Guillemot, 2007; Kessaris *et al.*, 2008). Upregulation of STAT1, STAT3, Smad1 and Smad4 in AHPCs following culture with CM strongly suggests that the astrogenic-promoting JAK-STAT and BMP signalling pathways are activated. There is even evidence to suggest that the components of these two pathways can crosstalk extensively, with STAT1/STAT3 and Smad1/Smad4 forming a complex (bound by co-activator P300) that is actively recruited to the GFAP promoter (Nakashima *et al.*, 1999). The situation is further complicated by a study reporting that the two signalling pathways function to generate distinctive cell populations, with JAK-STAT signalling resulting in a $GFAP^+$, astrocytic-like but immature phenotype and BMP signalling resulting in a $GFAP^+$ mature astrocytic phenotype (Bonaguidi *et al.*, 2005). AHPCs cultured with CM also upregulated expression of Sox9, a transcription factor reportedly expressed in immature astrocytes and throughout astrocyte maturation (Stolt *et al.*, 2003).

Expression of oligodendrocytic-promoting transcription factors

Expression of the oligodendrocytic-promoting transcription factors Olig1 and Olig2 were then examined in AHPCs cultured with CM compared to control. The crucial role these factors play in oligodendrogenesis have been highly documented in the literature and are extensively reviewed by Guillemot (Guillemot, 2007). Olig1 and Olig2 are members of the basic helix-loop-helix (bHLH) family of transcription factors and are key transcriptional determinants of oligodendrocytic fate (Lu *et al.*, 2002; Zhou and Anderson, 2002). Both factors are expressed in oligodendrocytic-committed precursors and more mature oligodendrocytes, with abolishment of either factor resulting in a

dramatic loss of oligodendrocytic material in the CNS (Zhou *et al.*, 2000; Lu *et al.*, 2002; Zhou and Anderson, 2002). Although data from this study reveals that AHPCs do not demonstrate any significant decrease in expression of Olig1 until the 7 day timepoint ($p<0.05$), continued expression of Olig1 is unlikely to be inhibitory to the astrogenic differentiation process as Olig1 is only involved in the very later stages of oligodendrogenesis (Lu *et al.*, 2002; Arnett *et al.*, 2004). It is Olig2 that plays the more crucial role in terms of commitment and specification of uncommitted precursors to the oligodendrocytic fate. Paradoxically, Olig2 is significantly upregulated in AHPCs cultured with CM at the early timepoints of 1 and 3 days ($p<0.001$), which is opposite to the predicted expression profile. Studies have even reported Olig2 as a potent inhibitor of astrogenesis (Fukuda *et al.*, 2004). However, evidence has been published to suggest a role for Olig2 in the development of astrocytes (Marshall *et al.*, 2005). Furthermore, since expression of Olig2 has been associated with inhibition of neuronal differentiation, it is perhaps this inhibitory activity that is supporting the astrocytic differentiation of AHPCs following culture with CM, rather than its oligodendrocytic-promoting activity (Lee *et al.*, 2005; Cai *et al.*, 2007; Guillemot, 2007).

Expression of neuronal-promoting transcription factors

AHPCs demonstrated significant downregulation of three of the four neuronal-promoting transcription factors examined in this study – Ngn1, Ngn3 and Mash1 – and these observations were significant at the statistical level at various timepoints examined. Expression of the fourth neuronal-promoting transcription factor examined – Ngn2 – was unchanged compared to control cultures. The crucial roles these factors play in neurogenesis have been highly documented in the literature and are extensively reviewed by Guillemot (Guillemot, 2007). Proneural genes such as Ngn1, Ngn2, Ngn3 and Mash1 play crucial roles in many of the processes involved in neuronal differentiation, from initial commitment of immature neural stem and progenitor cells to differentiation right through to maintenance of a mature neuronal phenotype (Nieto *et al.*, 2001; Sun *et al.*, 2001; Bertrand *et al.*, 2002; Britz *et al.*, 2006). Overexpression of proneural genes is sufficient to induce neuronal differentiation in uncommitted precursors (Farah *et al.*, 2000; Nakada *et al.*, 2004). Similarly, downregulation of proneural genes results in reduced neuronal differentiation concomitant with increased astrocytic differentiation, suggesting that such factors play an important role not only

in neuronal differentiation but also in preventing premature astrocytic differentiation (Nieto *et al.*, 2001).

Expression of neuronal-inhibitory factors

Expression of the five neuronal-inhibitory proteins examined in this study – Hes1, Hes5, Id1, Id2 and Id3 – were upregulated in AHPCs cultured with CM compared to control and these observations were significant at the statistical level at various timepoints examined. The crucial roles these factors play in astrogenesis have been highly documented in the literature and are extensively reviewed by Guillemot (Guillemot, 2007). Hes1 and Hes5 have been reported to play crucial roles in maintenance of an undifferentiated state in neural stem and progenitor cells (Nakamura *et al.*, 2000; Ohtsuka *et al.*, 2001). That said, however, there is also much evidence to suggest that both Hes1 and Hes5 play critical roles in promotion of astrocytic differentiation, most probably through inhibition of proneural gene activity such as those described above (Hojo *et al.*, 2000; Guillemot, 2007). Hes proteins may also have a more direct role in promoting astrogenesis as they have previously been reported to activate components of the JAK-STAT signalling cascade, itself a key requirement for astrogenesis (Kamakura *et al.*, 2004). Other neuronal-inhibitory proteins examined in this study included three members of the Inhibitor of differentiation (Id) family – Id1, Id2 and Id3. Id proteins are reportedly activated by BMP signalling pathways and are key determinants of astrocytic differentiation (Nakashima *et al.*, 2001). They inhibit neuronal differentiation by sequestering E proteins, which are the dimerisation partners of proneural bHLH transcription factors, blocking their neuronal-promoting activities (Vinals *et al.*, 2004). Similarly, Id proteins have been reported to dimerise with Olig transcription factors and their E protein dimerisation partners, thus blocking their oligodendrocytic-promoting activity also (Samanta and Kessler, 2004). Therefore, increased expression of Olig2 in AHPCs cultured with CM may be counteracted by increased expression of the sequestering Id proteins, providing a further possible mechanism whereby astrogenesis is observed at the expense of oligodendrogenesis.

Overall summary

Studies in this Chapter have demonstrated that MSCs cultured under conditions previously established for the propagation of neurospheres resulted in the formation of large, multicellular aggregates sharing many phenotypically similar traits to NSC-derived neurospheres. Aggregates were able to influence neural development by paracrine mechanisms involving the release of trophic factors and/or other metabolic products that induced the expression of an astrocytic phenotype in the AHPC system. Immature AHPCs were induced to express both structural and functional markers indicative of an astrocytic phenotype following culture with CM for 7 days and this effect was exclusively the result of culture with MSC-derived soluble factors. In terms of cellular and molecular mechanisms bringing about this astrocytic effect, kinetics of GFAP expression concomitant with the observation that proliferation of the $GFAP^+$ subpopulation was not influenced following culture with CM suggested that the soluble factor(s) present in CM were not positively selecting for astrocytic-committed precursors and, as such, suggested an instructive mechanism was operational. However, analysis of cell death demonstrated that culture of AHPCs with CM resulted in an increase in the rate of cell death specifically in the $GalC^+$ subpopulation. Further analysis confirmed the activation of apoptotic pathways specifically in the $GalC^+$ subpopulation following culture with CM. Therefore, selective apoptosis in the $GalC^+$ subpopulation could be a further contributing mechanism resulting in astrogenesis at the expense of oligodendrogenesis. From a transcriptional perspective, astrogenesis was induced in AHPCs by increased expression of astrocytic-promoting and neuronal-inhibitory transcription factors concomitant with decreased expression of neuronal- and oligodendrocytic-promoting transcription factors. As neural differentiation is such an intricate, complicated process *in vitro* and even more so *in vivo*, it is not surprising that multiple changes are required at the molecular level to bring about specific differentiation events. However, it must be acknowledged that this expression data is at the mRNA level and, although mRNA levels are not always indicative of actual protein levels, qRT-PCR is advantageous in that it provides a rapid and easy technology in which to screen many factors. Although transcription factor activity is often controlled at the post-translational level, changes at the transcriptional level are useful in themselves as indicators of cellular activity.

Chapter 5

CHARACTERISATION OF THE MSC SECRETOME USING TARGETED TRANSCRIPTIONAL PROFILING AND SHOTGUN PROTEOMICS

5.1 Introduction

A major fundamental flaw of *in vitro* stem cell-based assays is that the artificial environment of 2D tissue culture plastic in no way resembles the complex 3D environment in which stem cells reside *in vivo* – the 'stem cell niche'. At the basic physiological level, the stem cell niche can be described as a basic unit of tissue structure which serves a crucial role in maintenance of stem cell viability/function, physically protecting it from harm and integrating signals that mediate the appropriate response of the stem cell to the specific needs of the organism in terms of tissue generation, maintenance and repair (Scadden, 2006). At the more-detailed molecular level, the stem cell niche can be described as a dynamic entity in which complex interactions occur between the stem cell and its neighbouring cells (typically involving direct cell-cell interaction and/or the release of soluble factors/cytokines produced by identical cells (autocrine) or different cells (paracrine) within the niche), extracellular matrix and oxygen tension, amongst many other factors (Fuchs *et al.*, 2004; Scadden, 2006). Although the concept of the 'stem cell niche' and its crucial role in maintenance/regulation of stem cell behaviour/function is not a novel one – originally proposed by Schofield over 30 years ago – the concept remained largely theoretical in the years that followed and it is only recently, as understanding of the plethora of interacting factors at play within the stem cell niche has improved, that interest in this area has begun to intensify (Schofield, 1978; Presnell *et al.*, 2002; Fuchs *et al.*, 2004; Scadden, 2006).

As such, a more comprehensive understanding of the complex cellular and molecular mechanisms of neural differentiation will require a greater appreciation of the niche-specific signals governing neural stem cell behaviour *in vivo*. In mammals, neural stem cells are maintained in two main niches in the adult brain – the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Eriksson *et al.*, 1998; Gould *et al.*, 1999; Garcia *et al.*, 2004; Maslov *et al.*, 2004; Ehninger and Kempermann, 2007). That said, there is increasing evidence to suggest that neural stem cells may reside in additional niches located throughout the adult brain, including the amygdala, neocortex, striatum, subcallosal zone and substantia nigra (Gould *et al.*, 1996; Bernier *et al.*, 2002; Zhao *et al.*, 2003; Van Kampen *et al.*, 2004; Bedard *et al.*, 2006; Seri *et al.*, 2006; Gould, 2007). The

neural stem cell niche is highly complex and is composed of many different structural and functional components such as neurons, astrocytes, oligodendrocytes, microglia, immune cells, vasculature and extracellular matrix, amongst many other factors, with each of these components interacting extensively in order to modulate the function/behaviour of neural stem cells in terms of proliferation, migration and differentiation (Mercier *et al.*, 2002). Although considerable effort can be made (and has been) to mimic particular aspects of the neural stem cell niche *in vitro*, such as coating cellware with poly-L-ornithine and laminin to provide an environment more permissible to the culture of AHPCs, these efforts are generally unsatisfactory. For example, the extracellular matrix component of the neural stem cell niche *in vivo* consists of a highly intricate balance/combination of extracellular proteins – typically consisting of large glycoproteins such as fibronectin, collagen, laminin and proteoglycans – arranged purposely to provide a scaffold for cellular support and present at precise concentrations in order to induce specific responses in stem cells within the niche (Adams and Watt, 1993; Tate *et al.*, 2004; Ma *et al.*, 2008). Furthermore, it is now widely accepted that astrocyte- and vasculature-specific soluble factors/cytokines are likely to be the most significant factors within the niche involved in maintenance/regulation of neural stem cell function/behaviour (Palmer *et al.*, 2000; Alenghet and Ingber, 2002; Jin *et al.*, 2002; Song *et al.*, 2002; Wurmser *et al.*, 2004; Shapiro *et al.*, 2005; Barkho *et al.*, 2006; Plumpe *et al.*, 2006; Ehninger and Kempermann, 2008). That said, it is unwise to consider the roles of the various component(s) of the stem cell niche in isolation, as there is extensive overlap between them; for example, extracellular matrix proteins have been reported to bind specific growth factors and increase their local concentration within the niche whilst certain growth factors regulate the synthesis and breakdown of extracellular matrix proteins (Watt, 1986; Sporn *et al.*, 1987; Gordon, 1988; Roberts *et al.*, 1988; Rothenberg *et al.*, 1988). Accordingly, the stem cell niche should be considered as a dynamic entity (Scadden, 2006).

However, despite the clear overlap between the role(s)/function(s) of the various components of the stem cell niche, studies to date have concentrated on individual components of the niche and their influence on stem cell behaviour/function. For example, studies involving the *in vitro* culture of cells on 3D structures such as biodegradable scaffolds, hydrogels, sponges, microcarriers and polystyrene-based

scaffolds all share the common aim of encouraging cells to adopt conformations and form cell-cell interactions more representative of those that occur within their niche *in vivo* compared to the highly unnatural restraints imposed upon them during routine 2D culture (Mikos *et al.*, 1993; Takahashi *et al.*, 2005; Bokhari *et al.*, 2007; Bradley *et al.*, 2009; Lin and Anseth, 2009). Indeed, this was subject to investigation in Chapter 3 of this thesis. Likewise, the influence of extracellular matrix on cellular behaviour/function can be assessed by coating cellware with various combinations/concentrations of extracellular matrix proteins such as collagen, laminin and fibronectin; Reinnervate Ltd. has been involved in collaborations investigating the immobilisation of peptide fragments corresponding to putative functional domains of extracellular matrix proteins and determining their influence(s) on cellular attachment, proliferation and differentiation (Cooke *et al.*, 2008; Cooke *et al.*, 2009). However, it is perhaps the influence of diffusible molecules such as soluble factors and cytokines within the stem cell niche that are of most interest (Hall and Watt, 1989). The significance of soluble factor/cytokine signalling in the regulation of stem cell function/behaviour is best exemplified in the haematopoietic system where stromal cells (and other cell types) within the niche produce a complement of factors which promote haematopoietic cell survival, proliferation and differentiation (Clark and Kamen, 1987; Dexter and Spooncer, 1987). This phenomenon is discussed in further detail in Section 1.7.1. A similar phenomenon has been described in the epidermis, where keratinocytes secrete a complement of factors, including TGF-α and TGF–β, which function *via* autocrine mechanisms to regulate the growth rate of other keratinocytes within the niche; interestingly, the expression profiles of such factors in keratinocytes change in response to appropriate environmental stimuli (Coffey *et al.*, 1987; Akhurst *et al.*, 1988). Far less is known regarding the role of soluble factors/cytokines in modulating stem cell behaviour within the intestinal crypt, though one study reports that TGF-β is secreted by terminally-differentiated cells at the tip of the villus which functions to inhibit crypt cell proliferation (Barnard *et al.*, 1989).

Neural stem and progenitor cells within their niche are also under the influence of soluble factors and cytokines – the majority of which are astrocytic and/or vasculature-derived in origin – which function to provide structural and trophic support for various aspects of neural activity including proliferation, migration, differentiation, neurite extension and synaptogenesis (Qian *et al.*, 1992; Mercier *et al.*, 2002; Bachoo *et al.*,

2004; Deumens *et al.*, 2004; Hagg, 2005; Moore *et al.*, 2009). Astrocytes in particular have been demonstrated to secrete an intricate combination of extracellular matrix, such as laminin, and soluble factors/cytokines, such as FGF-2, which significantly influence neural behaviour both *in vitro* and *in vivo* (Costa *et al.*, 2002; Le Roux and Esquenazi, 2002; Moore *et al.*, 2009). There is considerable evidence to suggest that MSCs are able to function in an analogous manner to astrocytes following transplantation into the mammalian nervous system and this extends to the release of extracellular matrix components and neurotrophic factors within the neural niche (Azizi *et al.*, 1998; Tremain et al., 2001; Chen et al., 2002; Chopp and Li, 2002; Munoz et al., 2005; Chen and Chopp, 2006; Crigler et al., 2006; Deng *et al.*, 2006). Indeed, MSC-mediated paracrine activity is considered to be the major mechanism contributing to functional recovery following transplantation of MSCs into models of neurological and neurodegenerative disorders (Chen *et al.*, 2002; Chen *et al.*, 2005; Chen *et al.*, 2007).

Several efforts have been made to artificially reproduce the paracrine relationship between MSCs and neural stem/progenitor cells *in vitro*, predominantly using conditioned media-based approaches, though variant approaches such as co-culture have also been used successfully (Wislet-Gendebien *et al.*, 2004; Rivera *et al.*, 2006; Croft and Przyborski, 2009). Conditioned media assays are advantageous in that conditioned media is easily produced and biologically tested *in vitro*, particularly when a hypothesised autocrine/paracrine mechanism is under investigation. Co-culture assays have the added advantage of permitting intercellular signalling between the two cell systems under investigation, allowing for feedback mechanisms; however, the significant disadvantage of co-culture assays is that it is extremely difficult to determine the cellular origin of proteins within the culture media (Chen *et al.*, 2002; Croft and Przyborski, 2009). Indeed, even if the cell systems used were derived from different species, extensive homology between many growth factors/cytokines means extensive crosstalk during antibody-based techniques such as ELISA cannot be discounted (Chen *et al.*, 2002).

Ultimately, the aim of all conditioned media-based studies should be as follows:

a) Observation of a biological effect.

b) Identification of the bioactive factor(s) responsible for exerting the biological effect.

Chapter 4 addressed the first of these key issues, demonstrating that MSC-derived aggregates secreted a complement of soluble factors/cytokines that induced an astrogenic effect in AHPCs. It is therefore envisioned that MSCs may present as vehicles for the delivery of highly-efficacious levels of neurotrophic factors and cytokines to areas of neurological and neurodegenerative damage following transplantation. Identification of the bioactive factor(s) present in MSC aggregate-derived CM may bypass the need for cellular therapy at all, instead permitting exogenous application of such molecules directly to neurologically-damaged areas. Similarly, increased knowledge in this area may lead to the generation of new media supplements that can be used to direct the differentiation of neural stem cells in a highly controlled manner, providing a convenient method by which to generate *in vitro* culture of astrocytes.

The paracrine function(s) of MSCs in terms of their effect(s) on neural development and plasticity using an *in vitro* conditioned media (CM)-based model was examined previously in Chapter 4. The present study aims to extend upon the results generated in the previous Chapter in order to scrutinise the MSC aggregate-derived secretome in further detail, with the ultimate aim of identifying the bioactive factor(s) responsible for exerting the biological effects observed in the previous chapter. To date, most conditioned media studies make little effort to identify the bioactive factors present within the highly complex mixture of proteins in conditioned media and those that do typically pursue a traditional approach, involving characterisation of only one or a handful of secreted proteins (Moore *et al.*, 2009). For example, Rivera and colleagues adopted a random screening approach using recombinant proteins and blocking antibodies targeted against various soluble factors and cytokines, whilst Wislet-Gendebien and colleagues used targeted expression profiling using qRT-PCR and Western blotting, based on current knowledge within the literature (Wislet-Gendebien *et al.*, 2004; Rivera *et al.*, 2006).

In the present study, a combinatorial approach involving targeted transcriptional profiling using qRT-PCR and shotgun proteomics using LC-MS/MS was adopted in order to scrutinise the MSC aggregate-derived secretome in further detail, paying particular attention to those factors implicated in neural activities such as neural stem cell proliferation, migration and differentiation. Proteomic-based methodologies have previously been employed in several studies in order to scrutinise the MSC-derived secretome in detail; Sarojini and colleagues successfully used 2D LC-MS/MS to identify pigment epithelial-derived growth factor (PEDF) as the major fibroblast chemoattractant in MSC-derived CM, whilst De Kleijn and colleagues adopted a similar LC-MS and antibody array-based approach in order to identify the soluble factors/cytokines that could account for the therapeutic effect of MSC transplantation following myocardial infarction (De Kleijn *et al.*, 2008; Sarojini *et al.*, 2008). However, to our knowledge, the present study is the first to examine the MSC-derived secretome from a neural perspective.

Hypothesis

Induction of astrogenesis in AHPCs following culture in MSC aggregate-derived CM is a consequence of the activity of soluble factors and cytokines secreted directly into the medium by 3D MSC aggregates.

Aims of chapter

To identify the bioactive factor(s) present in MSC aggregate-derived CM responsible for the astrogenic effect in AHPCs observed in Chapter 4.

Objectives

1. Targeted transcriptional profiling against astrocytic-promoting and astrocytic-inhibitory soluble factors, using qRT-PCR, based on current knowledge within the literature.

2. Examine influence of oxygen tension on transcriptional activity of MSC-derived aggregates.

3. Shotgun proteomic analysis of MSC aggregate-derived CM, using LC-MS/MS, to identify secreted peptides.

5.2 Materials and methods

5.2.1 RNA isolation, reverse transcription and qRT-PCR analysis of soluble factor expression

Expression of a range of soluble factors/cytokines with known neural associations (based on current knowledge within the literature) were examined at the mRNA level, using a qRT-PCR approach, in MSCs cultured under standard conditions and as aggregates under physiological and atmospheric oxygen tensions. Briefly, MSCs were seeded out under standard conditions and for aggregate formation under physiological and atmospheric oxygen tensions, as described in Sections 2.1 and 4.2.1 respectively, and maintained for 3 days i.e. to the point where CM would be collected for subsequent downstream studies. Following 3 days culture, total cellular RNA was isolated using the TRIreagent method (Section 2.9) followed by reverse transcription to cDNA (Section 2.10) and finally qRT-PCR using SYBR Green chemistry (Section 2.11), as detailed in the relevant sections. All expressions were normalised to GAPDH as previous detailed in Section 2.11, with normalised levels of expression shown as a percentage of the maximum expresser. Specific primer sequences are shown in Table 2.1.

5.2.2 Concentration of CM samples and 1D SDS-PAGE

CM was concentrated approximately 10-fold to a final volume of 200-400µl (approximate concentration 1.5µg/µl) by centrifugation in a 2 kDa molecular weight cut-off filter (Vivaspin 15R) at 3000 x *g* for 2 hours. Protease inhibitors were added to a final concentration of 1:1000 to prevent degradation of proteins of interest in the concentrate. Total protein was quantified using the Bradford assay and equivalent amounts of protein for each sample (12µg) were loaded onto a 4-12% bis-tris 1D SDS-PAGE gel. Therefore, all comparisons within the 1D gel were made on an equivalently-loaded basis. Gels were then stained in three successive solutions of Coomassie Brilliant Blue R-250 – 1) 10% acetic acid, 25% isopropanol, 2% Coomassie Brilliant Blue R-250; 2) 10% acetic acid, 10% isopropanol, 0.25% Coomassie Brilliant Blue R-250; and 3) 10% acetic acid, 0.25% Coomassie Brilliant Blue R-250 – to allow visualisation of protein bands.

5.2.3 Preparation of samples for proteomic analysis by the North East Proteome Analysis Facility (NEPAF)

Three biologically-independent batches of MSCs were seeded out for aggregate formation in media supplemented with ITS and ITS+P+P to form pairwise samples, using an identical methodology to that described in Section 4.2.1 (please refer to Section 5.3.5 for explanation of ITS and ITS+P+P media). After 3 days, the CM was removed from the flasks, centrifuged at 2000rpm for 5 minutes to remove cellular debris and the supernatant collected and filtered using a 0.22μm pore filter to remove further contaminants. Samples were stored at -20^{o}C until required.

CM was concentrated approximately 10-fold to a final volume of 200-400μl (approximate concentration 1.5μg/μl) by centrifugation in a 2 kDa molecular weight cut-off filter (Vivaspin 15R) at 3000 x *g* for 2 hours. Protease inhibitors were added to a final concentration of 1:1000 to prevent degradation of proteins of interest in the concentrate. Total protein was quantified using the Bradford assay and equivalent amounts of protein for each sample (12μg) were loaded onto a 4-12% bis-tris 1D SDS-PAGE gel. Therefore, all comparisons within the 1D gel were made on an equivalently-loaded basis. Gels were stained in three successive solutions of Coomassie Brilliant Blue R-250 – 1) 10% acetic acid, 25% isopropanol, 0.025% Coomassie Brilliant Blue R-250; 2) 10% acetic acid, 25% isopropanol, 0.003125% Coomassie Brilliant Blue R-250; and 3) 10% acetic acid, 0.003125% Coomassie Brilliant Blue R-250 – to allow visualisation of protein bands. The gel was subsequently stored in destaining solution (10% acetic acid, 1% glycerol) prior to manipulation by the North East Proteome Analysis Facility (NEPAF), a state-of-the-art facility providing proteomic-based services to groups/companies in both the private and public sector in the UK (http://www.nepaf.com/home.html).

5.2.4 Preparation of gel slices, tryptic digestion, liquid chromatography and MALDI-TOF MS/MS

Following fractionation of paired CM samples on a 1D SDS-PAGE gel, this gel was passed onto NEPAF for high-throughput shotgun proteomics analysis. All subsequent steps were therefore performed by NEPAF; however, a consideration of the relevant methodologies will be considered here.

Each of the six sample lanes (3 x ITS-CM, 3 x ITS+P+P-CM) on the 1D gel were laterally sliced into 8 sections and prepared for in-gel tryptic digestion. Note that the regions of the gel corresponding to the transferrin and BSA bands (~60-80 kDa) were excluded from analysis (the presence of BSA being attributable to carry-over from serum-containing media). In-gel tryptic digestion results in the generation of shorter fragments i.e. peptides, which have distinctive masses, ultimately aiding in identification of the parental protein. Peptides were extracted from the gel matrix and resolved using reversed phase nano-HPLC in-line with the MS. Chromatography was performed on a Dionex Ultimate 3000 NanoLC System, Dionex Pepmap100 C18 Column (25cm x 75um i.d., 3 μm, with C18 trap column) at 60°C at a flow rate of 300 nl/minute over 102 minutes. Eluted peptides were then directly analysed using a tandem MS methodology (nanospray MS/MS, LTQ Orbitrap XL, ThermoScientific) in order to identify individual peptides.

5.2.5 Protein identification

MS/MS spectra generated by NEPAF were searched against the international X!Tandem search engine, available through the Global Proteome Machine Organisation interface (www.thegpm.org). This algorithm matches MS/MS spectra with peptide sequences on its database in order to identify the parent protein from which the peptide was generated and gives data in terms of a wide range of convenient parameters, including a description of the identified protein, ENSEMBL identifiers, description of protein function, an estimated molecular weight of the protein and a log(e) score, amongst others. Information for each protein was gathered using the description given in the GPM result file and also the corresponding ENSEMBL identifier (www.ensembl.org).

5.2.6 Spectral counting

Semi-quantitation of the shotgun proteomic data generated above was achieved using a spectral counting method. Here, it is assumed that the number of peptides detected for each protein is indicative of the relative abundance of that protein within the complex sample mixture, such that the greater the number of peptides detected for a particular protein, the more abundant that protein must be in the mixture. However, large proteins will generate a greater number of peptides compared to smaller proteins; therefore, normalisation against protein size is necessary. Abundance rankings were

calculated for each identified protein based on the number of peptides contributing to its positive identification. These figures were subsequently used to calculate ratios of upregulation/downregulation in biologically-inactive (ITS) versus biologically active (ITS+P+P) samples.

5.2.7 Data processing

In order to create a manageable list of putative candidate soluble factors from the 2429 hits generated using the above methodology, a number of stringent criteria were applied:

a) Protein must be identified from at least two peptides
b) Protein must be either exclusively detected or upregulated 2-fold or more in at least 2 out of 3 pairwise samples
c) Log(e) score must be $\leq$-3

Data is presented in terms of a number of parameters, including ENSEMBL identifier, protein description, pairwise comparisons, gel slice in which protein was identified, number of peptides contributing to each protein identification, log(e) score, normalised abundance ratio biologically active/biologically inactive samples, estimated molecular weight of protein and calculated molecular weight.

5.3 Results

5.3.1 MSCs demonstrate marked differences at the transcriptional level following culture under aggregate-inducing conditions compared to standard culture conditions; similarly, oxygen tension also presents as a critical determinant of transcriptional activity

Studies in the previous chapter demonstrated that MSCs cultured as large multicellular aggregates secreted a specific complement of soluble factors/cytokines that induced AHPCs to commit to and undergo astrogenesis following 7 days in culture. This differentiative effect was only observed following culture with aggregate-derived CM and could not be reproduced following culture with monolayer-derived CM. Adopting a CM approach to examine the secretory activities of various cell types *in vitro* is not a unique strategy to this investigation and many studies throughout the literature have employed similar tactics in order to examine autocrine and paracrine mechanisms of cellular behaviour and function. However, the major shortcoming of most studies is that few make any attempt to characterise the secretome of the cells in order to identify the soluble factor(s) responsible for bringing about the observed biological effect, whether this be a consequence of increased expression of active factors or a decrease in expression of inhibitory factors, or both. It is ultimately the identification of these active (or inhibitory) factors that will be of significant use in a research/industrial setting.

The principal aim of this Chapter is therefore to provide a detailed analysis of the MSC secretome – more specifically, the secretome of MSC-derived aggregates – in order to identify the bioactive soluble factor(s) responsible for the observed astrogenic effect in AHPCs. Initial assessment of the secretome was achieved using a transcriptional profiling approach. Here, expression of a range of soluble factors known to play key roles in neural differentiation and development were examined in MSC-derived aggregates vs. standard MSC monolayer cultures at the mRNA level using qRT-PCR. The effect of oxygen tension on the expression of soluble factors in aggregates was also determined as previous results demonstrated that oxygen tension significantly influenced the secretory activity of MSC-derived aggregates. Specific soluble factors were selected on account of previous observations and a review of the current literature, allowing identification and subsequent targeted analysis of potential

candidates. All expressions were normalised to GAPDH as previous detailed in Section 2.11, with normalised levels of expression shown as a percentage of the maximum expression level recorded.

The soluble factors examined were grouped into subcategories according to the specific protein family to which they belonged including BMP, gp130 (IL-6)-related, classic neurotrophins, EGF, PDGF, TGF and miscellaneous protein families. Results are shown in Figures 5.1, 5.2, 5.3, 5.4, 5.5, 5.6 and 5.7 respectively. In terms of members of the BMP family, expression of BMP2, BMP4 and BMP7 were determined at the mRNA level in MSCs cultured under standard conditions and as aggregates under physiological and atmospheric oxygen tensions. Results are shown in Figure 5.1. Expression of BMP2 was relatively uniform and transcript levels comparable in MSCs cultured under all three conditions (Figure 5.1A). The lowest levels of expression were detected in MSCs cultured under standard conditions with increased expression observed following subsequent culture in aggregate-inducing conditions, though this was not significant at the statistical level. Aggregates cultured under atmospheric oxygen conditions demonstrated greater levels of expression than aggregates cultured under physiological oxygen conditions though, similarly, this difference was very slight and not significant at the statistical level. BMP4, the BMP family member most frequently associated with astrogenesis, was expressed at maximum levels in MSCs cultured under standard conditions (Figure 5.1B). Levels of BMP4 transcript were detected at significantly lower levels in MSCs cultured as aggregates under both physiological ($p<0.01$) and atmospheric ($p<0.001$) oxygen tensions. Interestingly, although BMP4 expression appeared to be only very slightly increased in aggregates cultured under physiological oxygen compared to atmospheric oxygen tensions, this difference was highly significant at the statistical level ($p<0.001$). The expression profile of BMP7 mirrored that observed for BMP4, with maximum levels of expression detected in MSCs cultured under standard conditions followed by a dramatic reduction in expression following culture under aggregate-inducing conditions (Figure 5.1C). This decrease in expression was statistically significant for MSCs cultured as aggregates under both physiological ($p<0.05$) and atmospheric ($p<0.01$) oxygen tensions. Although the difference in transcript levels appeared to be far more dramatic than that observed for BMP4, this observation was not significant at the statistical level.

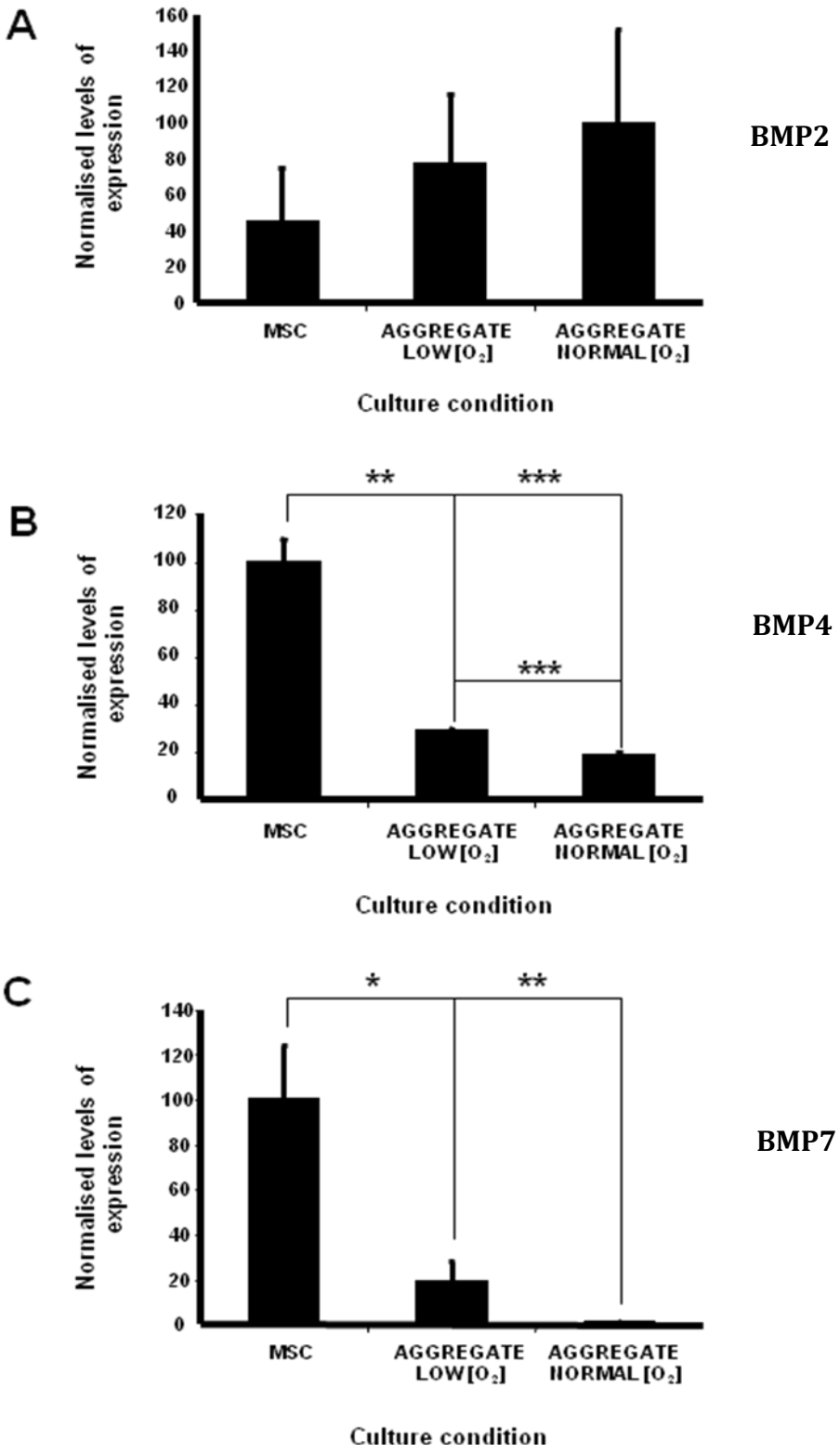


Figure 5.1. Expression of members of the BMP protein family at the mRNA level in MSC aggregates cultured under varying oxygen tensions and in MSCs cultured under standard conditions for 3 days. QRT-PCR analysis was performed to compare mRNA transcript levels of three members of the BMP protein family – **A:** BMP2; **B:** BMP4; **C:** BMP7 – in MSCs cultured as aggregates under varying oxygen tensions and in MSCs cultured under standard conditions for three days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, ** $p<0.01$, *$p<0.05$.

In terms of members of the gp130 (IL-6)-related family of cytokines, expression of CNTF, LIF and IL-6 were determined at the mRNA level in MSCs cultured under standard conditions and as aggregates under physiological and atmospheric oxygen tensions. Results are shown in Figure 5.2. The expression profile observed for CNTF mirrored that previously observed for BMP7, with maximum levels of expression detected in MSCs cultured under standard conditions followed by a dramatic reduction in expression following culture under aggregate-inducing conditions (Figure 5.2A). Decreased expression of CNTF in aggregates compared to MSCs cultured under standard conditions was significant at the statistical level for aggregates cultured under both physiological ($p<0.01$) and atmospheric ($p<0.01$) oxygen tensions. Differences in expression between aggregates cultured under varying oxygen tension were not significant at the statistical level. Expression of LIF was low in MSCs cultured under standard conditions and was significantly upregulated in aggregates cultured under both physiological ($p<0.001$) and atmospheric ($p<0.01$) oxygen tensions (Figure 5.2B). Maximum expression was observed in aggregates cultured under physiological oxygen conditions with aggregates cultured under atmospheric oxygen conditions demonstrating high levels of expression compared to standard MSCs but lower levels of expression compared to aggregates cultured under physiological oxygen conditions ($p<0.05$). The expression profile observed for IL-6 demonstrated extensive similarity to that observed for CNTF, with maximum levels of expression detected in MSCs cultured under standard conditions followed by a dramatic reduction in expression following culture under aggregate-inducing conditions (Figure 5.2C). Decreased expression of IL-6 in aggregates compared to MSCs cultured under standard conditions was significant at the statistical level for aggregates cultured under both physiological ($p<0.05$) and atmospheric ($p<0.05$) oxygen tensions. Although aggregates cultured under physiological oxygen tensions demonstrated slightly higher levels of IL-6 expression than counterparts cultured under atmospheric oxygen tensions, this difference was not significant at the statistical level.

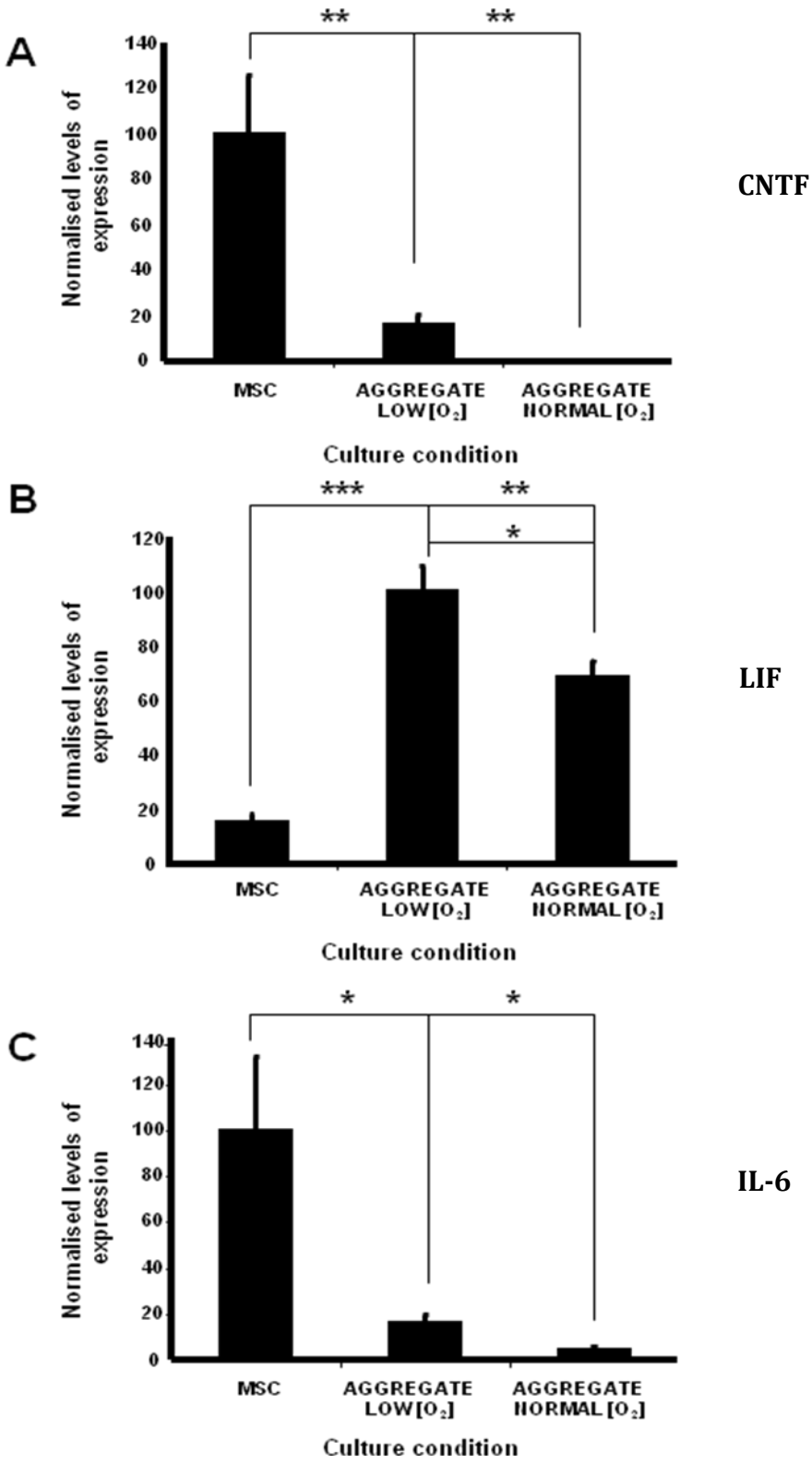


Figure 5.2. Expression of members of the gp130 (IL-6) family of cytokines at the mRNA level in MSC aggregates cultured under varying oxygen tensions and in MSCs cultured under standard conditions for 3 days. QRT-PCR analysis was performed to compare mRNA transcript levels of three members of this protein family – **A:** CNTF; **B:** LIF; **C:** IL-6 – in MSCs cultured as aggregates under varying oxygen tensions and in MSCs cultured under standard conditions for three days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, ** $p<0.01$, *$p<0.05$.

In terms of members of the classical neurotrophin family of cytokines, expression of NGF and BDNF were determined at the mRNA level in MSCs cultured under standard conditions and as aggregates cultured under physiological and atmospheric oxygen tensions. Results are shown in Figure 5.3. The expression profile observed for NGF mirrored that previously observed for LIF, with low levels of expression in MSCs cultured under standard conditions and significantly greater levels of expression in aggregates cultured under both physiological ($p<0.05$) and atmospheric ($p<0.05$) oxygen tensions (Figure 5.3A). Analogous to the LIF expression profile, maximum expression of NGF was detected in aggregates cultured under physiological oxygen conditions with aggregates cultured under atmospheric oxygen conditions demonstrating high levels of expression compared to standard MSCs but lower levels of expression compared to aggregates cultured under physiological oxygen conditions ($p<0.05$). Expression of BDNF was relatively uniform with no discernable differences in transcript levels being detected between MSCs cultured under standard or aggregate-inducing conditions (Figure 5.3B).

In terms of members of the EGF family of cytokines, expression of EGF and neuregulin were determined at the mRNA level in MSCs cultured under standard conditions and as aggregates cultured under physiological and atmospheric oxygen tensions. Results are shown in Figure 5.4. Expression of EGF was unique in that transcript levels were relatively uniform between MSCs cultured under standard conditions and as aggregates under physiological oxygen tensions, with significantly lower transcript levels being detected in aggregates cultured under atmospheric oxygen tensions (Figure 5.4A). Decreased expression of EGF following aggregate culture under atmospheric oxygen tension was moderately significant at the statistical level when compared to both other culture conditions ($p<0.01$ in both instances). Expression of neuregulin mirrored that previously observed for several other cytokines including BMP4, BMP7, CNTF and IL-6, with maximum levels of expression observed in MSCs cultured under standard conditions (Figure 5.4B). Although levels of transcript were detected at lower levels in MSCs cultured as aggregates under both physiological and atmospheric oxygen tensions, this decrease in expression was only significant at the statistical level following aggregate culture under atmospheric oxygen tensions ($p<0.01$). Expression of neuregulin was greater in aggregates cultured under

physiological oxygen tensions compared to atmospheric oxygen tensions and this observation was highly significant at the statistical level (p<0.001).

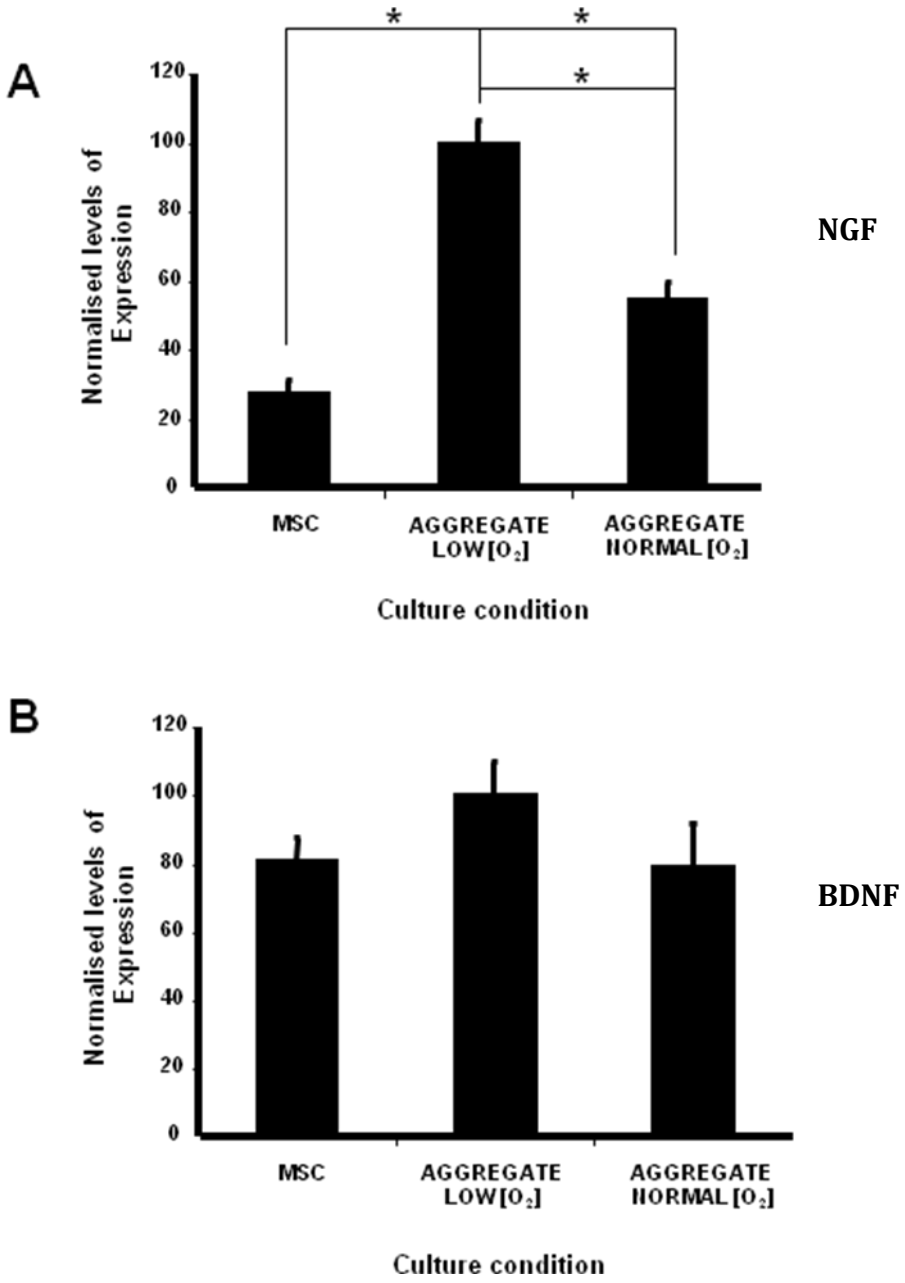


Figure 5.3. Expression of members of the classical neurotrophin family of cytokines at the mRNA level in MSC aggregates cultured under varying oxygen tensions and in MSCs cultured under standard conditions for 3 days. QRT-PCR analysis was performed to compare mRNA transcript levels of two members of this protein family – **A:** NGF; **B:** BDNF – in MSCs cultured as aggregates under varying oxygen tensions and in MSCs cultured under standard conditions for three days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *p<0.05.

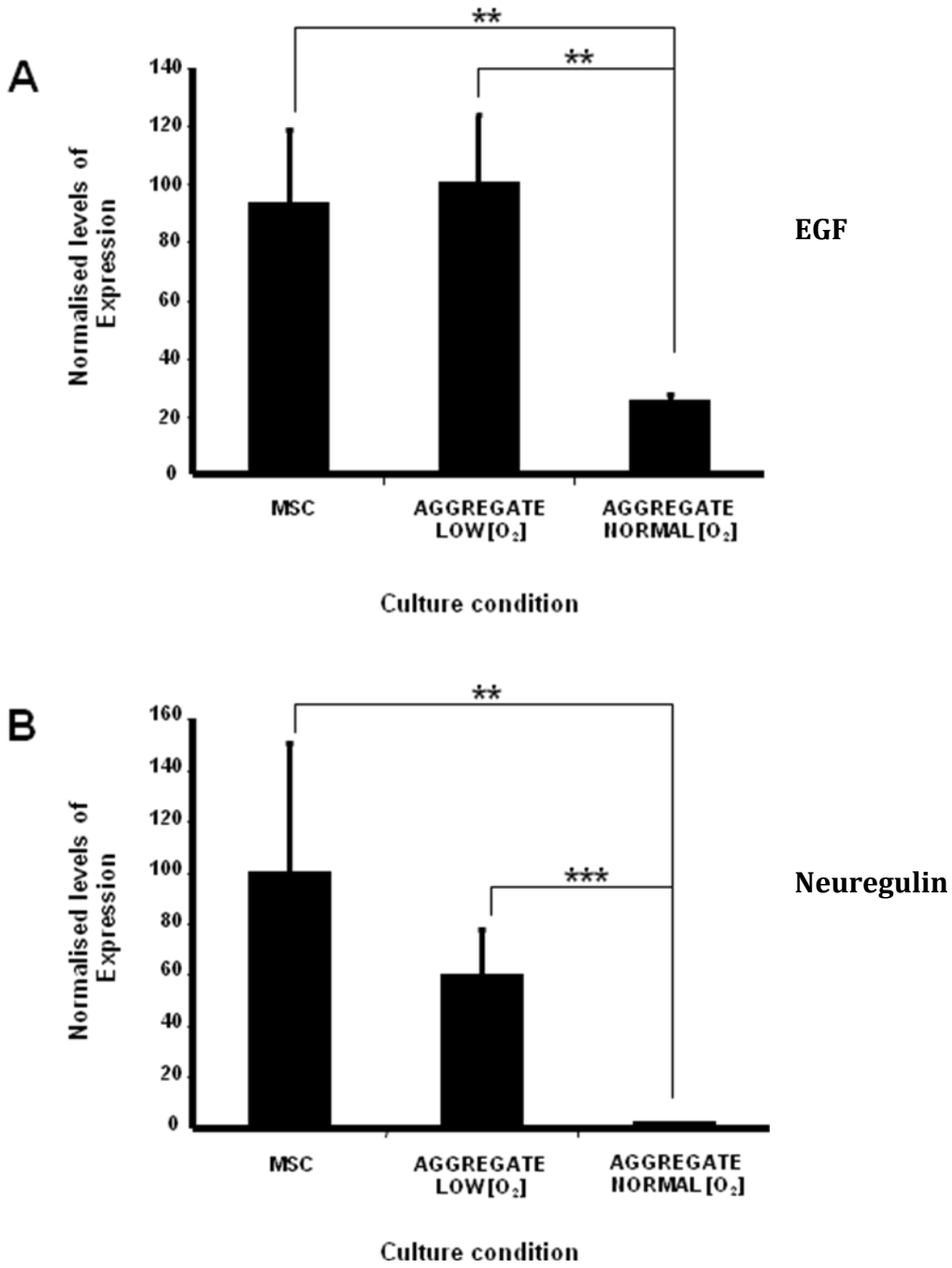


Figure 5.4. Expression of members of the EGF family of cytokines at the mRNA level in MSC aggregates cultured under varying oxygen tensions and in MSCs cultured under standard conditions for 3 days. QRT-PCR analysis was performed to compare mRNA transcript levels of two members of this protein family – **A:** EGF; **B:** Neuregulin – in MSCs cultured as aggregates under varying oxygen tensions and in MSCs cultured under standard conditions for three days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, ** $p<0.01$.

In terms of members of the PDGF family of cytokines, expression of PDGF-A and VEGF were determined at the mRNA level in MSCs cultured under standard conditions and as aggregates cultured under physiological and atmospheric oxygen tensions. Results are shown in Figure 5.5. Expression profile observed for PDGF-A was unique and antagonistic to that previously observed for NGF, with highest levels of expression observed in MSCs cultured under standard conditions and as aggregates under atmospheric oxygen tensions (Figure 5.5A). Lowest levels of expression were observed in MSCs cultured as aggregates under physiological oxygen tensions. However, no statistically significant difference was found in terms of PDGF-A expression between MSCs cultured under any of the various conditions. In terms of VEGF, maximum expression was observed in MSCs cultured under standard conditions, with MSCs cultured as aggregates under both physiological and atmospheric oxygen tensions demonstrating significantly lower levels of expression ($p<0.01$ in both instances) (Figure 5.5B). Although there was a slight tendency for expression to increase in aggregates cultured under atmospheric oxygen compared to physiological oxygen tensions, this observation was not significant at the statistical level.

In terms of members of the TGF family of cytokines, expression of activin-A and TGF-β1 were determined at the mRNA level in MSCs cultured under standard conditions and as aggregates cultured under physiological and atmospheric oxygen tensions. Results are shown in Figure 5.6. Expression profile observed for activin-A mirrored that previously observed for BMP7, CNTF and IL-6, with maximum levels of expression detected in MSCs cultured under standard conditions followed by a dramatic reduction in expression following culture under aggregate-inducing conditions (Figure 5.6A). This decrease in expression was statistically significant for MSCs cultured as aggregates under both physiological ($p<0.001$) and atmospheric ($p<0.001$) oxygen tensions, with transcripts being undetectable in aggregates cultured under the latter condition. The expression profile observed for TGF-β1 was very similar to that observed for activin-A, with maximum levels of expression also being detected in MSCs cultured under standard conditions followed by a dramatic reduction in expression following culture under aggregate-inducing conditions (Figure 5.6B). Similarly, this decrease in expression was statistically significant for MSCs cultured as aggregates under both physiological ($p<0.001$) and atmospheric ($p<0.01$) oxygen

tensions. However, whereas activin-A transcripts were undetectable in aggregates cultured under atmospheric oxygen tensions, TGF-β1 transcripts were actually detected at higher levels in aggregates cultured under atmospheric oxygen tensions compared to those cultured under physiological oxygen tensions, though this difference was very slight and not significant at the statistical level.

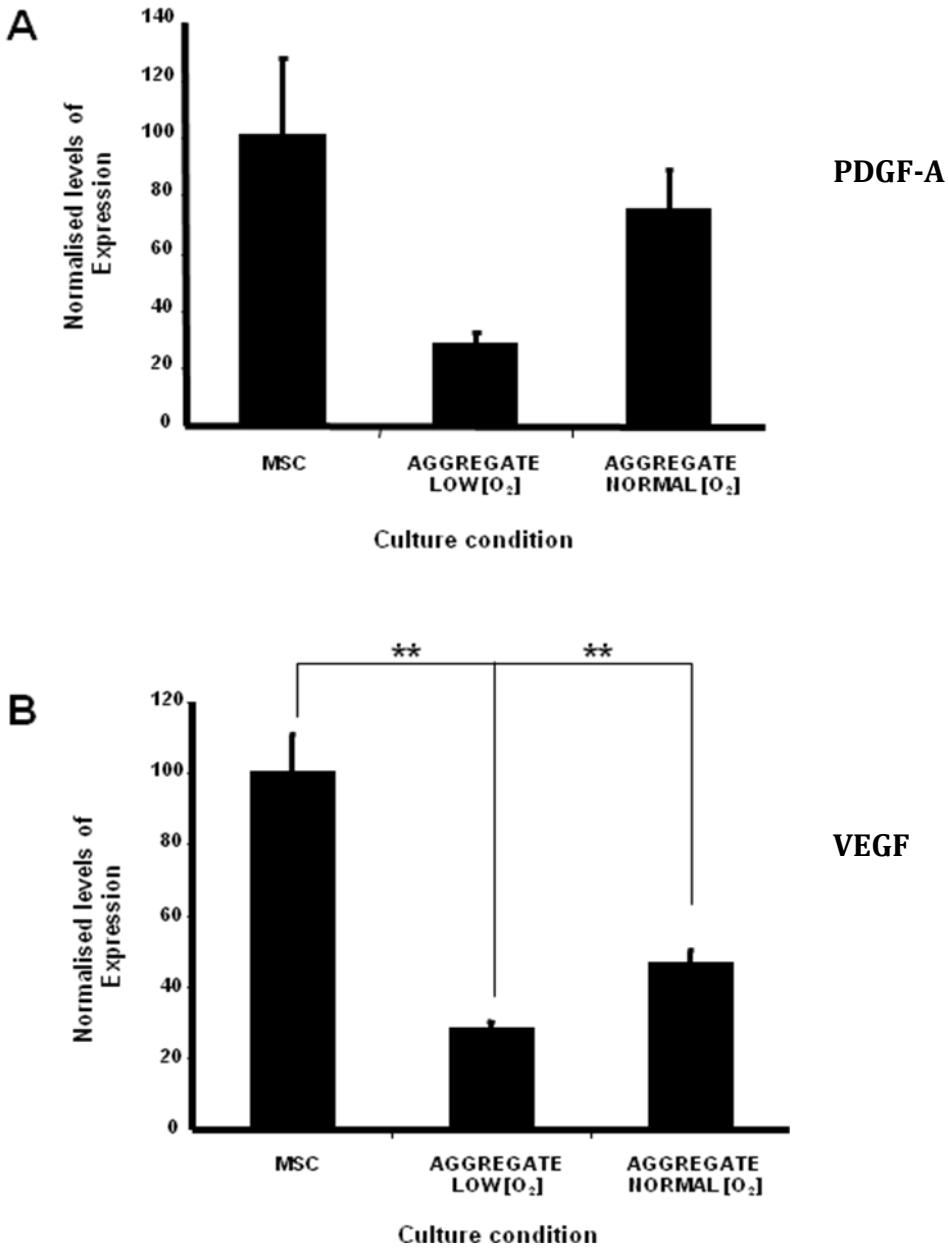


Figure 5.5. Expression of members of the PDGF family of cytokines at the mRNA level in MSC aggregates cultured under varying oxygen tensions and in MSCs cultured under standard conditions for 3 days. QRT-PCR analysis was performed to compare mRNA transcript levels of two members of this protein family – **A:** PDGF-A; **B:** VEGF – in MSCs cultured as aggregates under varying oxygen tensions and in MSCs cultured under standard conditions for three days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, ** $p<0.01$.

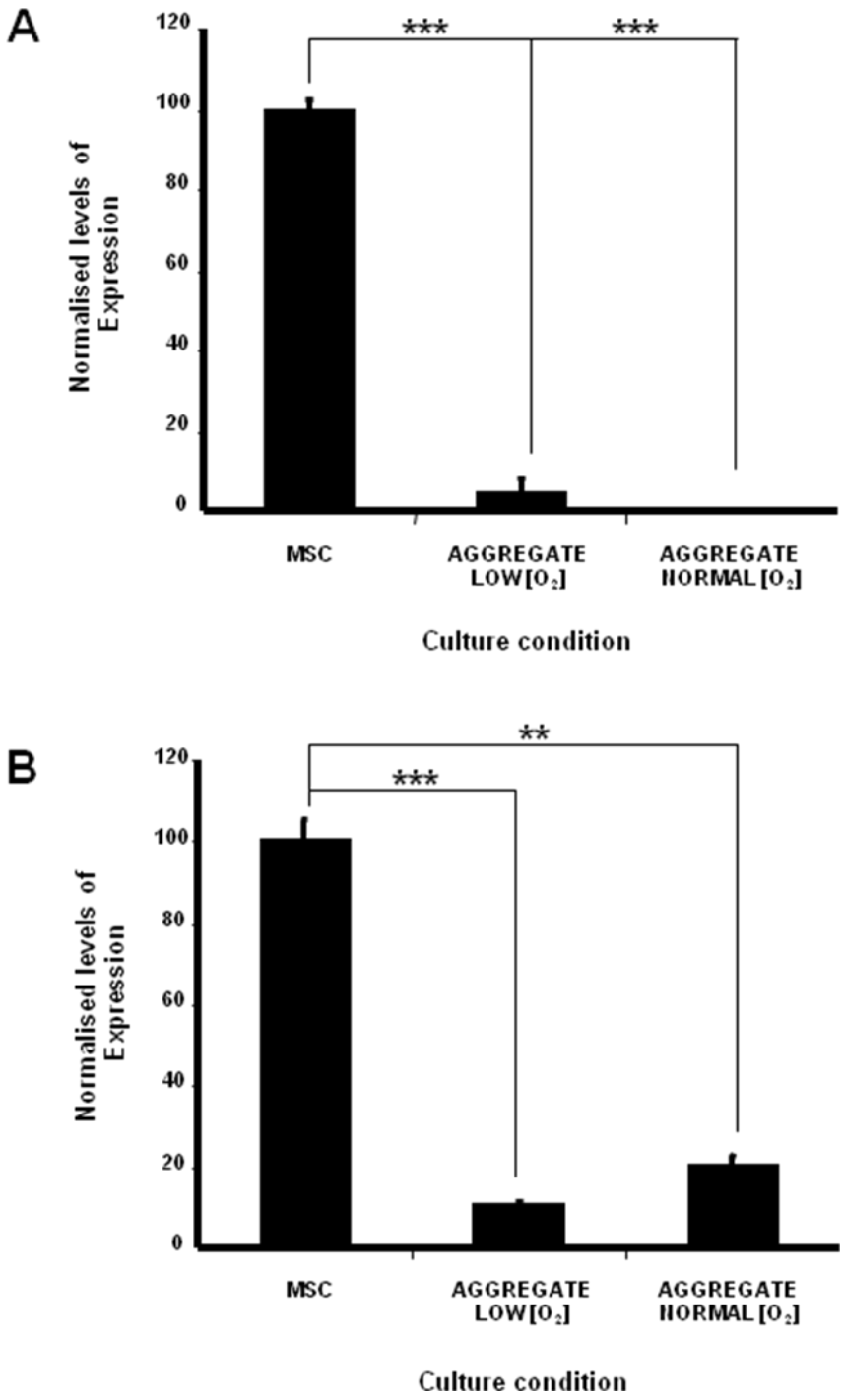


Figure 5.6. Expression of members of the TGF family of cytokines at the mRNA level in MSC aggregates cultured under varying oxygen tensions and in MSCs cultured under standard conditions for 3 days. QRT-PCR analysis was performed to compare mRNA transcript levels of two members of this protein family – **A:** Activin-A; **B:** TGF-β1 – in MSCs cultured as aggregates under varying oxygen tensions and in MSCs cultured under standard conditions for three days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** $p<0.001$, ** $p<0.01$.

The final group of cytokines examined were a miscellaneous selection consisting of cystatin C, FGF2, GDNF, IGF-1 and TNF. Expression of each of these factors was determined at the mRNA level in MSCs cultured under standard conditions and as aggregates cultured under physiological and atmospheric oxygen tensions. Results are shown in Figure 5.7. Expression profiles observed for cystatin C and TNF followed very similar trends and mirrored those previously observed for activin-A, BMP7, CNTF and IL6, with maximum levels of expression detected in MSCs cultured under standard conditions followed by a dramatic reduction in expression following culture under aggregate-inducing conditions (Figures 5.7A and 5.7E). For TNF, this decrease in expression was significant at the statistical level for MSCs cultured as aggregates under both physiological ($p<0.01$) and atmospheric ($p<0.01$) oxygen tensions, whereas for cystatin C, decreased expression was insignificant at the statistical level. When levels of expression were compared between aggregates cultured under physiological versus atmospheric oxygen tensions, aggregates cultured under physiological oxygen tension demonstrated significantly higher expression for both cystatin C ($p<0.05$) and TNF ($p<0.05$) compared to their atmospheric counterparts. The expression profile observed for FGF2 was extremely similar to that previously observed for EGF, with uniform levels of expression detected in MSCs cultured under standard conditions and as aggregates under physiological oxygen tension, with significantly lower levels of expression detected in aggregates cultured under atmospheric oxygen tension (Figure 5.7B). Decreased expression of FGF2 following culture as aggregates under atmospheric oxygen tension was moderately significant at the statistical level when compared to both other culture conditions ($p<0.01$ in both instances). Expression of GDNF demonstrated a degree of similarity to the expression profiles observed for NGF and LIF, with moderate levels of expression in MSCs cultured under standard conditions compared to significantly higher levels of expression in aggregates cultured under physiological oxygen tension (Figure 5.7C). This difference was significant at the statistical level ($p<0.01$). However, whereas expression of the cytokines NGF and LIF were also elevated in aggregates cultured under atmospheric oxygen tension compared to standard MSCs, the opposite trend was observed for GDNF, in that aggregates cultured under atmospheric oxygen tension demonstrated the lowest levels of expression. Reduced expression of GDNF in aggregates cultured under atmospheric oxygen tension was significant at the statistical level when compared to expression in standard MSCs ($p<0.01$) and aggregates cultured under physiological oxygen tensions

(p<0.001). Finally, the expression profile observed for IGF-1 demonstrated similarity to the expression profile previously observed for BMP4, with maximum levels of expression detected in MSCs cultured under standard conditions (Figure 5.7D). Levels of IGF-1 transcript were detected at significantly lower levels in MSCs cultured as aggregates under both physiological (p<0.01) and atmospheric (p<0.01) oxygen tensions. However, differences in expression between aggregates cultured under both oxygen tensions were only very slight and were not significant at the statistical level.

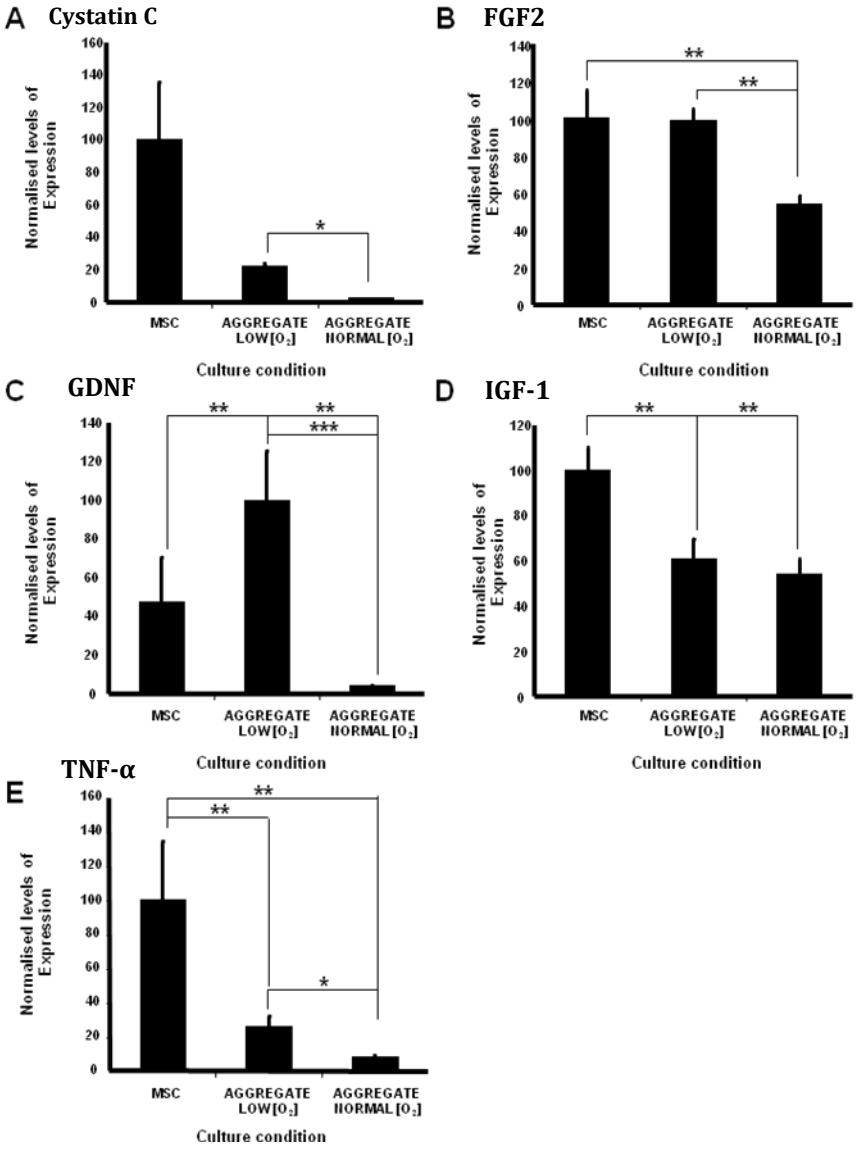


Figure 5.7. Expression of miscellaneous cytokines at the mRNA level in MSC aggregates cultured under varying oxygen tensions and in MSCs cultured under standard conditions for 3 days. QRT-PCR analysis was performed to compare mRNA transcript levels of five miscellaneous cytokines – **A:** cystatin C; **B:** FGF2; **C:** GDNF; **D:** IGF1; **E:** TNFα – in MSCs cultured as aggregates under varying oxygen tensions and in MSCs cultured under standard conditions for three days. All values were normalised to GAPDH to account for any differences in RNA input and/or reverse transcription efficiencies. Normalised levels of expression are shown as percentages of the maximum expresser. Each bar represents the mean (n=3)+SEM, *** p<0.001, ** p<0.01, * p<0.05.

5.3.2 Conditioned media is not amenable to proteomic analysis due to abundant levels of transferrin

Transcriptional profiling of MSC-derived aggregates and MSCs cultured under standard conditions, as performed in the first section of this Chapter, offers a convenient starting point for the detailed analysis of MSC-derived secretomes, particularly as prior knowledge of the biological activity of these secretomes allowed targeted analysis of soluble factors/cytokines specifically known to be mediators of such activity. However, this approach does have several disadvantages in that:

a) RNA levels are not always indicative of actual protein levels, a consequence of extensive RNA processing prior to protein synthesis.
b) Making the assumption that RNA levels are indicative of actual protein levels in this study, it is not possible to demonstrate that these proteins are secreted into the culture media.
c) Targeted analysis of soluble factors/cytokines introduces a bias into the study, such that resulting knowledge will be limited only to those factors examined i.e. the role of novel factors, or factors previously unassociated with this process, will remain undetected.

Therefore, to address these three important issues, an additional approach was adopted in this study in order to examine MSC-derived secretomes at the protein level in an unbiased manner. This involved application of proteomic-based techniques in order to identify proteins present in CM, making the assumption that a significant proportion of the proteins identified (which were not components of the basal culture media) were cell-derived secretory products as opposed to intracellular components released from lysed cells.

Conditioned media from MSCs cultured under standard conditions and as aggregates under physiological oxygen tensions were concentrated to a final volume of 200-400µl (approximate concentration 1.5µg/µl) by centrifugation in a 2 kDa molecular weight cut-off filter and equivalent amounts of protein for each sample loaded onto a 4-12% bis-tris 1D SDS-PAGE gel as described in Section 5.2.2. Gels were stained in three successive solutions of Coomassie Brilliant Blue R-250 as described to allow

visualisation of protein bands. Figure 5.8 shows a typical 1D-PAGE gel, with lanes 1-3 corresponding to molecular weight marker, standard MSC and MSC-derived aggregate respectively. The 1D-PAGE profiles observed for standard MSCs and MSC-derived aggregates were both dominated by a heavy single band located in the 59-109 kDa range of the gel (Figure 5.8). This band was not a consequence of gel overloading and the observation that the band was equal between the two samples strongly suggested that it was a common component of the media and not a result of cell secretion. Excision of this band followed by tryptic-based digestion and analysis by MALDI-TOF MS identified this protein as transferrin, the major constituent of N2 supplement, present in basal media. Such high abundance proteins can be problematic to proteomic analyses as they create a massive artificial dynamic range in the sample and may mask the detection of lower abundance proteins, which are often of much more interest.

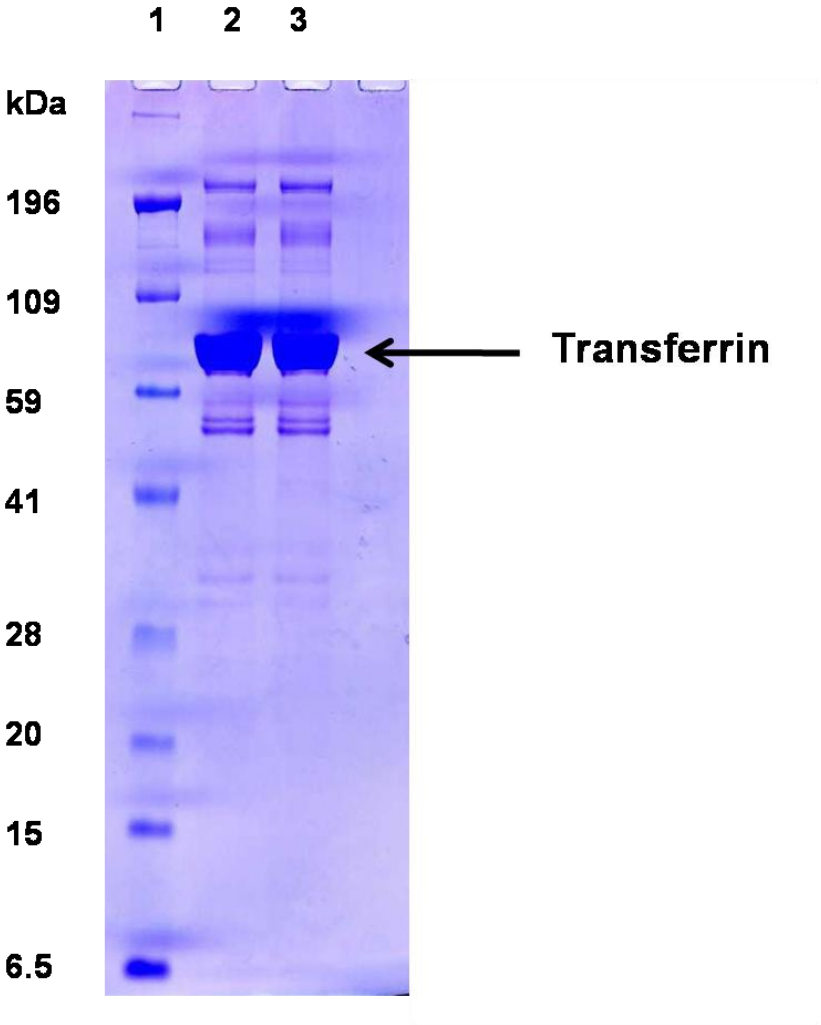


Figure 5.8. A typical 1D SDS-PAGE gel showing the protein profile of conditioned media derived from MSCs cultured under standard conditions (Lane 2) and as aggregates under physiological oxygen tension (Lane 3). Both protein profiles are dominated by a single, heavy band located in the 59-190 kDa range of the gel. This protein was subsequently identified as transferrin.

5.3.3 Substitution of N2 supplement with ITS supplement in basal media produces conditioned media that is more amenable to proteomic analysis due to reduced levels of transferrin

High abundance proteins such as transferrin present in CM pose many problems in terms of proteomic analysis, predominantly by obscuring detection of lower abundance proteins which usually are more likely to be relevant to the biological question addressed by the study. This is particularly true of this study, where the predicted molecular size of candidate growth factors/cytokines means they are likely to be masked by the large region covered by the heavy transferrin band on the gel. Although immunodepletion of transferrin is possible (several assays are commercially-available) this is not ideal as binding of candidate proteins to transferrin and, similarly, non-specific binding of candidate proteins to the antibody used in the immunodepletion assay, means some loss of target protein(s) is inevitable. Ideally, use of an alternative growth supplement with lower levels of transferrin would be better as it would allow exclusion of the transferrin band from further analysis without affecting a wide region of the gel. ITS supplement presents as a promising alternative to N2 supplement as it contains significant lower levels of transferrin compared to N2 (Table 5.1). ITS supplement contains approximately 20-fold lower levels of transferrin than N2 supplement, meaning that the dominant band observed in 1D-PAGE gels should be approximately 20-fold lower in density in gels using ITS-containing CM. This is shown in Figure 5.9. Other notable differences in composition are that ITS contains double the amount of insulin of N2 supplement and also lacks progesterone and putrescine.

	Transferrin	**Insulin**	**Selenium**	**Progesterone**	**Putrescine**
N2	100mg/L	5mg/L	5.2µg/L	6.3µg/L	16.11mg/L
ITS	5.5mg/L	10mg/L	5µg/L	–	–

Table 5.1. Composition of the commercially available cell culture supplements, N2 and ITS. ITS supplement contains approximately 20-fold lower levels of transferrin compared to N2 supplement which means that it will be more amenable to proteomic analysis as the transferrin band that dominates 1D SDS-PAGE gels should be of a density approximately 20-fold less. Other notable differences in

terms of composition of N2 and ITS supplements include that the ITS supplement contains 2-fold higher levels of insulin and lacks progesterone and putrescine.

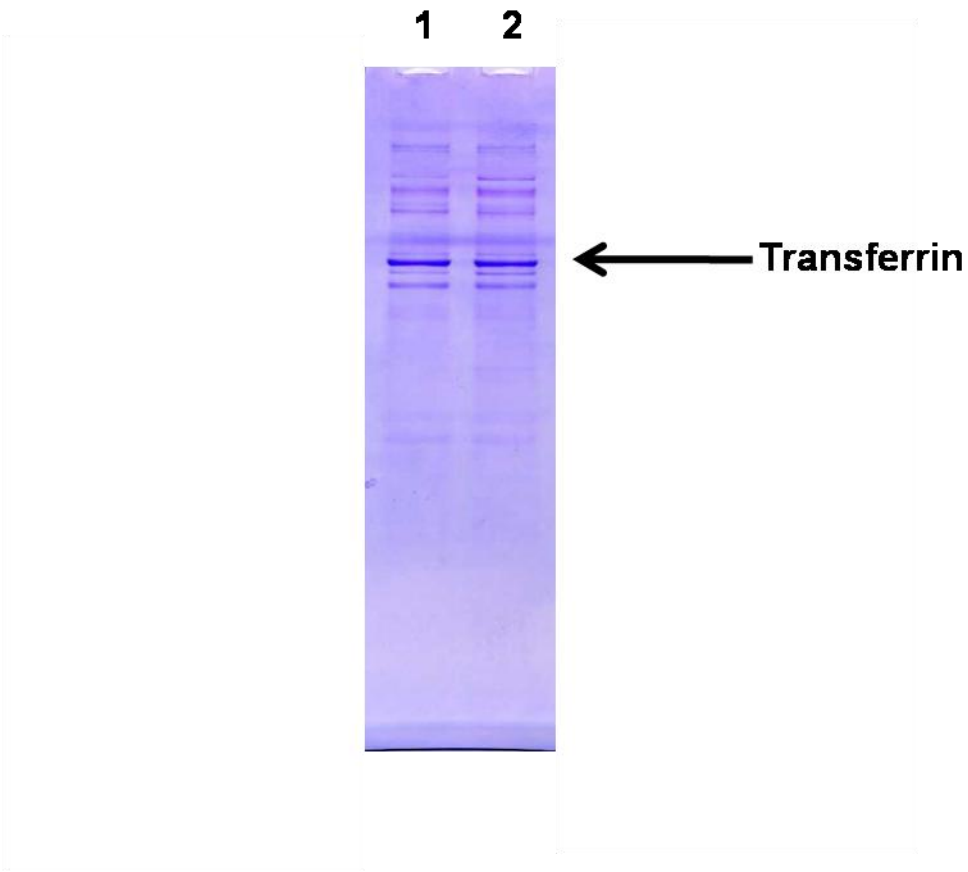


Figure 5.9. A typical 1D SDS-PAGE gel showing the protein profile of conditioned media derived from MSCs cultured under standard conditions (Lane 1) and as aggregates under physiological oxygen tension (Lane 2) where N2 supplement has been replaced by ITS supplement in both cases. Both protein profiles still possess a dominant single, heavy band located in the 59-190 kDa range of the gel corresponding to transferrin. However, the density of the transferrin band is significantly lower than that previously observed with N2 supplement meaning ITS-containing conditioned media will be more amenable to proteomic analysis.

5.3.4 Substitution of N2 supplement with ITS supplement in aggregate-derived CM abolishes the previously observed astrogenic effect in AHPCs, both in terms of morphological and marker expression analyses

Although substitution of N2 supplement with ITS supplement was advantageous in terms of producing CM with lower levels of transferrin, specific details of the biological activity of ITS-containing CM were not assessed at that point. As the composition of N2 and ITS supplements vary in terms of basic constituents and the concentration of common constituents (Table 5.1), it was essential to reassess biological activity of ITS-containing CM using methodologies analogous to those used in Chapter 4 for the initial characterisation of N2-containing CM, using the AHPC

system. Biological activity of ITS-containing CM was assessed in AHPCs following 7 days culture in ITS-containing CM and appropriate controls, both in terms of morphological phenotype and expression of mature neural markers. Neural marker expression was examined using flow cytometry due to its high sensitivity.

Briefly, MSCs were seeded out for aggregate formation and allowed to condition media for 3 days, as described in Section 4.2.2, differing only in terms of media supplementation – ITS instead of N2 – with all other exogenously-added media constituents remaining identical. After three days, medium was collected and prepared, as detailed previously, prior to application in the subsequent culture of AHPCs. As per the original CM assay (Section 4.2.3), AHPCs were seeded onto cellware coated with PLO and laminin at a density of 10,000 cells/cm^2 in their normal proliferative media and allowed 24 hours to settle (designated day –1). Following this 24 hour settling period (day 0), medium was removed and replaced with ITS-containing CM or appropriate control media (UC: unconditioned media; PC: proliferation control) and AHPCs cultured for a further 7 days. Medium was replenished on the 4th day. After 7 days, AHPCs were analysed for any obvious changes in cellular morphology (Figure 5.10).

As previously observed in the original CM assay, PC AHPCs were largely phase dark and very densely packed following 7 days culture, with evidence of extensive overgrowing (Figure 5.10A). Cell bodies were very rounded and showed no obvious evidence of process/cytoplasmic extension, indicating that cells in these cultures were largely undifferentiated. On the whole, these cultures appeared very unhealthy. In contrast, AHPCs cultured under UC and CM conditions appeared healthy and typically demonstrated phase-bright soma (Figures 5.10B and 5.10C). AHPCs cultured under UC conditions possessed numerous short processes, again in contrast to PC cultures, though these processes were short in length and did not demonstrate any obvious neuronal or glial characteristics (Figure 5.10B). This was consistent with previous observations from the original CM assay (Figure 4.4B). Interestingly, AHPC morphology following culture with ITS-containing CM differed significantly to that observed following culture with N2-containing CM in the original CM assay (Figures 4.4C and 5.10C respectively). AHPCs cultured with N2-containing CM generally displayed a mature neural morphology, with multipolar soma forming a stellate, star-

shaped morphology indicative of a glial morphology, in particular, an astrocytic morphology (Figure 4.4C). Culture of AHPCs with ITS-containing CM, however, did not induce the same morphological traits (Figure 5.10C). Although AHPCs cultured under these conditions also possessed multipolar soma as observed in the original assay, the processes themselves were very short and demonstrated little evidence of tertiary branching.

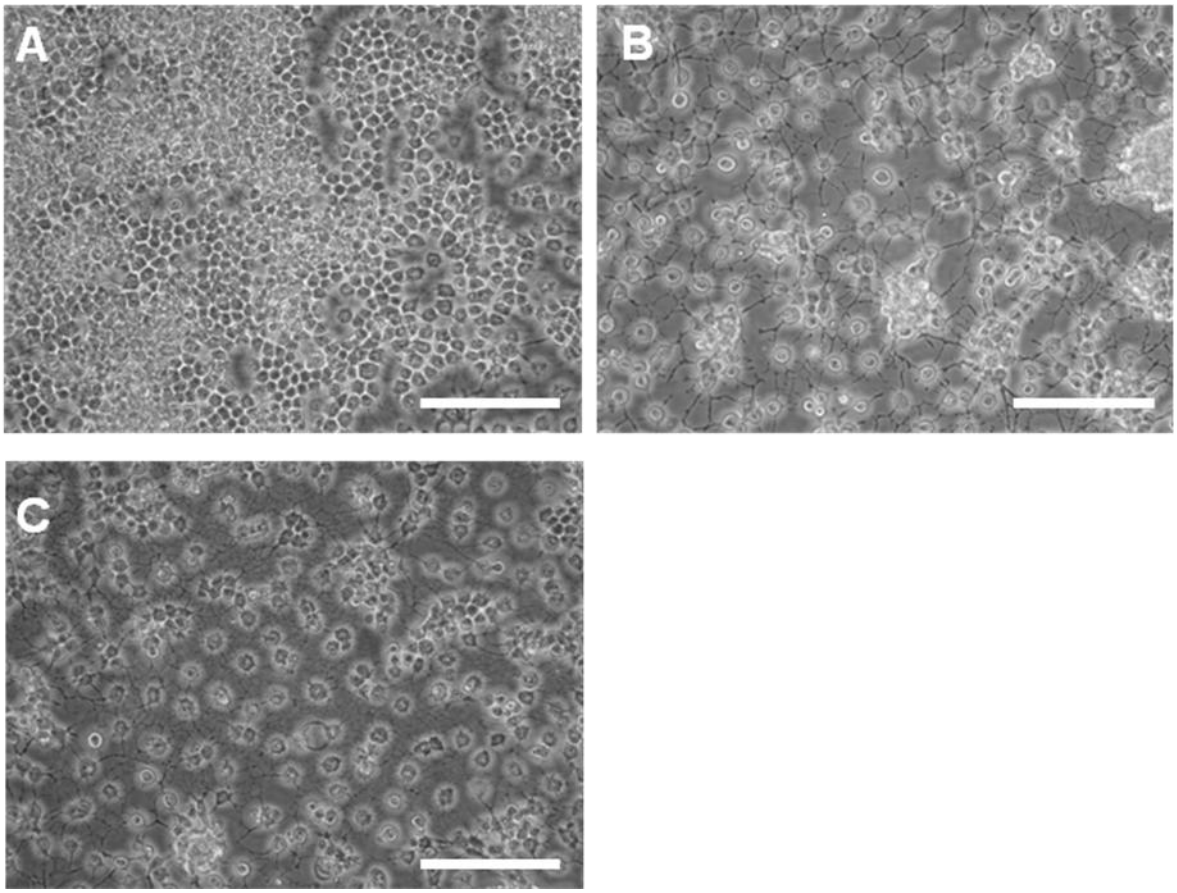


Figure 5.10. Morphology of AHPCs following 7 days culture with ITS-CM and appropriate controls. A: Following culture in normal proliferative media, AHPCs are largely phase dark, consisting of very rounded cell bodies and very few (if any) processes. After 7 days culture under these conditions, AHPCs appear undifferentiated and unhealthy. **B:** Following culture in unconditioned media (UC), AHPCs are largely phase bright and display numerous short processes. However, the morphology of these cells suggests they are largely undifferentiated. **C:** Following culture in ITS-CM, AHPCs demonstrate a similar morphology to that observed following culture in UC (**B**). These morphological data suggest that AHPCs cultured with ITS-CM remain largely undifferentiated following 7 days culture. Scale bar = 100μm.

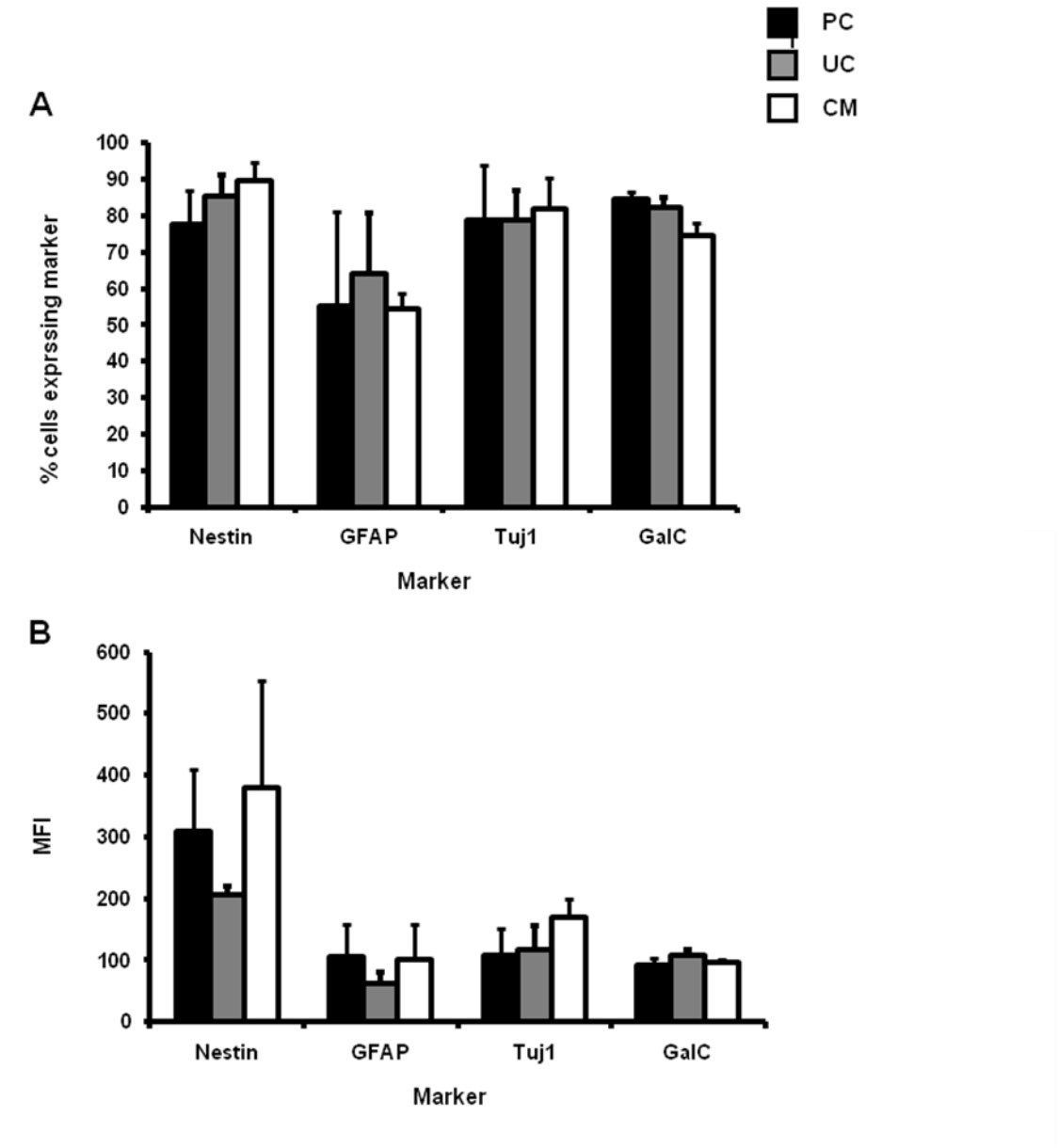


Figure 5.11. Flow cytometric analysis of marker expression in AHPCs treated with ITS-CM and appropriate controls. A: % cells expressing marker; **B:** mean fluorescence intensity of marker expression. In terms of the percentage of cell expressing each neural marker, there was very little difference between each of the samples. Similarly, in terms of intensity of marker expression, there was very little difference between each of the samples. These data suggest that the astrogenic effect observed following culture of AHPCs with N2-containing CM is abolished following substitution of N2 supplement with ITS supplement.

Although examination of cellular morphology is an easy and convenient starting point in terms of assessing cell phenotype, expression of a range of neural markers was examined at the flow cytometric level in order to provide a more accurate indication of AHPC behaviour following culture with ITS-containing CM and appropriate controls for 7 days. AHPCs were seeded out as described previously (Section 4.2.3) and cultured in ITS-containing CM and appropriate controls (PC and UC) for 7 days. Following 7 days culture, AHPCs were fixed in 2% PFA at 4^{o}C overnight and processed for flow cytometry against nestin, Tuj-1, GFAP and GalC, as per the original CM assay. Data in terms of the percentage of cells expressing marker and MFI of marker expression are shown graphically in Figure 5.11.

In terms of the percentage of cells expressing each neural marker, there was very little observable difference between each of the samples examined (Figure 5.11A). Any differences in expression that were apparent were very slight and not significant at the statistical level. Similarly, in terms of MFI of each neural marker, there was once again very little observable difference between each of the samples examined (Figure 5.11B). Any differences in MFI that were apparent were only very slight and not significant at the statistical level. Such trends in neural marker expression were generally consistent with those previously observed in the original CM assay, with the exception of MFI of GFAP expression. Soluble factor(s) present in ITS-containing CM did not induce the significant upregulation in intensity of GFAP expression observed following culture with N2-containing CM. Combined, such morphological observations and neural marker expression data suggested that the astrogenic effect of aggregate-derived soluble factor(s) was completely abolished following the substitution of N2 supplement with ITS supplement. Marker expression analyses also demonstrated that the astrogenic effect was not abolished at the expense of another effect taking its place. It must also be noted here that, although data has not been included in Figure 5.11, AHPCs cultured in equivalent N2-containing CM were included in each independent assay as positive controls to ensure that the experimental setup was functioning correctly at both the technical and biological level.

5.3.5 Reduced levels of transferrin in ITS supplement are tolerable during the conditioning process if the medium is also supplemented with progesterone and putrescine

Results in the previous section demonstrate that aggregate-derived CM exerts very different biological effects in AHPCs depending on whether N2 or ITS supplement is used as a basic component of the culture media. AHPCs cultured in N2-containing CM for 7 days underwent astrogenesis, an effect characterised by the acquirement of an astrocytic morphology concomitant with increased expression of both structural and functional markers indicative of astrocytic differentiation (Figures 4.4, 4.5, 4.7 and 4.9). However, following substitution of N2 supplement with ITS supplement, this astrogenic effect was completely abolished, with AHPCs demonstrating no astrocytic-like morphology and no upregulation in expression of GFAP in comparison to control cultures (Figures 5.10 and 5.11). Furthermore, expression of other neural markers (Tuj-1 and GalC) remained unchanged compared to control cultures, suggesting that the astrogenic effect had not been abolished at the expense of another neural effect taking its place.

Therefore, as the primary difference between the two experimental setups was in terms of the compositions of N2 and ITS supplements (as shown in Table 5.1), it was a reasonable assumption that the compositional differences between N2 and ITS supplements were the reason for the differences observed between the two experimental assays. In order to identify the critical component(s) in N2 supplement that were essential to generating the astrogenic effect in AHPCs, the CM assays were repeated using ITS supplement combined with various other additives (based on the published formulation for N2 supplement, as shown in Table 5.1). Briefly, MSCs were seeded out for aggregate formation as described previously (Section 4.2.1) in media supplemented with N2 supplement, ITS + transferrin + progesterone + putrescine (ITS+T+P+P), ITS + transferrin (ITS+T) and ITS + progesterone + putrescine (ITS+P+P). MSCs were also seeded out for aggregate formation in N2-containing media as a positive control. Following this, as per the original CM assay (Section 4.2.3), AHPCs were seeded onto cellware coated with PLO and laminin at a density of 10,000 cells/cm^2 in their normal proliferative media and allowed 24 hours to settle (designated day –1). Following this 24 hour settling period (day 0), media was removed and replaced with the various CMs described above and AHPCs cultured for

a further 7 days. Medium was replenished on the 4th day. After 7 days, AHPCs were processed for flow cytometric analysis and expression of the astrocytic marker GFAP examined in terms of percentage of cells expressing GFAP and MFI of expression (Figure 5.12).

Consistent with previous observations, expression of GFAP in terms of the percentage of cells positive for expression was very uniform and there was very little observable difference between each of the samples (Figure 5.12A). Any differences in expression that were apparent were extremely slight and were not significant at the statistical level. However, in the original CM assay, the astrogenic effect was characterised by a highly significant increase in MFI of GFAP expression (Figure 4.7). A similar trend was observed in all of the samples examined in the current assay in that AHPCs cultured with the various CMs for 7 days demonstrated dramatic increases in intensity of GFAP expression compared to PC control cultures (Figure 5.12B). AHPCs cultured in ITS+T+P+P – an almost identical formulation to N2 supplement – demonstrated almost identical levels of GFAP expression compared to AHPCs cultured in N2-containing CM, following 7 days culture. Similarly, AHPCs cultured in ITS+T and ITS+P+P also demonstrated a significant upregulation in intensity of GFAP expression, though the absolute values were slightly lower than those observed following culture in N2 or ITS+T+P+P. MFI of GFAP expression was lowest in AHPCs cultured in ITS+P+P; however, the difference in intensity of expression between AHPCs cultured in ITS+P+P compared to equivalent counterparts cultured in N2 was not significant at the statistical level. Therefore, the use of ITS+P+P CM provides an ideal culture system in which to analyse the MSC aggregate-derived secretome at the proteomic level because a) it contains low levels of transferrin compared to N2-containing CM and b) it still produces the astrogenic effect in AHPCs. Furthermore, the lack of biological activity of the original ITS-containing CM means that this can be used as a suitable control against which to compare ITS+P+P-CM.

5.3.6 Biological activity of ITS+P+P-CM was verified in terms of induction of GFAP expression in AHPCs prior to proteomic analysis

In order to examine the secretome of MSC-derived aggregates at the proteomic level and identify the astrocytic-inducing bioactive factor(s) secreted by aggregates, MSCs were seeded out for aggregate formation, as previously described (Section 4.2.1), in

media supplemented with ITS only (biologically inactive control) and ITS+P+P (biologically active sample of interest). Three biologically-independent paired samples were prepared and analysed using a shotgun proteomics approach by the North East Proteome Analysis Facility (NEPAF), based on LC-MS/MS, to allow scrutinisation of

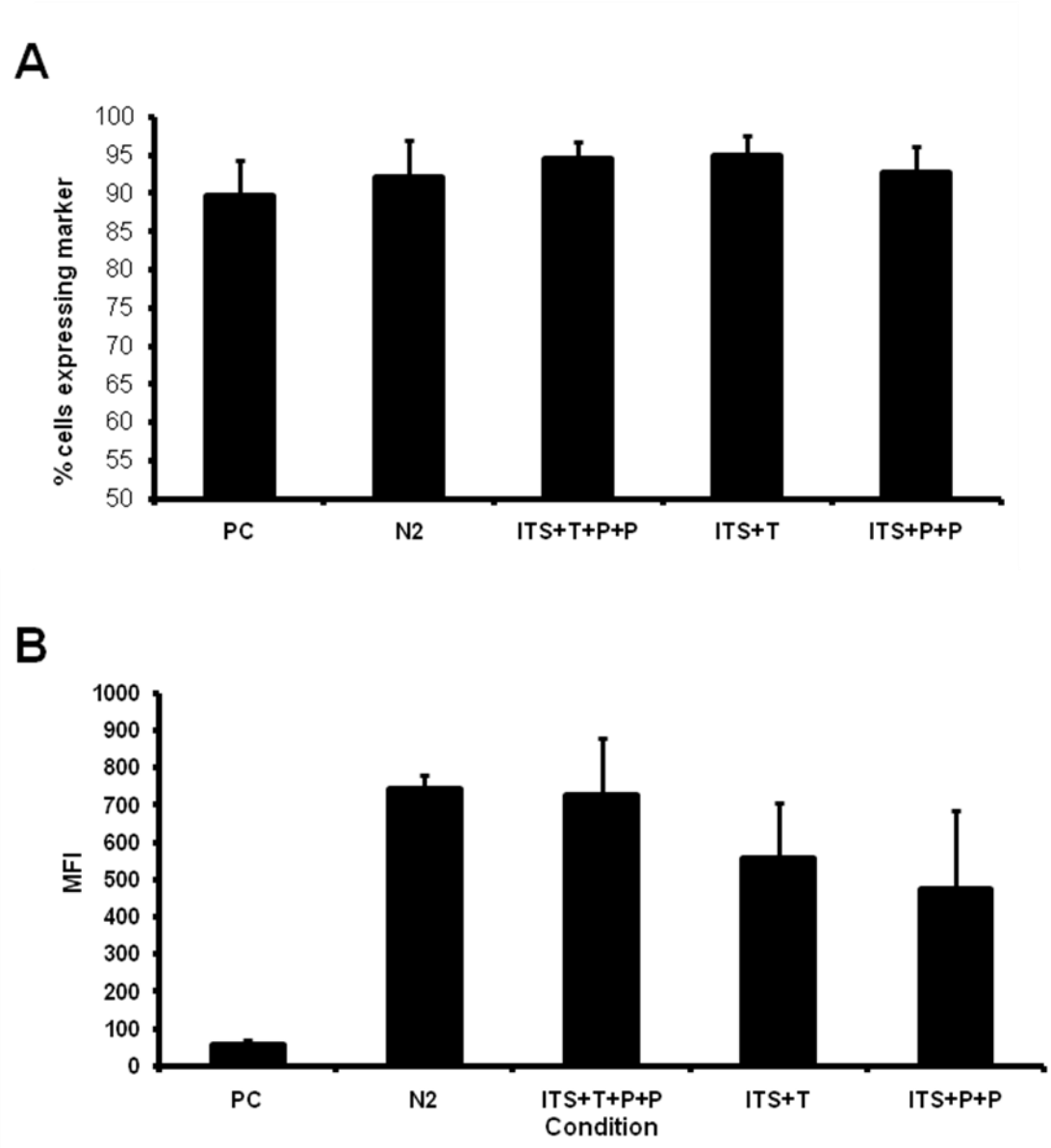


Figure 5.12. Flow cytometric analysis of GFAP expression in AHPCs cultured in conditioned media supplemented with ITS in combination with various additives, in keeping with the published formulation for N2 supplement. AHPCs were seeded out and cultured in conditioned media supplemented with ITS in combination with various additives, including transferrin/progesterone/putrescine, transferrin alone and progesterone/putrescine, for 7 days and processed for flow cytometry as previously described. Expression of GFAP was examined in terms of the percentage of cells expressing GFAP (**A**) and MFI of GFAP expression (**B**). Supplementation with progesterone and putrescine was sufficient to rescue the astrogenic effect previously observed in Section 4.7. Each bar represents the mean (n=3)+SEM.

the entire protein complement of MSC aggregate-derived CM produced under both conditions. However, before the paired samples were analysed by NEPAF, the biological activity of the samples were assayed by seeding AHPCs out followed by subsequent culture in the different CMs for 7 days, as per the previous CM assays (Section 4.2.3), followed by analysis of GFAP expression using immunocytochemical methods. Results are shown in Figure 5.13. Consistent with previous observations from this Chapter, AHPCs cultured in ITS-CM were biologically inactive (demonstrating only very weak, diffuse staining for GFAP; Figures 5.13D, 5.13E and 5.13F) whereas AHPCs cultured in ITS+P+P-CM were biologically active (demonstrating very strong, intense staining for GFAP; Figures 5.13A, 5.13B and 5.13C).

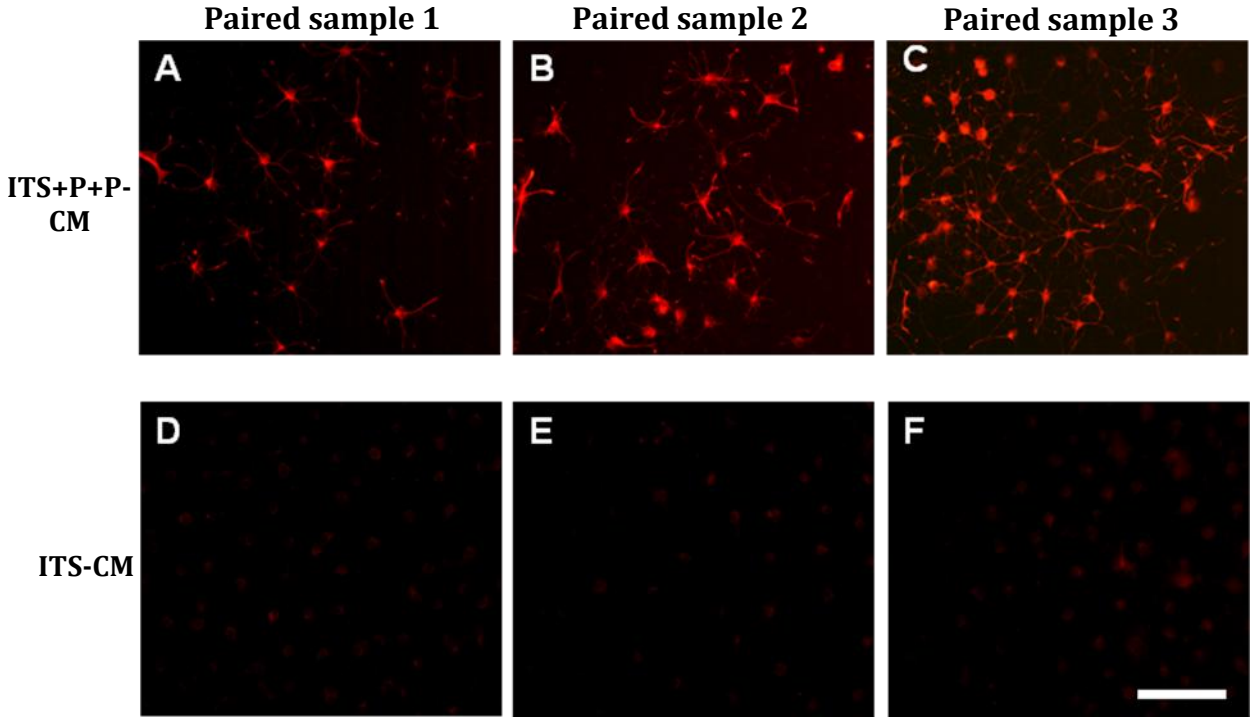


Figure 5.13. Validation of the biological activity of ITS-CM and ITS+P+P-CM paired samples prior to high-throughput shotgun proteomic analysis, performed by NEPAF. AHPCs were seeded out as described previously and cultured in paired ITS-CM (**D, E** and **F**) and ITS+P+P-CM (**A, B** and **C**) samples for 7 days, such that **A/D, B/E** and **C/F** are corresponding paired samples. Following 7 days culture, expression of GFAP was examined using immunocytochemical methods. AHPCs cultured in ITS+P+P-CM demonstrated significant upregulation of GFAP, whereas their counterparts cultured in ITS-CM did not demonstrate any upregulation of GFAP. This is in keeping with previous observations. Scale bar = 100μm.

5.3.7 The biologically-independent paired (ITS/ITS+P+P) CM samples were concentrated and fractionated by 1D SDS PAGE in-house prior to shotgun proteomic analysis by NEPAF

Following validation of the biological activity of the three biologically-independent paired (ITS/ITS+P+P) CM samples, in-house verification of sample integrity was achieved by concentration of each CM sample using a Vivaspin molecular weight cut-off filter followed by fractionation using 1D SDS-PAGE, as described in Section 5.2.3. Each sample was equivalently loaded onto a 1D SDS-PAGE gel, followed by staining with three successive Coomassie R-250 solutions. The gel was subsequently stored in destaining solution prior to shotgun proteomic analysis by NEPAF. Fractionation of complex protein mixtures such as CM is crucial prior to mass spectrometric analysis in order to enhance the dynamic range of analysis and improve identification of low abundance proteins, which are likely to be representative of candidate factors in this study. Figure 5.14 shows the 1D SDS-PAGE gel of the paired samples, along with a table describing protein concentration data.

Loading of the gel was organised to allow distinction between the three biologically-independent paired samples, such that samples 1, 3 and 5 corresponded to biologically-inactive ITS control cultures and samples 2, 4 and 6 corresponded to their equivalent biologically-active ITS+P+P cultures. There appeared to be a high degree of reproducibility between equivalent samples e.g. samples 1 vs. 3 vs. 5 and samples 2 vs. 4 vs. 6 and, even at the paired level, there were no obvious distinctions between the samples e.g. samples 1 vs. 2, 3 vs. 4 and 5 vs. 6. Putative transferrin bands were much lower in the six samples than previously observed in N2-containing media, which would ultimately aid in unmasking some of the lower abundance factors in the complex protein mixture.

5.3.8 Identification of proteins in CM using a shotgun proteomics approach based on LC-MS/MS, performed by NEPAF

The three biologically-independent paired CM samples fractionated on the 1D SDS-PAGE gel above were passed onto NEPAF for high-throughput shotgun proteomics analysis, based on LC-MS/MS. Detailed methods are described in the Materials and Methods section. Briefly, this involved an SDS-PAGE prefractionation prior to slicing each sample lane laterally into 8 equal sections. The gel region containing transferrin

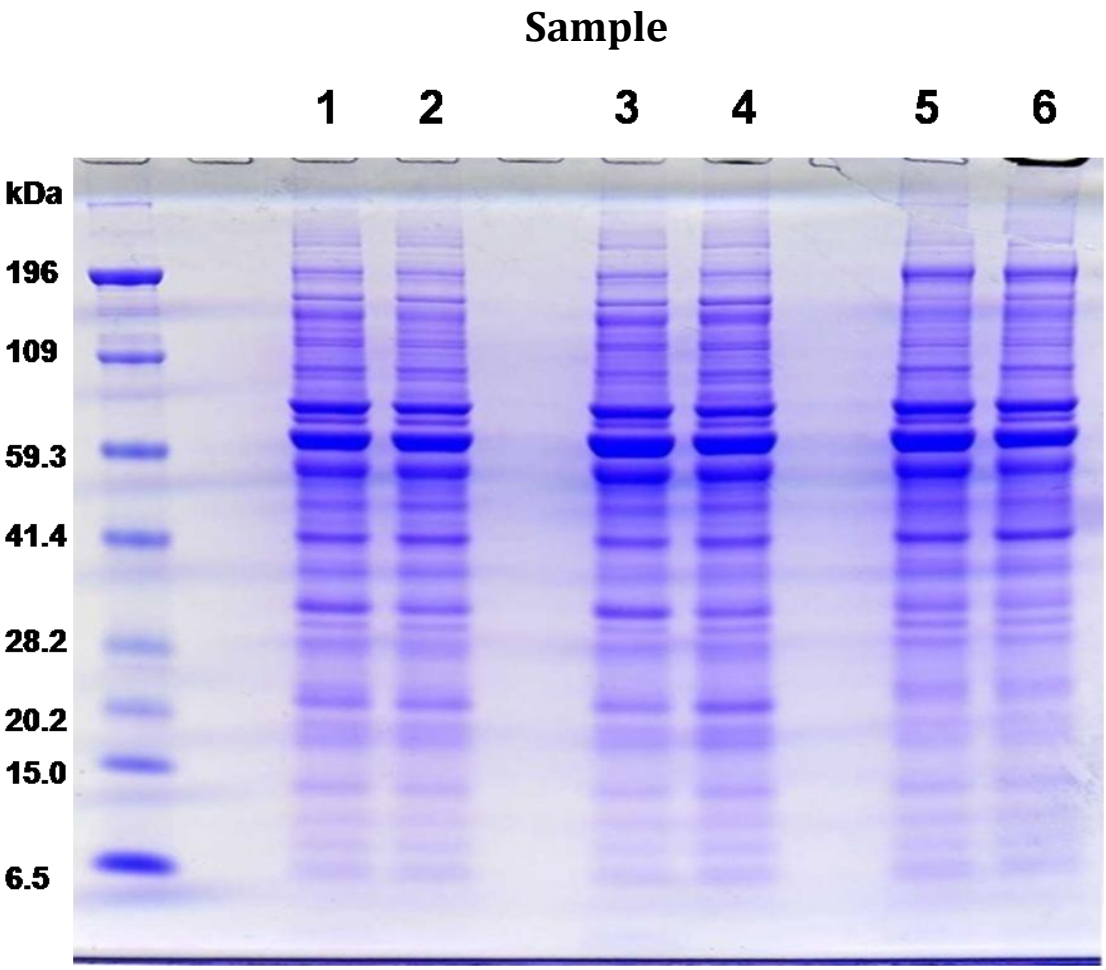
and BSA bands (~60-80 kDa) were excluded from the analysis (with the presence of BSA being attributable to carry-over from serum-containing media). Each gel slice was prepared for protein digestion with trypsin and the subsequently extracted peptides from each gel slice were separately resolved using reversed phase nano-HPLC in-line with the MS. Eluted peptides were directly analysed by nanospray MS/MS (LTQ Orbitrap XL, ThermoScientific). Tandem MS allowed identification of the individual peptide fragments by searching MS/MS spectra against the international X!Tandem search engine.

The raw data from these analyses generated an unmanageable number of hits – 2429 in total. However, a systematic approach was adopted in order to generate a more manageable list of putative candidate factors and this approach is shown diagrammatically in Figure 5.15. The original dataset contained considerable redundancy due to multiple peptides from the same protein being identified. Similarly, known media-based protein contaminants such as transferrin, albumin and keratin were also identified. Removal of redundancy/contaminants reduced the number of hits significantly, from 2429 to 621, though this was still an unmanageable dataset. Application of stringent selection criteria was then applied to reduce this dataset further. The criteria were as follows:

a) Protein must be identified from at least two unique peptides
b) Protein must be either exclusively detected or upregulated 2-fold or more in ITS+P+P-CM compared to ITS-CM, in at least 2 out of 3 pairwise samples
c) Log(e) score (significance score) must be $\leq$-3

Application of the above criteria narrowed down the number of hits from an unmanageable 621 to a more manageable 38 (Table 5.2). Detailed information regarding the reported function(s) of these 38 proteins offered further selection in terms of putative candidate factors responsible for exerting the astrogenic effect in AHPCs. A number of identified proteins are reported to be secreted soluble factors with roles in neural developmental processes, including gamma enolase (neuron specific enolase), glia-derived nexin precursor, calumenin precursor and cortistatin. Similarly, some intracellular proteins have been reported to undergo active secretion

from cells, including translationally controlled tumor protein (TCTP; or lens epithelial protein) and thioredoxin-dependent peroxide reductase **(Söderberg** *et al.*, 2000; Amzallag *et al.*, 2004).



Sample number	Sample type	Approx. volume of concentrate (µl)	Protein conc. (µg/µl)
1	ITS	250	1.50
2	ITS+P+P	250	1.73
3	ITS	400	1.22
4	ITS+P+P	400	1.11
5	ITS	300	1.43
6	ITS+P+P	300	1.29

Figure 5.14. 1D SDS-PAGE gel showing the protein profile of conditioned media derived from MSCs cultured as aggregates in ITS- (samples 1, 3 and 5) and ITS+P+P- (samples 2, 4 and 6) containing CM. Conditioned media samples are organised such that samples 1 and 2, 3 and 4, 5 and 6 are equivalent pairs seeded from the same batch of cells. Additionally, each pair are biologically independent of one another.

Also of considerable interest is the large number of extracellular matrix proteins/proteins involved in extracellular matrix remodelling identified, including collagens, fibronectins and metalloproteinase inhibitor 2 (TIMP-2). Such molecules are reported to play crucial roles in neural developmental processes (Jaworski and Perez-Martinez, 2006; Li *et al.*, 2009). The highly complex nature of neural differentiation means that the astrogenic effect observed in AHPCs is likely to result from interplay between several of these candidate factors as opposed to the effect(s) of a single factor.

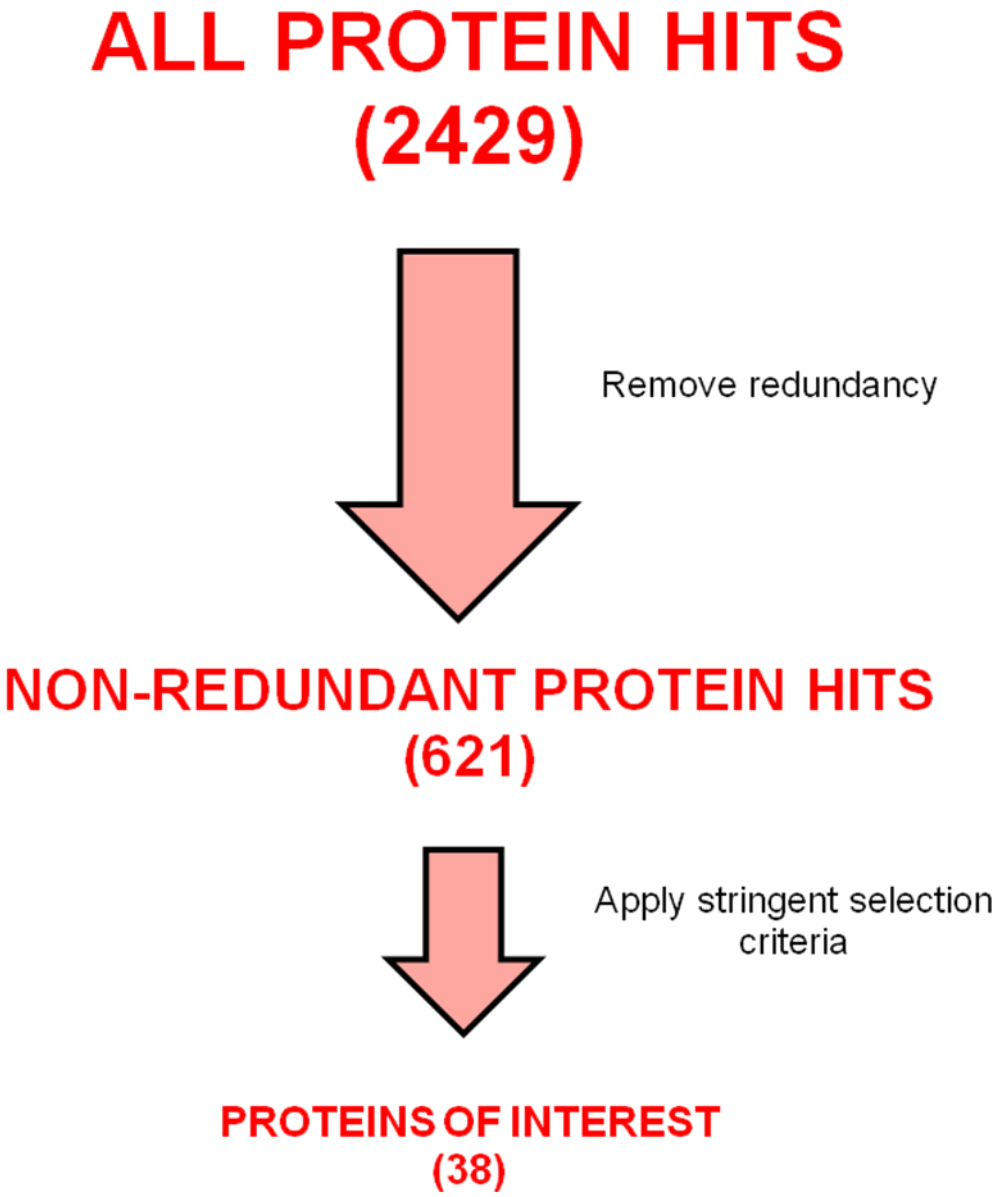


Figure 5.15. Diagrammatic representation of the data processing strategy adopted. The original dataset contained considerable redundancy due to multiple peptides from the same proteins being identified. These was removed, along with any hits corresponding to known contaminants e.g. transferrin, albumin and keratin. Stringent selection criteria was applied to narrow down the dataset further still. Firstly, the protein had to be identified from (or by) at least 2 peptides. Then identified proteins had to be either a) detected exclusively in active sample or b) upregulated by at least 2-fold in at least 2 out of 3 pairwise active vs. control samples. A total number of 38 candidate factors adhered to this selection criteria.

Table 5.2

Proteins exclusively detected or up-regulated by greater than 2-fold in at least 2 out of 3 pairwise active vs. control CM samples

Information is provided in terms of ENSEMBL identifier; protein description; result reproducibility; gel slice in which protein was identified; number of peptides contributing to each identification; log(e) significance score; normalised abundance ratio in active vs. control CM samples, based on total number of peptides normalised for dataset size/protein molecular weight; estimated Mr based on gel slice in which protein was identified; calculated Mr based on database interrogation; and function of protein, based on database interrogation and literature searches.

Ensembl database identifier	Protein description	Sample pairs in which elev/exclus in active sample	Gel slice	No. peptides	Score log(e)	Abundance ratio [active]/[cont]	Mr gel (kDa)	Mr calc (kDa)	Function
ENSRNOP00000001095	Tubulin beta-5 chain	2	3	5	-22	ndc	45-60	49.6	Microtubule protein, cytoskeleton
		3	3	45	-199	2.21			
ENSRNOP00000001383	Translationally-controlled tumor protein (Lens epithelial protein)	2	5	7	-36.5	ndc	25-35	19.4	Putative roles in calcium binding and microtubule stabilization
		3	5	4	-22	3.70			Neural differentiation of ESCs (PMID: 1804727)
ENSRNOP00000004091	Calreticulin precursor (CRP55) (Calregulin)	2	3	12	-37.5	4.01	45-60	48	Calcium binding protein chaperone
		1	3	12	-79.6	2.24			
ENSRNOP00000004520	ARP3 actin-related protein 3 homolog	2	3	6	-23.8	ndc	45-60	47.5	Regulation of actin polymerization Filopodia formation, growth cone motility and neuritogenesis (PMCID: PMC2291425) Neuronal morphology and formation of neural networks (PMCID: PMC1852583) Development of dendritic spines and synapses (PMID: 18430734)
		1	3	2	-14.8	2.62			
		3	3	7	-35.4	2.15			
ENSRNOP00000005601	Gamma-enolase (Neuron-specific enolase)	2	3	8	-19.7	ndc	45-60	47.1	Glycolytic enzyme with possible function as a neurotrophic factor
		3	3	4	-23.8	ndc			
ENSRNOP00000005987	Clathrin heavy chain	2	1	6	-67.2	ndc	150+	191.4	Vesicle-mediated transport Neurotrophin signalling (PMID: 11007890) CNS regeneration and homeostasis (PMID: 17376807) Component of neuromuscular junctions (PMID: 15133132)
		1	1	2	-11.3	2.62			
ENSRNOP00000006122	Melanoma-associated antigen MG50 (Fragment)	1	1	3	-17.1	ndc	150+	128.6	Response to oxidative stress No specific information on MG50 in terms of neural activity. However, family members (i.e. members of the melanoma antigen family) are implicated in neural processes: Necdin – expressed predominantly in post-mitotic neurons, promotes neuronal differentiation and neuronal survival (PMID: 18753379)
		2	1	7	-46	2.33			
ENSRNOP00000006322	Tubulin alpha-1A chain (Tubulin alpha-1 chain)	1	3	9	-53.5	ndc	45-60	50	Microtubule protein, cytoskeleton Expressed specifically in neurons (PMID: 7731546) Neuronal morphology (PMID: 7837290)
		3	3	44	-168	2.03			
ENSRNOP00000006607	Actin-related protein 2	2	4	8	-47.5	ndc	35-60	44.7	Regulation of actin polymerization
		3	3	6	-44.8	5.55			Clathrin-mediated endocytosis (PMID: 15085951)
ENSRNOP00000007398	Myosin-9 (Myosin heavy chain, non-muscle IIa)	3	2	13	-114	2.62	75-150	226.3	Roles in cell-cell adhesion and cell morphogenesis during differentiation Light chain has been implicated in various processes such as astrocyte growth/migration, but no evidence to suggest that the heavy chain is involved (PMID: PMC2289411)
		1	2	4	-35.1	2.62			
ENSRNOP00000008356	Calumenin precursor (Crocalbin)	2	4	14	-99.9	ndc	35-60	37	Calcium binding which also binds crotoxin, a neurotoxic phospholipase A2 Secreted by astrocytes (PMID: 19469553)
		3	3	13	-81.8	3.00			
ENSRNOP00000010720	Cullin-associated NEDD8-dissociated protein 1	1	2	3	-18.2	ndc	75-150	136.3	Regulation of protein ubiquitination with role in cell differentiation
		2	2	6	-44.7	ndc			
ENSRNOP00000011773	Actin, alpha cardiac muscle 1	2	4	39	-150	ndc	35-60	42	Actin filament protein, cytoskeleton, role in cell motility Extensive component of neurites (PMID: PMC2110351) Neural differentiation processes such as neurite extension (PMID: 15041197)
		3	3	95	-229	2.28			

ENSRNOP00000012334	Collagen alpha-1(V) chain precursor	3	1	5	-29.8	2.77	150+	184.8	Fibril forming collagen, ECM
		1	1	3	-18.2	2.09			
ENSRNOP00000012796	Filamin-B (Beta-filamin)	2	1	23	-206	7.68	150+	277.7	Cytosketon organization, predominantly expressed in brain including SVZ and lateral ventricles Expressed in soma and leading process of migratory neurons (PMID: 12393796) Determines shape of neurons during migration and maintains polarity (PMID: 15509752)
		1	1	10	-85.4	2.62			
ENSRNOP00000014859	Protein Family: Importin subunit beta 3 karyopherin beta 3	2	2	2	-16.3	ndc	75-150	123.8	Intracellular protein transport Important in trafficking various transcription factors to and from the nucleus, which is important in regulating neural differentiation (PMID: 17159997) Overexpression of importin 5 results in neural differentiation (**Science 5** January 2007 315: 18-19) Increased expression observed with neural differentiation (PMID: 17276333)
		1	2	2	-8.6	2.62			
ENSRNOP00000015186	Thioredoxin-dependent peroxide reductase, mitochondrial precursor	2	5	2	-13.1	ndc	25-35	28.3	Response to oxidative stress Neuroprotective against oxidative stress (PMID: 18262354) Involved in photoreceptor protection PMCID: (PMC2277329)
		3	5	5	-33.1	4.62			
ENSRNOP00000015598	Ras-related protein Rab-11A	2	5	3	-28.7	ndc	25-35	24.4	Regulation of endosomal trafficking
		3	5	3	-14.7	ndc			
ENSRNOP00000015956	Aspartate aminotransferase, mitochondrial precursor	2	4	7	-53.7	ndc	35-60	47.3	Amino acid metabolism and putative role in cellular uptake of long chain fatty acids Decreased aspartate aminotransferase activity in various brain regions associated with Huntington's disease PMID: 2988404
		3	3	7	-53.5	2.15			
ENSRNOP00000018310	Thioredoxin domain-containing protein 5 precursor (ER protein ERp46)	2	3	3	-22.8	ndc	45-60	46.3	Cell redox homeostasis
		1	3	2	-13	2.62			
ENSRNOP00000019152	Fibronectin precursor	1	1	81	-487	ndc	150+	262.6	ECM component implicated in regulation of cell adhesion and neurite outgrowth Fibronectin role in guidance of neurite outgrowth is a possible mechanism of MSC-mediated neurogenesis PMID: 19422806
		2	1	148	-804	ndc			
ENSRNOP00000020919	Glia derived nexin precursor (protease nexin 1, serpin E2)	3	3	2	-14.5	ndc	35-60	44	Cell differentiation, nervous system development, promotes neurite extension Known neurotrophin released by astrocyes PMID: 9160969 PN-1 has an anti-apoptotic effect on cultured neuronal cells, and its expression is up-regulated in the brain by injury or ischemia The potential functions of PN-1 are proposed to be neurotrophic support and inhibition of cell death during brain injury or alteration of the blood-brain barrier For references see PMID: 16797167
		2	4	3	-22.6	ndc			
ENSRNOP00000022007	Col4a1 protein (Fragment)	2	1	5	-40.3	ndc	150+	158.1	Network forming collagen, ECM Mutations in Col4a associated with hereditary cerebrovascular disorders PMID: 19506372; 19477666
		3	1	2	-12.7	ndc			
		1	1	2	-11.5	2.62			
ENSRNOP00000022401	Talin1 protein	2	1	17	-173	ndc	150+	270.9	Actin binding, cytoskeleton
		1	1	5	-40.2	6.55			
ENSRNOP00000022983	Serpin H1 precursor (Collagen-binding protein)	1	3	33	-190	3.93	45-60	46.5	Collagen biosynthetic process
		2	3	19	-76.4	3.17			
ENSRNOP00000024106	Alpha-enolase (Non- neural enolase)	2	3	36	-142	ndc	45-60	47.1	Multiple functions including glycolysis and growth control
		1	3	10	-88.2	2.62			
ENSRNOP00000024721	Tropomyosin alpha-1 chain	2	4	19	-126	ndc	35-45	32.5	Actin filament-binding protein implicated in nervous system development Brain specific isoforms may play important roles in neurogenesis via interaction with actin filaments PMID: 2320008
		3	4	39	-197	3.61			

ENSRNOP00000025939	Glutathione S-transferase Mu 2	2	5	4	-28.5	ndc	25-35	25.7	Role in olfactory processing Potential role in detoxification of neurotoxins PMID: 8090363, 7566694
		3	5	4	-17.5	ndc			
ENSRNOP00000026075	Olfactomedin-like 3	2	3	3	-6.8	ndc	45-60	45.9	Unknown, found throughout mammalian brain Olfactomedin protein family implicated as important regulators of nervous system development For refs see PMID: 18037275
		1	3	3	-9.5	1.96			
ENSRNOP00000026719	Procollagen, type VI, alpha 3	2	1	75	-642	ndc	150+	186.6	Beaded filament-forming collagen, ECM
		1	1	37	-308	2.10			
ENSRNOP00000026794	Alpha glucosidase 2 alpha neutral subunit	2	2	5	-43.7	ndc	75-150	109.4	Carbohydrate metabolism
		1	2	4	-23.2	5.24			
ENSRNOP00000032845	Collagen alpha-1(II) chain precursor	2	1	4	-19.2	ndc	150+	141.8	Fibril-forming collagen, ECM
		3	1	4	-10.5	ndc			
		1	1	2	-8	2.62			
ENSRNOP00000038110	Cortistatin precursor	2	3	10	-55.6	ndc	45-60	117.2	Neuropeptide, neuronal depressant properties For review see PMID: 17342027
		1	3	5	-34.9	3.27			
ENSRNOP00000041462	60S ribosomal protein L12	3	5	2	-7.8	ndc	15-35	17.8	Protein synthesis
		2	6	5	-18.7	ndc			
ENSRNOP00000042384	Metalloproteinase inhibitor 2 precursor (TIMP-2)	2	5	4	-25.9	ndc	25-35	24.4	Positive regulation of neuron differentiation Possible component of the neurogenic signaling cascade PMID: 16805810
		3	5	7	-25.9	ndc			
ENSRNOP00000044473	Tropomyosin beta chain	2	6	4	-15	ndc	15-45	32.9	Actin filament-binding protein Brain specific isoforms may play important roles in neurogenesis via interaction with actin filaments PMID: 2320008
		3	4	48	-189	3.70			
ENSRNOP00000053546	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 precursor	2	2	11	-89.1	ndc	75-150	87	Collagen stabilisation via lysine hydroxylation
		3	2	8	-53.8	ndc			
ENSRNOP00000057490	Septin 6	2	3	2	-8.5	ndc	45-60	48.9	Microtubule cytoskeleton organization and biogenesis
		3	3	8	-67.7	ndc			

ndc = not detected in corresponding control; Pair 1: lane 1 (control) vs lane 2 (active); Pair 2: lane 3 (control) vs lane 4 (active); Pair 3: lane 5 (control) vs lane 6 (active). Log(e) is the score for each identification i.e. gives an indication of how significant the hit is (a bit like BLAST) – ignore hits below the -3.0 cut off in the initial analysis. A = active; C = control.

5.4 Discussion

Stem cells as trophic mediators of differentiation

Stem cells are typically characterised at the functional level in terms of their potential to differentiate into a range of mature cell types. However, it is now largely acknowledged that, in addition to this differentiative function, many stem cell types also function as trophic mediators of differentiation, whereby they express and secrete a range of soluble factors and/or cytokines that are able to influence the proliferation and/or differentiation of other cells within their niche. This phenomenon has been described for both embryonic and adult stem cell types and is particularly relevant regarding MSCs, where their trophic role in the regulation of haematopoiesis has been documented for many years (Haynesworth et al., 1996; Lu et al., 2003, Guo et al., 2006; Cabanes et al., 2007). Furthermore, earlier studies in this investigation have demonstrated that MSCs are able to regulate osteogenic and adipogenic differentiation *via* autocrine mechanisms, involving increased expression of osteogenic- and adipogenic-promoting factors concomitant with decreased expression of inhibitory factors (Chapter 3) and, similarly, are able to regulate neural differentiation *via* paracrine mechanisms (Chapter 4). The aim of this chapter is to examine the paracrine effects of MSC-derived aggregates on neural differentiation in further detail, by scrutinising the MSC aggregate-derived secretome using a combination of targeted transcriptional profiling and shotgun proteomics.

Analysis of the protein complement of MSC conditioned medium

Although several studies have demonstrated that MSCs are able to function as paracrine mediators of neural differentiation, using CM assays similar to those used in this study, significant advancements in this field have been hindered due to technical difficulties in analysing the highly complex MSC secretome. Rivera and colleagues adopted a random screening approach using recombinant proteins and blocking antibodies targeted against various soluble factors and cytokines, based on current knowledge in the literature, in order to ascertain whether the oligodendrogenic effect observed following culture with CM could be reproduced (Rivera *et al.*, 2006). No reproducibility was observed. Wislet-Gendebien and colleagues, on the other hand, used targeted expression profiling using qRT-PCR and Western blotting in order to ascertain whether any astrocytic-promoting factors were upregulated in MSCs (Wislet-

Gendebien *et al.,* 2004). Secretion of the biologically-active form of BMP4 was detected in CM and addition of blocking antibodies directed against BMP4 into CM resulted in abolishment of the astrocytic effect, albeit not completely, suggesting that BMP4 may at least partially be responsible for the astrogenic effect observed in this study.

Transcriptional profiling of astrocytic-promoting and astrocytic-inhibitory factors

In the present study, a combinatorial approach involving targeted transcriptional profiling (qRT-PCR) and shotgun proteomics was adopted in order to scrutinise the MSC aggregate-derived secretome in detail. The astrogenic effect observed in AHPCs and subject to characterisation in Chapter 4 was a direct consequence of the biological activity of aggregate-derived soluble factors/cytokines present in the CM, by virtue of the nature of CM assays. Therefore, as a convenient starting point, expression of a range of astrocytic-promoting and astrocytic-inhibitory soluble factors were examined at the mRNA level, using qRT-PCR, in MSCs cultured under standard conditions and as aggregates under both physiological and atmospheric oxygen tensions, where it is anticipated that aggregates will demonstrate an upregulation in expression of a number of positive regulatory factors concomitant with downregulation of a number of negative regulatory factors. Specific soluble factors were selected based on current knowledge within the literature. The influence of oxygen tension on the transcriptional activity of MSC-derived aggregates was also examined here in order to corroborate previous findings in this study regarding the enhanced viability and functionality of aggregates cultured under physiological compared to atmospheric oxygen tensions (Chapter 4). One possibility to account for these observed functional differences was that aggregates cultured under varying oxygen tensions demonstrated different behaviour at the secretory level, in keeping with previously published studies (Aleyassine, 1970; Koos, 1986; Dionne *et al.*, 1989; Ohta *et al.*, 1990; Raff *et al.*, 1990; Dionne *et al.*, 1993; Papas *et al.*, 1996; Zhao *et al.*, 2009). It must be remembered that mRNA levels are not always indicative of actual protein levels and give no information as to whether the protein is actually secreted; however, mRNA studies are advantageous in that they provide a rapid and easy method in which to screen expression of many factors. Furthermore, changes at the transcriptional level are useful in themselves as indicators of cellular activity.

MSCs cultured under standard conditions and as aggregates under physiological and atmospheric oxygen tensions demonstrated markedly different expression profiles of a number of soluble factors at the mRNA level. LIF, NGF and GDNF were all upregulated in MSCs cultured as aggregates under physiological oxygen tension compared to those cultured under standard conditions and these observations were highly significant at the statistical level. The astrogenic role of LIF has been highly documented in the literature (Guillemot, 2007). LIF belongs to the gp130 (IL-6) family of cytokines and plays a key role in astrogenesis owing to its intrinsic ability to activate the JAK-STAT signalling pathway, one of the highly conserved signalling pathways implicated in astrogenesis (Bonni *et al.*, 1997; Rajan and McKay, 1998; He *et al.*, 2005; Guillemot, 2007). AHPCs cultured in aggregate-derived CM previously demonstrated significant upregulation of STAT1 and STAT3, downstream effectors of JAK-STAT signalling, strongly suggesting that JAK-STAT signalling was one of the predominant mechanisms causing astrogenesis in the AHPC system (Chapter 4). Therefore, increased expression/secretion of LIF by MSC-derived aggregates would be in keeping with activation of JAK-STAT signalling in AHPCs.

GDNF has been highly implicated in neuronal differentiation processes (Roussa and Krieglstein, 2004; Sun *et al.*, 2004; Huang *et al.*, 2005; Pozas and Ibanez, 2005; Murata *et al.*, 2008; Wissel *et al.*, 2008). However, it has yet to be documented as an astrocytic-promoting factor. Although it cannot be ruled out that GDNF is directly contributing to the astrocytic effect observed in the AHPC system in this thesis, it is unlikely. A more realistic possibility is that GDNF is making an indirect contribution to astrogenesis by inducing expression of LIF. One study examining GDNF-mediated neuronal differentiation in MPC cells observed that GDNF induced expression of LIF in these cells *via* activation of the MEK/ERK pathway, resulting in autocrine regulation of neuronal differentiation in these cultures (Park *et al.*, 2004). Although the differentiative effect reported in this study is different to that in the present study (neuronal as opposed to astrocytic differentiation) it is a possibility that cell-type specific action of LIF prevented activation of JAK-STAT signalling in MPC cells (an observation reported in the study) explaining why neuronal differentiation was observed at the expense of astrocytic differentiation.

Similarly to GDNF, NGF has been highly implicated in neuronal differentiation processes but has yet to be documented as an astrocytic-promoting factor (Klesse *et al.*, 1999; Stipic *et al.*, 2001; Lee *et al.*, 2005). Of particular interest to this investigation, however, is the reported role of NGF in promotion of apoptosis in oligodendrocytes *via* activation of caspases 1, 2 and 3 (Casaccia-Bonnefil *et al.*, 1996; Chenghua *et al.*, 1999). Analysis of cell death in AHPCs cultured with CM revealed the activation of apoptotic pathways specifically in the $GalC^+$ subpopulation (using expression of cleaved caspase 3 as an indicator of apoptosis); therefore, the association of NGF with apoptosis in oligodendrocytes concomitant with its increased expression in MSC-derived aggregates suggests that NGF may be mediating apoptosis in the $GalC^+$ subpopulation. However, this hypothesis is somewhat controversial as NGF has paradoxically been reported to protect oligodendrocytes from TNF-α-mediated apoptosis (Takano *et al.*, 2000).

In addition to significant upregulation of LIF, NGF and GDNF as discussed above, MSCs cultured as aggregates under physiological oxygen tension also demonstrated significant downregulation of a large subset of factors examined, including BMP4, BMP7, CNTF, IL-6, VEGF, Activin-A, TGF-β1, IGF-1 and TNF-α. These observations were highly significant at the statistical level. Of this subset, it is the downregulation of both BMP4 and BMP7 that is perhaps the most interesting, as both molecules have been reported to be potent inducers of astrogenesis (Gross *et al.*, 1996; Nakashima *et al.*, 2001; Guillemot, 2007; Imura *et al.*, 2008). This astrogenic effect is reported to be particularly pronounced in older neuroprogenitor cultures (>E14) such as the AHPC system used in this study, wherein BMP4 promotes astrogenesis at the expense of neurogenesis/oligodendrogenesis (Gross *et al.*, 1996; Nakashima *et al.*, 2001). This is in keeping with neural development *in vivo*, where neurogenesis is primarily associated with the embryonic period and gliogenesis is primarily associated with the postnatal period (Jacobsen, 1991). Furthermore, Imura and colleagues observe BMP7 production by endothelial cells *in vivo*, promoting astrogenesis in postnatal cortical progenitors; this effect was inhibited following the introduction of BMP antagonists into this system (Imura *et al.*, 2008). However, several studies have reported that BMPs can also function as potent inducers of neuronal differentiation (Li *et al.*, 1998; Mabie *et al.*, 1999; Lopez-Coviella *et al.*, 2000). A particularly interesting study by Chang and colleagues adopted a conditioned media approach in order to

investigate the effect(s) of both neuron- and astrocyte-derived soluble factors on neural differentiation (Chang *et al.*, 2008). Their study demonstrated that BMPs secreted by neurons induced neuronal differentiation whereas BMPs secreted by astrocytes induced astrocytic differentiation of cortical stem cells. Although this effect was concentration-dependent, with neurons secreting lower levels of BMPs than astrocytes, Chang and colleagues also demonstrated a context-dependent effect in that BMP combined with other neurogenic molecules promoted neuronal differentiation regardless of concentration. This study highlights the fundamental principle that the biological activity of soluble factors is highly dependent upon context and is very often system-specific. Downregulation of BMP4 and BMP7 expression in MSC-derived aggregates suggests that their astrogenic role in the AHPC system is secondary to the combined effects of LIF, GDNF and NGF. Although Smad1 and Smad4 – effectors of BMP signalling – are upregulated in AHPCs following culture in CM (Chapter 4), this effect could be attributable to extensive crosstalk between signalling pathways as BMP signalling is reported to be strongly enhanced by JAK-STAT signalling (Guillemot, 2007). Similarly, significantly reduced expression of Ngn1 in AHPCs following culture in CM (Chapter 4) could permit low concentrations of BMPs to be biologically effective, as high levels of Ngn1 repress the astrogenic effect of BMPs (Sun *et al.*, 2001).

MSCs cultured as aggregates under physiological oxygen tension also demonstrated significant downregulation of IL6 and CNTF, both members of the conserved IL6 (gp130) family of cytokines, which are reported to be potent inducers of JAK-STAT signalling, one of the main mechanisms promoting astrogenesis (Johe *et al.*, 1996; Bonni *et al.*, 1997; Rajan and McKay, 1998). Astrocytes have even been reported to regulate their own differentiation *via* an autocrine mechanism involving the production and secretion of CNTF (Chang *et al.*, 2003). Similarly to BMPs, downregulation of IL6 and CNTF expression in MSC-derived aggregates suggests that their astrogenic role in the AHPC system is secondary to the combined effects of LIF, GDNF and NGF. The fundamental principle recurring in this discussion is that the biological effect of soluble factor activity is highly context-dependent and this is likely to be the case here. As discussed previously, neural development *in vivo* is characterised predominantly by neurogenesis in the embryonic stage of development followed by a switch to gliogenesis in the postnatal stage; the precise mechanism controlling this

transition is largely unknown, though it is considered to be highly regulated by both intrinsic and extrinsic signals (Jacobsen, 1991; Temple, 2001). Barnabe-Heider and colleagues report that IL6 (gp130)-related cytokines such as CNTF are expressed by neurons and may therefore form a negative feedback mechanism whereby newborn neurons promote the neurogenesis-astrogenesis transition (Barnabe-Heider *et al.*, 2005). Therefore, it could be that IL6 and CNTF are more important in the early transition from neurogenesis to astrogenesis rather than promotion of astrogenesis in later developmental stages. Activin-A and TGF-β1, both members of the TGF-β superfamily of cytokines, are downregulated in MSCs cultured as aggregates under physiological oxygen tensions. Such observations are consistent with what would be predicted based on current knowledge within the literature, as activin-A is implicated in the promotion of neurogenesis rather than astrogenesis and TGF-β1 is largely reported to function as a neuroprotective factor (Henrich-Noack *et al.*, 1996; Zhu *et al.*, 2001; Abdipranoto-Cowley *et al.*, 2009). Likewise, downregulation of IGF-1 could be anticipated based on current knowledge within the literature, as IGF-1 is reported to be a potent inducer of oligodendrogenesis in adult neuroprogenitor cells and O2A bitpotent precursor cells (Kuhl *et al.*, 2002; Kuhl *et al.*, 2003; Hsieh *et al.*, 2004; Rivera *et al.*, 2006). Mature oligodendrocytes have been reported to provide trophic support to cortical neurons, an effect mediated predominantly by the production and secretion of IGF-1 (Wilkins *et al.*, 2001). There is currently no evidence to suggest that IGF-1 plays any role in astrogenesis or survival of mature astrocytes. Finally, although one study reported that TNF-α had the potential to induce astrogenesis in neural stem cells in response to injury, it was observed that this astrogenic effect was not attributable to TNF-α alone; rather, TNF-α in combination with two other cytokines, IL-1β and IFN-γ (Ricci-Vitiani *et al.*, 2006).

Effect of oxygen tension on expression of astrocytic-promoting and astrocytic-inhibitory factors

As discussed above, MSCs cultured under standard conditions and as aggregates under physiological oxygen tension demonstrated significantly different transcriptional profiles for astrocytic-promoting and astrocytic-inhibitory soluble factors, which could account (at least in part) for the astrogenic effect of MSC aggregate-derived CM. Previous studies described herein also demonstrated that oxygen tension is a crucial parameter to consider when culturing MSC-derived aggregates, with aggregates

cultured under physiological oxygen tension demonstrating enhanced viability and secretory activity compared to aggregates cultured under atmospheric oxygen tension (Chapter 4). Therefore, in order to ascertain whether changes at the transcriptional level were responsible for the observed differences in secretory behaviour, transcriptional profiling was also performed for MSCs cultured as aggregates under atmospheric oxygen tension, allowing a direct comparison of the expression profiles of aggregates cultured under both physiological and atmospheric oxygen tensions. Although the overall expression profiles did not demonstrate any particularly obvious differences at the graphical level, statistical analysis revealed that aggregates cultured under atmospheric oxygen tensions expressed significantly lower levels of BMP4, LIF, NGF, EGF, neuregulin, cystatin C, FGF2, GDNF and TNF-α, compared to aggregates cultured under physiological oxygen tensions. Although a subset of these soluble factors are expressed at low levels in aggregates cultured under physiological oxygen tension, they may be sufficient to exert a biological effect; decreased levels in aggregates cultured under atmospheric oxygen tension, conversely, may fall below a certain threshold required for biological activity. Thus, differences in expression of soluble factors/cytokines concomitant with differences in proliferation/viability are likely to account for the observed differences in secretory behaviour of MSCs cultured as aggregates under physiological and atmospheric oxygen tensions. The secretory activity of a range of cell types is reported to be significantly influenced by oxygen tension (Aleyassine, 1970; Koos, 1986; Dionne *et al.*, 1989; Ohta *et al.*, 1990; Raff *et al.*, 1990; Dionne *et al.*, 1993; Papas *et al.*, 1996; Zhao *et al.*, 2009).

Limitations of targeted transcriptional profiling

Targeted transcriptional profiling of MSCs cultured under standard conditions and as aggregates under physiological/atmospheric oxygen tensions revealed unique expression profiles for several genes encoding potentially-relevant soluble factors/cytokines which could account, at least in part, for the observed astrogenic effect of MSC aggregate-derived CM. Although targeted transcriptional profiling was a convenient starting point in the first instance and generated an interesting dataset, this approach was disadvantageous due to its reductionist nature in that the dataset was restricted to the subset of genes selected for investigation based on current knowledge within the literature. A more holistic, unbiased approach would involve global analysis of the complete collection of mRNAs (representing the combined outputs of

transcription, RNA processing and RNA turnover) in MSCs cultured under the various conditions, in order to define the genes which are upregulated/downregulated in aggregates versus standard cultures. This is known as whole transcriptome analysis and could easily be achieved using a DNA microarray-based approach and the total RNA isolated and used for the targeted transcriptional profiling experiments detailed above.

However, despite the obvious advantages of DNA microarray-based technologies and whole transcriptome analysis, such an approach would be somewhat unsuitable for the purposes of the biological issue being addressed by this study i.e. identification of the biologically-active soluble factors/cytokines secreted by MSC-derived aggregates, as whole transcriptome analysis would yield a massive dataset ascertaining to every single gene whose expression profile is altered, the large majority of which are likely to be highly irrelevant to the biological issue being addressed. Indeed, altering the culture conditions of MSCs from standard (10% serum, atmospheric oxygen tension) to aggregate-inducing (N2 supplement, physiological oxygen tension) conditions is likely to promote a huge number of transcriptional changes, consequences of altered cell-cell interactions, varying media constituents and varying oxygen tension, all of which have been reported to significantly affect gene expression in various systems (Kanoh *et al.*, 1991; Weaver *et al.*, 1997; Wang *et al.*, 1998; Bacon *et al.*, 2003; Rinaudo *et al.*, 2003; Harvey *et al.*, 2004; Csete, 2005; Schofield and Radcliffe, 2005; Sullivan *et al.*, 2006). Additionally, although many of the identified genes will be highly characterised, some may be orphan genes to which no biological function has been assigned; such genes would therefore be missed due to a lack of current knowledge. A final consideration is the often poor correlation between mRNA levels and protein levels concomitant with extensive post-translational modification of proteins (Abbott, 1999). Direct analysis of protein complement rather than RNA is therefore more likely to be representative of the biological situation.

Shotgun proteomic analysis using LC-MS/MS to identify secreted factors

Accordingly, the MSC aggregate-derived secretome was further scrutinised in this study using a shotgun proteomic-based approach in order to indiscriminately examine the protein complement of MSC aggregate-derived CM. This approach is immediately advantageous over transcriptional profiling in that it is performed directly on the CM,

in contrast to transcriptional profiling, which is performed at the cellular level; as such, any data generated by the shotgun proteomic approach is more specific than transcriptional profiling, with all identified proteins immediately presenting as candidate factors. As proteomic-based technologies become more accessible and routinely available to the everyday cell biology laboratory, the number of studies adopting a proteomic-based approach to CM analysis are increasing, using methods ranging from basic 1D SDS-PAGE to more sophisticated mass spectrometric-based techniques (Prowse *et al.*, 2005; Volmer *et al.*, 2005; Chiellini *et al.*, 2008; Kang *et al.*, 2008; Sardana *et al.*, 2008; Bendall *et al.*, 2009). In particular, there appears to be significant interest in scrutinising the protein complement of CM produced by cancer cell lines, as novel proteins secreted from cancer cells present as potential biomarkers of the disease, which would be invaluable from a clinical/diagnostic perspective (Sardana *et al.*, 2007; Gunawardana *et al.*, 2009; Srisomsap *et al.*, 2010).

High transferrin levels in N2 supplement are problematic during proteomic analysis

In terms of the proteomic analysis of MSC aggregate-derived CM, an unanticipated problem manifested during an initial 1D SDS-PAGE screen using concentrated CM samples, with all profiles being dominated by a heavy, single band located in the 59-109 kDa range of the gel. Further analysis revealed that this band was due to high levels of transferrin, a key component of N2 supplement used in the serum-free culture of these cells. It has been well documented that high abundance serum proteins such as albumin, transferrin and IgG are highly problematic for proteomic analysis due to their masking effect over truly-secreted proteins, which are typically present at much lower concentrations (Lim and Bodnar, 2002; Xue *et al.*, 2008). However, contaminating media constituents were not anticipated to be a problem for proteomic analysis of the cultures used in this study as the protocol for aggregate formation stipulated that MSCs be seeded out in serum-free conditions in the presence of EGF and FGF (Hermann *et al.*, 2004; Hermann *et al.*, 2006). Similarly, MSCs were washed thoroughly in PBS prior to seeding out for aggregate formation in order to remove high abundance serum proteins as far as reasonably possible and without compromising cellular integrity. Although the observed levels of transferrin were lower than would be expected following culture under standard conditions (10% serum), they were still sufficient to mask lower abundance proteins of interest. There were two possible solutions:

a) Increase the number of sample preparation steps to include a transferrin-immunodepletion step; may result in loss of low abundant proteins of interest due to non-specific binding to transferrin/antibody.
b) Use an alternative cell culture supplement that contains a lower concentration of transferrin.

A transferrin-immunodepletion strategy would be highly advantageous in that it would not require any significant alterations to the current experimental setup; however, the latter strategy involving the use of an alternative cell culture supplement was adopted in this study due to the high possibility of losing candidate soluble factors/cytokines through non-specific protein/antibody interactions. ITS supplement, a serum-free media supplement commercially available from Sigma-Aldrich, presented as the most promising alternative to N2 supplement, containing approximately 20-fold lower levels of transferrin (Table 5.1). Indeed, the intensity of the transferrin band was significantly reduced following 1D SDS-PAGE analysis of ITS-containing CM, strongly suggesting that ITS-containing CM would be far more amenable to proteomic analysis than its N2-containing counterpart. However, a further unanticipated problem was encountered during the biological validation of ITS-containing CM, in that the well-characterised astrogenic effect of N2-containing media could not be reproduced in AHPCs, either at the morphological level or in terms of GFAP expression, following culture under analogous conditions. As the only difference between the two experimental setups was in terms of the basic compositions of N2 and ITS supplements, it was therefore reasonable to assume that a component unique to N2 and lacking in ITS supplement was essential during the conditioning process. In keeping with this, the original study describing the production and characterisation of N2 supplement, using the rat neuroblastoma B104 cell line as a model system, made two key observations (Bottenstein and Sato, 1979):

- The absence of any component of N2 supplement resulted in sub-optimal cell growth.
- Transferrin was the most stringently-required component of N2 supplement.

These two key observations suggested that either a) an absence of progesterone and putrescine or b) significantly reduced levels of transferrin, in ITS supplement were responsible for abolishing the astrogenic effect in AHPCs following 7 days culture with ITS-containing CM. Either scenario is as likely as the other, as all three molecules have been demonstrated to be essential for viable cell growth/proliferation in culture; progesterone and putrescine have been demonstrated to be essential for cell proliferation, whilst transferrin has been demonstrated to be essential for sustaining cell division in a range of cell types (Herbst and Snell, 1949; Ham, 1964; Pohjanpelto and Raina, 1972; Mather and Sato, 1977). An additional consideration is that ITS supplement may not be suitable for longer-term cultures due to its very simple composition; a review of the current literature reveals that ITS supplement is predominantly used to generate CM samples that are highly amenable to proteomic analysis and, in the majority of such studies, cells are cultured in ITS-containing medium for less than 24 hours (Lim *et al.*, 2002; Volmer *et al.*, 2005). Furthermore, the manufacturer's instructions even recommend the addition of 2% serum to ITS-containing medium for optimal cell culture conditions (www.sigma-aldrich.com).

In order to investigate the importance of the individual components of N2 supplement and their influence on the secretory activity of MSC-derived aggregates, equivalent cultures were set up such that MSCs were seeded out for aggregate formation in ITS-containing media in combination with various other additives, based on the published formulation of N2 supplement (Table 5.1) and allowed to condition the media accordingly. Interestingly, aggregates cultured in ITS-containing media supplemented with progesterone and putrescine (ITS+P+P) demonstrated paracrine behaviour analogous to that observed following culture in N2-containing media, such that AHPCs significantly upregulated GFAP expression following 7 days culture in the equivalent CM. Therefore, ITS+P+P-CM provides an ideal system in which to scrutinise the MSC aggregate-derived secretome, for a number of reasons:

a) Levels of transferrin in ITS+P+P-CM are significantly lower than levels observed in N2-containing CM.

b) Astrogenic effect observed in AHPCs following culture in N2-containing CM is easily reproduced by additional supplementation of ITS-containing media with progesterone and putrescine.
c) Progesterone and putrescine are unlikely to complicate proteomic analysis, as both are non-proteins (progesterone is a steroid hormone, putrescine is a diamine).
d) Lack of biological activity of the original ITS-CM sample means that this can be used as a suitable control against which to compare ITS+P+P-CM (provides a basis for distinction between non-specific aggregate-derived proteins and biologically-active proteins responsible for inducing an astrogenic effect in AHPCs)

38 putative candidate factors were identified by proteomic analysis

Following the optimisation of aggregate culture conditions to produce CM samples more amenable to proteomic analysis, the final aim of this study was to scrutinise the aggregate-derived secretome using a shotgun proteomic-based approach in order to indiscriminately examine the protein complement of aggregate-derived CM and identify the biologically active soluble factor(s)/cytokine(s) responsible for the observed astrogenic effect in AHPCs. The secretome of aggregates cultured in ITS+P+P media (biologically active) and that of ITS media (biologically inactive) were examined as three biologically-independent paired samples using LC-MS/MS. The raw data from these analyses generated an unmanageable number of hits – 2429 in total – though removal of redundancy/known media contaminants and application of stringent selection criteria (as detailed in Section 5.2.7) helped to narrow the dataset down to a more manageable number of hits corresponding to those proteins significantly upregulated/found exclusively in ITS+P+P-CM – 38 in total (Table 5.2). These proteins therefore present as candidate factors.

The 38 proteins demonstrated to be significantly upregulated/exclusively detected in biologically-active ITS+P+P-CM were organised into five broad categories accordingly to their reported function and/or subcellular localisation – extracellular matrix, metabolic proteins, cytoskeletal components, chaperones/heat shock proteins and soluble factors/classically secreted proteins – and the distribution of the 38

proteins within these categories is shown diagrammatically in Figure 5.16. These categories and the distribution of proteins within them demonstrate the considerable functional diversity of the MSC aggregate-derived secretome. Interestingly, cytoskeletal proteins such as actin, tubulin and filamin were the most abundantly-represented category, accounting for 32% of total protein. This, combined with the high representation of metabolic proteins (23%), suggested that over half of the total protein complement upregulated/exclusively detected in ITS+P+P-CM was intracellular in origin. Although such high levels of intracellular protein in CM may seem somewhat paradoxical, this phenomenon is not uncommon and is frequently observed in proteomic-based CM studies. For example, Moore and colleagues reported that 36% of proteins secreted by astrocytes in culture were intracellular in origin, whilst a similar study by Lafon-Cazal and colleagues reported a figure of 58% (Lafon-Cazal *et al.*, 2003; Moore *et al.*, 2009). Even more extreme figures are reported in CM studies using cancer cell lines; Kulasingam and Diamandis reported that only 34% of the secretome of a breast cancer cell line corresponded to *bona fide* extracellular/membranous proteins, whilst an even lower figure of 23% was reported by Sardana and colleagues following proteomic analysis of a prostate cell line (Kulasingam and Diamandis, 2007; Sardana *et al.*, 2007).

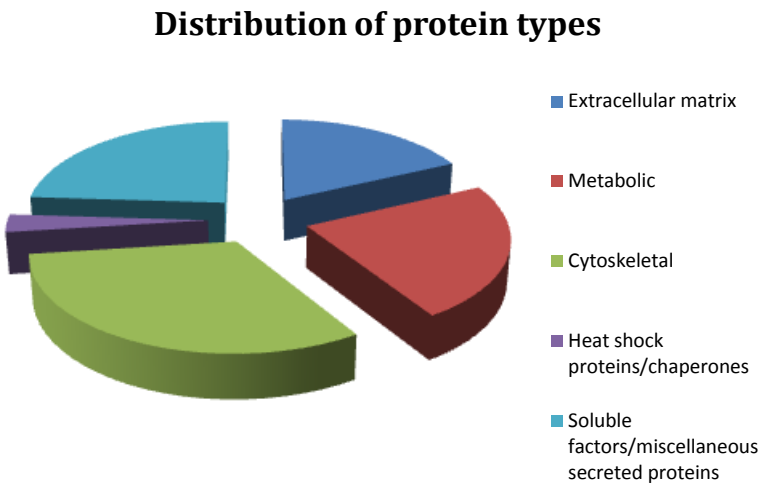


Figure 5.16. Summary of the protein distribution and functional classifications of the 38 candidate proteins identified in MSC aggregate-derived conditioned media using a shotgun proteomics approach. For the purposes of discussion, the candidate factors identified in MSC aggregate-derived CM using proteomics were classified into five broad categories in keeping with their distribution and reported functions. Classification of certain candidates into a single category was often difficult due to considerable overlap between the categories and multifunctionality of many of the candidate factors.

One possible reason to account for the high detection rate of intracellular proteins in CM samples is cell lysis secondary to cell death and subsequent release of intracellular contents into the media (Xue *et al.*, 2008). Cell culture in serum-free conditions, as is routinely performed to generate CM samples amenable to proteomic analysis, is characterised by particularly high levels of cell death and autolysis, with several independent studies reporting an optimal conditioning time of between 18-24 hours, after which extensive contamination with intracellular proteins is observed (Mauri *et al.*, 2005; Mbeunkui *et al.*, 2006). As the protocol adopted throughout this investigation utilised a conditioning time of three days, it could be anticipated that the high levels of intracellular proteins detected in CM were attributable to extensive cell death/autolysis during the conditioning period; however, no significant evidence of this was apparent during routine observation of cultures. Furthermore, as knowledge continues to improve regarding the intricate processes and pathways involved in cell secretion, the definition of the term *secretome* will have to evolve accordingly, as the contribution of proteins released through non-classical (endoplasmic reticulum/Golgi-independent) secretory pathways and the secretion of exosomes are largely overlooked in favour of proteins secreted by classical secretory pathways e.g. growth factors, cytokines and extracellular matrix (Volmer *et al.*, 2005). In particular, there is increasing interest in the biological role(s) of exosomes, membranous vesicles of approximately 40-100nm diameter which are generated within the endosomal system and released by most cell types following fusion of multivesicular bodies with the plasma membrane (Johnstone, 2006; Simons and Raposo, 2009; Simpson *et al.*, 2009). Exosomes have a plethora of known biological functions, including immune response, antigen presentation and intra-/inter-cellular communication. Proteomic methods have been employed in several studies in order to scrutinise the protein composition of exosomes and a variety of protein types have been detected, including metabolic enzymes, cytoskeletal proteins, elongation factors and chaperones, with exact protein compositions being largely representative of host cell origin (Thery *et al.*, 2001; van Niel *et al.*, 2001; Pisitkun *et al.*, 2004; Volmer *et al.*, 2005; Simons and Raposo, 2009; Simpson *et al.*, 2009).

Additionally, it is often difficult to assign each of the 38 candidate factors to a single category based on their reported biological function(s)/subcellular localisation(s), as the functional diversity of many of the factors is such that they could be assigned to

multiple categories. For example, metabolic enzymes were considered to be of intracellular origin for the purposes of the preceding discussion. Although this is an accurate statement, there have been several reports of metabolic enzymes such as thioredoxin proteins undergoing active secretion from a range of different cell types (Rubartelli *et al.*, 1993; Rubartelli *et al.*, 1995; Bäckman *et al.*, 2007). Further intracellular proteins identified as candidate factors in this study have been reported to be actively secreted from different cell types, including translationally controlled tumor protein (TCTP; or lens epithelial protein) and thioredoxin-dependent peroxide reductase **(Söderberg** *et al.*, 2000; Amzallag *et al.*, 2004).

Therefore, one of the fundamental limitations of CM studies using proteomic-based techniques such as those employed in this thesis is the inherent inability to distinguish between truly-secreted proteins and artifactual products of cell culture. As such, a significant contribution of non-classical/exosome secretion cannot be discounted as attributing towards the high representation of intracellular cytoskeletal and metabolic proteins identified in this study. Numerous reports of intracellular proteins undergoing active secretion from cells further blurs this distinction. Therefore, the intracellular proteins identified in this study must be considered as putatively-secreted.

However, due to the lack of data regarding the secretion of intracellular cytoskeletal and metabolic proteins in the CM system used in this study, the remainder of this discussion will focus on the more classically-secreted proteins and extracellular matrix proteins identified, accounting for 24% and 18% of the total complement of candidate factors respective, as both have been reported to significantly contribute towards the beneficial effects of MSC-mediated paracrine activity in the nervous system (Tremain et al., 2001; Chen et al., 2002; Wislet-Gendebien et al., 2004; Chen and Chopp, 2006; Crigler et al., 2006; Rivera et al., 2006; Chen et al., 2007; Schinkothe et al., 2008).

TIMP2

A comprehensive review of the current literature combined with gene ontology analysis identified a number of particularly strong candidates from within this group that presented as potentially-important determinants of the astrogenic effect in AHPCs. Of these, TIMP2 (metalloproteinase inhibitor 2) presented as a particularly strong candidate owing to its previously recognised role as a component of neurogenic

signalling pathways controlling the transition of neuroprogenitors from immature, proliferative entities to mature, terminally-differentiated neural cell types (Jaworski and Perez-Martınez, 2006). TIMP2 is one of four members of the highly conserved TIMP protein family – tissue inhibitor of metalloproteinases – a group of proteins of approximately 20-30kDa which function predominantly as natural inhibitors of matrix metalloproteinases (MMPs) which, in turn, are a group of endopeptidases involved in remodelling/degradation of the extracellular matrix. Expression profiling of TIMP2 reveals specificity to neurons and astrocytes throughout the adult nervous system, strongly suggesting a functional role for TIMP2 in neural development and/or maintenance of neural integrity (Fager and Jaworski, 2000; Crocker *et al.*, 2004). In their interesting and detailed study, Jaworski and Perez-Martinez observed significant upregulation of TIMP2 in four neural cell lines – PC12, Neuro-2a, N1E-115 and P19 – following induction of neuronal differentiation in these cultures, strongly suggesting a role for TIMP2 in the differentiative process (Jaworski and Perez-Martinez, 2006). The direct influence of TIMP2 was observed to be mediated by interactions with cell surface-bound integrin molecules; in particular, $\alpha_3\beta_1$ integrin. This observation is in keeping with additional studies reporting that TIMP molecules demonstrate greater functionally diversity than previously anticipated and are able to directly influence cellular behaviour in addition to indirectly influencing cellular behaviour through their MMP-inhibitory role (Baker *et al.*, 2002; Crocker *et al.*, 2004). Therefore, a direct contribution of TIMP2-mediated signalling to the astrogenic effect in AHPCs cannot be excluded; similarly, TIMP2 may contribute to the astrogenic effect *via* a more indirect mechanism, namely its effect on MMP-mediated extracellular matrix remodelling. In keeping with the latter possibility, MMP2, MMP3 and MMP13 were detected in both ITS- and ITS+P+P-CM; therefore, upregulation of TIMP2 in biologically-active ITS+P+P-CM may inhibit the activity of MMP2/3/13 such that the extracellular matrix is not remodelled and remains permissive to astrocytic differentiation. Furthermore, MMPs have been reported as regulators of diverse cellular functions such as proliferation, migration, differentiation and neurite outgrowth; TIMP2 may influence these cellular processes indirectly *via* its interaction with MMP molecules (Zuo *et al.*, 1998; Diaz-Rodriguez *et al.*, 1999; Lee *et al.*, 2001).

Glia-derived nexin (serpin E2)

Glia-derived nexin (serpin E2) also presented as a strong putative candidate factor, being exclusively detected in biologically-active ITS+P+P-CM samples and previously being reported as a neurotrophin that is actively secreted by astrocytes (Stone *et al.* 1987; Brenneman *et al.*, 1997). This protein belongs to the serpin protein family (serine proteinase inhibitor family) and several studies have demonstrated that it has an anti-apoptotic effect in the brain, undergoing significant upregulation following injury/ischaemia/alterations to the blood-brain barrier (Meier *et al.*, 1989; Hoffman *et al.*, 1992; Brenneman *et al.*, 1997; Turgeon and Houenou, 1997). The neuroprotective function of glia-derived nexin has been attributed to its protease inhibitory function (Onuma *et al.*, 2006). Additionally, glia-derived nexin has demonstrated neurite-inducing properties in neuroblastoma cell lines and hippocampal neurons (Meier *et al.*, 1989). Although a direct role in promoting differentiation in neural stem/progenitor cells is not known with any certainty, there is evidence to suggest a role in promoting neuronal differentiation (Lin *et al.*, 2005).

Gamma enolase (neuron-specific enolase)

Gamma enolase (neuron-specific enolase, or enolase 2) is one of three enolase isoenzymes present in mammals and is an interesting candidate factor owing to its restricted expression to mature neurons and other neural cell types during the later stages of development; indeed, Cervello and colleagues observed a three-fold increased in gamma enolase levels following differentiation of the N115 neuroblastoma cell line for 24 hours (Cervello *et al.*, 1993). Expression of gamma enolase was previously thought to be restricted to neuronal cell types only; however, it has since been detected in astrocytes, oligodendrocytes and has even been indicated as a marker of prostatic neuroendocrine cells (Vinores and Rubinstein, 1985; Xu *et al.*, 1995; Deloulme *et al.*, 1997; Daddi *et al.*, 2004; Untergasser *et al.*, 2005; Raposio *et al.*, 2007). However, despite its use as a neural-specific marker in cell differentiation studies, gamma enolase has yet to be reported as an inducer of neural differentiation, though it has been reported as an actively-secreted protein (Lawton *et al.*, 1986).

Calumenin (crocalbin)

Another potentially-interesting candidate factor identified in this study is calumenin (crocalbin) which is observed to be significantly upregulated in biologically active ITS+P+P-CM compared to biologically-inactive ITS-CM. Calumenin is a member of

the CREC protein family, a small yet rapidly-expanding protein family encoded by five genes in total and characterised by multiple highly-conserved EF-hand domains (Hansen *et al.*, 2009; Honoré, 2009). Functionally, members of the CREC family are highly diverse but, at the cellular level, all localise to various stages of the secretory pathway; however, only calumenin has been reported to undergo active secretion from cells into the culture media (Vorum *et al.*, 1999; Hansen *et al.*, 2009). Indeed, a recent study observed the secretion of calumenin from astrocytic cells in culture, therefore presenting the possibility that calumenin could have an as-yet uncharacterised role in neural development (Dowell *et al.*, 2009). Upregulation of calumenin has been suggested to reflect Schwann cell activation and proliferation (Jiménez *et al.*, 2005). In their interesting study, Østergaard and colleagues examined the effect(s) of calumenin on cellular behaviour by culturing fibroblasts in its presence/absence followed by characterisation of the cellular response using 2D SDS-PAGE MS/MS (Østergaard *et al.*, 2006). The presence of extracellular calumenin altered the organisation of the actin cytoskeleton and also influenced cell cycle status of the cultured fibroblasts; therefore, it can be envisioned the calumenin functions in an analogous manner in the culture system used in the present study, as both cytoskeletal rearrangement and modulation of cell cycle status are both key processes in neural differentiation.

Translationally-controlled tumour protein (lens epithelial protein)

Translationally-controlled tumour protein (lens epithelial protein) presents as a potentially interesting candidate factor as its expression is restricted exclusively to lens epithelial cells, where it is co-expressed with the astrocytic marker, GFAP (Hatfield *et al.*, 1984). Although lens epithelial cells are of a different embryological origin to astrocytic cells, they share many similar phenotypic traits in terms of cellular shape, morphology and functionality (O'Rahilly and Meyer, 1959; Hertz, 1977; Levitt *et al.*, 1981).

Melanoma-associated antigen MG50

Additionally, melanoma-associated antigen MG50 is a potentially interesting candidate factor. Melanoma-associated antigen MG50 (interleukin 1 receptor antagonist) is reported to be one of the only melanoma-associated antigens that is not a differentiation antigen or mutated protein (Mitchell *et al.*, 2000). Although there is currently no published data on MG50 and its biological relevance to the nervous

system, other members of the melanoma-associated antigen family have been implicated in neural processes; for example, necdin is expressed predominantly in post-mitotic neurons and has been described as a promoter of neuronal differentiation/survival (Pisarra *et al.*, 1998; Kuwajima *et al.*, 2006).

Olfactomedin-3

Olfactomedin-3 is a secreted glycoprotein observed to be upregulated in biologically-active ITS+P+P-CM and, although the precise biological function of olfactomedin-3 is unknown, other members of the olfactomedin protein family – including noelin, tiarin, pancortin, Omf1 and gliomedin – have been significantly implicated in regulation of the mammalian nervous system; in particular, gliomedin is reported to be secreted by and incorporated into the extracellular matrix surrounding Schwann cells, whilst Omf1 has been observed to promote neuronal differentiation in *Xenopus* models (Zeng *et al.*, 2004; Moreno and Bronner-Fraser, 2005; Eshed *et al.*, 2007; Lee *et al.*, 2008).

Cortistatin

Cortistatin is a neuropeptide which bears significant sequence homology to somatostatin – sharing 11 of its 14 amino acids – and has an important role in signalling throughout the nervous system (Rubinfield and Shimon, 2006). Although the vast majority of cortistatin studies focus on its predominant role in deep sleep and other related processes, cortistatin has previously been reported to induce neurite outgrowth in PC12 cells (Kim *et al.*, 2007). The same study also demonstrated that cortistatin could induce neuronal differentiation in the same cell system in the presence of retinoic acid; however, retinoic acid is the gold-standard neuronal differentiation reagent and it is therefore likely that the differentiative effects observed were predominantly a consequence of retinoic acid rather than cortistatin. That said, a combinatory effect cannot be discounted.

Extracellular matrix proteins

Also of considerable interest were the abundant number of extracellular matrix proteins – including various collagens and fibronectins – that were detected either exclusively or significantly upregulated in biologically-active ITS+P+P-CM compared to biologically-inactive ITS-CM. Such molecules have previously been reported to play crucial roles in neural developmental processes (Jaworski and Perez-Martinez,

2006; Li *et al.*, 2009). The crucial role of extracellular matrix in basic neural stem cell biology and maintenance of neural stem cell integrity is highlighted by the necessity to coat cellware with various combinations/concentrations of extracellular matrix proteins to provide an environment more permissive to the culture of neural cell types (Carbonetto and Cochard, 1987). Although this is a useful and convenient compromise allowing the *in vitro* culture of neural cell types, coating cellware with individual/basic combinations of extracellular matrix proteins is not truly representative of the *in vivo* niche of neural cell types, where extracellular matrix reflects a dynamic combination of proteins arranged purposely and present at precise concentrations.

In addition to provision of structural support and organisation within the niche, extracellular matrix components also provide key regulatory signals that promote cellular processes such as proliferation, migration and differentiation (Tate *et al.*, 2004; Hayashi *et al.*, 2007; Tanentzapf *et al.*, 2007). Such phenomena can be a consequence of direct interaction between extracellular matrix ligands and integrin receptors on the cell surface, or indirectly as a consequence of binding and presentation of growth factors within the niche to increase their local concentration (Watt, 1986; Ferri and Levitt, 1995; Conover and Notti, 2008; Ma *et al.*, 2008). However, despite the undoubtedly important contribution of extracellular matrix to neural stem cell biology within the neural niche, the function of such components are poorly understood at present. Studies examining the spatial and temporal expression patterns of extracellular matrix components within the neural stem cell niche have revealed that extracellular matrix is present in abundant quantities during developmental processes such as differentiation, migration and neurite extension etc.; however, expression of such components are significantly reduced at the end of development (Letourneau *et al.*, 1994; Lathia *et al.*, 2007; Ma *et al.*, 2008). Therefore, it could be envisioned that the extracellular matrix lattice secreted and emplaced by MSC aggregates cultured in ITS+P+P-supplemented media provides a culture substratum amenable to astrocytic differentiation (Koslova *et al.*, 1993). It is anticipated that extracellular matrix components play a particularly prominent role in the later stages of neural differentiation, involving processes such as neurite outgrowth and synaptogenesis etc. (Li *et al.*, 2009). The lack of current understanding in how interactions between extracellular matrix and neural stem cells contribute to differentiative processes means that this would be difficult to verify.

Overall summary

Neural differentiation is a complex, highly intricate and multifaceted process, and is regulated extensively by both spatial and temporal environmental cues *in vivo*; therefore, the astrogenic effect observed in AHPCs following culture in MSC aggregate-derived CM for 7 days is likely to be a consequence of extensive interplay between several of the candidate factors identified in this chapter, as opposed to the effect(s) of a single factor. Within the stem cell niche there is extensive crosstalk between diffusible soluble factors and extracellular matrix components and it can be envisioned that similar cross-mechanisms are occurring in the AHPC system used throughout this thesis. The major limitation of conditioned media studies to date has been the lack of effort to characterise the cellular secretome and identify the bioactive factor(s) responsible for the observed cellular effects. Traditional approaches have involved targeting a single factor or a handful of factors predicted to be present within the complex secretome based on the observed cellular effect(s) and current knowledge within the literature. Studies within this Chapter adopted a traditional approach in the first instance, involving targeted transcriptional profiling using qRT-PCR and then adopted a more technically-advanced approach of shotgun proteomics using LC-MS/MS to indiscriminately examine the MSC aggregate-derived secretome. A number of potential candidate factors have presented as ideal candidates for further investigation.

Chapter 6

GENERAL SUMMARY AND OVERVIEW

6.1 General Discussion

Stem cells are ideal candidates for potential clinical application in regenerative cellular-based therapies and tissue engineering strategies against a wide range of clinically-important disorders. In particular, adult stem cells – such as MSCs used in studies throughout this investigation – are garnering significant interest as they are widely accessible and circumvent the considerable ethical issues surrounding the use of embryonic stem cells. The intrinsic ability of MSCs to differentiate into mesodermal derivatives such as bone and fat means that they immediately present as ideal candidates for cellular-based therapies targeted against osseous and adipose tissue defects, whilst an increasing number of studies suggest that MSCs may also be used for cellular-based therapies against a wider range of clinically-important disorders, including those of the cardiac, renal and nervous systems. It is the latter that is of particular significance to this thesis.

However, despite the intense excitement surrounding stem cells and their potential to revolutionise modern regenerative medicine, translation of stem cell research from the laboratory bench to a clinical setting has been slower than anticipated and somewhat disappointing. One of the main reasons to account for this lag is the assumption made in the majority of studies that stem cells cultured in the artificial 2D environment of tissue culture plastic behave in an analogous manner to which they would when functioning as part of a living tissue *in vivo*. This is highly unlikely to be the case, particularly in studies involving stem cells, where the contribution of niche-specific signals towards stem cell function/behaviour is equally as significant as the intrinsic properties of the stem cells themselves (Scadden, 2006). Therefore, although culturing cells using traditional methods is convenient and has increased our knowledge of biological processes, a greater appreciation of niche-specific signals and a better understanding of the balance between stem cell intrinsic properties, niche-specific signals are required before stem cells are successfully introduced into a clinical setting.

Research conducted for this study has investigated numerous facets of the stem cell niche and the effect(s) of such facets on various aspects of MSC behaviour/functionality *in vitro*. In the first instance, cellular viability and differentiative potential of MSCs cultured using traditional methods i.e. 2D tissue

culture plastic was compared to that of counterparts cultured on the novel, polystyrene 3D scaffold, Alvetex^{TM}, with the hypothesis that MSCs cultured in 3D would be encouraged to adopt conformations/morphologies and form cell-cell interactions more representative of those that occur *in vivo*. Indeed, MSCs cultured on the 3D scaffold demonstrated excellent cellular morphology concomitant with enhanced viability/differentiative potential compared to counterparts cultured in the traditional environment of 2D tissue culture plastic. At the molecular level, the enhanced differentiative potential of MSCs cultured on 3D was determined to be a consequence, at least in part, of enhanced autocrine signalling; however, differences in expression of soluble factors/cytokines observed at the transcriptional level were not verified at the protein level. The contribution of autocrine and paracrine signalling, mediated by cellular-derived soluble factors and cytokines, represents another facet of the stem cell niche that is often overlooked; in fact, soluble factors/cytokines are likely to represent the most significant factors within the niche involved in maintenance/regulation of stem cell behaviour/function. Combined, results from Chapter 3 demonstrate that culturing cells in 3D and encouraging the formation of conformations/geometries more representative of those that occur *in vivo* and provides a more physiologically-relevant system in which to study the complex cellular and molecular mechanism(s) by which MSCs commit to a specific fate and undergo appropriate differentiation programs. The ultimate aim is to exploit this knowledge in order to control stem cell behaviour in clinical applications, and develop new and improved reagents for cell culture.

An alternative method of culturing MSCs in 3D was introduced in Chapter 4. Previous studies have demonstrated that MSCs form large, multicellular aggregates when cultured under conditions described for the propagation of neurospheres, which promotes structural interactions/re-establishment of mutual contacts between cells without the use of external scaffolds such as that used in Chapter 3 (Hermann *et al.*, 2004; Hermann *et al.*, 2006; Pampaloni *et al.*, 2007; Croft and Przyborski, 2009). Of particular relevance to this thesis is the latter study by Croft and Przyborski, which observed that MSC-derived aggregates were able to influence the cell fate decisions of primary embryonic neural stem cells when in a co-culture system; this effect was a consequence of paracrine signalling between the two cell populations (Croft and Przyborski, 2009). In this co-culture model, MSC-derived aggregates released a complement of soluble factors/cytokines that induced embryonic neural stem cells to

commit to and undergo a neuronal differentiation program. These observations are in support of many previously-published studies reporting that MSC transplantation in numerous models of neurological and neurodegenerative disorders results in beneficial effects as a consequence of the paracrine action(s) of MSC-derived soluble factors/cytokines on host neural stem/progenitor cells (reviewed in Chapter 1). Further understanding of the paracrine relationship between MSCs and NSCs is highly desirable from a clinical perspective, as NSCs are inaccessible and technically challenging to culture *in vitro*; therefore, if MSCs can be manipulated to release a complement of soluble factors/cytokines that directs the differentiation of host neural stem cells as required, this may provide a potential therapeutic avenue for the treatment of neurological and neurodegenerative disorders. Detailed dissection of the MSC secretome may circumvent the need for cellular delivery of these bioactive molecules at all, instead permitting exogenous administration of such factors.

Studies in Chapter 4 aimed to investigate the paracrine effect(s) of MSC-derived aggregates in further detail, using a conditioned media approach and the AHPC system. Following 7 days culture in MSC aggregate-derived CM, AHPCs were induced to commit to and undergo an astrocytic differentiation program, as determined by expression of structural/functional astrocytic markers and upregulation of astrocytic-promoting transcription factors. It was originally anticipated that the effects observed in this study would mirror those observed by Croft and Przyborski (2009); however, this was not the case, with MSC aggregate-derived soluble factor/cytokines inducing astrogenesis at the expense of neurogenesis in this system. The reasons for such discrepancies are highly likely to be a consequence of the obvious differences in experimental system between the present study and that of Croft and Przyborski (2009):

- The present study used the AHPC cell line, which was originally propagated from adult rats; the study by Croft and Przyborski (2009), conversely, used primary E14 neural progenitor cells. It is not uncommon for the same molecule to exert highly different effects in cells of embryonic vs. adult origin (Guillemot, 2007).

- The present study used a conditioned media approach, in which cellular communication is unidirectional; the study by Croft and Przyborski, conversely, used a co-culture approach, in which cellular communication is bidirectional. Therefore, it cannot be ascertained with any certainty that MSCs produce and secrete the same complement of soluble factors/cytokines into conditioned media that they do when under the influence of neural stem cell-derived soluble factors/cytokines in co-culture; indeed, MSCs have previously been reported to modify their secretome depending on the microenvironment in which they are cultured (Chen *et al.*, 2002).

It can therefore be envisioned that the co-culture system adopted by Croft and Przyborski (2009) is a more physiologically-relevant system in which to study the paracrine relationship between MSCs and NSCs, as cellular communication is likely to be multi-directional *in vivo* rather than unidirectional, as is the case in conditioned media approaches. However, a conditioned media approach was adopted in this study as characterisation of the MSC aggregate-derived secretome is a crucial step towards understanding the interactions between MSCs and NSCs and how this can be exploited for therapeutic applications in a clinical setting; crucially, co-culture assays do not allow determination of the cellular origin of proteins in the media.

A combinatorial approach involving targeted transcriptional profiling using qRT-PCR and shotgun proteomics using LC-MS/MS was adopted in Chapter 5 in order to scrutinise the MSC aggregate-derived secretome in further detail, with the ultimate aim of identifying the bioactive factor(s) responsible for exerting the biological effects observed in Chapter 4. To our knowledge, this is the first large-scale study targeting identification of neurotrophic factors in MSC-derived conditioned media. Targeted transcriptional profiling identified significant upregulation of LIF, NGF and GDNF at the mRNA level in MSC-derived aggregates; however, these transcriptional changes were not verified at the protein level. Shotgun proteomic analysis of MSC aggregate-derived CM identified further putative candidate factors encompassing a wide range of general cellular functions, including extracellular matrix, metabolic proteins, cytoskeletal components, chaperones/heat shock proteins and soluble factors/classically-secreted proteins. Of these, TIMP2, glia derived nexin (serpine 2)

and calumenin (crocalbin) presented as particularly interesting putative candidate factors, though a significant contribution of extracellular matrix proteins such as collagens and fibronectins are also likely to be important contributing factors. Such is the complex nature of the neural differentiation process, that extensive interplay between several of the candidate factors identified in Chapter 5 is likely to be causative of the astrogenic effect in AHPCs.

Studies in this thesis have examined the bifunctionality of MSCs in terms of their intrinsic ability to differentiate into mesodermal derivatives such as bone and fat, and also their paracrine behaviour involving the release of soluble factors/cytokines that influence the behaviour of other stem cell types; in the case of this thesis, neural stem cells. Of most interest is the observation that soluble factors/cytokines produced and secreted by MSC-derived aggregates induce astrogenesis in AHPCs following 7 days culture. Until recently, astrocytes were considered to be passive cells whose main function was to provide structural and functional support to neurons; however, this is no longer considered to be the case, as studies continue to be published reporting more widespread roles/functions of astrocytes (Svendsen, 2002; He and Sun, 2007). These are discussed in Chapter 4. Renewed interest in astrocyte biology means there is high demand for convenient *in vitro* assays, as astrocytes are notoriously difficult to study *in vitro* due to technical challenges in their culture. MSC aggregate-derived CM may therefore provide an easy source of astrocytes from neural stem/progenitor cells from which to perform further *in vitro* studies. However, further verification of the effect(s) of MSC aggregate-derived CM on neural stem and progenitor cells from other sources would be highly beneficial.

6.2 Summary of key findings

- MSCs isolated from the bone marrow of postnatal rats demonstrate an MSC-like phenotype in terms of cell surface and intracellular marker expression and potential to differentiate into the mesodermal derivatives, bone and fat.

- MSCs cultured on the novel, polystyrene 3D scaffold manufactured by Reinnervate Ltd. – Alvetex^{TM} – demonstrate enhanced viability and functionality in terms of their ability to differentiate into osteogenic and adipogenic lineages.

- Enhanced osteogenic and adipogenic differentiation of MSCs cultured on the scaffold is attributable, at least partially, to autocrine behaviour.

- MSCs can form large, multicellular aggregates resembling neurospheres following culture under previously-published conditions described for the propagation of neurospheres. MSC-derived aggregates are positive for neural antigen expression.

- MSC-derived aggregates secrete a complement of soluble factors/cytokines into their culture media that induces AHPCs to undergo astrocytic differentiation.

- The astrogenic effect observed in AHPCs occurs independently of effects on cell proliferation; however, increased cell death in putative oligodendrocytic-precursors may contribute to the astrogenic effect.

- Increased cell death in putative oligodendrocytic-precursors is a direct consequence of activation of apoptotic pathways.

- LIF, NGF, GDNF, TIMP2, glia derived nexin (serpine 2) and calumenin (crocalbin) all present as particularly interesting candidate factors identified following targeted transcriptional profiling and shotgun proteomic analysis of MSC aggregate-derived CM.

- Extracellular matrix proteins secreted by MSC-derived aggregates are also likely to be important contributors to the astrogenic effect.

6.3 Future work

Studies in this investigation have examined several aspects of MSC behaviour and function, particularly the ability of MSCs to function as paracrine mediators of neural differentiation, using a conditioned media approach and the AHPC system as a model of neural development. Although the cellular and molecular mechanisms responsible for the astrogenic effect in AHPCs were studied in extensive detail in this thesis, a major limitation of this work is that the conditioned media was characterised using only a single cell line. As such, further verification of the effect(s) of MSC aggregate-derived CM on neural stem and progenitor cells from other sources would be highly beneficial. Preliminary results achieved following culture of the human embryonic neural stem cell line, REN 197VM, in MSC aggregate-derived CM for 7 days are shown in Figure 6.1.

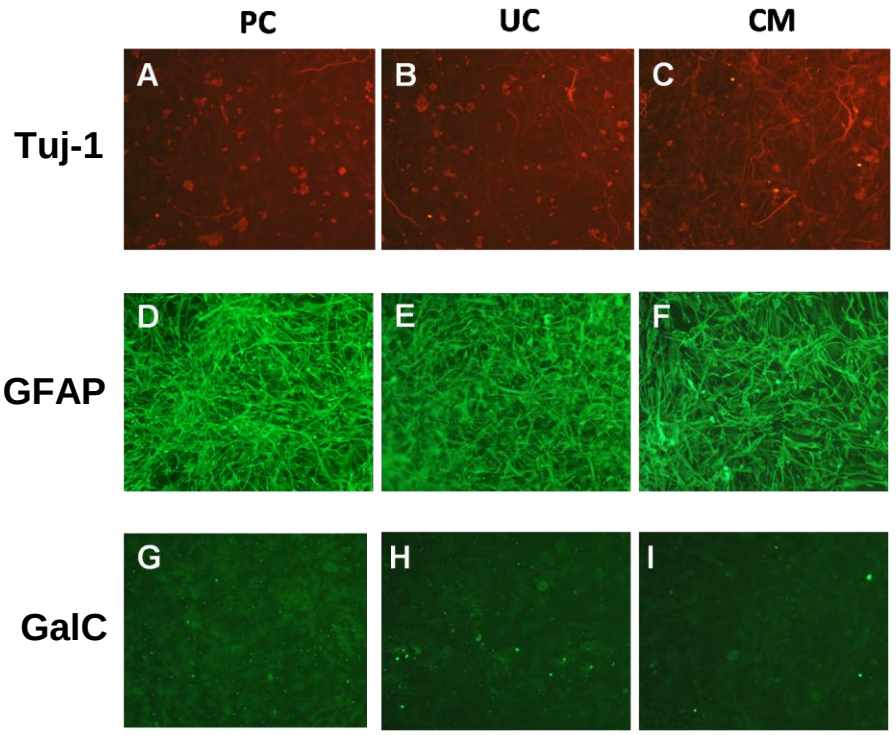


Figure 6.1. Expression of neural markers in REN 197VM cells following 7 days culture with MSC aggregate-derived CM and appropriate controls. A, B and **C:** expression of Tuj-1; **D, E** and **F:** expression of GFAP; **G, H** and **I:** expression of GalC. Treatment of REN 197VM cells with conditioned media results in the development of more extensive and intricate processes indicative of neuronal differentiation compared to control cultures. However, extensive co-expression of neural markers in the REN 197VM cell line makes phenotypic determination difficult. Scale bar = 100µm.

One key feature of the above study was that the proliferation control (PC), unconditioned media control (UC) and conditioned media (CM) cultures all demonstrated significant co-expression of the neural markers Tuj-1 and GFAP, and poor expression of GalC (Figure 6.1). Significant co-expression of Tuj-1 and GFAP in undifferentiated REN 197VM cultures has previously been demonstrated in our laboratory (data not shown). However, morphological analysis suggested that REN 197VM cells cultured in MSC aggregate-derived CM for 7 days were beginning to develop more extensive and intricate processes indicative of neuronal differentiation compared to control cultures (Figure 6.1C). These morphological changes were very subtle, but apparent, and can be observed at higher magnification in Figure 6.2. Although induction of neuronal differentiation in REN 197VM cells differs to the astrogenic effect observed in AHPCs, such an effect in this system would support the observations of Croft and Przyborski, who observed neuronal differentiation in embryonic neural stem cells following co-culture with MSC-derived aggregates (Croft and Przyborski, 2009). These results therefore serve to highlight the importance of cell-intrinsic properties in the conditioned media effect.

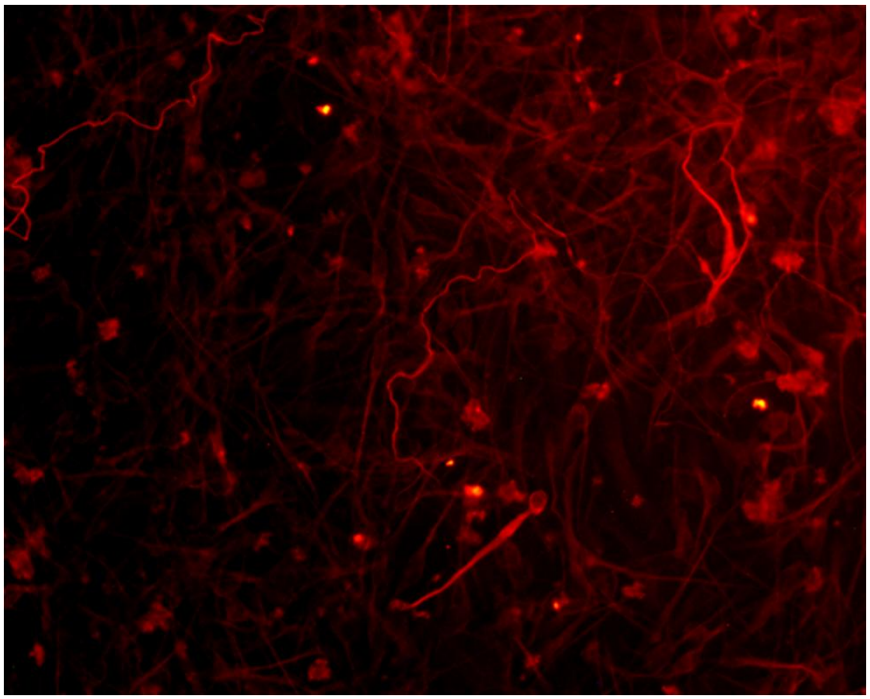


Figure 6.2. Morphology of Tuj-1^{+} REN 197VM cells following 7 days culture with MSC aggregate-derived CM. Although proliferation control (PC), unconditioned media control (UC) and conditioned media (CM) cultures all demonstrated significant co-expression of Tuj-1, morphological analysis suggested that CM cultures were beginning to develop more extensive and intricate processes indicative of neuronal differentiation. Magnification is X40.

Several additional areas of this thesis would also benefit from further verification work; in particular, verification of mRNA data at the protein level. Transcriptional profiling using qRT-PCR was used at several points in this thesis to examine expression of soluble factors and cytokines in MSCs cultured on the 3D scaffold (Chapter 3) and also in MSCs cultured as large, multicellular aggregates (Chapter 5). Although transcriptional profiling serves as a convenient and cost-effective starting point for such studies, mRNA levels are not always indicative of actual protein levels due to extensive post-transcriptional and post-translational processing. Having said that, changes at the transcriptional level are useful in themselves as indicators of cellular activity. In order to verify the qRT-PCR work performed throughout this thesis, several avenues of investigation could be performed. In the first and perhaps simplest instance, analysis of corresponding protein levels could be performed using Western blot analysis on cell lysates; however, if the protein being assayed is a truly-secreted protein, then intracellular levels may not be truly reflective of actual protein levels due to active secretion into the media. Therefore, a better approach could be concentration of the protein content of culture media followed by Western blot analysis, or determination of protein concentration in the media using an ELISA-based assay.

Results in Chapter 4 demonstrated that soluble factors/cytokines produced and secreted by MSC-derived aggregates were able to induce apoptosis in putative oligodendrocytic-precursors; however, this effect was not reproduced at a statistically-significant level in the 33B oligodendrocytoma cell line. It would be worthwhile investigating whether culture conditions could be modified appropriately such that MSC aggregate-derived CM induced apoptosis in this cancerous cell line at a statistically-significant level e.g. concentration of CM may result in a more potent induction of apoptosis.

Verification and functional testing of putative candidate factors identified in Chapter 5 would add significant impetus to this study. Although transcriptional profiling is limited in that mRNA levels are not always indicative of actual protein levels, targeted transcriptional profiling based on current knowledge within the literature was considered a convenient and necessary starting point because growth factors and cytokines are rarely detected by proteomic methods due to their low molecular weight,

low abundance and low stability (Moore *et al.*, 2009). Immunological validation of the targeted transcriptional profiling and shotgun proteomic data would be the next logical step for continuation of this study. This could be achieved directly *via* Western blot and/or ELISA-based methods on conditioned media samples. Following this, biological validation and functional testing of the putative candidate factor(s) could be performed by using either a) blocking antibodies/antagonists against the relevant factor(s) in order to ascertain whether the biological effect is maintained/abolished, or, b) recombinant proteins in order to ascertain whether the biological effect can be reproduced. Following successful validation and functional testing of the putative candidate factor(s), efforts could be made to exploit this by transfecting MSCs to overexpress any biologically-important factors.

One final point to consider is that the identification of putative candidate factors in Chapter 5 focussed predominantly on proteins that were significantly upregulated/exclusively detected in biologically-active ITS+P+P-CM, with little impetus placed on proteins that were downregulated. Indeed, if such factors were inhibitory to neural differentiation, then it could be anticipated that downregulation of these factors in biologically-active ITS+P+P-CM is equally as important as upregulation of promoting factors in determination of an astrocytic fate in AHPCs. Analysis of the original dataset revealed that 13 proteins were significantly downregulated in ITS+P+P-CM. These are: nucleoside diphosphate kinase A, nucleoside diphosphate kinase B, profilin-1, cystatin C, proteasome subunit α6, FK506-binding protein 1A, galectin-1, S100-A4, thioredoxin, eukaryotic translation initiation factor 5A-1, eukaryotic translation elongation factor 1 β2, myosin and cytochrome C.

Research conducted for this study has investigated numerous facets of MSC behaviour and function, including their intrinsic ability to differentiate into the mesodermal derivatives bone and fat, concomitant with their ability to mediate neural differentiation *via* the release of soluble factors and cytokines. Although much of the data generated throughout this study is promising, further verification of certain aspects and continued investigation, as detailed above, would add significant impetus to these findings and facilitate the generation of novel pharmacological products and cell culture reagents.

ANTIBODY	MANUFACTURER	SPECIES IN WHICH ANTIBODY RAISED	ANTIBODY SPECIFICITY	DILUTION
CD9	ABCAM ab92726	RABBIT	RAT, HUMAN, MOUSE	1:50
CD13	SANTA CRUZ sc-166105	MOUSE	RAT, HUMAN, MOUSE	1:50
CD15	ABCAM ab16285	MOUSE	RAT	1:50
CD34	SANTA CRUZ sc-7324	MOUSE	RAT, HUMAN	1:50
CD44	ABCAM ab24504	RABBIT	RAT, HUMAN, MOUSE	1:50
CD45	ABCAM ab33923	MOUSE	RAT	1:50
CD71	ABCAM ab65189	MOUSE	RAT	1:50
CD90	ABCAM ab225	MOUSE	RAT, GUINEA PIG, RABBIT	1:50

CD105	SANTA CRUZ sc-20632	RABBIT	RAT, HUMAN, MOUSE	1:50
CD138	ABCAM ab60199	RABBIT	RAT, HUMAN, MOUSE, HAMSTER	1:50
α-SMA	ABCAM ab5694	RABBIT	RAT, HUMAN, MOUSE, CHICKEN, COW, PIG	1:400
VIMENTIN	ABCAM ab8978	MOUSE	RAT, HUMAN, MOUSE, CHICKEN, COW, DOG, GOAT, HAMSTER, ZEBRAFISH	1:400
FIBRONECTIN	ABCAM ab6328	MOUSE	RAT, HUMAN, CHICKEN, COW, DOG, PIG	1:200
β-ACTIN	ABCAM ab8226	MOUSE	RAT, HUMAN, MOUSE, CHICKEN, COW, DOG, PIG	1:400
NESTIN	BD BIOSCIENCES 611658	MOUSE	RAT, HUMAN, MOUSE	1:200

TUJ-1	ABCAM ab7751	MOUSE	RAT, HUMAN, MOUSE, CHICKEN, COW, HAMSTER, PIG	1:600
GFAP	ABCAM ab7260	RABBIT	RAT, HUMAN, MOUSE, ZEBRAFISH	1:200
GALC	ABCAM ab83752	RABBIT	RAT, HUMAN, MOUSE, DOG	1:200
BrdU	ABCAM ab8152	MOUSE	N/A	1:40
CLEAVED CASPASE 3	ABCAM ab52294	RABBIT	RAT, HUMAN, MOUSE	1:200

APPENDIX A. Details of the primary antibodies used throughout this thesis. Information provided in this appendix includes antigen targeted by antibody, manufacturer, host species, antibody specificity and dilution used. Details of the secondary antibodies used to detect these primary antibodies are given in the relevant methods sections in the main body of the thesis, as the secondary antibodies used differed depending on cell type (MSC vs. AHPC) and application (flow cytometry vs. immunofluorescence).

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