ALPHA-L-IDURONIDASE TRANSDUCED MESENCHYMAL STEM CELLS AS A THERAPY FOR THE TREATMENT OF CNS DEGENERATION IN MUCOPOLYSACCHARIDOSIS TYPE I MICE

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Abstract

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disorder that is characterised by a deficiency in the α-*L*-iduronidase (IDUA) enzyme, resulting in the accumulation of undegraded heparan sulphate and dermatan sulphate glycosaminoglycans (gags) within the lysosome of nearly every cell. MPS I is a multi-tissue and organ disease, presenting with profound mental retardation and skeletal abnormalities. Haematopoietic stem cell (HSC) transplant and enzyme replacement therapy, two clinically available forms of treatment, are able to correct the soft tissue aspects of MPS disease, but have had a limited effect on the more complex skeletal and neurological symptoms. Stem cell therapy utilizing mesenchymal stem cells (MSC) has the potential to overcome these limitations due to their ability to differentiate into cells that are the major sites of MPS pathology.

MSCs naturally produce and secrete significantly higher levels of multiple MPS enzymes than HSCs *in vitro* and can be engineered to over-express multiple MPS enzymes using a lentiviral system. MSCs were found to secrete up to 5,559 fold greater IDUA enzyme after lentiviral transduction *in vitro*, suggesting a greater potential to cross-correct MPS pathology than HSCs. Lentiviral transduction was stable and persistent *in vitro*, and over-expression of MPS enzyme did not affect MSC *in vitro* differentiation down osteogenic, adipogenic, chondrogenic or neurogenic lineages.

Systemically administered human derived MSCs distribute widely, to multiple MPS I affected organs, including the brain, and persist *in vivo* for at least two months post administration. Significantly elevated brain and serum IDUA activity was observed two and six months post administration, respectively, and was associated with sustained functional improvements in

neuromuscular strength, motor control, coordination and spatial learning. MSCs were found to limit astroglial activation and modulate brain inflammatory gene expression of *Cd68*, *Gfap* and *Tnf in vivo*. Vertebral body width also returned towards normal. However, no improvement in gag storage or elevations of IDUA were observed in other tissues.

For the first time, this thesis has investigated the biochemical and behavioural changes due to *i.v.* administered hMSCs in MPS I mice. This thesis demonstrates that MSCs can exert added neurological improvements in MPS I pathology through exhibiting a combined effect between their superior enzyme secretion and anti-inflammatory effects. While minimal changes were noted in MPS I associated somatic pathology, MSCs could be administered in combination with already implemented ERT and/or BMT, which have both shown resolution in patients stored gag, therefore providing additional clinical benefits to MPS I children.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abbreviations

IDUA/*IDUA*/*Idua*	α-L-iduronidase
α-MEM	Minimum essential medium (α modified)
AAV	Adeno-associated-virus
BMT	Bone marrow transplant
BBB	Blood brain barrier
Cd68	CD68 antigen
CNS	Central nervous system
Ccl3	Chemokine (C-C motif) ligand 3
CS	Chondroitin sulphate
CycpA	Cyclophilin A
DAPI	4’,6-diamidino-2-phenylindole
DS	Dermatan sulphate
DMEM	Dulbecco’s modified eagle medium
ERT	Enzyme replacement therapy
FCS	Foetal calf serum
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GT	Gene therapy
GFAP/*Gfap*	Glial fibrillary acidic protein
gag(s)	Glycosaminoglycan(s)

GvHD	Graft-versus-host disease
Hep	Heparin
HSC(s)	Haematopoietic stem cell(s)
HS	Heparan sulphate
hBM	Human derived bone marrow
hDP	Human derived dental pulp
HA	Hyaluronan
i.v.	Intravenous
Ifnγ	Interferon gamma
Il1β	Interleukin 1 beta
KS	Keratan sulphate
M6P	Mannose-6-phosphate
M6PR	Mannose-6-phosphate receptor
MSC(s)	Mesenchymal stem/stromal cell(s)
MPS	Mucopolysaccharidosis
MPS I	Mucopolysaccharidosis type I
PBS	Phosphate buffered saline
SDT	Substrate deprivation therapy
SHIRPA	SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment
SUMF1	Sulphatase modifying factor 1
Tgfβ1	Transforming growth factor beta 1

Tnfα	Tumor necrosis factor alpha

Chapter One: Introduction

1.0 Overview

Mucopolysaccharidosis (MPS) is a genetically inherited disorder that is characterised by the deficiency or loss of a specific lysosomal enzyme activity, which results in the accumulation of glycosaminoglycans (gags) within the lysosomes of cells. MPS type I (MPS I) is a multi-tissue and multi-organ disease which is considered the prototypical MPS disease; characterised by profound neurological deficits and skeletal abnormalities (Scott, Guo et al. 1992; Scott, Litjens et al. 1993; Neufeld and Muenzer 2001; Reolon, Braga et al. 2006). The relentless nature of disease progression, and the number of organ systems affected present numerous obstacles to developing an effective treatment.

One novel therapy that is beginning to be explored is the use of transplanted mesenchymal stem/stromal cells (MSCs). These cells can be used alone or transduced with a viral vector encoding the deficient gene of interest to introduce functional therapeutic enzyme to patients with MPS. Cultured MSCs are a heterogeneous mixture of multipotent stem cells and committed stromal progenitor cells that can be found in a range of tissues and have previously been used as target cells for gene therapy for various pre-clinical applications (Meyerrose, Roberts et al. 2008; Ghaedi, Soleimani et al. 2011). The immunomodulatory properties associated with MSCs reduces the risk of graft-versus-host disease (GvHD) or post engraftment inflammation following infusion of allogeneic MSCs; two complications often seen in allogeneic haematopoietic stem cell (HSC) transplantation patients receiving ablative therapies (Majhail 2010; Lin, Lairson et al. 2011). MSCs are also capable of forming multiple cell types that are affected in MPS I disease, including neuronal-like cells (Gronthos, Brahim et al. 2002; Gronthos, Zannettino et al. 2003; Arthur, Rychkov et al. 2008).

In this project, human derived MSCs will be transduced with lentivirus encoding murine *Idua*, the enzyme deficient in MPS I, and permitted to over-express the lysosomal enzyme in culture before being intravenously administered into an immunosupressed *Idua* -/- murine model of MPS I. Over-expression of IDUA will enable transduced MSCs to secrete excess enzyme, facilitating widespread cross-correction of cells/tissues that do not undergo engraftment. This is a novel approach to try and combat the progressive, multi-faceted disease associated with MPS I.

1.1 The Mucopolysaccharidoses

The mucopolysaccharidoses (MPS) are a group of 11 heritable lysosomal storage disorders that are categorized by a deficiency in, or absence of, an enzyme responsible for the degradation of distinct glycosaminoglycans (gags) (Neufeld and Muenzer 2001). Depending on the enzyme that is affected, the catabolism of heparan sulphate (HS), dermatan sulphate (DS), chondroitin sulphate (CS), keratan sulphate (KS) or hyaluronan (HA) may be independently or jointly blocked (Neufeld and Muenzer 2001). MPS affects one in 22,500 Australian children and as a whole can be a very debilitating disease (Meikle, Hopwood et al. 1999).

MPS are inherited in an autosomal recessive manner, with the exception of MPS type II which is X-linked. Each MPS subtype has a distinguishing eponym (except MPS IX), enzyme deficiency, and range of clinical manifestations (Table 1.1). However, several clinical features are common to multiple MPS subtypes, these include; a chronic and progressive neurodegenerative course, organomegaly and dysostosis multiplex. Vision, hearing, airways, cardiovascular function and joint mobility may also be affected. Central nervous system (CNS) involvement is present in 2/3 of MPS disorders and usually results in profound

Table 1.1: Classification of the Mucopolysaccharidoses.

MPS	Eponym	Enzyme deficiency	Stored gag	Clinical pathology
I	Hurler/ Scheie	α-*L*-iduronidase	HS, DS	CNS, skeletal & somatic
II	Hunter	iduronate-2-sulphatase	HS, DS	CNS, skeletal & somatic
IIIA	Sanfilippo A	sulphamidase	HS	CNS & somatic
IIIB	Sanfilippo B	α-N-acetylglucosaminidase	HS	CNS & somatic
IIIC	Sanfilippo C	acetyl-CoA: α-glucosamide N-acetyltransferase	HS	CNS & somatic
IIID	Sanfilippo D	glucosamine-6-sulphatase	HS	CNS & somatic
IVA	Morquio A	galactose-6-sulphatase	KS	Skeletal & somatic
IVB	Morquio B	β-D-galactosidase	KS	Skeletal & somatic
VI	Maroteaux-Lamy	N-acetylgalactosamine-4-sulphatase	DS	Skeletal & somatic
VII	Sly	β-*D*-glucuronidase	DS, CS, HS	CNS, skeletal & somatic
IX	-	hyaluronidase	HA	Skeletal & somatic

The Mucopolysaccharidoses (MPS) are a group of 11 lysosomal storage disorders; each characterised by an enzyme deficiency responsible for the degradation of unique glycosaminoglycans (gags). The catabolism of heparan sulphate (HS), dermatan sulphate (DS), chondroitin sulphate (CS), keratan sulphate (KS) and hyaluronan (HA) may be individually or collectively blocked, resulting in a combination of CNS, skeletal and somatic pathology.

Adapted from Neufeld and Muenzer (2001).

intellectual handicap (Scott, Bunge et al. 1995; Neufeld and Muenzer 2001). There is also a strong correlation among MPSs linking residual enzyme activity to the age of diagnosis, disease severity and life expectancy (Meikle, Hopwood et al. 1999; Haley, Fragala Pinkham et al. 2006).

1.1.1 Mucopolysaccharidoses type I

MPS I results from a deficiency or the loss of IDUA (Matalon and Dorfman 1972; McKusick, Howell et al. 1972) enzyme activity, required for the stepwise degradation of two gag chains; HS and DS (Figure 1.1) (Scott, Guo et al. 1992; Scott, Litjens et al. 1993; Reolon, Braga et al. 2006). MPS I is representative of common manifestations associated with MPS storage disorders and is the most frequent form of MPS, affecting one in 88,000 Australian children (Meikle, Hopwood et al. 1999). Hurler syndrome, the most severe form of MPS I, is characterized by developmental delay and intellectual disability, heart disease, airway obstruction, corneal clouding, short stature, joint contractures, organomegaly and death during early childhood. The attenuated form, Scheie syndrome, results in corneal clouding and stiff joints, while intelligence and life span are normal. Patients with Hurler-Scheie syndrome exhibit an intermediate phenotype (Neufeld and Muenzer 2001).

Mutation analysis following the cloning of the *IDUA* gene has provided some molecular justification for the range of MPS I phenotypes (Scott, Bunge et al. 1995) and hence clinical heterogeneity; directly linking IDUA catalytic capability to the severity of the associated disease in most cases (Bunge, Clements et al. 1998). Of the 119 mutations present in the *IDUA* gene (Stenson, Cooper et al. 2009), mutations that produced less than 0.13% residual enzyme activity, effectively knocking out the normal activity of the *IDUA* gene, result in

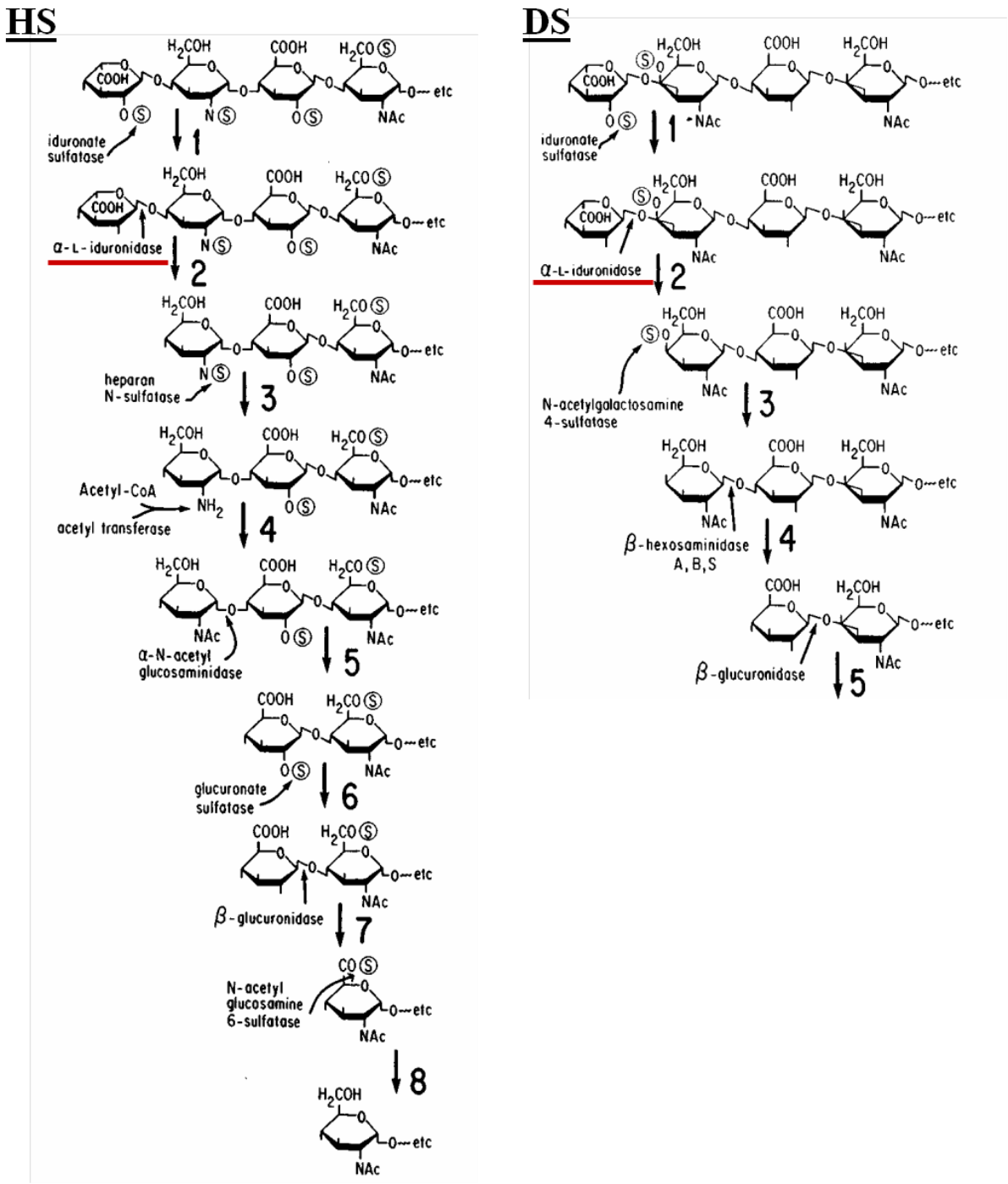


Figure 1.1: Stepwise degradation of HS and DS gags.

a-*L*-iduronidase (step 2) hydrolyses the terminal α-*L*-iduronic acid residues of both HS and DS gags in their stepwise degradation. In MPS I, this process is inhibited or reduced, resulting in the accumulation of undegraded HS and DS gags within every cells lysosomes.

Adapted from Neufeld and Muenzer (2001).

patients suffering from Hurler's syndrome (Bunge, Clements et al. 1998). Mutations resulting in enzyme activity of 7% of normal are usually associated with the milder medical manifestations of MPS I (Scott, Litjens et al. 1993; Bunge, Clements et al. 1998), while carriers (50% of enzyme activity) are phenotypically normal (Clarke and Heppner 2002). The range of severity seen within individual lysosomal storage disorders may in part be attributed to different types of mutations occurring within the same gene, with residual enzyme data and the rate of gag turnover being combined to determine the correlation between the clinical genotype and biochemical phenotype and the clinical course of the disease (Bunge, Clements et al. 1998). However, environmental and other currently undetermined genetic background factors can influence disease development and as a result genotype-phenotype associations do not always stand (Bunge, Clements et al. 1998; Meikle, Hopwood et al. 1999).

1.1.2 Central Nervous System (CNS) pathology

Profound intellectual deficits are only present in the most severe form of MPS I, while patients who suffer from either Scheie or Hurler-Scheie syndrome exhibit normal intellect (Neufeld and Muenzer 2001; Venturi, Rovelli et al. 2002). Substantial physical changes are observed in the MPS I brain; thickened adventitia of blood vessels, thickened leptomeninges and dura, a marked increase in surrounding periadventitial space and hydrocephalus is observed (Young, Wolfe et al. 1966; Constantopoulos, Iqbal et al. 1980). The large nerve cells of both the brain stem and cerebral cortex are severely affected with ubiquitous neuronal changes and related gliosis (Dekaban and Constantopoulos 1977). Accumulated gag levels are five times greater in MPS I neurons, while there is a five to seven fold increase in HS and DS in other cell types of the brain (Figure 1.2) (Constantopoulos, Iqbal et al. 1980).

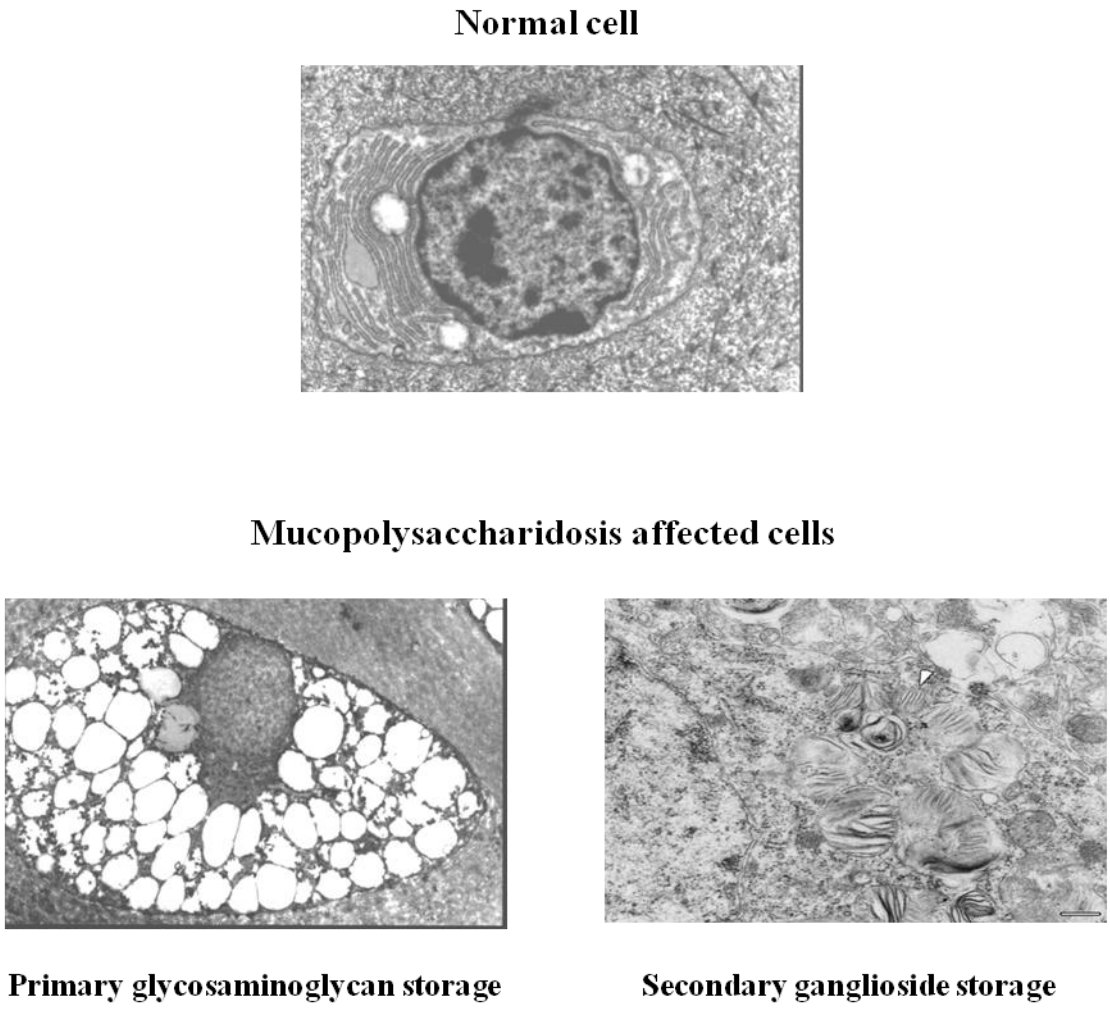


Figure 1.2: Primary and secondary storage seen in mucopolysaccharidoses type I.

MPS I is characterised by the primary storage of HS and DS gags, resulting in large, distended lysosomes, giving the cytoplasm a foamy appearance. While secondary storage of gangliosides GM2, GM3 and of cholesterol is also observed in the brain of HS-storing MPSs, including MPS I, as striped zebra bodies.

Adapted from Matrix Biology Unit, Department of Genetic Medicine, WCH, Adelaide

Meganeurites are expanded axon hillocks, found to stain intensely for lipids (Dekaban and Constantopoulos 1977), which have been identified as a combination of gangliosides G_{M2}, G_{M3} and of cholesterol (Figure 1.2) (Constantopoulos and Dekaban 1978; Constantopoulos, Iqbal et al. 1980). Gangliosides are a class of lipids predominately found in the brain, with G_{M2} and G_{M3} gangliosides associated with normal brain function and development. G_{M2} and G_{M3} are significantly elevated in the brain of MPS patients that have a primary accumulation of HS, such as MPS I, with CNS symptoms of neuronal deformities and intellectual handicap similar between HS-storing MPS diseases and the gangliosidoses (Constantopoulos, McComb et al. 1976; Constantopoulos and Dekaban 1978; Constantopoulos, Iqbal et al. 1980; Walkley, Zervas et al. 2000; Walkley 2004) .While this finding is unexpected, the elevated level of gangliosides has been shown to correlate with the growth of ectopic dendrites on affected neurons, which is consistent with the role of G_{M2} in dendritogenesis during development; and hence has severe consequences for brain and neuronal function (Walkley, Zervas et al. 2000; Walkley 2004). Although MPS I children meet some early developmental milestones, the clinical consequence of the above changes is a progressive loss of acquired skills.

1.1.3 Bone pathology

A range of skeletal abnormalities (collectively termed dysostosis multiplex) exist that affect both the growth and stature of MPS I patients. Bone abnormalities include; high lumbar kyphosis, hip dysplasia which can lead to dislocation, sclerosis and marked lateral angulations of the limbs (Schmidt, Ullrich et al. 1987; Masterson, Murphy et al. 1996; Tandon, Williamson et al. 1996). Bone formation is reduced (Tandon, Williamson et al. 1996), joint irregularities are common (Schmidt, Ullrich et al. 1987) and children fall below the 3rd percentile for height by approximately two years of age (Tandon, Williamson et al. 1996; Polgreen, Tolar et al. 2008). As MPS I children age, their movements become restricted due to thickening of the synovial capsule and membrane, leading to a decrease in range of

movement, which hinders routine tasks such as feeding and hygiene (Sifuentes, Doroshow et al. 2007). Children often undergo numerous orthopaedic interventions to correct their associated bone pathology; however, most severely affected children still become wheelchair bound late in their first decade of life.

1.2 Therapies

Finding an effective multi-tissue treatment for MPS I has been difficult, due to both the severity and progressive nature of pathology. Bone marrow transplantation (BMT) and enzyme replacement therapy (ERT) are currently the only multi-tissue treatments available; however, each has its own limitations (discussed below). The associated risks and benefits of either BMT or ERT must be individually assessed for each patient prior to designing a treatment plan, with consideration of severity of clinical disease, patient age, potential for growth and developmental quotient (Muenzer, Wraith et al. 2009). Despite being placed on ERT or BMT, children may still require ongoing neurological and orthopaedic support.

Gene therapy (GT), substrate deprivation therapy (SDT) and stem cell therapy (SCT) are three new approaches that aim to reduce the disease manifestations associated with MPS I. GT works by introducing the desired therapeutic enzyme to the patient by utilizing a viral vector, while SDT acts by reducing the initial synthesis of the storage material (Kobayashi, Carbonaro et al. 2005; Jakobkiewicz-Banecka, Wegrzyn et al. 2007; Roberts, Rees et al. 2007). SCT utilizes engraftment of multi- or pluri- potent stem cells from a healthy donor to introduce functional enzyme; using either haematopoietic, embryonic, induced pluripotent or mesenchymal stem cells. SCT is often done in combination with either GT or SDT to synergistically improve systemic enzyme activity (Biswas and LeVine 2002; Meyerrose, Roberts et al. 2008). Although GT, SDT and SCT are showing potential efficacy, all new

treatment approaches are still in animal trials for MPS I, and hence the research stage of development.

1.2.1 Current therapies

MPS I therapies were initially developed to introduce functional enzyme to deficient cells to overcome defective gag metabolism. The basis of these treatments was to target the enzyme to the lysosome, where undegraded gags accumulate, allowing internalisation and subsequent cleavage of the precursor protein to produce the active form of the enzyme.

1.2.1.1 Mechanisms for enzyme targeting

Initial studies investigating the kinetics of MPS I storage molecules concluded that the accumulation of HS and DS gags was due to a defect in their degradation, and excluded the possibility that their biosynthesis or secretion was altered (Fratantoni, Hall et al. 1968; Fratantoni, Hall et al. 1968). Subsequent *in vitro* studies found that when Hurler derived fibroblasts were co-cultured or supplemented with medium extracted from normal cells, the storage and kinetic pattern of HS and DS degradation was restored to normal. Cell-to-cell contact was not required for normalisation, but was mediated by uptake of IDUA, then termed 'Hurler corrective factor', from the surrounding medium (Fratantoni, Hall et al. 1968; Fratantoni, Hall et al. 1968; Di Ferrante, Donnelly et al. 1971; Bach, Friedman et al. 1972; Shapiro, Hall et al. 1976; Spellacy, Shull et al. 1983). Correction was inhibited by the addition of mannose-6-phosphate (M6P), but not mannose-1-phosphate, indicating that secreted enzyme is taken up by MPS I defective cells specifically through the M6P receptor (M6PR) (Spellacy, Shull et al. 1983), later shown to be the cation-independent M6PR ($M6PR^{CI}$) (Roberts, Weix et al. 1998; Olson, Peterson et al. 2010).

Lysosomal enzymes are glycosylated in the endoplasmic reticulum before being post-translationally modified within the Golgi apparatus to acquire M6P residues, which target enzymes to lysosomes via binding to M6PR at physiological pH (Kornfeld 1992; Di Natale, Di Domenico et al. 2002). In addition, there are motifs at the N-terminus of the protein that target to the lysosome in conjunction with $M6PR^{CI}$ (Roberts, Weix et al. 1998). The enzyme-receptor complex is then targeted to the mature lysosome through the endosomal system where the enzyme, in its precursor form (87kDa), is dissociated from the receptor and aspartic and glutamic acid residues are cleaved at acidic pH to produce the mature, active form (67kDa) (Shapiro, Hall et al. 1976; Schuchman, Guzman et al. 1984; Brooks, Fabrega et al. 2001; Brooks 2002). In the normal situation a small amount of enzyme may be diverted from the endosomal/lysosomal system and secreted from the cell. Phosphorylated enzyme can bind to the M6PR on the cell surface or on the surface of other cells, while some unphosphorylated enzyme binds the mannose receptor (Sands and Davidson 2006). However, as MPS I affects reticuloendothelial cells as well as other cell types, phosphorylated enzymes are currently used in ERT to target affected cells, preferentially exploiting the M6PR over the mannose receptor (Kakkis, Muenzer et al. 2001). Endocytosis and trafficking of secreted enzyme to the lysosome to allow normal catabolic activity within another cell can be mediated by either receptor. Many therapeutic approaches, such as ERT and GT, are based on this enzyme secretion and uptake process, termed cross-correction (Figure 1.3) (Kornfeld 1992; Di Natale, Di Domenico et al. 2002). Forced over-expression of MPS enzymes from either plasmid or viral vectors overwhelms the normal biosynthetic pathway and increased levels of the precursor form containing M6P residues can be recovered from the extracellular medium/matrix.

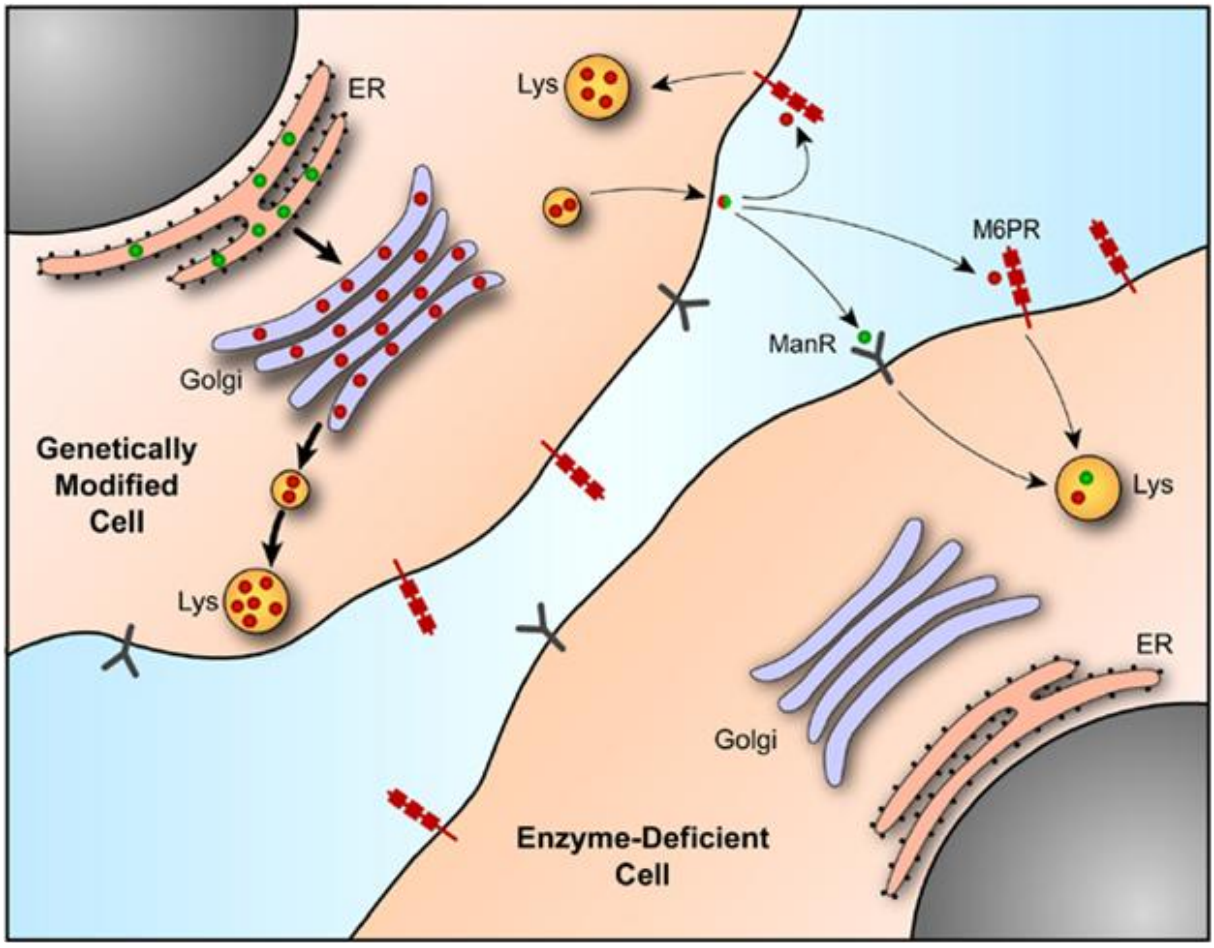


Figure 1.3: Principles of lysosomal enzyme trafficking and cross correction.

Lysosomal enzymes are glycosylated (green circles) within the endoplasmic reticulum (ER) of normal and/or genetically modified cells, before being transported to the Golgi apparatus for acquisition of mannose-6-phosphate (red circles). This modification allows the enzyme to bind either the plasma membrane localised mannose-6-phosphate receptor (M6PR – ubiquitously expressed) or the mannose receptor (ManR – limited to cells of the reticuloendothelial system). Majority of the enzymes released from the Golgi apparatus are trafficked to mature lysosomes (lys), while a minority of the enzymes are secreted from the cell where they bind either M6PR (phosphorylated) or ManR (unphosphorylated). Endocytosis and subsequent lysosomal targeting of exogenous enzymes can be mediated by both receptors.

Adapted from Sands and Davidson (2006).

1.2.1.2 Bone marrow transplant

Allogeneic matched donor BMT is a once off procedure for the introduction of hematopoietic stem cells (HSCs) that produce functional IDUA. However, a 12-24 month lag period is generally associated with BMT, where slow replacement of fixed tissue macrophages and microglial populations by the HSCs occurs in contrast to the quick progression of disease (Rovelli 2008). Therefore prior to treatment, disease progression and current clinical status must be assessed to allow extrapolation of clinical condition and subsequent eligibility for transplantation. The more severe the phenotype and the longer the interval between the onset of symptoms, subsequent diagnosis and the time taken to find a suitable matched donor to transplantation, often leads to a poorer BMT outcome (Rovelli 2008).

When successful, BMT can prolong the life of the patient by preventing, slowing or halting progression of many, but not all, of the clinical manifestations associated with MPS I (Boelens 2006). However, the CNS improvement coupled with BMT is generally only seen when transplantation occurs before two years of age, with 6-12 months being optimal (Church, Tylee et al. 2007), and thus before the onset of significant neurological impairment. If transplantation does not occur until later in life, CNS pathology cannot be stabilized and treatment is often associated with ongoing neurological deterioration in addition to CNS complications such as vascular disease, metabolic disturbance, infection and chemotherapy-induced neurotoxicity (Yoshida, Hayakawa et al. 2008).

The first successful MPS I BMT was performed in 1980, and to date over 400 patients with severe MPS I have received HSCs transplants (Muenzer, Wraith et al. 2009). Although success rates associated with the transplant itself have improved with time, the mortality rates and risks linked to the procedure are still considerable. Factors that affect the success of the

procedure include: age at the time of transplant, degree of clinical involvement, donor compatibility, neurological development, cardiopulmonary status and the ability to achieve stable engraftment without rejection (Wynn, Wraith et al. 2009). MPS I patients who have undergone successful BMT have shown a rapid reduction of obstructive airway symptoms, preservation of intellectual development, growth and decreased gag storage.

Bone pathology and cardiac valvular deformities do not seem to respond as well to the transplant and often have to be corrected after BMT with additional orthopaedic and cardiovascular interventions (Boelens 2006; Muenzer, Wraith et al. 2009). BMT does not result in donor chimerism of MSCs, cells which give rise to bone forming osteoblasts, even under conditions where total haematopoietic engraftment occurs (Koc, Peters et al. 1999). At least 20-25% of patients also require a second transplant due to incomplete donor chimerism or graft failure (Rovelli 2008). The limited efficacy of BMT is most likely associated with the restricted synthesis and secretion capacity of HSCs to produce high enough circulating levels of IDUA and the ability of cells to cross the blood-brain-barrier (BBB). BMT is therefore used primarily in young patients with CNS involvement, but not in those with milder disease (Wynn, Wraith et al. 2009).

An animal study has shown that transduction of HSCs with a viral construct encoding *IDUA* leads to improved resolution of pathology due to increased synthesis and secretion of functional enzyme from engrafted cells that is available for cross-correction of adjacent and distant cells via diffusion through the circulation (Visigalli, Delai et al. 2010). However, this approach is yet to be tested in patients.

1.2.1.3 Enzyme replacement therapy

ERT replaces the deficient IDUA enzyme with laronidase; a recombinant form of IDUA (Aldurazyme®, Genzyme), and has been clinically available since April 2003 (Thomas, Jacobs et al. 2006). However, with a molecular weight of 87 kDA (Barton and Neufeld 1971), recombinant IDUA is too large to diffuse across the BBB from the peripheral circulation into the CNS, or into avascular tissues such as the cornea and joints. MPS I Hurler patients, who have severely established CNS involvement, are not usually treated with ERT as the therapy is ineffective at improving cognitive or CNS function. However, MPS I patients with Scheie or Hurler-Scheie phenotypes, without CNS involvement, who are placed on weekly intravenous (*i.v.*) laronidase have improved heart and lung function, decreased urinary gag output and decreased spleen and liver size. Some patients also showed improved shoulder flexion, endurance, activity, visual acuity and sleep apnea/hypoapnea index (Neufeld and Muenzer 2001; Sifuentes, Doroshow et al. 2007; Clarke, Wraith et al. 2009; Muenzer, Wraith et al. 2009).

Bone and CNS pathology is not reversed by ERT, although early initiation of ERT, prior to onset of disease or before irreversible damage has occurred, has been shown to be more effective in slowing the development of skeletal and neurological deficits associated with MPS I (Clarke, Wraith et al. 2009; Muenzer, Wraith et al. 2009). The annual cost of ERT treatment is approximately $340,000 USD per year per child (Beutler 2006), often with subordinate doses of recombinant enzyme being received by sufferers; shown through a vast improvement in disease pathology when laronidase dosage is doubled in a canine model (Dierenfeld, McEntee et al. 2010).

1.2.2 Potential therapies

GT and SCT, like BMT and ERT, aim to replace the deficient lysosomal enzyme to aid in the degradation of accumulating gags, while SDT acts to reduce the initial synthesis of gag chains to provide a lesser burden on the residual enzyme. Both GT and SDT are often seen in combination with SCT, to better combat lysosomal distension associated with MPS and associated disease phenotype.

1.2.2.1 Gene therapy

GT is the insertion of a normal functioning gene into affected cells using a viral vector delivery system. Both lentiviral and AAV vectors have the ability to integrate foreign DNA into the target cells' chromosome and transduce non-dividing cells, resulting in stable, long-term expression of the gene of interest. Additional clinical advantages of the lentivirus include low immune response activation, capacity to be readily produced in large titres and easy pseudotyping to permit extensive cellular tropism (Naldini, Blomer et al. 1996; Naldini, Blomer et al. 1996; Miao, Nakai et al. 2000). Vector delivered via intrathecal injection allows effective and widespread distribution of the target gene to the brain with some benefit to somatic tissues, as the vector is able to cross the ependymal lining of the ventricles and interchange between the cerebral spinal fluid and interstitial fluid due to lack of tight junctions (Bielicki, McIntyre et al. 2010; McIntyre, Byers et al. 2010).

In vitro evaluation of a late generation lentivirus encoding human *IDUA* found that the vector was able to mediate and sustain high levels of IDUA expression (1.5 fold above normal) within MPS I fibroblasts, leading to clearance of intracellular gag chains (Di Natale, Di Domenico et al. 2002). Excess IDUA enzyme secreted from transduced cells in precursor form is endocytosed and correctly cleaved and processed to its mature form via the M6PR in

non-transduced cells, representing cross-correction. This study demonstrated that the lentivirus provides a successful mode of delivery and subsequent expression of the *IDUA* gene to deficient cells (Di Natale, Di Domenico et al. 2002).

Lentiviral-mediated or AAV-mediated administration of IDUA in the MPS I mouse can lead to sustained expression of enzymatically active IDUA in multiple organs, with the liver generally receiving the highest level of vector (Kobayashi, Carbonaro et al. 2005; Herati, Ma et al. 2008). Associated markers of MPS I disease are almost normalised in mice treated as neonates, while symptoms are only partially improved when treatment is initiated in young adults. These results indicate that early intervention allows prophylactic treatment rather than post-hoc management (Kobayashi, Carbonaro et al. 2005; Herati, Ma et al. 2008). However, an attempt to reproduce these results in a larger animal model was less successful; with an increase in injections required and the addition of immunosuppression to prevent clearance of the vector and immune recognition of human IDUA (Ciron, Desmaris et al. 2006). These results highlight the difficulty associated with the application of new approaches to treatment and the complexity of translating successful results from one animal model to another.

1.2.2.2 Substrate deprivation therapy

Unlike ERT, BMT and GT which aim to introduce new functional enzyme, SDT works by inhibiting the initial synthesis of gag chains in order to decrease their accumulation within lysosomes. Due to the small molecular size of SDT inhibitors, these molecules have the capacity to cross the BBB and hence directly target CNS pathology (Roberts, Rees et al. 2007). SDT inhibitors are believed to limit gag chain synthesis, directly or indirectly, by preventing sugar precursor formation and/or the activity of glycosyl transferases. Rhodamine B, a SDT inhibitor, has been shown to be effective in reducing the level of gag storage in the

liver and brain of MPS IIIA mice, leading to an improvement in disease pathology and CNS function (Roberts, Rees et al. 2007).

SDT has potential for MPS treatment as it helps to address the balance between synthesis and degradation of gags, which leads to a decreased burden on the deficient enzyme and also has the major advantage of being able to cross the BBB. For SDT to be an effective stand-alone treatment there must be some residual enzyme activity, and is therefore more suited to patients suffering an attenuated form of MPS. However, when combined with other forms of therapy which introduce new therapeutic enzyme to the host; such as SCT, SDT would become more applicable to patients with a wider range of residual enzyme activities.

1.2.2.3 Stem cell therapy

While HSCs (BMT) are currently being used in the clinical setting, other potential approaches utilizing stem cells that are in preclinical trials are embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs). ESCs are pluripotent cells derived from the inner cell mass of an embryo at the pre-implantation stage (Thomson, Itskovitz-Eldor et al. 1998) and were first isolated from the inner cell mass of a mouse blastocyst (Evans and Kaufman 1981; Martin 1981). Murine ESC lines genetically enhanced to express high levels of stable sulphamidase are currently being trialed in the mouse model of MPS IIIA to replace the deficient lysosomal enzyme in the brain and combat CNS degeneration (Robinson, Zhao et al. 2010). Although ESCs possess pluripotency and unlimited capacity to proliferate, ethical considerations currently limit their clinical use (Thomson, Itskovitz-Eldor et al. 1998).

Human iPSCs share common traits with human ESCs, but bypass the ethical constraints as they are able to be generated from neonatal or adult dermal fibroblasts through the introduction of four defined transcription factors; Oct4, Sox2, Nanog and Lin-28 (Jiang, Lv et al. 2011; Wada, Wang et al. 2011). Currently, iPSCs are being investigated as a transplantation therapy for stroke in a rodent model of middle cerebral artery occlusion (Jiang, Lv et al. 2011) and also as amyloid β-specific clearing macrophages to help reduce inflammation and plaque formation in Alzheimer's disease (Senju, Haruta et al. 2011).

1.3 Mesenchymal stem cells

MSCs are clonogenic, non-haematopoietic stem/progenitor cells that reside in a range of tissues and are characterized by their intrinsic self-renewal capacity which allows them to differentiate into multiple lineages, including; osteoblasts, chondrocytes, adipocytes, smooth muscle cells and myelo-supportive fibroblasts (Figure 1.4) (Gronthos, Brahim et al. 2002; Jiang, Jahagirdar et al. 2002; Gronthos, Zannettino et al. 2003; Le Blanc 2003). Recent studies have also shown that MSCs are able to form myocytes, cardiomyocytes, epithelial cells, hepatocytes and neurons *in vitro*, however, this may be dependent upon the tissue source of MSCs and/or the associated growth factors available during differentiation (Majumdar, Thiede et al. 2000; Jiang, Jahagirdar et al. 2002; Fox, Chamberlain et al. 2007; Arthur, Rychkov et al. 2008). The specific lineage that a MSC becomes committed to is fundamentally influenced by environmental conditions and chemical cues which are present within specific organs and/or tissues; which can be mimicked *in vitro* by altering culture conditions and available growth factors (Liu, Zhuge et al. 2009). Given the effect of environmental conditions on their differentiation, MSCs potential *in vivo* is dictated by their site of engraftment due to the effect of surrounding cells.

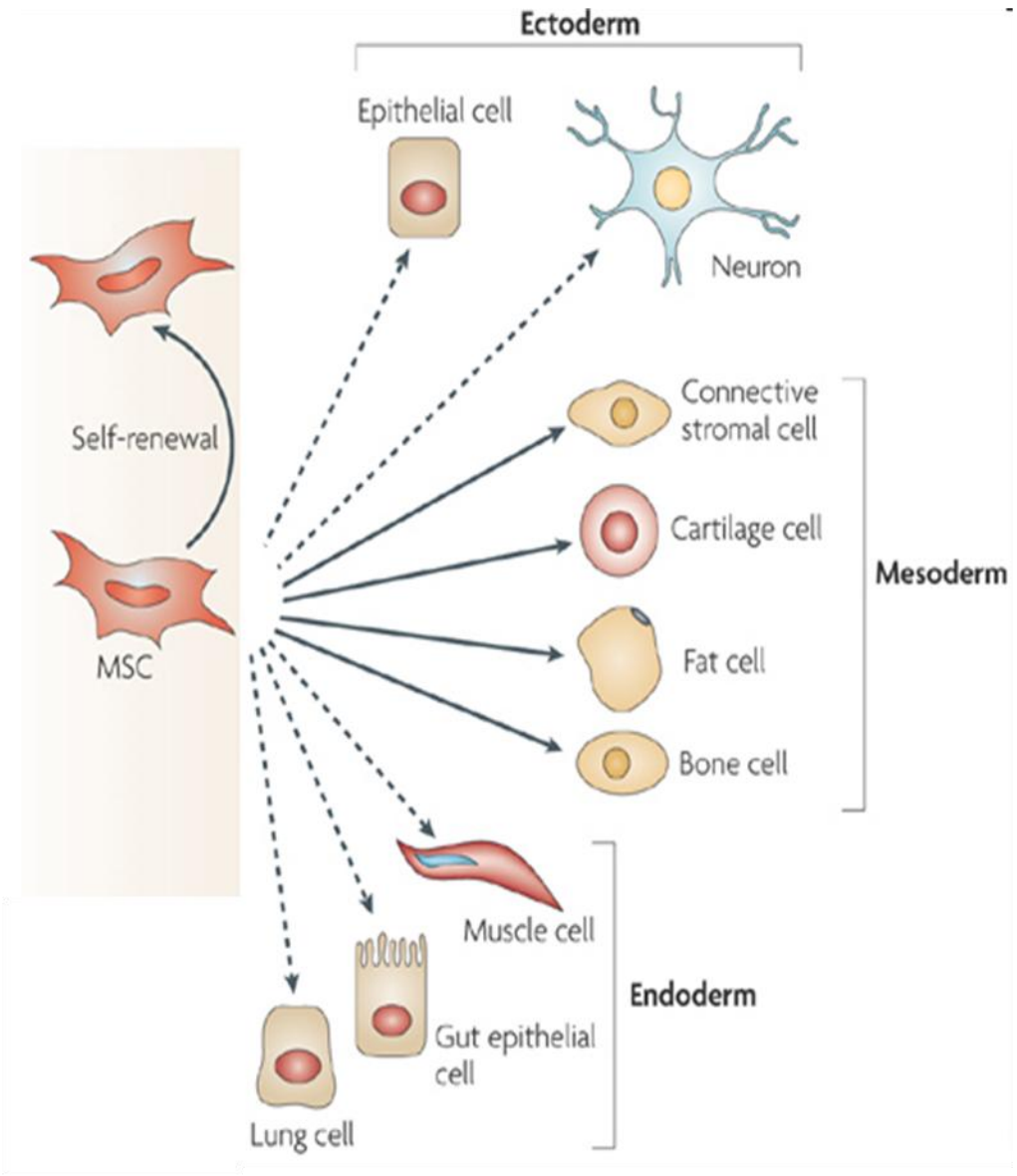


Figure 1.4: Mesenchymal stem cells have a multipotent differential capacity.

Mesenchymal stem cells are multipotent, non-haematopoietic stem cells that have the capacity to self renew (curved arrow) and differentiate down multiple lineages; primarily into cells of the mesoderm lineage (solid lines). MSCs are reported to be able to transdifferentiate into cells of the ectoderm and endoderm *in vitro* (dashed lines), however this is still controversial *in vivo*.

Adapted from Uccelli *et al.* (2008).

MSCs from the bone marrow were first isolated in 1974 by Friedenstein *et al*. (Friedenstein, Chailakhyan et al. 1974) and have since become the tissue of choice in many studies, however, MSCs have since been isolated from a range of tissues, including; placental tissue (Fazekasova, Lechler et al. 2010), peripheral blood (Kuznetsov, Mankani et al. 2001; Cao and Feng 2009), adipose tissue (Zuk, Zhu et al. 2001), umbilical cord blood (Bieback, Kern et al. 2004), dental pulp (Gronthos, Mankani et al. 2000; Yamaza, Kentaro et al. 2010) and connective tissue of the dermis and skeletal muscle (Jiang, Vaessen et al. 2002). MSCs have been isolated from various species, including; human, mouse, guinea pig, rabbit, pig, dog, sheep and chick (Castro-Malaspina, Gay et al. 1980; Jessop, Noble et al. 1994; Kadiyala, Young et al. 1997; Pittenger, Mackay et al. 1999; Martin, Cox et al. 2002; Ringe, Kaps et al. 2002; Gronthos, Zannettino et al. 2003; Shao, Goh et al. 2006; Abdallah, Boissy et al. 2007). While similarities exist between MSCs obtained from different species, prior to clinical application, knowledge gained from experimenting with animal models must first be validated in the human system (Wagner and Ho 2007).

For a population of cells to be classified as MSCs they must meet the minimal requirement as stipulated by the International Society for Cytotherapy and satisfy three criteria, including; i) greater than 95% of cells in culture must express CD105, CD90 and CD73, while 95% of cells in culture must lack the expression of haematopoietic antigens such as; CD34, CD14, CD45 or CD11b, CD79α or CD19 and HLA-DR, ii) cells in culture must exhibit plastic adherence, and iii) cells must be able to differentiate into fat, bone and cartilage (Dominici, Le Blanc et al. 2006). However, these criteria are not unique to MSCs, where fibroblast populations derived from skin, gingival and other tissues express the MSC associated markers at similar levels and exhibit many of the functional properties described above (Wada, Menicanin et al. 2009; Wada, Wang et al. 2011).

Prospective isolation of human clonogenic MSC from bone marrow and other tissues based on the immunophenotype; STRO-1^{bright}/STRO-3^{+}/STRO-4^{+}/$CD106^{+}$/$CD146^{+}$/$CD14^{-}$/$CD34^{-}$/$CD45^{-}$/Glycophorin-A^{-}, has led to the characterization of purified multi-potential MSC-like populations and determined that their progeny are a heterogeneous mix of committed progenitors with a minor fraction of cells retaining a "stem cell state" following *ex vivo* expansion (Gronthos, Zannettino et al. 2003; Shi and Gronthos 2003; Zannettino, Paton et al. 2007; Gronthos, McCarty et al. 2009). In the case of murine MSCs, Sca-1 has been utilized for cell identification and isolation (Xu, De Becker et al. 2010).

1.3.1 Isolation and characterisation

Various surface antigens (listed above) can be utilized for positive selection and isolation of a population of MSCs (Gronthos, Graves et al. 1994; Gronthos, Zannettino et al. 2003; Shi and Gronthos 2003; Nadri and Soleimani 2007; Zannettino, Paton et al. 2007; Gronthos, McCarty et al. 2009), alternatively haematopoietic surface markers (listed above) can also be used to negatively select stem cell populations (Baddoo, Hill et al. 2003; Zannettino, Paton et al. 2007). MSCs isolated from various tissues exhibit similar phenotypic characteristics, however, isolated cells do show different tendencies to differentiate and proliferate in response to stimulation by different growth factors (Sakaguchi, Sekiya et al. 2005). While isolation methods, culture medium, seeding density, culture surface, and growth and chemical factors influence the expansion, differentiation and immunogenic properties of the isolated MSCs (Sotiropoulou, Perez et al. 2006). *In vitro* bone marrow derived MSCs are limited to approximately 25-40 population doublings (Shi, Gronthos et al. 2002), or about 8-15 passages, before their proliferation ability stops and cells become senescent, after which cells proliferate slower and become less packed in culture due to their increase in cell size (Digirolamo, Stokes et al. 1999; Fehrer, Laschober et al. 2006).

It is believed that MSCs utilize selectins, chemokines and integrins secreted on the surface of cells to home to damaged tissues (Fox, Chamberlain et al. 2007), however, a cascade of events is involved, including; rolling of MSCs within the blood vessels, endothelial cell surface adhesion, transendothelial migration, blood vessels extravasation, and migration through the extracellular matrix to the site of damage (Liu, Zhuge et al. 2009).

1.3.2 MSCs for therapeutic use

MSCs have been used in a wide range preclinical studies and clinical trials due to recent advances in regenerative medicine and biotechnology, allowing the use of the body's own stem cells to overcome associated ethical concerns. MSCs have been implemented in preclinical studies for their use in heart disease (Itescu, Schuster et al. 2003; Amado, Saliaris et al. 2005), stroke or spinal cord injury (Chopp, Zhang et al. 2000; Mezey, Chandross et al. 2000; Chen, Li et al. 2001), diabetes (Chen, Jiang et al. 2004; Oh, Muzzonigro et al. 2004; Tang, Cao et al. 2004), kidney and liver disease (Ito, Suzuki et al. 2001; Kale, Karihaloo et al. 2003; Fang, Shi et al. 2004; Abdel Aziz, Atta et al. 2007), Crohn's disease (Gonzalez, Gonzalez-Rey et al. 2009; Akiyama, Chen et al. 2012), osteogenesis imperfect (Pereira, O'Hara et al. 1998; Guillot, Abass et al. 2008; Jones, Moschidou et al. 2012), graft versus host disease (GvHD) (Jang, Kim et al. 2013; Tobin, Healy et al. 2013), osteoarthritis (Augello, Tasso et al. 2007; van Buul, Siebelt et al. 2014), and to a lesser extent MPS (Meyerrose, Roberts et al. 2008; da Silva, Pereira et al. 2012; Martin, Stilhano et al. 2014).

MSCs were first trialled in cancer remission patients in 1995, to determine infusion safety of *ex vivo* expanded BM MSCs (Lazarus, Haynesworth et al. 1995). Since 1995, nearly 400 clinical trials have been registered for their use with MSCs (www.clinicaltrials.gov) (Wang, Qu et al. 2012), largely due to findings from animal models of disease. Clinical trials can be

separated into 12 pathological conditions, with major areas of interest including: bone and cartilage disease, including osteoarthritis and osteogenesis imperfect (Horwitz, Prockop et al. 1999; Horwitz, Prockop et al. 2001; Horwitz, Gordon et al. 2002; Le Blanc, Gotherstrom et al. 2005; Orozco, Munar et al. 2013; Wong, Lee et al. 2013; Vangsness, Farr et al. 2014), heart disease (Chen, Fang et al. 2004; Chen, Liu et al. 2006; Janssens, Dubois et al. 2006; Hare, Traverse et al. 2009; Jeevanantham, Afzal et al. 2013; Rodrigo, van Ramshorst et al. 2013), liver disease (Lin, Zhang et al. 2012; Shi, Zhang et al. 2012; Zhang, Lin et al. 2012; Mohamadnejad, Alimoghaddam et al. 2013; Wang, Li et al. 2013) and GvHD (Le Blanc, Rasmusson et al. 2004; Zhang, Liu et al. 2009; Perez-Simon, Lopez-Villar et al. 2011; Calkoen, Vervat et al. 2014; Herrmann and Sturm 2014).

Numerous phase I/II trials have also been conducted in diabetes (Bhansali, Upreti et al. 2009; Lin, Wang et al. 2009; Jiang, Han et al. 2011), brain disease (Bang, Lee et al. 2005; Lee, Hong et al. 2010; Han, Chang et al. 2011), multiple sclerosis (Bonab, Sahraian et al. 2012; Hou, Liu et al. 2013), cancer (Koc, Gerson et al. 2000), spinal cord damage (Karamouzian, Nematollahi-Mahani et al. 2012; Dai, Liu et al. 2013), Crohn's disease (Ditschkowski, Einsele et al. 2003; Garcia-Olmo, Garcia-Arranz et al. 2005; Garcia-Olmo, Herreros et al. 2009) and lung disease (Tzouvelekis, Paspaliaris et al. 2013; Weiss, Casaburi et al. 2013). MSCs have also been trialled for their safety and immunomodulation of GvHD in MPS I patients following HSC transplantation (Koc, Day et al. 2002).

MSCs are capable of differentiating down multiple cell lineages; including both neural and bone, and therefore have the potential to overcome previous therapy drawbacks that do not adequately address the skeletal and neurological disease associated with MPS. IDUA over-expressing MPS I derived MSCs that were injected into the left ventricle of MPS I affected mice resulted in improved functional performance and brain gag resolution. However, due to

the CNS directed administration, somatic pathology was not assessed, suggesting that this route of administration would not combat the widespread pathology associated with MPS I (da Silva, Pereira et al. 2012). Meyerrose *et al*. (2008) (Meyerrose, Roberts et al. 2008) showed that human MSCs transduced with a lentivirus encoding β-glucuronidase and injected intraperitoneally into a murine model of MPS VII can increase circulating serum levels of β-glucuronidase to nearly 40% of normal. This elevation led to an increase in enzyme present within all organs surveyed, reduced lysosomal distension and normalisation of secondary elevated enzymes. More importantly, brain pathology was minimised and a high level of bone marrow correction was seen. The superior results were attributed to the greater secretory capacity of MSCs when compared to other cell types, allowing widespread uptake by non-engrafted cells via the M6PR. However, MSCs were administered to neonatal mice, prior to established pathology, which is not generally applicable to MPS I patient diagnosis (Meyerrose, Roberts et al. 2008).

1.3.3 Homing and immunomodulation

MSCs can be therapeutically administered via several different approaches, most commonly either directly to the target site or via systemic intravascular delivery. In the case of target site administration, MSCs can directly interact with the identified environment, therefore mobilization and/or homing mechanisms are not vital, but survival and proliferation signals are paramount. However, when MSCs are systemically administered their homing capability and recruitment mechanisms play a crucial role in their ability to reach the target site (Liu, Zhuge et al. 2009). MSCs, when injected *i.v.*, have been found to engraft within the brain, indicating that MSCs are able to cross the BBB (Chen, Li et al. 2001; Honma, Honmou et al. 2006; Omori, Honmou et al. 2008; Li, Liao et al. 2011). However, studies have also shown that intra-arterial infusion of MSCs provides a widespread distribution of MSCs into target organs, when compared to *i.v.* infusion which leads to MSCs becoming trapped within the

lung (Love, Wang et al. 2007; Hale, Dai et al. 2008; Togel, Yang et al. 2008; Walczak, Zhang et al. 2008). MSCs have also been detected in a wide variety of tissues between nine and 21 months after systemic infusion, indicating that MSCs are able to engraft and proliferate within a host and maintain distribution long-term (Devine, Cobbs et al. 2003).

GvHD is a common risk factor associated with the introduction of foreign immune cells into an immunosupressed host (Sato, Ozaki et al. 2010; Kebriaei and Robinson 2011); however, MSCs possess intrinsic immunomodulatory properties that allow the normalization of the immune system following transplantation through the inhibition of T-cell proliferation (Le Blanc 2003). Due to their apparent lack of immunogenicity, introduction of MSCs into the host can be either from an autologous or allogeneic source (Reiser, Zhang et al. 2005; Chamberlain, Fox et al. 2007; Le Blanc, Frassoni et al. 2008; Scherer, van Pel et al. 2010). These factors make MSCs a prime vehicle to be coupled with gene therapy to allow the introduction, engraftment and distribution of functional enzyme to a wide range of deficient and affected tissues. The immunomodulatory properties associated with MSCs will mean that patients undergoing MSC based therapy will not require acute immunosuppression prior to transplantation, and a perfect matched donor will not be required, as allogeneic cells are compatible (Le Blanc 2003).

1.3.4 Immunosuppression

MSCs are known to have immunomodulatory properties when administered into an allogeneic host (Le Blanc 2003; Kebriaei and Robinson 2011; De Miguel, Fuentes-Julian et al. 2012), however, their ability to function across species without rejection is still largely debated (Niemeyer, Vohrer et al. 2008; Li, Ezzelarab et al. 2012; Kim, Lee et al. 2013). Majority of studies conclude that MSCs from one species can engraft and promote tissue recovery in

another species (Jiang, Cui et al. 2003; Allers, Sierralta et al. 2004; Niemeyer, Vohrer et al. 2008; Lin, Chern et al. 2011; Li, Ezzelarab et al. 2012), however, 75% of studies that assessed MSCs xenotransplant potential were performed in fully or partially immunodeficient mice receiving human MSCs (Li, Ezzelarab et al. 2012).

1.3.4.1 Cyclosporin mediated immunosuppression

Cyclosporin is a wide-acting immunosuppressive agent that reduces the activity of the immune system by inactivating calcineurin and inhibiting the growth and activity of T cells and their soluble mediators (Epstein 1996; Byun, Kim et al. 2012). Cyclosporin administration is commonly used to prevent rejection, or GvHD, following organ transplantation (Goring, Levy et al. 2014; Levy, Villamil et al. 2014; Silva, Yang et al. 2014).

Cyclosporin has previously been observed to prevent neuronal cellular damage and reperfusion injury following traumatic brain injury through preventing the destruction of mitochondria and the subsequent release of caspases by unbinding cyclophilin from the mitochondrial pore and transiently preventing the influx of calcium, which ultimately leads to mitochondrial swelling (Xiong, Gu et al. 1997; Albensi, Sullivan et al. 2000; Sullivan, Rabchevsky et al. 2000; Sullivan, Thompson et al. 2000; Starkov, Chinopoulos et al. 2004; Mazzeo, Brophy et al. 2009).

1.4 MPS animal models

In vitro studies where the deficit is mirrored in tissue culture, as in MPS I, unlimited material is accessible to examine and investigate the biochemical defect and for the subsequent development of prospective therapeutic approaches. However, application of acquired

understanding and knowledge from *in vitro* studies to treatment of patients remains complex, making animal models a fundamental aspect for bridging the gap between preliminary assessment and potential therapies.

Animal models are a vital component of MPS I disease analysis as they permit characterisation of clinical pathology, examination of genotype-phenotype correlations and also allow the development and assessment of disease therapies before their use in humans. Canine (Shull, Munger et al. 1982), feline (Haskins, Jezyk et al. 1979) and murine (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998; Ohmi, Greenberg et al. 2003) models of MPS I exist; with canine and feline occurring naturally. Although the animal models provide a good account of disease progression and associated biochemistry of MPS I, it must be noted that persistence to sexual maturity is seen in murine (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998), canine (Shull, Munger et al. 1982) and feline (Haskins, Jezyk et al. 1979) models which indicates that animals die at a later point in life than humans with Hurler syndrome (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998; Neufeld and Muenzer 2001).

1.4.1 MPS I murine model

The murine model of MPS I provides many advantages over both the feline and canine animal models as: i) analysis of murine development and behaviour has been well characterised and documented, ii) large colony numbers can be generated in a short period of time and iii) progeny exhibit less genetic variation between generations. The murine model has also been shown to be an accurate model of human disease (Rogers, Fisher et al. 1997; Neufeld and Muenzer 2001; Rogers, Peters et al. 2001).

The *Idua* knockout mouse was created by neo cassette insertion into exon VI of the *Idua* gene, producing homozygous mice that have no residual IDUA enzymatic activity (Clarke, Russell et al. 1997). Selective disruption of the *Idua* gene was essential during the establishment of the murine model, as contained within and partially overlapping the murine *Idua* gene is a canalicular sulphate transporter gene; *Sat-1*. Disruption of the *Idua* gene alone, without affecting other surrounding genes, was essential in order to mimic the human genetic disease profile (Clarke, Russell et al. 1997).

Homozygous affected mice show severe physical and skeletal abnormalities, elevated lysosomal storage, increased urinary gag output and progressive neuronal loss within the cerebellum (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998). Comprehensive examination of *Idua* -/- mouse brain reveals evidence of progressive Purkinje cell loss with age, abnormal lysosomal storage within glial cells of the cortex, increase in neurons size within the caudate nucleus and cytoplasmic vacuolation within; Purkinje cells of the cerebellum, neurons of the cerebral cortex, leptomeninges and glial cells (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998). From four weeks of age bone morphology and physiology changes are evident; with *Idua* -/- mice showing foreshortening of the premaxillary bones and enlargement of the cranium. The zygomatic, squamosal and malar process appear smaller and denser, while the rib cage appears wider and thicker. With age, regular thickening of the diaphysis of long bones becomes apparent, and widening of the zygomatic arch and malar processes becomes amplified. Joint deformity occurs in the paws which manifests as shortened phalanges, while the pelvis is usually poorly formed which is often evident by an altered gait (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998; Neufeld and Muenzer 2001).

Idua -/- mice show behaviour and learning deficits when compared to normal mice in; marble burying task, novel object recognition, rotarod, open field analysis and spatial learning and memory tasks (Reolon, Braga et al. 2006; Pan, Sciascia et al. 2008; Baldo, Mayer et al. 2012). These findings closely correlate to behavioural findings of MPS I patients and indicate that murine *Idua* (-/-) knockout is an accurate animal model of human disease and will be a valuable tool for the assessment of new disease therapies.

1.5 Behaviour testing in rodents

The three-stage SHIRPA protocol is a semi-quantitative analysis that was developed to allow standardised assessment of mouse behaviour (Rogers, Peters et al. 2001). It provides the framework for a full clinical characterisation of the appearance and normal behaviour of the animal in a manner consistent between different research groups and animal models (Rogers, Fisher et al. 1997). Stage one is used for high throughput analysis which bases assessment on observations to provide a behavioural and functional profile, while stages two and three are adapted to examine a particular biological system; such as the nervous system, therefore providing a more comprehensive behavioural and pathological assessment (Table 1.2) (Rogers, Fisher et al. 1997; Rogers, Peters et al. 2001).

1.5.1 Inverted grid

Neuromuscular strength is measured using the inverted grid test; which falls into the first tier of the SHIRPA protocol (Table 1.2) (Rogers, Fisher et al. 1997; Rogers, Peters et al. 2001). The inverted grid is used to quantitatively assess mice with CNS degeneration and/or skeletal deformities by placing the animal upside-down on a wire grid suspended above a cushioned surface, and recording the number of hind limb movements and latency to fall of each animal (Rogers, Fisher et al. 1997; Jeyakumar, Butters et al. 1999; Rogers, Peters et al. 2001). While

Table 1.2: Three stage SHIRPA protocol and related parameters.

Tier	Observations	Our assessment
First (primary)	Behavioural Observation Profile: - Gait/Posture - Motor control/Coordination - Excitability/Aggression - Muscle tone/Body weight	Inverted grid Weight analysis Daily monitoring
Second (secondary)	Locomotor Activity Food and Water Intake Balance and Coordination Analgesia Histology Biochemistry	Rotarod Open field Post-mortem analysis
Third (tertiary)	Anxiety Learning and Memory Prepulse Inhibition Electromyography Electroencephalography Nerve Conduction Magnetic Resonance Imaging	Open field Water cross maze

Primary analysis is based on a scored observational assessment to develop a behavioural and functional profile, the secondary stage of investigation provides a comprehensive pathological and behavioural appraisal, while the tertiary stage of examination is focused on neurological disease.

Adapted from Rogers *et al.* (1997) and Rogers *et al*. (2001).

the inverted grid has not been used previously used to determine neuromuscular strength in MPS I mice, neurological and/or skeletal involvements have been investigated in murine models of MPS type IIIA (Hemsley and Hopwood 2005; Langford-Smith, Langford-Smith et al. 2011), IIIB (Langford-Smith, Malinowska et al. 2011) and VII (Macsai, Derrick-Roberts et al. 2012; Derrick-Roberts, Pyragius et al. 2014).

1.5.2 Rotarod

Motor function, coordination and balance can be evaluated using the rotarod, which comes under the second tier of the SHIRPA protocol (Table 1.2) (Rogers, Fisher et al. 1997; Rogers, Peters et al. 2001). Animals are placed on a rotating drum, at either constant or accelerating speeds, and their latency to fall and speed achieved is recorded. The rotating drum stimulates mice to perform each trial, while operator bias is removed as individual performance is recorded by an automated system. Both neurological deficits and skeletal pathology will affect the animals performance on the rotarod (Dunham and Miya 1957). The rotarod has been previously used to detect differences between MPS I mice and age-matched normal controls (Garcia-Rivera, Colvin-Wanshura et al. 2007; Nan, Shekels et al. 2012), as well as other MPS murine models (Heldermon, Hennig et al. 2007; Polito, Abbondante et al. 2010; Macsai, Derrick-Roberts et al. 2012; Mohammed, Snella et al. 2012; Derrick-Roberts, Pyragius et al. 2014; Frohbergh, Ge et al. 2014).

1.5.3 Open field

The open field test is used to measure the exploratory activity and anxiety of mice; which comes under both the second and third tier of the SHIRPA protocol (Table 1.2) (Rogers, Fisher et al. 1997; Rogers, Peters et al. 2001). The number of horizontal and vertical beam crosses can be converted into total distance travelled and number of rearing events, assessing

the spontaneous locomotor activity of each mouse which falls into tier two of the SHIRPA protocol. Total time spent in centred versus marginal regions of the apparatus, in conjunction with hyperactivity, can suggest anxiety, which falls into the third tier of the SHIRPA protocol (Rogers, Fisher et al. 1997; Rogers, Peters et al. 2001). The open field test has been previously utilised to determine behavioural changes between MPS I mice and age-matched normal controls (Reolon, Braga et al. 2006; Pan, Sciascia et al. 2008; Baldo, Mayer et al. 2012), as well as other murine models of MPS (Hemsley and Hopwood 2005; Bielicki, McIntyre et al. 2010; McIntyre, Byers et al. 2010; Polito, Abbondante et al. 2010; Langford-Smith, Langford-Smith et al. 2011; Langford-Smith, Malinowska et al. 2011; Macsai, Derrick-Roberts et al. 2012; Derrick-Roberts, Pyragius et al. 2014).

1.5.4 Water cross maze

Spatial learning and memory; or hippocampus function, can be measured using a water maze, which falls into the third tier of the SHIRPA protocol (Table 1.2) (Rogers, Fisher et al. 1997; Rogers, Peters et al. 2001). This behavioural test employs water as a negative stimulus and incorporates a submerged platform as a positive reward, which promotes learning and mouse participation. Mice are released directly into a circular pool (Morris water maze) or within a perspex cross (water T/cross-maze) filled with opaque water and required to find the submerged platform by using constant visual cues. Time taken to locate the submerged platform and number of correct/incorrect entries (water T/cross-maze only) are recorded. Previous MPS I murine studies have used the Morris water maze (Pan, Sciascia et al. 2008; Wolf, Lenander et al. 2011) and water T-maze (Ou, Herzog et al. 2014) to test spatial learning and memory deficits. Spatial learning deficits have also been determined in other murine models of MPS using both the Morris water maze (Bastedo, Sands et al. 1994; O'Connor, Erway et al. 1998; Frisella, O'Connor et al. 2001; Gliddon and Hopwood 2004; Sakurai, Iizuka et al. 2004; Crawley, Gliddon et al. 2006) and water-cross maze (Roberts, Rees et al.

2007; Macsai, Derrick-Roberts et al. 2012; Derrick-Roberts, Pyragius et al. 2014). The water cross-maze allows CNS deficits to be analysed separately from skeletal aspects of MPS I disease, by providing a buoyant testing environment which limits skeletal involvement, and the introduction of correct/incorrect entry parameters in addition to time latency to reach the platform.

1.6 Hypothesis and Aims

MSCs not only have the ability to differentiate down a wide range of lineages (Pittenger, Mackay et al. 1999; Jiang, Jahagirdar et al. 2002; Gronthos, Zannettino et al. 2003; Fox, Chamberlain et al. 2007; Arthur, Rychkov et al. 2008) and promote healing through suppression of immune cells, but are also capable of homing to sites of damage (Reiser, Zhang et al. 2005; Chamberlain, Fox et al. 2007; Fox, Chamberlain et al. 2007; Scherer, van Pel et al. 2010); making them an ideal candidate to link with the current gene therapy approach. This leads to the hypothesis and aim of my project:

Hypothesis: CNS pathology associated with MPS I will be alleviated following *i.v.* administration of human derived MSCs transduced with murine *Idua*.

Aims: To treat MPS I knockout mice with human derived MSCs transduced with murine *Idua* and to assess whether the over-expression of the deficient enzyme, utilizing MSCs as the vehicle, reduces MPS I associated pathology and leads to an improvement in lysosomal gag storage, CNS function or skeletal malformations.

1.6.1 Significance

MPS I is a very debilitating disease that results in CNS degeneration, skeletal malformations and somatic tissue damage; leading to premature death in the early teens. Current therapies available for MPS I help stabilise the somatic tissue and organ damage, but fail to fully address the CNS degeneration and skeletal deformities. MSCs are known to be able to: differentiate into many cell types affected by MPS (Jiang, Jahagirdar et al. 2002; Fox, Chamberlain et al. 2007; Arthur, Rychkov et al. 2008), have the ability to over-express lysosomal enzymes (Meyerrose, Roberts et al. 2008), are capable of crossing the BBB (Chen, Li et al. 2001) and also exhibit immunomodulatory properties; lowering the risk of GvHD (Sato, Ozaki et al. 2010; Kebriaei and Robinson 2011). These factors make MSCs a complementary vehicle for the trafficking, expression and secretion of murine *Idua* to help combat the incapacitating symptoms associated with MPS I and provide a novel therapy approach.

Chapter Two: Materials and Methods

2.1 Materials

Materials used are listed in Appendix A.

2.2 *In vitro* experimentation

2.2.1 Cell line basal growth media

Human derived MSCs, ranging from passage three through six, were sourced from either bone marrow (hBM MSCs) or dental pulp (hDP MSCs) of healthy donor patients (Gronthos, Mankani et al. 2000; Gronthos, Zannettino et al. 2003). MSCs were cultured in basal growth media consisting of α-MEM supplemented with; 10% (v/v) foetal calf serum (FCS), 2mM L-glutamine, 100μM L-ascorbate-2-phosphate, 1mM sodium pyruvate, 50U/mL penicillin and 50μg/mL streptomycin. Human bone marrow CD34+ (HSCs) cells were cultured in StemSpan supplemented with; 10% (v/v) FCS, 2mM L-glutamine, 50U/mL penicillin and 50μg/mL streptomycin. Human MPS I, human MPS IIIA and murine MPS VII skin fibroblasts were cultured in DMEM supplemented with; 10% (v/v) FCS, 2mM L-glutamine, 50U/mL penicillin and 50μg/mL streptomycin.

2.2.1.1 Cell line culture

Cells were maintained at 37°C in 5% CO2 and 90% humidity, with media changed twice weekly. Upon reaching 90% confluency, T75 flasks (Cellstar, Sigma-Aldrich Pty Ltd) containing either MSCs or fibroblasts were washed three times in phosphate buffered saline (PBS) and cells harvested with 10% (v/v) trypsin (0.12% porcine trypsin, 0.02% EDTA-2Na, 0.04% glucose, without Ca2+ or Mg2+). Cells were maintained with a 1:3 split.

2.2.2 Determination of enzyme activity

2.2.2.1 Sample collection

Enzyme assays were performed on the cell layer and media of both hBM and hDP MSCs and HSCs in triplicate. Cells were seeded at 10,500 cells per cm^2 in basal growth media. Cells were fed twice weekly; with HSCs being spun at 300 x *g* for five minutes to pellet cells prior to removal of old media. After one week, cells were fed and media collected 24 hours post-feeding and stored at -20°C, pending analysis. The cell layer was lysed for ten minutes at room temperature by the addition of 0.1% (v/v) triton X-100 in PBS, before being stored at -20°C, pending analysis.

2.2.2.2 Fluorogenic substrates

β-*D*-glucuronidase, IDUA and sulphamidase enzyme activity was determined as previously described by (Wolfe and Sands 1996), (Clarke, Russell et al. 1997) and (Karpova, Voznyi Ya et al. 1996), respectively. β-*D*-glucuronidase and IDUA samples were read on a LS 50B luminescence spectrometer using an AS 91 auto-sampler (Perkin Elmer, Waltham, Massachusetts, USA) at excitation 366nm and emission 446nm, while sulphamidase samples were read on a Wallac Plate reader at excitation 355nm and emission 460nm.

2.2.2.3 Radio-labelled substrates

Radio-labelled substrates for 2-sulfatase, 4-sulfatase, and 6-sulfatase were employed as previously described by (Hopwood 1979), (Hopwood, Elliott et al. 1986) and (Hopwood and Elliott 1983), respectively. The conversion of substrate to product was determined by HPLC using an Alltima C18-LL 5µm column (Adelab Scientific, Therbarton, South Australia, Australia). Samples were run on a 1200 series HPLC machine (Agilent Technologies,

Mulgrave, VIC, Australia) using a HPLC-LS-pump (raytest, Wilmington, NC, USA) and ChemStation for LC system software (B.04.02 [96]; Agilent Technologies).

2.2.2.4 Bradford protein assay

Enzyme activity was normalised to total protein using the Bradford protein assay as previously described by (Bradford 1976).

2.2.3 Virus production

Lentiviral vectors pHIV-EF1αmm*Gus*, pHIV-EF1αmm*Cos*, pHIV-EF1αrn*4S*, pHIV-EF1αeYFP and pHIV-EF1αluciferase were already available in our laboratory; however, pHIV-EF1αmm*Idua* was required to be constructed.

2.2.3.1 Plasmid preparation

Mouse codon optimized α-*L*-iduronidase was source from GenScript (Piscataway, US) contained within a pUC57 vector. pHIV-EF1αluciferase (4µg), containing chloramphenicol resistance, and pUC57-mm*Idua* (4µg) underwent NdeI/KpnI (New England Biolabs) digestion overnight at 37ºC, containing: 70 units NdeI, 70 units KpnI, 1x NEB 4 buffer (New England Biolabs) and 5ng bovine serum albumin (BSA).

2.2.3.1.1 pUC57-mmIdua purification

Following overnight digestion, entire pUC57-mm*Idua* sample was run in conjunction with SPP1 molecular weight ladder (Geneworks) on 0.8% (w/v) agarose, at 75V for 35 minutes, to

allow digested bands to be excised. Excised bands were purified using HiYield Gel/PCR DNA extraction kit (Real Genomics) as per the manufacturer's instructions.

2.2.3.1.2 pHIV-EF1αluciferase purification

Following overnight digestion, pHIV-EF1αluciferase vector was purified by the addition of one volume phenol/chloroform, vortexed and centrifuged at 13,000 *x g* for five minutes. 1/10th the volume 3M sodium acetate, pH 5.2, and three volumes absolute ethanol were added to the aqueous phase and DNA precipitated at room temperature for 30 minutes. Following precipitation, the sample was centrifuged at 13,000 *x g* for ten minutes at 4ºC, before the pellet was washed with 75% (v/v) ethanol. Following centrifugation at 13,000 *x g* for ten minutes, the supernatant was discarded, the pellet air dried and resuspended in 20µL 0.5% (v/v) TE buffer. Following resuspension, 2µL alkaline phosphatase calf intestinal enzyme (CIP; New England Biolabs) was added to prevent excised ends from rejoining.

2.2.3.2 Vector and insert ligation

Purified pHIV-EF1α vector (250ng) and mm*Idua* insert (100ng) were ligated together using 2U T4 DNA ligase (Roche) and 10x T4 ligase buffer (Roche) overnight at room temperature. Following overnight ligation, the ligation mixture was strata cleaned (Roche) to remove any salts; 0.5 volumes of strata clean resin was added to 20µL ligation mixture and vortexed for 15 seconds, before being centrifuged at 2,000 *x g* for one minute.

2.2.3.3 Transformation of sure E.coli cells

Sure *E*.coli cells were transformed with 250ng strata cleaned pHIV-EF1αmm*Idua* and electroporated at 1.7kV. Transformed cells were resuspended in 40 volumes TY broth and

incubated with shaking at 37ºC for one hour. Transformed culture broth was spread onto agar plates containing 30µg/mL chloramphenicol using aseptic techniques and placed in 37ºC incubator overnight.

2.2.3.4 Miniprep

After overnight incubation, colonies were picked using aseptic techniques and incubated with shaking at 37ºC overnight in 2mL TY broth and 30µg/mL chloramphenicol. Cultured pHIV-EF1αmm*Idua* was isolated by centrifugation at 13,000 *x g* for one minute and resuspended in 100µL of lysis buffer (2.5M lithium chloride, 50mM tris, 4% (v/v) triton X-100, 62.5mM EDTA, pH 8.0). An equal volume of phenol/chloroform/TN (Sigma) was added and vortexed for 30 seconds, before centrifugation at 13,000 *x g* for five minutes. Aqueous phase was precipitated at room temperature for two minutes by the addition of 0.65 volumes of isopropanol and centrifuged at 13,000 *x g* for ten minutes. Remaining pellet was washed with 75% (v/v) ethanol, before being resuspended in 20µL 0.5% (v/v) TE buffer.

2.2.3.4.1 Restriction enzyme digest of plasmid

Following miniprep purification, pHIV-EF1αmm*Idua* plasmid was digested with 5 units BstBI (New England Biolabs) and 10x NEB 4 buffer and incubated overnight at 65ºC. Following overnight digestion, 1µL digested plasmid was run in conjunction with SPP1 (Geneworks) on 0.8% (w/v) agarose at 100V for 35 minutes to determine if correct ligation and transformation had occurred.

2.2.3.5 Midiprep

Plasmid was incubated overnight with shaking at 37ºC in 100 volumes TY broth with 30µg/mL chloramphenicol. Plasmid was purified using Genopure plasmid midi kit as per the low copy number plasmid protocol (Roche applied Sciences). Equal volumes of culture and sterile glycerol were combined and stored at -80ºC for future analysis, pending confirmation of correct vector.

2.2.3.5.1DNA sequencing

Plasmid sequencing (350ng) was carried out by the IMVS sequencing centre (Adelaide, Australia) to determine correct vector ligation was achieved.

2.2.3.6 Gigaprep

Small amount of the glycerol stock (Section 2.2.3.5) was streaked onto an agar plate containing 30µg/mL chloramphenicol and incubated overnight at 37ºC. A single colony was picked and cultured in 2mL TY broth containing 30µg/mL chloramphenicol at 37ºC overnight with shaking. Miniprep purification (Section 2.2.3.4) and subsequent restriction enzyme digest (Section 2.2.3.4.1) were carried out to confirm correct plasmid had been selected. Purification of pHIV-EF1αmm*Idua* plasmid was performed using Endofree plasmid mega and giga kit (Qiagen) as per the manufacturer's protocol; except a 1:100 dilution was used from the starter culture to increase yield.

2.2.3.7 Virus preparation

Virus was produced in HEK broad 293T cells and purified by anion exchange. Briefly, 1.6 x 10^6 cells/cm^2 HEK broad 293T cells were transient transfected by calcium phosphate co-

precipitation using 2x HeBS and a DNA mix containing: 170µg PpHIV-EF1αmm*Idua* vector construct, 3.16µg expression constructs (pcDNA3Tat101ml and pHCMVRevmlwhvpre), 2µg helper construct (pHCMVgagpolmllstwhv), 7.9µg envelope glycoprotein (pVSV-G) and 2.5M calcium chloride. Cells were exposed to the precipitate for eight hours, before media was replaced with Opti-Pro SFM (Gibco, Life Technologies) containing 4mM glutamine and 4mM penicillin/streptomycin for an additional 40 hours. Medium containing virus was clarified at a flow rate of 6mLs per minute through a 50mm polydisc AS 0.45µM filter (Whatman, UK) and bound to two MustangQ Acrodiscs (Pall corporation, UK) via anion exchange, using a Watson Marlow 323 pump (Watson Marlow, UK). Virus was eluted from the discs by the addition of 1.5M sodium chloride and the eluate ultracentrifuged at 42,000 *x g* at 4ºC for 90 minutes using a Beckman Coulter optima L-100K ultracentrifuge and SW60 rotor. Resulting pellet was resuspended in 0.9% (w/v) saline and stored at -80ºC. Virus concentration was determined as p24 protein using the HIV-1 p24 ELISA kit.

2.2.3.8 Viral transfection

MSCs were seeded in α-MEM/10% (v/v) FCS and HSCs were seeded in StemSpan/10% (v/v) FCS at 10,500 cells per cm^2 and transfected three hours later with the addition of 4µg/mL polybrene and 50µg/mL gentamycin along with 0.0007µg/mL - 0.07µg/mL p24 protein of pHIV-EF1αeYFP (transduction efficiency), or 0.056µg/mL p24 protein of pHIV-EF1αmm*Idua*, pHIV-EF1αmm*Gus*, pHIV-EF1αmm*Cos* and pHIV-EF1αrn*4S* (cross correction and over-expression study). Cells were exposed to the virus for 24 hours before the media was removed and replaced with basal growth media. Non-transduced replicates underwent the same experimental protocol, but did not receive any lentiviral vector.

2.2.3.9 Transduction efficiency

Transduction efficiency of pHIV-EF1αeYFP was determined on duplicate wells using FACS analysis for both HSCs and MSCs. Two days post transfection duplicate wells for each viral concentration (listed in 2.2.3.8) were harvested using 10% (v/v) trypsin in PBS and transferred to a tube containing 1% (v/v) FCS in PBS before centrifugation at 390 *x g* for five minutes. The cell pellet was resuspended in 1% (w/v) paraformaldehyde in PBS and stored in the dark prior to analysis on a FACSCalibur (Becton Dickinson; BD, North Ryde, NSW, Australia) flow cytometry system equipped with CELLQuest software (Becton Dickinson; BD, Australia).

2.2.4 Mannose-6-phosphate mediated cross correction

2.2.4.1 Over-expression of enzyme in hDP or hBM MSCs

hBM and hDP MSCs were transfected with 0.56µg/µL p24 protein of pHIV-EF1αmm*Gus*, pHIV-EF1αmm*Idua* and pHIV-EF1αmm*Cos* as described in Section 2.2.3.8. After 24 hours of virus exposure, media was removed and replaced with basal growth media without FCS for 48 hours, before media was stored under sterile conditions at 4°C. Enzyme activity in the media was determined for each MPS enzyme (Section 2.2.2.2).

2.2.4.2 Cross-correction by addition of hDP or hBM derived enzyme

Human MPS I, human MPS IIIA and mouse MPS VII skin fibroblasts were incubated with DMEM/ 10% FCS containing 5µCi/mL $^{35}SO_4$ (Perkin Elmer) for 24 hours to label lysosomal gags. Cells were then harvested (Section 2.2.1.1) and seeded in 6-well plates at 10,500 cells per cm^2. After three hours, 5mM mannose-6-phosphate was added and cells incubated for a further 16 hours. The corresponding MPS fibroblasts were then incubated in the presence or

absence of medium containing IDUA, sulphamidase or β-*D*-glucuronidase (total of 50pmol/min, 10pmol/min and 50pmol/min enzyme activity respectively, determined using fluorogenic substrates; Section 2.2.2.2) derived from either hBM or hDP MSCs transduced with lentivirus encoding the appropriate MPS enzyme (Section 2.2.3.8). After 24 hours the medium was removed and the cell layer harvested by scraping into 20mM Tris 0.5M NaCl pH 7.0.

2.2.4.3 $35SO_4$ incorporation

Cells were freeze-thawed six times and an aliquot taken for scintillation counting on a Tri-Carb 2910 TR liquid scintillation analyser (Perkin Elmer). Cell protein was determined using the Bicinchoninic acid (BCA) protein assay kit with standards spiked with relevant volume of 20mM Tris 0.5M NaCl pH 7.0. Results expressed as counts per minute (cpm) per mg protein, with all analysis performed in duplicate.

2.2.5 Stable β-D-glucuronidase transduction without splitting

Replicate plates of hDP and hBM MSCs were seeded at 10,500 cells per cm^2 in α-MEM/10% FCS; 50% of wells were transfected three hours later (Section 2.2.1.1 and 2.2.3.8) with 0.56μg/μL p24 protein pHIV-EF1αmm*Gus*, and additional wells served as non-transduced controls. Cells were maintained in cell culture for eight weeks; with twice weekly feeding with basal growth media. Each week, triplicate wells were harvested 24 hours post feeding, and β-*D*-glucuronidase enzyme activity determined in both the medium and cell layer (Section 2.2.2.1 and 2.2.2.2).

2.2.5.1 Stable β-glucuronidase transduction with continual splitting

Replicate plates of hBM MSCs were seeded at 10,500 cells per cm^2 in α-MEM/10% FCS and transfected three hours later (Section 2.2.1.1 and 2.2.3.8) with varying concentrations of pHIV-EF1αmm*Gus*; 0.0007μg/μL - 0.07μg/μL p24 protein in duplicate, with additional wells serving as non-transduced controls. Upon reaching confluency, one plate was fixed with chloral-formal acetone for 30 minutes at 4°C and stained for β-*D*-glucuronidase (Sigma-Aldrich Pty Ltd) activity, as previously described (Macsai, Derrick-Roberts et al. 2012). Transduction efficiency was determined by counting pink (transduced) and clear (non-transduced) cells for each well. Wells of one plate were harvested with 10% (v/v) trypsin in PBS and passaged 1 in 3 in basal growth media. This process was repeated over an eight-week period *in vitro*, with replicate plates undergoing six passaging events.

2.2.5.2 Vector copy number per cell

Genomic DNA was isolated from the cell layer at each passage event using the Promega SV wizard genomic DNA purification kit, and vector copy number determined, as previously described (Derrick-Roberts, Pyragius et al. 2014).

2.2.6 Stem cell differentiation

2.2.6.1 Osteogenic differentiation

Replicate plates of hBM and hDP MSCs were seeded with 10,500 cells per cm^2 in α-MEM/10% (v/v) FCS. Three hours later 50% of the wells were transfected with 0.056μg/mL p24 protein of either pHIV-EF1αmm*Gus* or pHIV-EF1αmm*Idua* (Section 2.2.1.1 and 2.2.3.8), while additional wells served as non-transduced controls. After 24 hours, media was removed and replaced with either basal growth media or osteogenic induction media as per

Gronthos *et al.* (2011) with no β-mercaptoethanol (Gronthos and Zannettino 2011). Media was replaced twice weekly.

2.2.6.1.1 Osteogenic quantification

Once weekly, over a five week period, triplicate wells underwent von Kossa staining (BD technical bulletin #444, BD, Australia), β-*D*-glucuronidase staining (Macsai, Derrick-Roberts et al. 2012) or lysed in 0.1% (v/v) triton X-100 in PBS for determination of enzyme activity, or were extracted in 0.6M HCl for calcium quantification via σ-cresolphthalein-calcium reaction, for both transduced (± osteogenic induction media) and non-transduced (± osteogenic induction media) controls.

2.2.6.2 Adipogenic differentiation

Replicate plates of hBM and hDP MSCs were seeded with 10,500 cells per cm^2 in α-MEM/10% (v/v) FCS; 50% of wells were transfected three hours later with 0.056μg/mL p24 protein of either pHIV-EF1αmm*Gus* or pHIV-EF1αmm*Idua* (Section 2.2.1.1 and 2.2.3.8), while additional wells served as non-transduced controls. After 24 hours, media was removed and replaced with either basal growth media or adipogenic induction media as per Gronthos *et al.* (2011) with no β-mercaptoethanol (Gronthos and Zannettino 2011). Media was replaced twice weekly.

2.2.6.2.1 Adipogenic quantification

Once weekly, over a five week period, triplicate wells underwent oil red staining (Tang, Otto et al. 2003) or β-glucuronidase staining (Macsai, Derrick-Roberts et al. 2012) or lysed in 0.1%

(v/v) triton X-100 in PBS for determination of enzyme activity, for both transduced (± adipogenic induction media) and non-transduced (± adipogenic induction media) controls.

2.2.6.3 Chondrogenic differentiation

Replicate cell pellets for hBM and hDP MSCs were created by seeding 3.0 x 10^5 cells in DMEM/10% (v/v) FCS in a screw cap microfuge tube and centrifuging at 800 *x g* for five minutes. Three hours later, 50% of the cell pellets were transfected with 0.056μg/mL p24 protein pHIV-EF1αmm*Gus* (Section 2.2.3.8), while additional cell pellets served as non-transduced controls. After 24 hours, media was removed and replaced with either basal growth media or chondrogenic induction media. Chondrogenic induction media consisted of DMEM-high glucose supplemented with 50U/mL penicillin, 50μg/mL streptomycin, 1X ITS+ premix stock, 2mM L-glutamine, 1mM sodium pyruvate, 100μM L-ascorbate-2-phosphate, 0.1μM dexamethasone and 10ng/mL TGFβ3. Medium was replaced twice weekly.

2.2.6.3.1 Chondrogenic quantification

Once weekly, over a five week period, triplicate cell pellets were either paraffin embedded and sectioned for alcian blue staining (30 minutes in 1% (w/v) alcian blue pH 1.0 and counterstained for two minutes in haematoxylin) or were snap frozen in OCT and sectioned for β-*D*-glucuronidase staining (Macsai, Derrick-Roberts et al. 2012), for both transduced (± chondrogenic induction media) and non-transduced (± chondrogenic induction media) controls.

2.2.6.4 Neurogenic differentiation

Replicate plates of hBM and hDP MSCs were seeded with 16,000 cells per cm^2 in α-MEM/10% (v/v) FCS on 10µg/mL poly-L-ornithine and 5µg/mL laminin coated plates. Three hours later, 50% of the wells were transfected with 0.056µg/mL p24 protein pHIV-EF1αmm*Gus* (Section 2.2.3.8), while additional wells served as non-transduced controls. After 24 hours, media was removed and replaced with basal growth media. Neurogenic differentiation was performed as described by Arthur *et al.* (2008); following media B conditions (Arthur, Rychkov et al. 2008). Control samples were maintained in basal growth media throughout.

2.2.6.4.1 Neurogenic quantification

RNA was collected from replicates (18 day protocol (seven days in retinoic acid) and 25 day protocol (14 days in retinoic acid)) using Trizol, before being converted to cDNA by QuantiTect Reverse Transcription kit. Real-time PCR was performed, as previously described (Arthur, Rychkov et al. 2008), on the equivalent of 350ng RNA, using previously described primers to nestin, β-tubulin, neurofilament-M and GFAP with cyclophillin A as a housekeeping gene. Data was normalised to cyclophillin A and the fold change to samples cultured in basal growth media calculated using the ΔΔCt method (Livak and Schmittgen 2001).

2.3 MPS I husbandry

Mice were kept in same sex group housing on a 14 hour light / 10 hour dark cycle. Sterile food and water were available *ad libitum*; sterile filter cages were cleaned and changed once weekly. Mating occurred between six to 15 week old heterozygous pairs, or heterozygous

females and MPS I affected males; producing a maximum of three litters. Pups were toe tagged on day seven, and weaned at three weeks of age after genotyping had been performed (Section 2.3.1). All mice were weighed and checked daily to monitor condition and healthy appearance. Urine collection was carried out on a monthly basis through the use of metabolic cages (Hatteras Instruments Inc., Cary, North Carolina) with sterile food and water available *ad libitum*. All animal studies were approved by the CYWHS institutional animal ethics committees.

2.3.1 MPS I genotyping

At seven days of age pups were toe tagged for identification purposes. Amputated toes were then used for genotyping to determine if the pup was normal, heterozygous or MPS I affected at exon VI of the *IDUA* gene. This involved the generation of a crude lysate followed by exon VI amplification by polymerase chain reaction (PCR) and analysis by gel electrophoresis.

2.3.1.1 Toe lysis

A total tissue lysate was generated using Viagen lysis buffer containing 0.4 mg/mL proteinase K as per the manufacturer's instructions (Viagen Biotech Inc., Los Angeles, CA). If PCR was not performed straight away, samples were stored at -20°C.

2.3.1.2 Polymerase chain reaction

The PCR was performed using 5.2μM dNTPs, 50pmol wild type (wt) forward (5'-GGA ACT TTG AGA CTT GGA ATG AAC CAG-3') and reverse primer (5'-CAT TGT AAA TAG GGG TAT CCT TGA ACT C-3'), 50pmol MPS I exon VI mutant primer (5'-GGA TTG GGA AGA CAA TAG CAG GCA TGC T-3'), 1.25U Taq polymerase and 10x Roche PCR buffer (100mM Tris-HCL, 15mM

$MgCl_2$ and 500mM KCl) and made up to 23μL with deionised water. Reactions were placed in a BioRad thermocycler for one cycle at 94°C for three minutes, 35 cycles at 94°C for 30 seconds, 65°C for one minute, 72°C for one minute and one cycle at 72°C for two minutes. PCR products were held at 4°C before running on an agarose gel.

2.3.1.3 Gel electrophoresis

DNA was run on 2.5% (w/v) agarose gel in TAE buffer (40mM Tris-acetate, 2nM EDTA pH 8.5) for 35 minutes at 100V alongside pUC19/HpaII molecular weight marker. Samples were pre-mixed with loading buffer (20% (v/v) Ficoll 400, 0.1M Na_2EDTA, 0.25% (w/v) bromophenol blue, 1.0% (w/v) SDS) before being loaded onto the gel. After electrophoresis, gels were stained for five minutes in 50μg/mL ethidium bromide, followed by 30 minutes of destaining in deionised H_2O. Gels were then visualised under UV using Uvitec GelDoc system version 11.01 and images were captured for interpretation.

2.3.2 Treatment administration

Following genotype determination, animals were allocated to a treatment group; normal saline, normal cyclosporin, MPS I saline, MPS I cyclosporin, MPS I cyclosporin receiving hBM MSCs and MPS I cyclosporin receiving hBM MSCs transduced with IDUA (Table 2.1).

2.3.2.1 Cyclosporin immunosuppression

From seven weeks of age, animals allocated to cyclosporin treatment groups (Table 2.1) received daily subcutaneous injections of 10mg/kg (v/v) cyclosporin in sterile saline. Animals allocated to saline treatment groups (Table 2.1) received an equivalent dose of daily

Table 2.1: Allocation of animals to treatment groups.

Age (at analysis)	Normal				MPS I									
	Saline		Cyclosporin		Saline		Cyclosporin		Cyclosporin + hBM MSCs		Cyclosporin + hBM IDUA MSCs		Cyclosporin + hDP IDUA MSCs	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
2 months + 2 days (distribution)	-	-	1	2	-	-	1	2	-	-	1	2	1	2
Total	-		**3**		-		**3**		-		**3**		**3**	
4 months (short term)	4	4	4	4	4	4	3	5	4	4	6	3	-	-
Total	**8**		**8**		**8**		**8**		**8**		**9**		-	
8 months (long term)	4	4	4	5	4	4	5	4	4	4	5	4	-	-
Total	**8**		**9**		**8**		**9**		**8**		**9**		-	

subcutaneous injection of sterile saline. The subcutaneous site was rotated daily through four different zones on the mouse; left shoulder, right shoulder, left hip and right hip.

2.3.2.2 Stem cell transfection and harvest

Prior to animal administration, hBM MSCs were expanded until reaching passage five, after which they were seeded at 6,700 cells per cm^2 in αMEM/10% FCS and allocated to a treatment group; hBM MSCs (untransfected) or hBM MSCs transfected with 0.56µg/mL p24 pHIV-EF1αmm*Idua* (Section 2.2.3.8). Twenty-four hours post virus removal, cells were harvested in 10% (v/v) trypsin in PBS, centrifuged at 390 *x g* and rinsed in sterile saline before being re-centrifuged to create a cell pellet. A cell count was performed using 0.4% trypan blue stain (Invitrogen, Carlsburg, CA, USA) and countess automated cell counter (Invitrogen, Carlsburg, CA, USA). A total of $1x10^6$ cells were resuspended in 100µL sterile saline and transferred to separate sterile microfuge tube for animal administration.

2.3.2.3 Stem cell administration

At eight weeks of age, mice allocated to either hBM MSCs or hBM IDUA MSCS treatment groups (Table 2.1) received a once off tail vein injection of 1mg/kg (v/v) sodium nitroprusside in sterile saline. After five minutes, mice received an additional tail vein injection of $1x10^6$ MSCs resuspended in 100µL sterile saline (Section 2.3.2.2). Prior to injection, stem cells were pipette mixed using sterile techniques, to ensure no cell clumps had formed since the time of harvest. Mice were allowed to recover in sterile filter cages placed within a 29°C humidicrib.

2.4 Behaviour testing

2.4.1 Animal usage

Animals subjected to behaviour testing from two to four months of age included 16 normal saline (eight male and eight female), 17 normal cyclosporine (eight male and nine female), 16 MPS I saline (eight male and eight female), 18 MPS I cyclosporine (eight male and ten female), 16 MPS I hBM treated (eight male and eight female) and 18 MPS I hBM IDUA treated (eleven male and seven female). Animals subjected to behaviour testing from five to eight months of age included eight normal saline (four male and four female), nine normal cyclosporine (four male and five female), eight MPS I saline (four male and four female), seven MPS I cyclosporine (two male and five female), seven MPS I hBM treated (three male and four female) and eight MPS I hBM IDUA treated (four male and four female). Behavioural testing and subsequent biochemical analysis (Section 2.5) timeline is shown in Figure 2.1.

2.4.2 Inverted grid

Neuromuscular strength was measured using the inverted grid test, initially at two month of age and then at monthly intervals. The mouse was placed onto a 10cm by 18cm wire grid, which was turned upside-down and placed approximately 50cm above a padded surface. The amount of time spent holding onto the inverted grid, to a maximum of 120 seconds was recorded (Figure 2.2).

2.4.3 Rotarod

Motor function, coordination and balance were evaluated using an accelerating rotarod (Harvard Apparatus), initially at two month of age and then at monthly intervals. Mice were

Figure 2.1: Timeline of treatment initiation, behavioural testing and biochemical analysis.

Mice were treated from seven weeks of age with either daily saline or cyclosporin subcutaneous injections, depending on group classification (Table 2.1). After one week of daily injections, mice allocated to hBM MSCs, with or without IDUA over-expression, or hDP IDUA MSC treatment groups (Table 2.1) received a tail vein injection of a vasodilator (1mg/kg nitroprusside). Five minutes post vasodilation, treated mice received a once off injection of $1x10^6$ MSCs also via the tail vein.

Behaviour testing was initiated from two months of age to generate a baseline prior to treatment (short and long term treatment groups only), and repeated monthly; except for the water cross maze, which was only performed prior to two (short term) and six months (long term) post groups being humanely killed. Biochemical and *ex vivo* analysis was performed on dissected tissues to determine treatment efficacy.

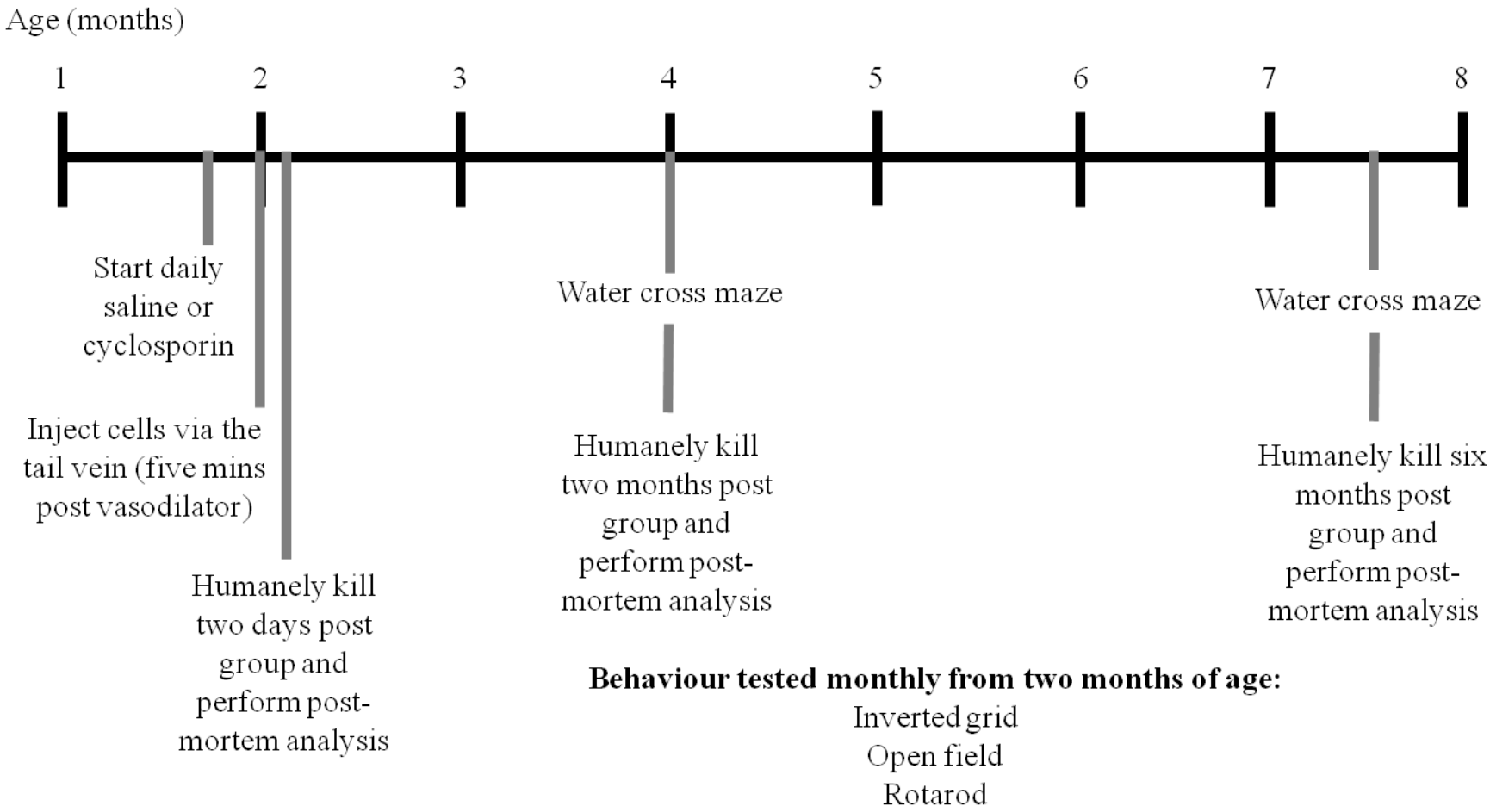

Age (months)
1
2
3
4
5
6
7
8
Start daily saline or cyclosporin
Inject cells via the tail vein (five mins post vasodilator)
Humanely kill two days post group and perform post-mortem analysis
Water cross maze
Humanely kill two months post group and perform post-mortem analysis
Water cross maze
Humanely kill six months post group and perform post-mortem analysis
Behaviour tested monthly from two months of age:
Inverted grid
Open field
Rotarod

Figure 2.2: Inverted grid

Neuromuscular strength was measured using the inverted grid test at monthly intervals from two months of age. The mouse was placed onto a 10cm by 18cm wire grid, which was turned upside-down and placed approximately 50cm above a padded surface. The amount of time spent holding onto the inverted grid, to a maximum of 120 seconds was recorded

placed into separate compartments on a rotating drum that was approximately 30cm from the ground and subjected to an accelerating speed of 5rpm to 35rpm over 120 seconds with an additional 60 seconds at a constant speed of 35rpm (Figure 2.3). When the mouse fell from the rotating drum, a touch sensor was activated prompting the latency (time) to fall and the speed achieved to be stored and relayed to CUB 2005 software version 3.0.15 (Ugo Basile S.r.l., Italy, 2005). The mice were rested for 30 minutes before the test was repeated. This protocol was performed over three consecutive days, by which time the animals were performing at their maximum. The second trial on day three was used for monthly comparison between treatment groups.

2.4.4 Open field

The open field test was used to measure the anxiety and exploratory activity of mice, initially at two month of age and then at monthly intervals. Each mouse was placed in the front left hand corner of the automated activity monitoring system (Harvard Apparatus), facing the corner (Figure 2.4). Horizontal and vertical infra-red beam crosses over a 180 second time frame were recorded by the Harvard Apparatus and relayed to Versamax software version 4.12-125E (Accuscan Instruments, Inc., USA, 2005). Beam crosses were converted to total distance travelled, zone entries, resting time and rearing activity via Versadat software version 3.02-125E (Accuscan Instruments, Inc., USA, 2005).

2.4.5 Water cross maze

Spatial learning was measured using a modified version of the Morris water maze, the water cross maze, as previously described (Roberts, Rees et al. 2007). A 72cm wide Perspex cross (arm width 20cm, arm length 26cm) was placed in the middle of a 130cm diameter circular pool and filled to 14cm with water containing 500g of pre-dissolved milk powder; with water

Figure 2.3: Rotarod

Motor function, coordination and balance were evaluated using an accelerating rotarod at monthly intervals from two months of age. Mice were placed into separate compartments on a rotating drum and subjected to an accelerating speed of 5rpm to 35rpm over 120 seconds with an additional 60 seconds at a constant speed of 35rpm. Latency (time) to fall and the speed achieved, to a maximum of 180 seconds, were recorded.

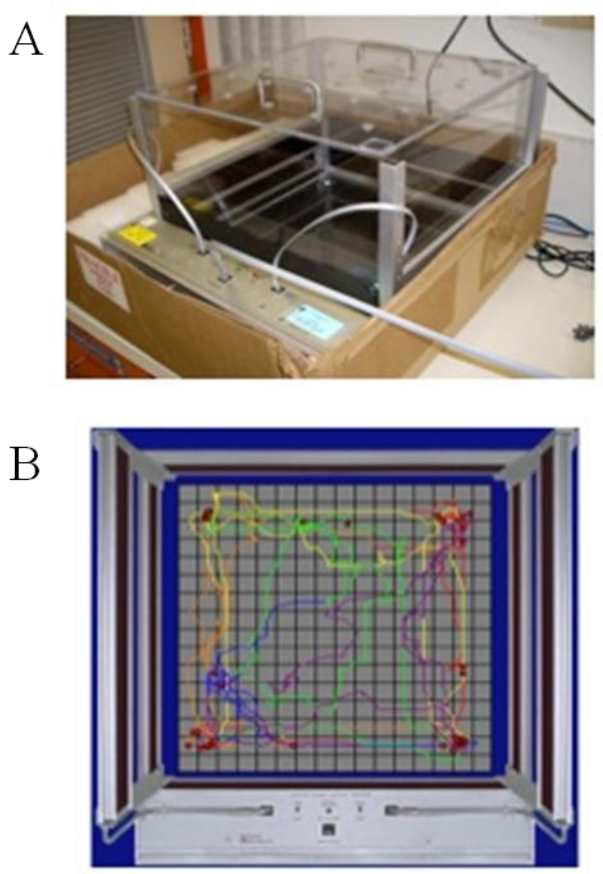


Figure 2.4: Open field

The open field test was used to measure the anxiety and exploratory activity of mice at monthly intervals from two months of age. Each mouse was placed facing the corner in the front left hand corner of the automated activity monitoring system (Harvard Apparatus) **(A)**. Horizontal (coloured lines) and vertical (red dots) infra-red beam crosses over a 180 second time frame were recorded and converted to total distance travelled, zone entries, resting time and rearing activity **(B).**

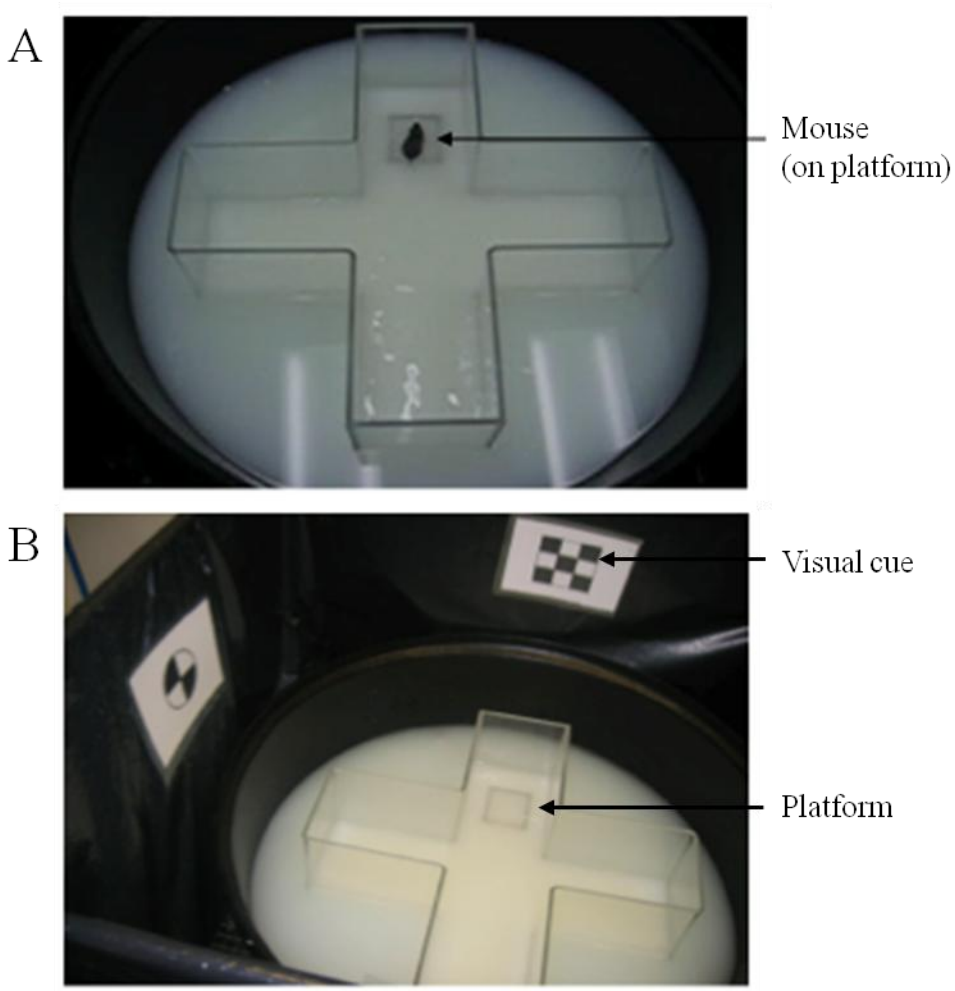


Figure 2.5: Water cross maze

Spatial learning was measured using the water cross maze at four and eight months of age. A 72cm wide Perspex cross was placed in the middle of a 130cm diameter circular pool and filled to 14cm with water containing milk powder **(A)**. Visual cues (on A4 laminated paper) were placed on blacked out panels at the each end of the cross arms, which mice used to locate the submerged platform **(B)**.

temperature adjusted to between 23.5-24.5°C. Visual cues (on A4 laminated paper) were placed on blacked out panels at each end of the cross arms; organised in cube orientation to prevent interference by external cues, with the submerged platform always located in the same position (Figure 2.5).

Mice were initially habituated to the apparatus, with the platform removed, by being released in the perspex cross in one arm facing the wall and allowed to swim for 60 seconds before being removed, rinsed in sterile water and dried. The following six days comprised the learning phase of the test. Mice were released within different cross arms and given 60 seconds to locate the submerged platform using the visual cues. Each mouse performed six trials per day, and learning was assessed using measures of escape latency (time taken to reach the submerged platform), correct arm entries (when the animal finds the submerged platform directly from the release point) and incorrect arm entries (number of entries or re-entries into cross arms which do not contain the submerged platform). Animal groups were tested at either four or eight months of age for a learning period of six days.

2.5 Biochemical analysis

2.5.1 Mouse sacrifice and tissue collection

Animals were humanely killed at either two, four or eight months of age by carbon dioxide asphyxiation before blood was collected via cardiac puncture. Blood was stored at 4°C overnight before serum was separated by centrifugation at 1500 *x g* for 20 minutes and stored at -80°C for future analysis. The following tissues were collected, weighed and stored at -80°C pending analysis; brain, liver, kidney, spleen, heart and lung. The brain was cut in half sagittally along the cerebral fissure; a central slice from the right hand side was collected and snap frozen in OCT (two days post treatment group) or stored at 4°C in 10% neutral buffered

formalin for paraffin embedding and light microscopy (LM) (short and long-term treatment groups), while the remaining tissue was minced and a representative 20mg section stored at -80°C for real-time PCR analysis. A central slice from the left hand side was also collected stored at 4°C in EM fixative (1.25% (v/v) glutaraldehyde, 4% (w/v) paraformaldehyde and 4% (w/v) sucrose in PBS) for resin embedding and electron microscopy (EM). Cross sections of remaining somatic organs were snap frozen in OCT (two days post treatment group) or stored in 10% neutral buffered formalin or EM fixative respectively (short and long-term treatment groups). LM samples were transferred into 70% ethanol the following day, while EM samples remained in their fixative. The right hand side femur, lumbar vertebrae (L4-6), skull and tibias (cut in half longitudinally) were removed and fixed in 10% buffered formalin overnight and then stored in 70% ethanol for bone morphology analysis.

2.5.2 Sample usage

Samples subjected to biochemical analysis (Figure 2.1) included eight normal saline (four male and four female), eight normal cyclosporin (four male and four female), eight MPS I saline (four male and four female), eight MPS I cyclosporin (three male and five female), eight MPS I hBM treated (four male and four female) and nine MPS I hBM IDUA treated (six male and three female) at four months of age. At eight months of age eight normal saline (four male and four female), nine normal cyclosporin (four male and five female), eight MPS I saline (four male and four female), seven MPS I cyclosporin (three male and four female), six MPS I hBM treated (three male and three female) and seven MPS I hBM IDUA treated (six male and one female) animals samples underwent biochemical analysis.

2.5.3 Tissue extraction

Tissues were thawed and homogenised using a Potter-Elalihjem test tube and pestle apparatus with 0.1% (v/v) Triton X-100. Tissues were homogenised in weight to 0.1% (v/v) Triton X-100 volume ratios as follows; liver 1:1, kidney 1:1, heart 1:5 and lung 1:5. The remaining left hand side brain and spleen samples were homogenised in set volumes of 500µL and 250µL of 0.1% (v/v) Triton X-100 respectively. Homogenised samples were transferred to a sterile microfuge tube before being centrifuged at 10,000 *x g* for 20 minutes. Supernatant volume was recorded and transferred into a fresh tube; with 1:50 dilutions made for liver and kidney, 1:20 dilutions for spleen, heart and lung and a 1:10 dilution made for the brain.

2.5.4 α-L-iduronidase enzyme assay

IDUA enzyme assay was performed as per Clarke *et al.* (1997), briefly samples were incubated with 25µM 4-umbelliferyl-α-L-iduronide substrate for four hours at 37°C in citrate-phosphate buffer (0.1M citric acid, 2M disodium hydrogen phosphate, pH 4.8)(Clarke, Russell et al. 1997). The reaction was stopped by the addition of an excess of 0.2M glycine buffer, pH 10.7. Samples were read on a LS 50B luminescence spectrometer using an AS 91 autosampler (Perkin Elmer, Massachusetts) at excitation 366nm and emission 446nm.

2.5.5 β-hexosaminidase enzyme assay

β-hexosaminidase enzyme assay was performed as per Leaback and Walker (1961). Briefly samples were incubated with 0.264mm 4-methylumbellifryl-2-acetamindo-2-deoxy-β-D-glucopyranoside substrate for 30 minutes at 37°C in citrate-phosphate buffer (0.1M citric acid, 2M disodium hydrogen phosphate, pH 4.8)(Leaback and Walker 1961). The reaction was stopped by the addition of an excess of 0.2M glycine buffer, pH 10.7. Samples were read

on a LS 50B luminescence spectrometer using an AS 91 autosampler (Perkin Elmer, Massachusetts) at excitation 366nm and emission 446nm.

2.5.6 Uronic acid assay

Tissue gags were precipitated by the addition of 2.5 volumes of 0.1% cetylpridinium chloride in 0.054M citrate buffer, pH 4.8 for 30 minutes at 37°C. After ten minutes centrifugation at 450 *x g*, supernatant was aspirated and the pellet was resuspended in 2M LiCl and 5.3 volumes of absolute ethanol added and the samples left at -80°C for one hour to precipitate gags. Samples were centrifuged for ten minutes at 450 *x g* before supernatant was aspirated and pellets were allowed to air dry overnight. Pellets were resuspended in 100µL of Milli-Q water before being centrifuged for one minute at 450 *x g*, after which the supernatant was collected and used in uronic acid assay (Blumenkrantz and Asboe-Hansen 1973). Standards were prepared using D-glucuronolactone and 0.0125M tetraborate in sulphuric acid was added to all samples and standards. Samples were incubated at 100°C for ten minutes before being allowed to cool on ice. 0.15% (w/v) 3-phenylphenol was added as a colour reagent and allowed to develop for five minutes. Absorbance was read at 520nm for 0.1 seconds using 1234 Delfia Research Fluorometer (Wallac, Finland) and uronic acid levels were determined against a standard curve.

2.5.7 Bicinchoninic acid protein assay

Enzyme activity and uronic acid was normalised to total protein using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Briefly, a 50:1 working solution of BCA : 4% copper (III) sulphate pentahydrate was added at a ratio of 8:1 to a known bovine serum albumin standard curve and to protein samples being analysed. Samples were incubated at 37°C for 30 minutes, before absorbance was read at 560nm for one second using 1234 Delfia

Research Fluorometer (Wallac, Finland) and protein levels were determined against the standard curve.

2.6 Real-time PCR

2.6.1 Genomic DNA purification

Genomic DNA was purified from 20mg sections of tissue using the Wizard SV genomic DNA purification system (Promega) as per the manufacturer's instructions for a microcentrifuge.

2.6.2 Human genome specific detection

Detection and quantification of the number of transplanted human cells remaining in various tissues from mice that received either hBM MSCs alone or hBM MSCs transduced with IDUA was performed as per Song *et al*. (2012), using gDNA (Section 2.6.1) and the human specific primer-based Real-Time PCR (RT-PCR) method (Song, Xie et al. 2012).

2.6.3 Beta-2-microglobulin staining

Immunohistochemical detection of transplanted human cells within the brain was performed to confirm human genome specific detection (Section 2.6.2) and to determine the location of remaining cells. For the two day distribution study 5µm thick OCT embedded brain sections (Section 2.5.1) on superfrost plus slides were post fixed in 10% (v/v) formalin for 30 minutes prior to antigen retrieval and staining protocol. While two month post 5µm thick paraffin embedded brain sections (Section 2.5.1) on silane coated slides were deparaffinised in xylene for three consecutive washes of five minutes each. Sections were rehydrated through two

three minute washes of absolute ethanol and one minute in 95% (v/v) ethanol, before being rinsed twice in dH_2O for five minutes each.

Antigen retrieval was performed using 1% (w/v) SDS for five minutes at room temperature, after which slides were rinsed three times in PBS for five minutes each. Sections were blocked (2% (w/v) bovine serum albumin (BSA) in PBS) for one hour at room temperature, before being incubated with 1:200 dilution of human polyclonal anti-beta-2-microglobulin (Novacastra, Leica) in blocking solution for 30 minutes at room temperature. Negative controls remained in blocking solution under the same conditions. Slides were washed three times with PBS for five minutes each, before being incubated with 1:1000 dilution sheep anti-rabbit FITC (Silenus) in blocking solution for one hour at room temperature in the dark. Slides were washed three times in PBS for five minutes each at room temperature in the dark, before being mounted using ProLong Gold antifade with DAPI and allowed to cure overnight in the dark. Staining was viewed using an upright fluorescence microscope and photographed at a magnification of 100x (1.25 oil lens).

2.7 Inflammatory mediators in the brain

2.7.1 Real-Time PCR

2.7.1.1 RNA extraction and cDNA generation

RNA was extracted from a thin sagittal section of brain in Trizol reagent as per the manufacturer's instructions (Invitrogen, USA) and quantified using a nanodrop spectrophotometer (Thermo Scientific, USA). 1µg RNA was converted to cDNA using the Qiagen QuantiTect reverse transcriptase kit as per the manufacturer's instructions (Qiagen, Germany).

2.7.1.2 Real-Time PCR analysis

The expression levels of five inflammatory mediators were determined using exon-exon boundary gene specific primers; *Gfap*, CD68 antigen (*Cd68*), chemokine (C-C motif) ligand 3 (*Ccl3*), tumour necrosis factor alpha (*Tnfα*) and interleukin 1 beta (*Il1β*). For each Real-Time PCR reaction; 0.5µL brain cDNA (0.5µg RNA equivalent), 1x SYBR green real-time PCR master mix, 45pmol forward primer and 45pmol reverse primer (one inflammatory gene primer set per reaction; Table 2.2). Cyclophillin A (*CycpA*) was used as a housekeeping gene to normalise data (Table 2.2). Real-time reactions were carried out on a thermocycler (Applied Biosystems 7300) with one cycle for two minutes at 50°C, one cycle for ten minutes at 95°, 40 cycles of 15 seconds at 95°C and one minute at °C. A dissociation step of one cycle for 15 seconds at 95°C, 30 seconds at 60°C and 15 seconds at 95°C was added after the elongation step. The $2^{-\Delta\Delta Ct}$ method was used to calculate the gene expression fold change compared to normal control mice (Livak and Schmittgen 2001).

2.7.2 Glial fibrillary acidic protein (GFAP) immunohistochemistry

Immunohistochemistry of GFAP was performed on 5µm thick, paraffin embedded brain sections on silane coated slides as per Section 2.6.3 with minor changes. Blocking was performed under the same conditions using 5% (w/v) BSA in PBS and a 1:1000 dilution of polyclonal rabbit anti-GFAP (DAKO, Denmark) in blocking solution was used as the primary antibody.

Table 2.2: Inflammatory genes primer sets.

Gene symbol	Gene name	Genbank access number	Function	Forward primer	Reverse primer
Gfap	Glial fibrillary acidic protein	NM_010277.2	marker of astroglial activation	TGGAGCTCAATGACCGCTTT	TCTCCTCCTCCAGCGATTCA
Cd68	CD68 antigen	NM_009853.1	expressed by monocytes, granulocytes and activated T-cells	CTTAAAGAGGGCTTGGGGCA	ACTCGGGCTCTGATGTAGGT
Ccl3	Chemokine (C-C motif) ligand 3	NM_011337.2	macrophage chemoattractant involved in acute inflammation	AGGATACAAGCAGCAGCGAG	TCAGGAAAATGACACCTGGC
Tnfa	Tumor necrosis factor alpha	NM_013693.2	expressed by astroglia and microglia, involved in regulation of immune cells	ATGGCCTCCCTCTCATCAGT	CTTGGTGGTTTGCTACGACG
Il1b	Interleukin 1 beta	NM_008361.3	cytokine secreted by astroglia and microglia, mediatory of the inflammatory response	AGCTTCCTTGTGCAAGTGTC	TGGGTGTGCCGTCTTTCATT
CycpA	Cyclophilin A	NM_008907.1	housekeeping gene	GGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG

2.8 Electron microscopy

2.8.1 Electron microscopy embedding

Tissue sections that had been preserved in EM fix (1.25% (v/v) glutaraldehyde, 4% (w/v) sucrose and 4% (w/v) paraformaldehyde in PBS) upon mouse dissection were resin embedded according the Adelaide Microscopy (Adelaide, Australia) resin embedding protocol. Prior to processing sagittal brain sections were cut into six pieces; firstly into thirds vertically and then in half horizontally, with orientation being maintained; while other cross sectional tissue samples were cut into $3mm^3$ pieces. Samples were washed for ten minutes in 4% (w/v) sucrose in PBS, before being post-fixed in 2% osmium tetroxide for one hour. Samples were then dehydrated through 70% (v/v) ethanol, 90% (v/v) ethanol and 100% (v/v) ethanol with three changes of 20 minutes each, before a final 20 minute dehydration in propylene oxide. Resin infiltration was initiated with 1:1 of propylene oxide: epoxy resin (57% (v/v) dodecenyl succinic anhydride, 26% (v/v) procure 812, 15.5% (v/v) araldite 502 and 1.5% (v/v) 2,4,6-tridimethylaminomethyl phenol) for two hours, before overnight infiltration of epoxy resin. Samples underwent two additional epoxy resin changes of three to four hours, before being embedded in fresh epoxy resin and polymerised in a 70°C oven for at least 24 hours.

2.8.2 Electron microscopy cutting

Resin embedded samples were cut into 1µm thick sections using an UltraCut S Ultramicrotome (Reichert Inc., Buffalo, USA) and glass knife. Sections were transferred onto glass slides and allowed to dry on a 90°C hot plate, before being stained with 1% toluidine blue for approximately ten seconds and washed with deionised water. Slides were then coverslipped and visualised, captured and analysed at 40x (0.75 lens) and 100x (1.25 oil lens) magnification using an Olympus BX41 microscope, Olympus XC50 camera and AnalySIS LS

Research version 3.1 computer software (Olympus Australia Pty. Ltd., Gulfview Heights, SA).

2.9 Bone morphology

2.9.1 Animal usage

Quantitative lumbar (L5) vertebral measurements were carried out on two normal saline (one male and one female), two MPS I saline (one male and one female), four normal cyclosporin (two male and two female), four MPS I cyclosporin (two male and two female), eight MPS I hBM MSC treated (four males and four females) and nine MPS I hBM IDUA (six males and three females) treated mice at four months of age.

2.9.2 Radiographs

Lumbar vertebrae (L4-L6) were scanned using a SkyScan 1174 (Bruker-MicroCT, Kontich, Belgium) with the following settings: 0.25 aluminium filter, repeat scanning = 1, 6.48µM image pixel size, 0.4 rotation step, flat field correction ON, 40% sharpening, random movement OFF, 2600msecs exposure, 50kV source voltage, 800uA source current and a 1.3Mp FW camera. Scans were reconstructed using NRecon (SkyScan, Belgium) using the following settings: misalignment OFF, smoothing OFF, 12% ring artefacts, 20% beam hardening, reconstruct ON, scales OFF, and 0-0.08 histogram range. Reconstructed scans were analysed and orientated using Dataviewer version 1.4.3 (SkyScan, Belgium) before ten subsequent sagittal sections are selected in CTAn version 1.10.1.0 (SkyScan, Belgium) showing the whole bone body intact. Lumbar width and length were measured using the measure tool, while trabecular number, trabecular thickness and bone mineral volume were calculated by tracing around the bone plan and analysing the region of interest. The following

settings were used for trabecular analysis: 3D analysis of the mean, radius = 1, constant = 0, dark background, pre-smoothing ON, pre-threshold ON, 85 lower grey threshold, 255 upper grey threshold and a despeckling of less than 10 voxels to remove both white and black speckles.

2.10 Statistics

The statistical significance of differences between means was determined using either a one-way or two-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test in SigmaStat version 3.0 (SigmaStat, SPSS Inc., USA).

Chapter Three: MSCs *in vitro* capacity to over-express MPS lysosomal enzymes and maintain differentiation potential

3.1 Introduction

The two clinically available forms of treatment for MPS, BMT and ERT, improve many of the soft tissue aspects of MPS disease but fail to adequately address the skeletal and neurological systems (Muenzer, Wraith et al. 2009; Nan, Shekels et al. 2012). Systemically administered ERT does not affect neurological disease, as the recombinant enzymes do not cross the blood brain barrier (BBB) (Barton and Neufeld 1971; Noh and Lee 2014). BMT is a once only procedure for the introduction of HSCs that produce functional enzyme, but is only applicable for MPS I patients with neurological involvement (Kingma, Langereis et al. 2013), with optimal efficiency see in younger patients (Church, Tylee et al. 2007). Human trials of BMT in other MPS types, such as MPS IIIA, have shown an increase in lifespan but no improvement in neurological function (Shapiro, Lockman et al. 1995; Sivakumur and Wraith 1999; Prasad, Mendizabal et al. 2008; Lau, Hannouche et al. 2010; Langford-Smith, Wilkinson et al. 2012).

Bone also responds poorly to BMT due to the inadequate penetration of leukocyte derived enzyme into the musculoskeletal system (Field, Buchanan et al. 1994; Vellodi, Young et al. 1997; Souillet, Guffon et al. 2003), and may also be attributed to the lack of donor chimerism for bone forming cells even under conditions of total haematopoietic engraftment (Koc, Peters et al. 1999; Koc, Day et al. 2002). Animal studies indicate that enzyme production by lymphoid and myeloid lineages is not sufficient to combat the multi-tissue symptoms associated with MPS, but that improved efficacy can be achieved by transducing HSCs with viral vectors to over-produce MPS enzymes (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012).

In addition to HSCs, the bone marrow contains another population of stem cells, MSCs. MSCs are multi-potent stromal cells that are characterised by their intrinsic self-renewal capacity and ability to differentiate into cells of the endoderm, ectoderm or mesoderm (Pittenger, Mackay et al. 1999; Gronthos, Brahim et al. 2002; Jiang, Jahagirdar et al. 2002; Gronthos, Zannettino et al. 2003; Le Blanc 2003), with recent studies also showing that MSCs are able to form additional cell types, including neurons *in vitro* (Majumdar, Thiede et al. 2000; Jiang, Jahagirdar et al. 2002; Jiang, Vaessen et al. 2002; Fox, Chamberlain et al. 2007; Arthur, Rychkov et al. 2008). MSCs have been isolated from a range of readily available tissues (Friedenstein, Chailakhyan et al. 1974; Gronthos, Mankani et al. 2000; Kuznetsov, Mankani et al. 2001; Zuk, Zhu et al. 2001; Jiang, Vaessen et al. 2002; Bieback, Kern et al. 2004; Seo, Miura et al. 2004; Cao and Feng 2009; Fazekasova, Lechler et al. 2010), and are also able to differentiate into many of the cell types which are affected by MPS. MSCs could therefore potentially be used to replace the non-functional cell populations in MPS patients.

The capacity of MSCs to produce MPS enzymes underpins their effectiveness as an MPS therapy. In this study we assessed the ability of both bone marrow and dental pulp derived MSCs to produce the enzymes deficient in MPS I, II, IIIA, IVA, VI and VII, and to over-express the enzymes deficient in MPS I, IIIA, VI and VII. Stable, long-term transduction of MSCs with the MPS VII enzyme β-*D*-glucuronidase was determined, after which transduced cells were induced down osteogenic, chondrogenic, adipogenic or neurogenic pathways.

3.2 Endogenous production of MPS lysosomal enzymes

The production of MPS enzymes by MSCs would underpin their effectiveness as an MPS therapy. We have assessed the ability of both bone marrow (hBM MSCs) and dental pulp (hDP MSCs) derived human MSCs versus bone marrow HSCs (HSCs) to produce the

enzymes deficient in MPS I, II, IIIA, IVA, VI and VII. One week post-plating lysosomal enzyme activities were determined on the cell layer and media (Chapter 2.2.2) to determine their baseline production and secretion capacities.

Both MSCs and HSCs produced MPS enzymes in the cell layer and secreted low levels into the surrounding media (Table 3.1). However, MSCs were found to produce significantly higher levels of the majority of MPS enzymes assayed when compared to HSCs (one-way ANOVA, Tukey's HSD). In the cell layer, hBM MSCs contained significantly more IDUA (111 fold), 2-sulphatase (2 fold), sulphamidase (136 fold), 6-sulphatase (17.5 fold), 4-sulphatase (2.5 fold) and β-*D*-glucuronidase (6 fold) than HSCs (one-way ANOVA, Tukey's HSD; Table 3.1). While hDP MSCs produced significantly more IDUA (100 fold), sulphamidase (34 fold), 6-sulphatase (14 fold), 4-sulphatase (3 fold) and β-*D*-glucuronidase (4 fold) than HSCs (one-way ANOVA, Tukey's HSD; Table 3.1), with only a small (non-significant) increase in 2-sulphatase production (1.2 fold).

Low levels of enzyme were secreted by both HSCs and MSCs into the surrounding media, however, hBM MSCs secreted significantly higher levels of endogenous IDUA (71.8 fold), sulphamidase (2,420 fold) and β-*D*-glucuronidase (3 fold) into the surrounding media when compared to HSCs (one-way ANOVA, Tukey's HSD; Table 3.1). No statistical significance was seen when comparing hBM MSC and HSC endogenous secretion of 2-sulphatase (1.2 fold decrease), 4-sulphatase (108 fold increase) or 6 sulphatase (755 fold increase). Similarly, hDP MSCs secreted significantly higher levels of endogenously produced 2-sulphatase (4 fold), 6-sulphatase (246 fold) and β-*D*-glucuronidase (5 fold) compared to HSCs (one-way ANOVA, Tukey's HSD; Table 3.1), however, no statistical significance was seen when comparing IDUA (27 fold increase), sulphamidase (820 fold increase) and 4-sulphatase (91 fold increase) secretion into the surrounding media. hBM MSCs produced significantly more

Table 3.1: Endogenous production of lysosomal enzymes.

		HSC		hBM MSC		hDP MSC	
MPS	**Enzyme**	**Media**	**Cell layer**	**Media**	**Cell layer**	**Media**	**Cell layer**
I	**α-*L*-iduronidase**	0.005 ± 0.001	67.09 ± 0.728	0.359 ± 0.158	7491 ± 1476	0.136 ± 0.072	6758 ± 665.0
II	**2-sulphatase**	0.551 ± 0.029	9.101 ± 0.964	0.45 ± 0.450	18.94 ± 2.250	2.245 ± 0.521	11.2 ± 1.203
IIIA	**sulphamidase**	UD	2.107 ± 0.602	0.242 ± 0.068	286.2 ± 130.2	0.082 ± 0.059	71.87 ± 27.62
IVA	**6-sulphatase**	0.008 ± 0.008	5.093 ± 0.225	6.042 ± 3.050	89.03 ± 10.33	1.97 ± 0.357	72.38 ± 5.826
VI	**4-sulfphtase**	0.004 ± 0.004	12.38 ± 0.035	0.433 ± 0.246	31.41 ± 5.720	0.364 ± 0.364	36.6 ± 3.058
VII	**β-*D*-glucuronidase**	0.064 ± 0.002	1663 ± 19.71	0.193 ± 0.163	10175 ± 2543	0.35 ± 0.106	6556 ± 1007

*Lysosomal enzyme activity in cell layer expressed as nmol/hr/mg protein and. as nmol/hr/mL for the media. Results are expressed as Mean ± Standard Error (n=4). UD = undetected. * Indicates significant difference between MSC and HSC, # indicates significant difference between hBM and hDP MSCs ($p<0.05$; one-way ANOVA, Tukey's HSD).*

2-sulphatase (1.69 fold) within their cell layer than hDP MSCs (one-way ANOVA, Tukey's HSD; Table 3.1), however, no other differences were seen between the MSCs derived from the two tissue sources.

3.3 Over-expression of α-*L*-iduronidase, sulphamidase, 4-sulfatase and β-*D*-glucuronidase in MSCs versus HSCs

Both hBM and hDP MSCs produced and secreted higher levels of multiple MPS lysosomal enzymes endogenously when compared to the currently used HSCs. Coupling their superior endogenous production with gene therapy, via the use of different lentiviral vectors encoding MPS lysosomal enzymes, to induce over-expression may provide further advantages when assessing their therapeutic potential (Chapter 2.2.2 and 2.2.3).

3.3.1 Transduction efficiency

Due to HSCs being non-adherent in culture, a comparison of transduction efficiency between MSCs and HSCs was performed using a lentiviral construct encoding enhanced yellow fluorescent protein (pHIV-EF1αeYFP) coupled with fluorescence-activated cell sorting (FACS) using viral concentrations ranging from 0-0.014µg/µL p24 protein pHIV-EF1αeYFP (Chapter 2.2.3.8 and 2.2.3.9).

Transduction with pHIV-EF1αeYFP demonstrated that MSCs were more readily transducible than HSCs (one-way ANOVA, Tukey's HSD; Figure 3.1). With the addition of 0.07µg/µL p24 protein pHIV-EF1αeYFP 80% of MSCs were transduced, nearly 3.5 fold greater than of HSCs (23%). When doubling the amount of lentivirus added *in vitro* (0.14µg/µL p24 protein

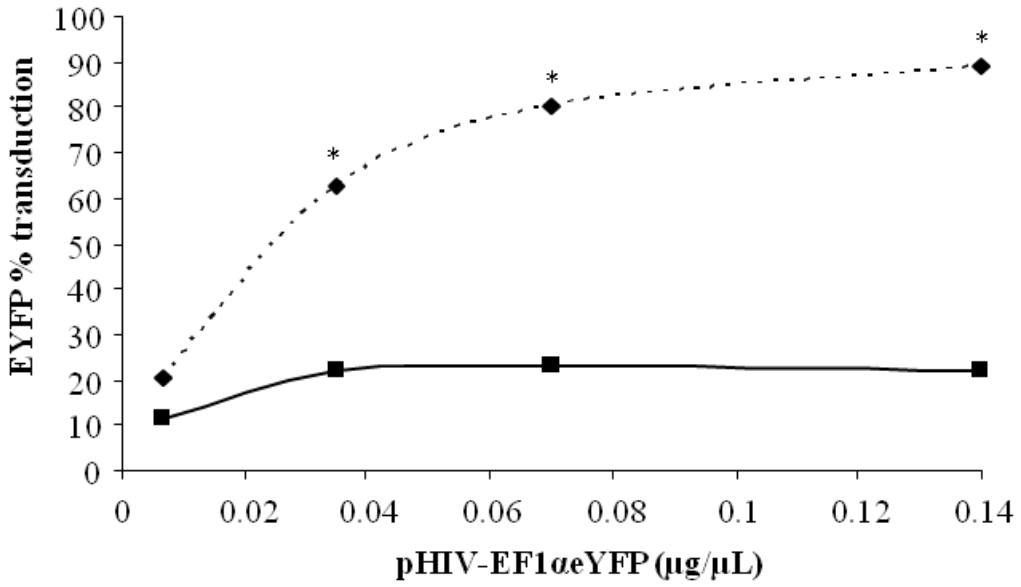


Figure 3.1: Transduction efficiency of MSCs versus HSCs.

hBM MSCs (dashed line) and HSCs (solid black) were incubated with pHIV-EF1αeYFP (0.0007-0.14µg/µL p24 equivalent) for 24 hours. Percentage transduction was determined by FACs analysis two days post transfection. Results are expressed as mean ± SEM (n=3).

* Indicates significant difference between MSC and HSC ($p<0.05$; one-way ANOVA, Tukey's HSD).

pHIV-EF1αeYFP), HSCs transduction remained constant at 22%, while MSCs increased to 89%, indicating that HSCs transduction had reached its maximum.

3.3.2 Over-expression of MPS lysosomal enzymes

Transduction of HSCs, hBM and hDP MSCs with a lentiviral construct encoding the enzymes deficient in MPS I, MPS IIIA, MPS VI or MPS VII (Chapter 2.2.3.8 and 2.2.3.9) resulted in significantly higher levels of IDUA (10, 805 and 562 fold), sulphamidase (346, 180 and 433 fold), 4-sulphatase (25, 865 and 513 fold) and β-*D*-glucuronidase (3, 120 and 217 fold) in the cell layer of HSCs, hBM MSCs and hDP MSCs, respectively (Figure 3.2), when compared to their corresponding non-transduced controls (one-way ANOVA, Tukey's HSD; Table 3.1). In addition, a significant increase in the secretion of IDUA (56188, 4257584 and 5085778 fold), sulphamidase (1559684, 57222 and 90082 fold), 4-sulphatase (17599, 213689 and 1125953 fold) and β-*D*-glucuronidase (11242, 1375645 and 827084 fold) into the media of respective HSC, hBM MSCs and hDP MSCs was observed when compared to their corresponding non-transduced controls (one-way ANOVA, Tukey's HSD; Figure 3.2).

When directly comparing HSCs with hBM or hDP MSCs, both MSC types produced significantly more IDUA (9303 and 5861 fold), sulphamidase (247 and 149 fold), 4-sulphatase (1714 and 868 fold) and β-*D*-glucuronidase (236 and 273 fold) within their respective cell layer than transduced HSCs (one-way ANOVA, Tukey's HSD; Figure 3.2). Enzyme secretion in the surrounding media was also significantly higher in hBM and hDP MSCs when transduced with IDUA (5559 and 2508 fold respectively), sulphamidase (88 and 47 fold respectively), 4-sulphatase (203 and 175 fold respectively) and β-*D*-glucuronidase (371 and 405 fold respectively), compared to transduced HSCs (one-way ANOVA, Tukey's

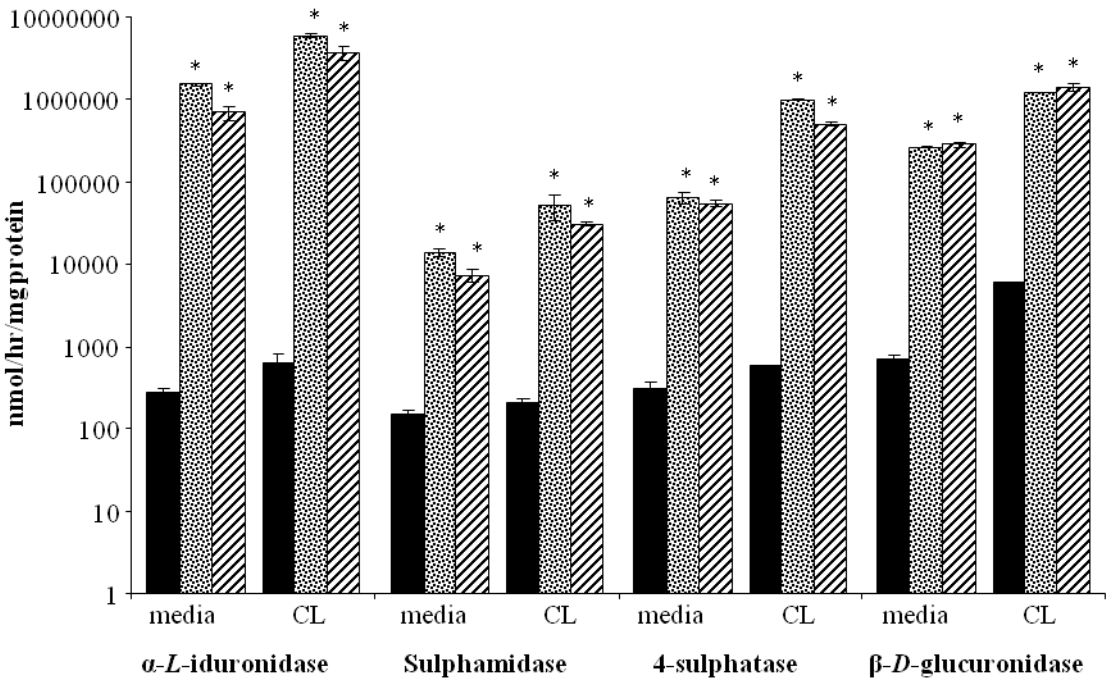


Figure 3.2: Over-expression potential of MSCs versus HSCs.

HSCs (solid black), hBM MSCs (dot fill) and hDP MSCs (striped fill) were transfected with lentiviral constructs encoding either α-L-iduronidase, sulphamidase, 4-sulphatase and β-D-glucuronidase for 24 hours. Fluorogenic substrates were used to measure enzyme activity which is expressed as nmol/hr/mg protein in the cell layer or nmol/hr/mL in the media. Results are expressed as mean ± SEM (n=3).

All transduced values were significantly elevated above endogenous levels ($p<0.05$, one-way ANOVA, Tukey's HSD) * Indicates significant difference to transduced HSCs ($p<0.05$; one-way ANOVA, Tukey's HSD).

HSD; Figure 3.2). There was no significant difference between hBM and hDP MSCs in the level of their MPS enzyme over-expression.

3.3.3 Mannose-6-phosphate mediated cross-correction of MPS I, IIIA and VII fibroblasts with hDP and hBM MSC derived enzyme

The enzymes, IDUA, sulphamidase and β-*D*-glucuronidase were over-expressed in hBM and hDP MSCs and the secreted enzymes tested for their ability to cross-correct MPS I, IIIA and VII fibroblasts respectively (Chapter 2.2.4).

Gag storage, as assessed by $^{35}SO_4$ incorporation, was significantly decreased in replicates that received enzyme (hBM and hDP MSC derived IDUA, sulphamidase or β-*D*-glucuronidase) indicating uptake of enzyme (one-way ANOVA, Tukey's HSD; Table 3.2). This reduction in gag storage was inhibited by the addition of M6P to the culture medium (Table 3.2). No significant difference was observed between the ability of enzyme secreted from transduced hBM or hDP MSCs to correct storage.

3.3.4 β-D-glucuronidase over-expression and transduction over eight weeks in culture

The readily transducible nature of MSCs (Chapter 3.3.1) and their ability to over-express multiple MPS lysosomal enzymes (Chapter 3.3.2) makes them a potential therapeutic option over the currently used HSCs. To assess the stability of transduction, hBM MSCs and hDP MSCs were incubated with lentivirus encoding β-*D*-glucuronidase and analysed over eight weeks of continuous culture, either with or without passaging (Chapter 2.2.5).

Table 3.2: Correction of gag storage by secreted MSC enzyme.

		Gag storage ($^{35}SO_4$cpm/mg cell protein)	
	Media containing enzyme derived from:	**hBM MSC**	**hDP MSC**
MPS I		4630, 3378 (4004) *	4370, 4039 (4203) *
	+ α-*L*-iduronidase	2679, 2858 (2768)	2402, 1955 (2178)
	+ α-*L*-iduronidase and M6P	5145, 4912 (5028) *	4648, 4151 (4400) *
MPS IIIA		4246, 4932 (4589) *	5624, 4855 (5239) *
	+ sulphamidase	2371, 2309 (2340)	2734, 2344 (2539)
	+ sulphamidase and M6P	6675, 4828 (5752) *	5238, 5611 (5425) *
MPS VII		14871, 14468 (14670) *	14836, 13104 (13970) *
	+ β-*D*-glucuronidase	9363, 9513 (9438)	8695, 10541 (9618)
	+ β-*D*-glucuronidase and M6P	11956, 14584 (13270) *	11660, 11779 (11719) *

The gags stored in MPS I, IIIA and VII fibroblasts were labelled with $^{35}SO_4$ *and the cells then incubated with medium containing the appropriate MPS enzyme derived from either hBM or hDP MSCs in the presence and absence of mannose-6-phosphate. Results are expressed as individual values, followed by the average in brackets. * indicates significant difference to MPS fibroblasts receiving appropriate MPS enzyme ($p<0.05$, one-way ANOVA, Tukey's HSD).*

3.3.4.1 Over-expression of β-D-glucuronidase in MSCs over eight weeks in vitro

Human BM and DP derived MSCs were capable of producing and secreting significantly higher levels of β-*D*-glucuronidase enzyme activity over an eight week, non-passaged period *in vitro* when compared to non-transduced controls (one-way ANOVA, Tukey's HSD; Figure 3.3A and 3.3B respectively). Enzyme production in the cell layer reached a maximum two weeks post transduction in hBM MSCs and three weeks post transduction in hDP MSCs, after which both remained constant. Secretion into the surrounding media reached its maximum one week post transduction for both hBM and hDP MSCs. At the end of the eight week period pHIV-EF1αmm*Gus* transduced hBM and hDP MSCs had on average a 127 and 211 respective fold increase in the amount of β-*D*-glucuronidase produced within the cell layer, which resulted in a 9,842 and 4,228 respective average fold increase in the amount secreted into the media, when compared to their non-transduced controls.

3.3.4.2 Stable transduction of β-D-glucuronidase over eight weeks in vitro

To assess stability during cell division, hBM MSCs were transduced with 0-0.07µg/µL p24 protein pHIV-EF1αmm*Gus* and subcultured over six passages during the eight week period *in vitro* (Chapter 2.2.5). A correlation was observed between virus concentration and transduction efficiency at all passage events (data not shown). Three different concentrations of lentivirus were assessed, that resulted in an initial transduction of 78% of cells (0.07µg/µL p24, black line), 50% of cells (0.035µg/µL p24, dashed line) or 30% of cells (0.007µg/µL p24, dotted line) (Figure 3.4A). A small decrease in the number of transduced cells (90-95% of initial percentage transduction) was observed in cells incubated with the two higher levels of virus over the eight week period. However, at the lowest concentration of virus tested no detectable transduced cells were observed after six passage events (Figure 3.4, dotted line). Staining for β-*D*-glucuronidase activity provided visual conformation of transduction

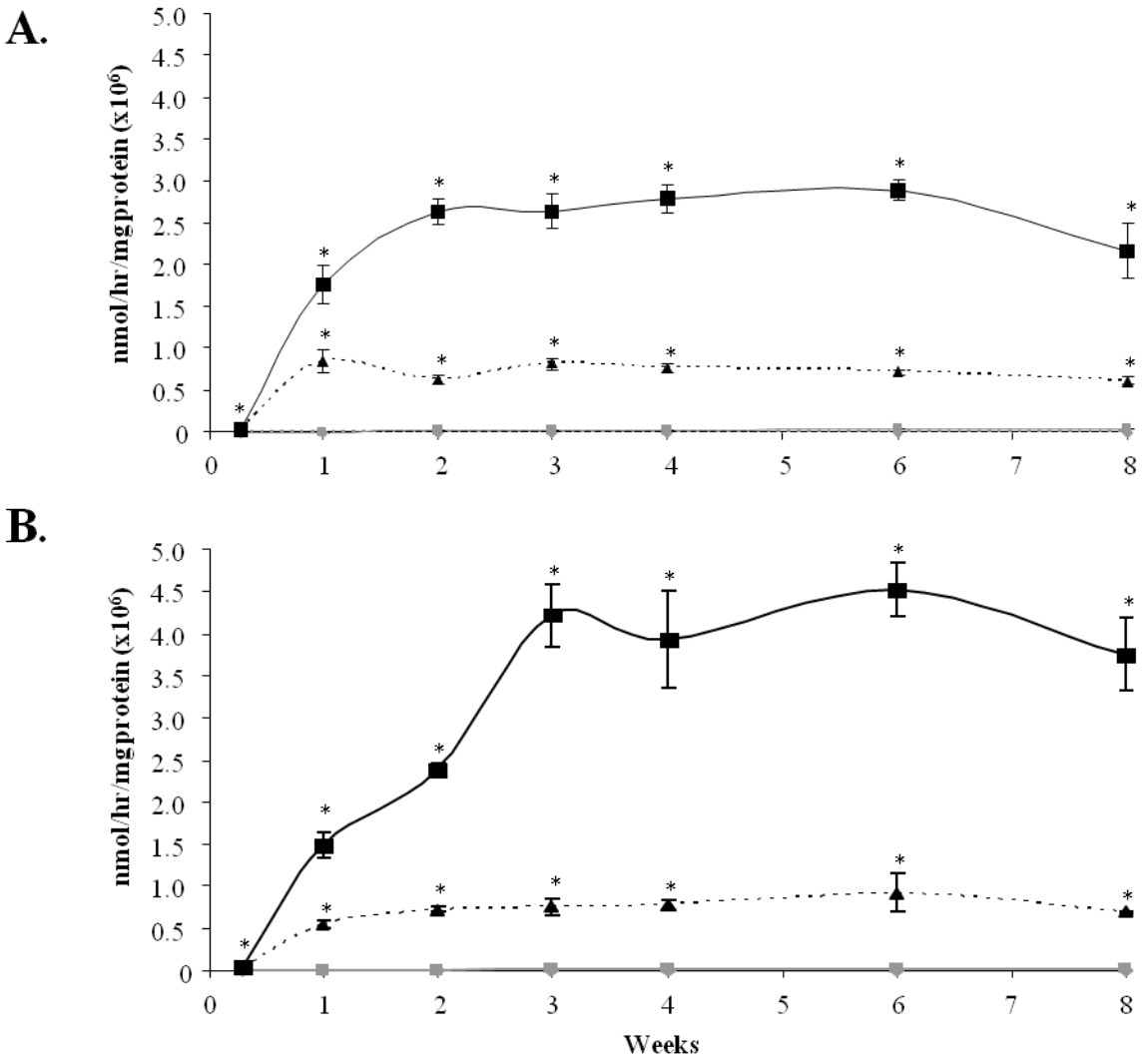


Figure 3.3: Over-expression potential of MSCs eight weeks *in vitro*.

hBM MSCs **(A)** and hDP MSCs **(B)** were transduced with a lentiviral construct encoding β-D-glucuronidase (pHIV-EF1αmm*Gus*) for 24 hours and maintained in growth media for eight weeks *in vitro*. Cell layer and media was collected once weekly, 24 hours post feeding. Transduced cell layer (solid black), transduced media (dashed black), non-transduced cell layer (solid grey) and non-transduced media (dashed grey). Activity in cell layer expressed as nmol/hr/mg protein or nmol/hr/mL in the media. Results are expressed as mean ± SEM (n=4).

* Indicates significant difference between non-transduced and transduced cell layer or media ($p<0.05$; one-way ANOVA, Tukey's HSD).

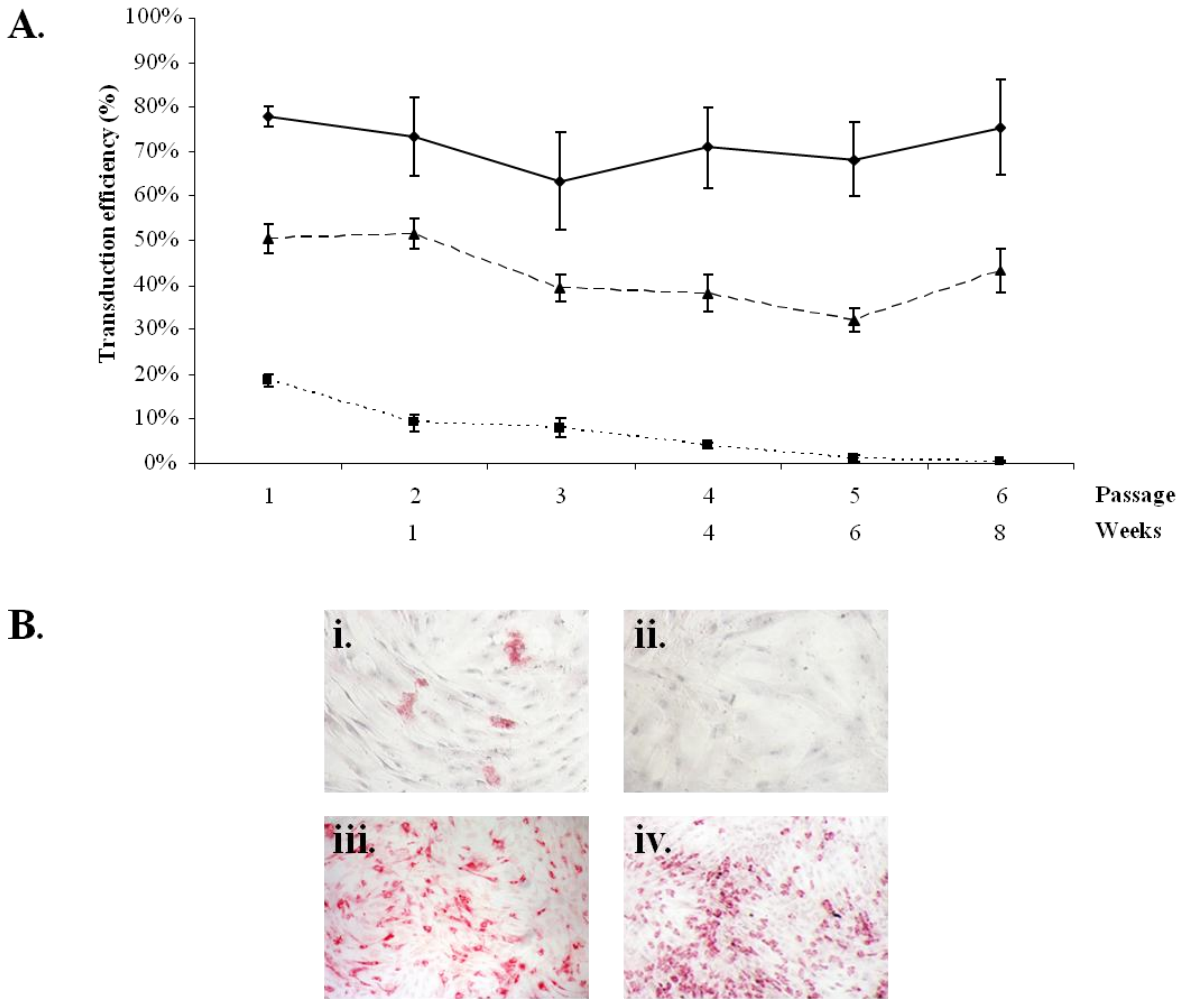


Figure 3.4: Stable transduction of β-*D*-glucuronidase over eight weeks in culture.

hBM MSCs transduced with (A) 0.007μg/μL (dotted line), 0.035μg/μL (dashed black) and 0.07μg/μL (solid black) p24 pHIV-EF1αmm*Gus* for 24 hours. Cells were passaged on reaching confluency, undergoing six passage events over eight weeks *in vitro.* Percent transduction is expressed as the mean ± SEM (n=4). (B) Representative pictures of β-*D*-glucuronidase staining for passage one (i and iii) and six (ii and iv) transduced with 0.007μg/μL (i and ii) and 0.07μg/μL pHIV-EF1αmmGUS (iii and iv) at 40x magnification.

efficiency (Figure 3.4B). Although transduction efficiency decreased at the lowest dose (0.007μg/μL p24) tested, vector copy number was maintained with 91% of original vector copy number detected after six passages, indicating stable integration of the vector.

3.3.5 Tri-lineage differentiation of transduced MSCs

To determine whether over-expression of an MPS enzyme affects the ability of MSCs to differentiate, hBM MSCs (Figure 3.5) and hDP MSCs (data not shown) were transduced with a lentiviral vector encoding β-*D*-glucuronidase (pHIV-EF1αmm*Gus*) and induced towards different mesenchymal lineages (osteogenic, adipogenic and chondrogenic), or towards a neuronal lineage (Chapter 2.2.6). This was performed using pHIV-EF1αmm*Gus* due to our ability to stain for β-glucuronidase activity *in vitro* (Chapter 2.2.5). However, osteogenic and adipogenic differentiation were repeated using a lentiviral vector encoding *Idua* (pHIV-EF1αmm*Idua*), used in subsequent *in vivo* studies (Chapter Four, Five and Six), to ensure comparable results with multiple vectors.

Strong staining for β-*D*-glucuronidase activity was observed at all time points by transduced cells, regardless of whether they had been induced to differentiate down osteogenic, adipogenic or chondrogenic lineages, when compared to non-transduced controls (Figure 3.5A, column 2 compared to column 1, week 4 data shown). The pHIV-EF1αmm*Idua* transduced cells produced significantly higher IDUA levels at all weeks analysed when compared to their non-transduced controls, regardless of whether they had been induced down the osteogenic or adipogenic lineages (Table 3.3, week 4 data shown).

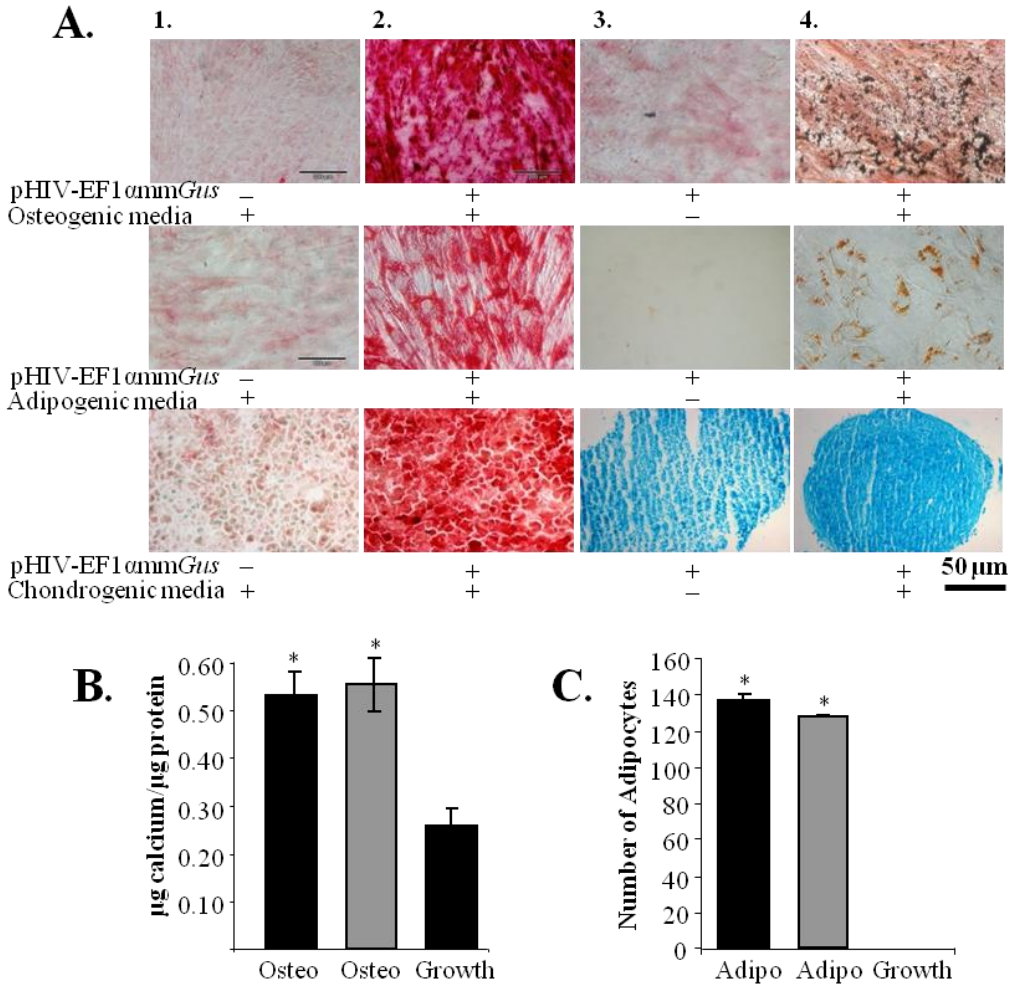


Figure 3.5: Osteogenic, adipogenic and chondrogenic differentiation of MSCs over-expressing β-*D*-glucuronidase.

(A) Strong staining for β-*D*-glucuronidase activity was observed in transduced cultures compared to non-transduced cultures (column 2 versus column 1) four weeks after the addition of differentiation media. Positive staining for von Kossa, Oil red O or alcian blue was observed in differentiation media induced cultures (column 4) compared to growth media controls (column 3). Visualisation performed by light microscopy at 40x magnification. (B) Quantification of mineralisation (calcium content) in non-transduced (grey) or pHIV-EF1αmm*Gus* transduced (black) cells. (C) Adipocyte number in non-transduced (grey) or pHIV-EF1αmm*Gus* transduced (black) cells. Results are expressed as mean ± SEM (n=3-4).

* Indicates significant difference between osteogenic or adipogenic induced cultures and growth controls ($p<0.05$; one-way ANOVA, Tukey's HSD).

Table 3.3: α-*L*-iduronidase transduced MSCs differentiation.

		OSTEOGENIC		ADIPOGENIC	
		IDUA enzyme level	**Calcium level**	**IDUA enzyme level**	**Adipocyte count**
hBM MSCs	Trans control	61230588 ± 6895960 #	0.4570 ± 0.005	10305740 ± 4815625 #	0 ± 0
	Trans differentiation	40684614 ± 2779966 #	0.8767 ± 0.002 *#	7278691 ± 1389410 #	159 ± 5 *
	Untrans control	15720 ± 1994	0.4978 ± 0.049	319069 ± 142567	0 ± 0
	Untrans differentiation	62582 ± 7668 *	0.6353 ± 0.012	310953 ± 55928	156.5 ± 4.5 *
hDP MSCs	Trans control	22191719 ± 3729900 #	0.5294 ± 0.001	17680716 ± 1902217 #	0 ± 0
	Trans differentiation	39275073 ± 6529816 #	0.9399 ± 0.004 *#	5156823 ± 72487 *#	163 ± 4 *
	Untrans control	100465 ± 361	0.5902 ± 0.061	291941 ± 10513	0 ± 0
	Untrans differentiation	232459 ± 29860	0.7080 ± 0.011	1610963 ± 6660	150.5 ± 0.5 *

*α-L-iduronidase (IDUA) enzyme levels in cell layer expressed as nmol/hr/mg protein for fluorogenic substrates. Calcium levels expressed as µg calcium/µg protein. Results are expressed as mean ± SEM of n=3. * Indicates significant difference between differentiation and control, # indicates significant difference between transduced and untransduced (p<0.05; one-way ANOVA, Tukey's HSD).*

3.3.5.1 Osteogenic differentiation

Mineral deposition, as determined by von Kossa staining (Chapter 2.2.6.1.1) was observed in both transduced and non-transduced cultures starting three weeks after the addition of osteogenic inductive medium and increased for the remaining two weeks in culture (Figure 3.5A, week 4 data shown). No difference was observed in staining between over-expressing β-*D*-glucuronidase and non-transduced cultures, indicating that osteogenic differentiation was not affected in β-*D*-glucuronidase over-expressing hBM and hDP MSCs (Figure 3.5A). This was confirmed by quantification of calcium content (Chapter 2.2.6.1.1). No significant difference in calcium levels for transduced and non-transduced cells for hBM (0.53 ± 0.03 and 0.55 ± 0.04µg calcium/µg protein respectively; Figure 3.5B) or hDP MSCs (1.07 ± 0.33 and 0.76 ± 0.22µg calcium/µg protein respectively) was observed when cultured under osteogenic inductive conditions. However, both were significantly elevated above cells maintained in growth media (0.26 ± 0.026 and 0.19 ± 0.09µg calcium/µg protein respectively; one-way ANOVA, Tukey's HSD; Figure 3.5B, hBM MSC only data shown).

Conversely, pHIV-EF1αmm*Idua* transduced hBM and hDP MSCs produced significantly higher calcium levels (0.8767 ± 0.002 and 0.9399 ± 0.004µg calcium/µg protein respectively) than their corresponding non-transduced controls (0.6353 ± 0.012 and 0.7080 ± 0.011µg calcium/µg protein respectively; one-way ANOVA, Tukey's HSD; Table 3.3), when induced down the osteogenic lineage. However, both were significantly elevated above cells maintained in standard growth media (0.4570 ± 0.005 and 0.5294 ± 0.001µg calcium/µg protein respectively; one-way ANOVA, Tukey's HSD; Table 3.3).

3.3.5.2 Adipogenic differentiation

Positive Oil red O staining (Chapter 2.2.6.2.1) of lipid was observed in differentiating cells following three weeks of adipogenic induction in both over-expressing β-*D*-glucuronidase (Figure 3.5A, week 4 data shown) and non-transduced cells (data not shown). Quantification of the number of adipocytes indicated no significant difference in the number of adipocytes formed between transduced and non-transduced hBM (127.5 ± 0.5 and 136 ± 4 adipocytes respectively) or hDP MSC (132 ± 1.5 and 135 ± 7 adipocytes respectively). However, both were significantly elevated above cells maintained in standard growth media, which did not contain any positive Oil red O staining (one-way ANOVA, Tukey's HSD; Figure 3.5C; hBM MSC only data shown).

Similar results were seen when cells were over-expressing IDUA (pHIV-EF1αmm*Idua*) (Table 3.3). Quantification of the number of adipocytes formed showed no significant difference between transduced and non-transduced hBM (159 ± 5 and 156.5 ± 4.5 adipocytes respectively) and hDP MSCs (163 ± 4 and 150.5 ± 0.5 adipocytes respectively) when induced down the adipogenic lineage (one-way ANOVA, Tukey's HSD; Table 3.3). Again, both were significantly elevated above cells maintained in standard growth media, which did not contain any Oil red O positive adipocytes (one-way ANOVA, Tukey's HSD; Table 3.3).

3.3.5.3 Chondrogenic differentiation

hBM and hDP MSCs over-expressing β-*D*-glucuronidase were also differentiated down the chondrogenic lineage (Figure 3.5A), as assessed by increased alcian blue staining of gag (Chapter 2.2.6.3.1) following three weeks of culture under chondrogenic inductive conditions. No difference in alcian blue staining was observed between transduced (Figure 3.5A, week 4 data shown) and non-transduced (data not shown) cell pellets following chondrogenic

induction. The chondrogenic induced pellet cultures contained spherical, condensed cells at a high cell density compared to those cultured in basal growth media which contained loose aggregates of cells with non-uniformed morphology and organisation.

3.3.5.4 Neuronal differentiation

The gene expression of an immature neuronal marker, nestin, and two mature neuronal markers, β-tubulin and NF-M, were measured at two different time points after the addition of neurogenic inductive medium to monitor the time course of neurogenesis (Chapter 2.2.6.4).

Seven days after the addition of retinoic acid, an up-regulation of the immature marker nestin (7.03 ± 2.46 fold increase) and a downregulation of β-tubulin and NF-M (6.22 ± 2.15 and 6.01 ± 2.79 fold decrease respectively) was observed in non-transduced hBM MSCs compared to cells maintained in basal growth media (one-way ANOVA, Tukey's HSD; Figure 3.6A). After an additional week in retinoic acid supplemented neuronal media this pattern was reversed. A downregulation of nestin (17.3 ± 7.88 fold decrease) and an up-regulation of β-tubulin and NF-M gene expression (3.94 ± 3.47 and 26.77 ± 6.78 fold increase respectively) was observed, indicating a maturation of the neuronal phenotype (one-way ANOVA, Tukey's HSD; Figure 3.6A).

Gene expression in hBM MSCs over-expressing β-*D*-glucuronidase followed a similar pattern. Nestin gene expression increased (3.78 ± 0.66 fold increase) seven days after retinoic acid addition, while β-tubulin expression decreased (2.28 ± 0.46 fold decrease; one-way ANOVA, Tukey's HSD; Figure 3.6A). After an additional seven days in retinoic acid supplemented neuronal media nestin gene expression decreased (1.87 ± 0.80 fold decrease), while β-tubulin and NF-M expression increased (2.864 ± 0.27 and 4.424 ± 2.66 fold increase

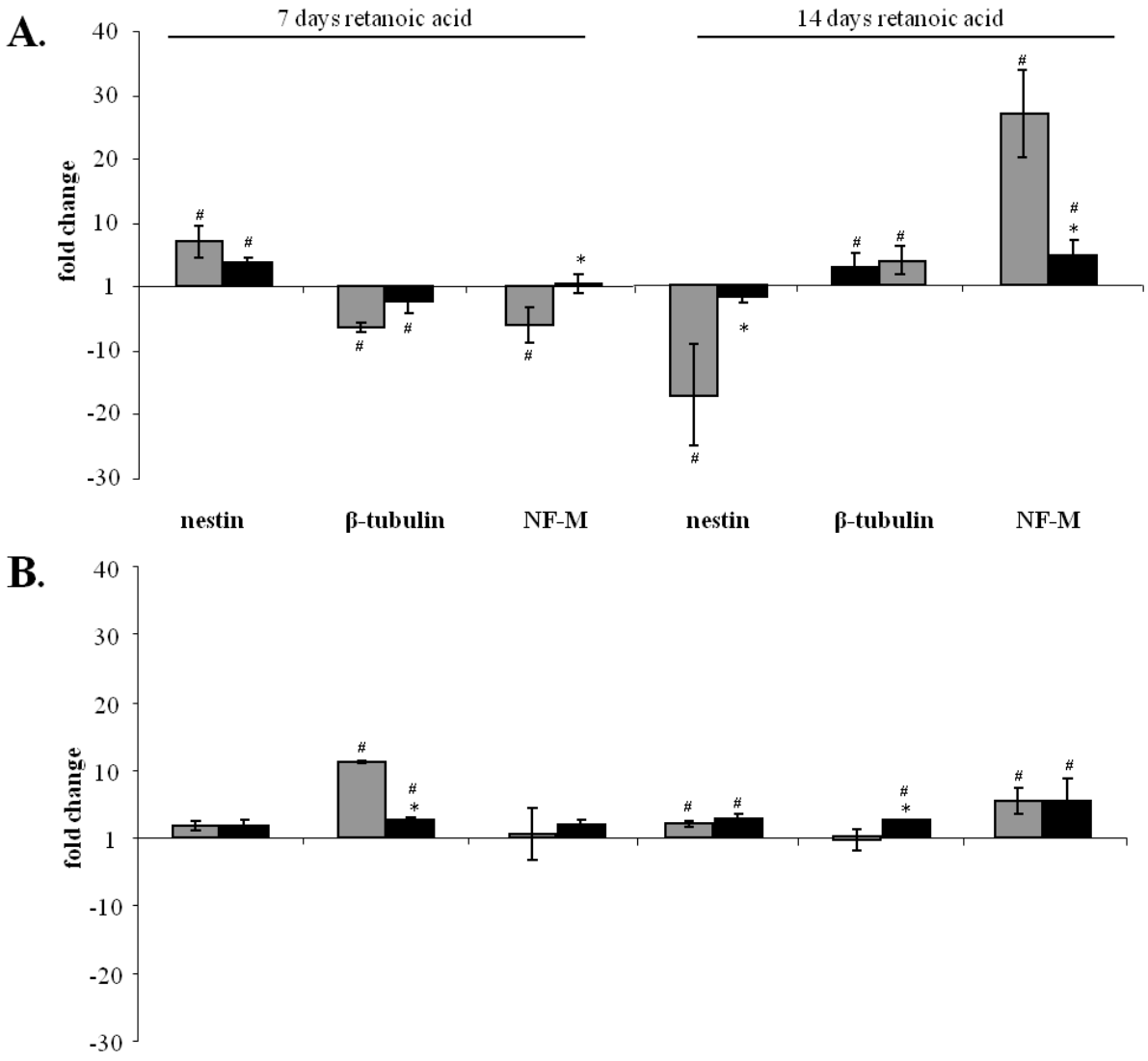


Figure 3.6: Neuronal differentiation of MSCs following β-*D*-glucuronidase transduction

Expression of neuronal genes seven and 14 days after retinoic acid addition by hBM MSCs (A) and hDP MSCs (B). Gene expression was normalised to cyclophilin A and the fold change in non-transduced (grey) and transduced (black) cells compared to cells cultured in basal growth media calculated using the ΔΔCt method. Results are expressed as mean ± standard deviation (n=3).

* Indicates significant difference between transduced and non-transduced controls, # Indicates significant difference between neuronal induced and growth controls ($p<0.05$; one-way ANOVA, Tukey's HSD).

respectively; one-way ANOVA, Tukey's HSD; Figure 3.6A). However, the extent of gene expression change for both nestin and NF-M was significantly different in hBM MSCs over-expressing β-*D*-glucuronidase compared to non-transduced neuronal controls 14 days post retinoic acid addition (one-way ANOVA, Tukey's HSD; Figure 3.6A).

In comparison, seven days after retinoic acid addition no significant change in nestin or NF-M expression was observed in non-transduced hDP MSCs, while β-tubulin expression increased (11.23 ± 0.22 fold increase), when compared to cells maintained in basal growth media (one-way ANOVA, Tukey's HSD; Figure 3.6B). Both nestin and NF-M gene expression increased after an additional week in retinoic acid supplemented neuronal media (2.16 ± 0.41 and 5.41 ± 1.92 fold increase respectively; one-way ANOVA, Tukey's HSD; Figure 3.6B).

Gene expression in hDP MSCs over-expressing β-*D*-glucuronidase followed a similar pattern, seven days after retinoic acid addition β-tubulin gene expression increased (2.53 ± 0.30 fold increase), however, expression was significantly less than in non-transduced hDP MSCs (one-way ANOVA, Tukey's HSD; Figure 3.6B). After an additional seven days in retinoic acid supplemented neuronal media, the increase in nestin and NF-M expression (2.66 ± 0.61 and 5.32 ± 3.28 fold increase respectively) was similar to that observed in non-transduced hDP MSCs, while β-tubulin gene expression was significantly up-regulated (2.37 ± 0.156 fold increase) in comparison to non-transduced neuronal controls (one-way ANOVA, Tukey's HSD; Figure 3.6B).

3.4 Discussion

Stem cell transplant using HSCs has been trialled in a number of MPS types, but is currently indicated only for the neurological form of MPS type I, where it stabilises CNS function

(Church, Tylee et al. 2007; Noh and Lee 2014). Skeletal symptoms persist in treated children and CNS symptoms in other MPS types progress even when total engraftment has been achieved (Koc, Day et al. 2002). MSCs have received increasing attention as therapeutic agents for bone and cartilage disease, including osteoarthritis and osteogenesis imperfect (Horwitz, Prockop et al. 1999; Horwitz, Prockop et al. 2001; Horwitz, Gordon et al. 2002; Le Blanc, Gotherstrom et al. 2005; Orozco, Munar et al. 2013; Wong, Lee et al. 2013; Vangsness, Farr et al. 2014), heart disease (Chen, Fang et al. 2004; Chen, Liu et al. 2006; Janssens, Dubois et al. 2006; Hare, Traverse et al. 2009; Jeevanantham, Afzal et al. 2013; Rodrigo, van Ramshorst et al. 2013), liver disease (Lin, Zhang et al. 2012; Shi, Zhang et al. 2012; Zhang, Lin et al. 2012; Mohamadnejad, Alimoghaddam et al. 2013; Wang, Li et al. 2013) and GvHD (Le Blanc, Rasmusson et al. 2004; Zhang, Liu et al. 2009; Perez-Simon, Lopez-Villar et al. 2011; Calkoen, Vervat et al. 2014; Herrmann and Sturm 2014), with MSCs currently being investigated in around 400 clinical trials (http://clinicaltrials.gov)(Wang, Qu et al. 2012).

The interest in MSCs has been further enhanced by their ability to be readily obtained from patients, easily expanded in culture and are generally non-tumorigenic when transfused (Prockop and Oh 2012). Several preliminary studies of MSCs in the genetic disorder OI have demonstrated safety and efficacy of these cell types in children (Horwitz, Prockop et al. 1999; Horwitz 2001; Horwitz, Prockop et al. 2001; Horwitz, Gordon et al. 2002) and when transplanted *in utero* (Le Blanc, Gotherstrom et al. 2005). A single report of MSC therapy in five MPS I children, who had previously undergone successful HSC transplant, reported no adverse effects or toxicities associated with the MSC transfusion, and all patients showed either a stabilisation or improvement in bone mineral density (Koc, Day et al. 2002). As the primitive cell type that differentiates into many of the mature cell types affected by MPS, MSCs would appear a logical choice to investigate for their efficacy in improving MPS

patient outcome. This chapter therefore determined MPS enzyme levels produced by hBM and hDP derived MSCs compared to HSCs, their ability to be transduced by a lentiviral vector incorporating MPS enzymes and differentiation capacity while over-expressing two MPS enzyme as a precursor to their use in animal studies and clinical trials.

3.4.1 Endogenous ability of MSCs succeeds HSCs

MSCs derived from hBM produced significantly higher intracellular levels of all six MPS enzymes tested, while hDP MSCs produced significantly higher levels of five MPS enzymes than $CD34^+$ HSCs. MSCs also secreted higher or equal levels of all enzymes into the culture medium than HSCs. Although enzyme secretion was higher in MSCs, whether the level achieved is sufficient to cross-correct in the clinical setting remains to be tested. Similarly, Koc *et al.* (2002) found significantly higher IDUA in normal MSCs (122 ± 69nmol/hr/mg protein) when compared to normal leukocytes (HSC derived; 77 ± 25nmol/hr/mg protein) levels *in vitro* (Koc, Day et al. 2002). Third passage cells were analysed using a slightly different enzyme assay (Hopwood, Muller et al. 1979), with large variations in both enzyme levels and isolated cell numbers noted between donors (Koc, Day et al. 2002). MPS animal studies using HSCs therapies showed that the level of enzyme produced correlated to treatment efficacy (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012), where HSCs needed to be engineered to over-express enzymes to reach maximum therapeutic benefit via cross-correction. A single study in the MPS VII mouse model noted that similar circulating enzyme levels were achieved when injecting non-transduced MSCs (Meyerrose, Roberts et al. 2008) and transduced HSCs (Hofling, Devine et al. 2004), suggesting that the higher levels achieved by MSCs would be more likely to correct widespread MPS pathology.

3.4.2 MSCs are more amenable to lentiviral over-expression

In agreement with previous reports (Leuci, Mesiano et al. 2011; Varma, Janic et al. 2011; Frecha, Costa et al. 2012), we observed that transduction efficiency of human MSCs was significantly higher than HSCs; with 90% and 25% of cells transduced respectively. In line with the greater transduction efficiency of MSCs, they over-expressed all MPS enzymes tested to a greater extent than HSCs. Significantly, MSCs from both tissue sources secreted higher levels of enzyme into the culture medium when compared to HSCs. As a source of MPS enzyme *in vivo*, transduced MSCs have the potential to generate higher circulating levels of MPS enzyme than HSCs.

Different levels of up-regulation of enzyme production and secretion capacity were observed between the different enzymes analysed. In particular, the levels of IDUA and β-*D*-glucuronidase produced in the over-expression system exceeded those of the sulphatases (4-sulphatase and sulphamidase). All sulphatases require post-translational modification of a highly conserved cysteine residue within the active site by sulphatase modifying factor 1 (SUMF1) (Schmidt, Selmer et al. 1995; Miech, Dierks et al. 1998). Over-expression of sulphatases can therefore be further manipulated by concurrent over-expression of SUMF1 (McIntyre, Byers et al. 2010) or by using a secretory signal in the vector construct (Sorrentino, D'Orsi et al. 2013). These varied over-expression results confers with the limitations associated with current therapy approaches for MPS, where the treatment of choice for one MPS type does not always transfer to an improvement in pathology in another MPS type.

A previous study over-expressing IDUA in BM MSCs isolated from MPS I knockout mice found significantly elevated IDUA enzyme expression one week post transfection at two

different viral concentrations *in vitro* when compared to non-transduced fibroblasts (95 and 225 fold increase respectively). However, while IDUA enzyme levels increased until four weeks post for the lower viral concentration (127 fold increase), levels were found to decline immediately at the higher viral concentration, with both viral concentrations producing IDUA levels below non-transduced controls after six weeks in culture (da Silva, Pereira et al. 2012). Over-expression of IDUA was lower than observed in the current study for normal donor hBM MSCs (805 fold increase), which may be attributed to cell source (murine versus human MSCs) or vector choice (MLV-SV40 versus pHIV-EF1α). However, a previous study showed that MPS IIIA affected HSCs were capable of reaching similar over-expression levels of sulphamidase when transduced with a lentiviral vector *in vitro* when compared to normal donor HSCs (Langford-Smith, Wilkinson et al. 2012), suggesting that disease status may not be the main contributing factor to loss of enzyme over-expression.

Production of transgene was consistent over eight weeks in non-cycling hBM and hDP MSCs. A small decline in the percentage of cells transduced (5-10%) was observed over six passages in culture, at the two highest doses of lentivirus tested, indicating that integration and transgene expression was stable over an extended period *in vitro* if the initial transduction efficiency was above 50%. This supports previous findings where 98.4% of cells maintained expression when an initial transfection of 90% was achieved (Zhang, La Russa et al. 2002; McGinley, McMahon et al. 2011). However, transgene suppression was evident at the lowest initial transduction efficiency, demonstrated by lack of positive β-D-glucuronidase staining after six passages *in vitro*, in contrast to the persistent detection of the viral *gag* gene by RT-PCR. Previous studies have shown that while lentiviral vectors have high genome stability and transducing ability, they are still subject to transgene silencing, due to epigenetic transgene suppression, choice of promoter and/or inactivation by target cells (Yao, Sukonnik et al. 2004; Ellis 2005; Xia, Zhang et al. 2007; McGinley, McMahon et al. 2011). This is

particularly evident in embryonic stem cells and HSCs, but is not well documented in MSCs (Challita and Kohn 1994; Ramezani, Hawley et al. 2003; Yao, Sukonnik et al. 2004; Xia, Zhang et al. 2007). However, a greater loss of transgene expression has been shown to correlate with lower initial transduction efficiencies (McGinley, McMahon et al. 2011).

During normal processing MPS enzymes are glycosylated in the endoplasmic reticulum before being post-translationally modified within the Golgi apparatus to acquire M6P residues, which target enzymes to lysosomes via binding to M6P receptor at physiological pH (Kornfeld 1992; Di Natale, Di Domenico et al. 2002). Enzymes secreted by MSCs transduced with lentivirus encoding IDUA, sulphamidase and β-*D*-glucuronidase were capable of cross-correcting the respective MPS fibroblasts in a M6P dependent manner. Thus, lentiviral transduced MSCs produced functional precursor protein that can be correctly targeted to the lysosome. In addition to their ability to differentiate into cells affected by MPS, the over-production of mannose-6-phosphorylated MPS enzymes by transduced MSCs could be expected to generate circulating levels of MPS enzyme *in vivo* capable of cross-correcting affected host cells in a manner analogous to ERT.

3.4.3 Lentiviral manipulation does not alter pluripotency of MSCs

The ability of MSCs to maintain pluripotency and differentiate into cells affected by MPS gag storage while over-expressing MPS enzymes was examined by assessing positive β-*D*-glucuronidase staining and IDUA enzyme activity following lentiviral transduction. Staining for β-*D*-glucuronidase activity indicated that lentiviral mediated transduction was stable during osteogenic, adipogenic and chondrogenic differentiation, with no significant adverse effects to lineage commitment. Similarly, significantly elevated IDUA enzyme activity was maintained during both osteogenic and adipogenic differentiation, which did not inhibit hBM

or hDP MSCs from lineage commitment. While previous studies have shown that MSCs are capable of osteogenic and adipogenic differentiation following lentiviral transduction (McMahon, Conroy et al. 2006; Van Damme, Thorrez et al. 2006; Wang, Dennis et al. 2009; Qian, Zhang et al. 2010), this is one of the first studies that has characterised chondrogenic (Wang, Dennis et al. 2009) and neuronal differentiation following lentiviral transduction. As neurological and skeletal pathology are the current problematic areas in MPS treatment, differentiation potential down these two lineages following lentiviral transduction is necessary for MSC engraftment and repair within these two systems.

Both transduced and non-transduced cells showed up-regulation of mature neuronal markers, however, differentiation down the non-mesodermal lineage appeared to be slightly delayed, or less pronounced, in the transduced cells. Dental pulp derived MSCs are thought to be of neural crest origin (Chai, Jiang et al. 2000; Mead, Logan et al. 2013), expressing markers of both mesenchymal and neuroectodermal stem cell lineages (Gronthos, Mankani et al. 2000; Miura, Gronthos et al. 2003; Sakai, Yamamoto et al. 2012). Recent evidence suggests hDP MSCs exhibit a greater propensity to form functional neurons that other tissue sources (Sakai, Yamamoto et al. 2012; Mead, Logan et al. 2013); consistent with the earlier up-regulation of mature neuronal β-tubulin in hDP MSCs than hBM MSCs.

3.4.4 Chapter conclusions and future directions

Murine studies incorporating lentiviral transduced HSCs in both MPS I (Visigalli, Delai et al. 2010) and MPS IIIA (Langford-Smith, Wilkinson et al. 2012) have shown vast improvements in many aspects of MPS disease pathology, however, both studies showed that neurological improvements were correlated with achieving supranormal enzyme levels. HSCs are only capable of differentiating into cells of the blood lineage, and hence the neurological

improvements associated with HSC transplants, lentiviral transduced or not, are due to infiltration of macrophages into the brain producing the defective enzyme available for uptake (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012). However, substantial physical changes are evident in the brains of MPS patients, including; dilation of the periventricular spaces, brain atrophy, widening of cortical sulci, abnormal signal intensity in the white matter and enlargement of the extra-ventricular spaces (Palmucci, Attina et al. 2013). Suggesting that repairing the multifaceted aspects of MPS brain disease most likely requires more than infiltrating macrophages and enzyme secretion, but also the repair of functional neurons. As both hBM and hDP MSCs were able to be induced into a neuronal-like state through the up-regulation of mature neuronal associated markers, β-tubulin and NF-M, their transition into preclinical trials may produce functional improvements in the neurological degeneration associated with multiple MPS subtypes.

In this chapter we have shown that MSCs hold many advantages over the currently used HSCs, most importantly, MSCs secrete significantly higher amounts of MPS enzymes than currently used HSCs, both endogenously and in an over-expression system; with previous studies showing a correlation between enzyme secretion and improved neurological correction (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012). Excess secreted enzyme can be internalised by non-engrafted/corrected cells via the M6P receptor, which would allow more widespread gag clearance. Adding to this, MSCs hold added immunological benefits due to their ability to inhibit T-cell proliferation and hence lower the immune response of the host. GvHD or a delay in treatment due to finding a HLA identical donor, both of which are associated with HSC transplants, are therefore circumvented.

HSCs can only give rise to cells of the blood, and a successful transplant therefore relies on the migration of transplant-derived leukocytes into the organ to lower gag storage and

produce functional lysosomal enzymes. However, MSCs can form multiple cell types including bone, cartilage and neuronal-like cells, potentially covering the current problematic areas associated with MPS therapy. MSCs also possess the ability to cross the BBB, which could prove fundamental for combating CNS deterioration in multiple MPS types (Chen, Li et al. 2001). This study warrants the further use of MSCs in an *in vivo* study to determine if they exhibit to cause an improvement in MPS related disease. A MPS I animal trial was therefore performed using hDP and hBM MSCs over-expressing IDUA to determine the biodistribution and any associated biochemical improvements for each tissue source (Chapter Four). The findings from this initial trial would determine what tissue source had any additional benefits for combating MPS I pathology for translation into a larger animal study looking at both functional improvement (Chapter Five) and long-term biochemical outcomes (Chapter Six).

Chapter Four: Biodistribution of hDP IDUA and hBM IDUA MSCs in MPS I mice

4.1 Introduction

To date, HSC based therapy is the treatment of choice for MPS I children presenting with neurological damage (Peters, Balthazor et al. 1996; Peters, Shapiro et al. 1998; Boelens 2006; Wynn, Wraith et al. 2009; Langereis, Borgo et al. 2013), however, the results in Chapter Three demonstrated that the endogenous levels and over-expression of IDUA by MSCs *in vitro* were significantly greater when compared to HSCs. As nearly every MPS I cell is engorged with gags, resulting in widespread inflammation and disease, how hDP or hBM MSCs biodistribute following *i.v.* administration in MPS I mice is currently unknown, but would provide valuable insight into their application as a potential multi-tissue treatment alternative.

MSCs, when administered systemically, are known to become trapped within the lung, mainly attributed to the difference in diameter of MSCs and lung capillaries (Gao, Dennis et al. 2001; Barbash, Chouraqui et al. 2003; Allers, Sierralta et al. 2004; Bentzon, Stenderup et al. 2005; Schrepfer, Deuse et al. 2007; Fischer, Harting et al. 2009). Cells that evade the lung vasculature diffuse to the liver, spleen and kidney within two days (Gao, Dennis et al. 2001), where they expand and migrate to other tissues over time (Allers, Sierralta et al. 2004). However, total or site specific irradiation (Pereira, Halford et al. 1995; Pereira, O'Hara et al. 1998; Francois, Bensidhoum et al. 2006; Francois, Usunier et al. 2014), vasodilator pre-treatment (Gao, Dennis et al. 2001; Schrepfer, Deuse et al. 2007), or site induced injury (Barbash, Chouraqui et al. 2003) have been used to manipulate the distribution and homing of MSCs.

MSCs were first isolated from the bone marrow (Friedenstein, Chailakhyan et al. 1974) and are considered the tissue source of choice for many studies. However, dental pulp derived

MSC-like cells (Gronthos, Mankani et al. 2000; Gronthos, Brahim et al. 2002; Miura, Gronthos et al. 2003; Shi and Gronthos 2003) are thought to be of neural crest origin (Chai, Jiang et al. 2000; Arthur, Rychkov et al. 2008; Arthur, Shi et al. 2009; Mead, Logan et al. 2013), with recent evidence suggesting they are more effective and capable of promoting functional recovery of neurons after spinal cord damage (Sakai, Yamamoto et al. 2012; Mead, Logan et al. 2013) or following stroke (Leong, Henshall et al. 2012).

Both hDP and hBM MSCs were capable of expressing mature neuronal markers β-tubulin and NF-M *in vitro* (Chapter Three), however, this chapter will assess their ability to distribute to the MPS I affected brain and other organs *in vivo* within two days of *i.v.* administration. To increase lung evasion, a once off injection of sodium nitroprusside, a strong vasodilator, was administered to MPS I treated mice prior to MSC injection to help promote widespread distribution. All mice, both normal and MPS I, underwent daily subcutaneously injections of cyclosporin one week prior to MPS I treated mice receiving MSCs via the tail vein. MSC biodistribution and biochemical parameters of MPS I disease were analysed two days post administration (Figure 4.1), to determine which cell source should be used for future experiments (Chapter Five and Six).

4.2 Tissue weights

MPS I patients are known to suffer from organomegaly, and more specifically hepatosplenomegaly; the enlargement of the liver and spleen (Muenzer, Wraith et al. 2009). Wet tissue weights were determined when two month old mice were humanely killed two days post MSC *i.v.* infusion (Figure 4.1).

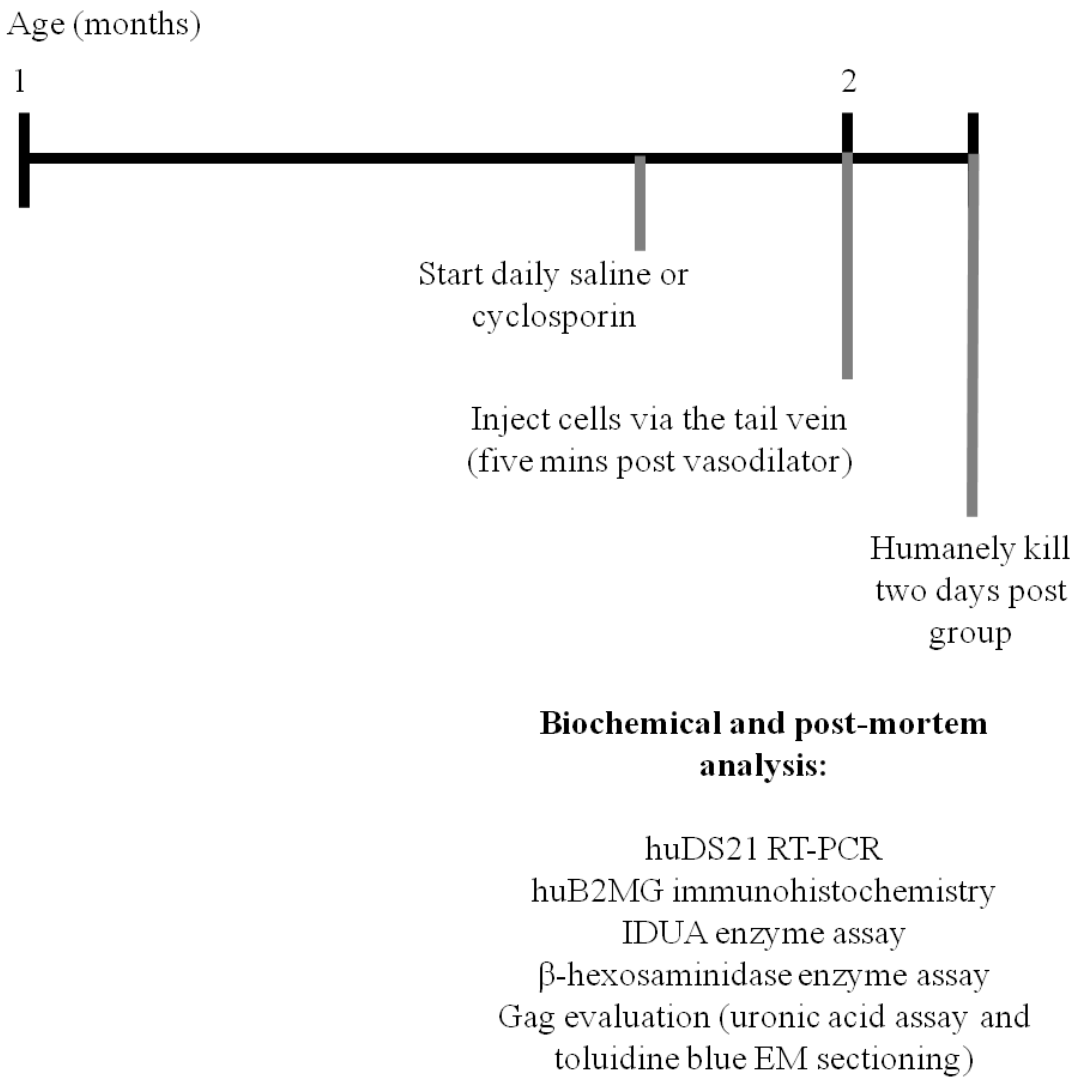


Figure 4.1: Timeline of treatment initiation and biochemical analysis.

Twelve (three normal and nine MPS I affected) mice were treated from seven weeks of age with daily cyclosporin subcutaneous injections. After one week of daily injections, MPS I affected mice allocated to hBM IDUA MSCs (three mice) or hDP IDUA MSC (three mice) treatment groups (Table 2.1) received a tail vein injection of a vasodilator (1mg/kg nitroprusside). Five minutes post vasodilation, treated mice received a once off injection of $1x10^6$ hBM IDUA MSCs or hDP IDUA MSCs also via the tail vein.

All mice, including three normal and three MPS I affected untreated controls, were humanely killed two days post MSC administration to determine the biodistribution of MSCs. Dissected tissues underwent numerous biochemical and post-mortem testing.

Wet tissue weights of the brain, kidney, heart and lung were not significantly altered in MPS I cyclosporin treated mice when compared to age matched normal cyclosporin treated controls (Figure 4.2). However, the wet tissue weights of the liver was significantly elevated in MPS I cyclosporin, hDP IDUA MSC and hBM IDUA MSC treated mice when compared to normal cyclosporin controls (123%, 169% and 174% of normal respectively; one-way ANOVA, Tukey's HSD; Figure 4.2). While the spleen of MPS I cyclosporin treated mice was slightly enlarged when compared to normal cyclosporin controls (108% of normal), this did not reach significance. However, the additional treatment of both hDP IDUA MSCs and hBM IDUA MSCs resulted in a significant enlargement of the spleen when compared to normal cyclosporin controls (167% and 175% of normal respectively; one-way ANOVA, Tukey's HSD; Figure 4.2).

4.3 Biodistribution of mesenchymal stem cells

The biodistribution of human MSCs in each mouse organ was determined by RT-PCR targeting a highly specific primer pair that is present once in the human genomic DNA sequence, Down syndrome region on chromosome 21 (huDS21) as per Song *et al*. (2012) (Chapter 2.6.1). Immunohistochemical analysis of human specific beta-2-microglobulin (huB2MG; Novacastra, Leica), a major histocompatibility complex class I antigen present on the surface of most nucleated cells was used to visualise the biodistribution of human cells within each organ (Chapter 2.6.3).

4.3.1 Human specific real-time PCR

Real-time PCR gene expression of huDS21 (Chapter 2.6.1) indicated that hDP IDUA MSCs and hBM IDUA MSCs distributed widely to multiple MPS I affected organs, however, cell numbers detected within each organ varied between each treated mouse (Table 4.1). hDP

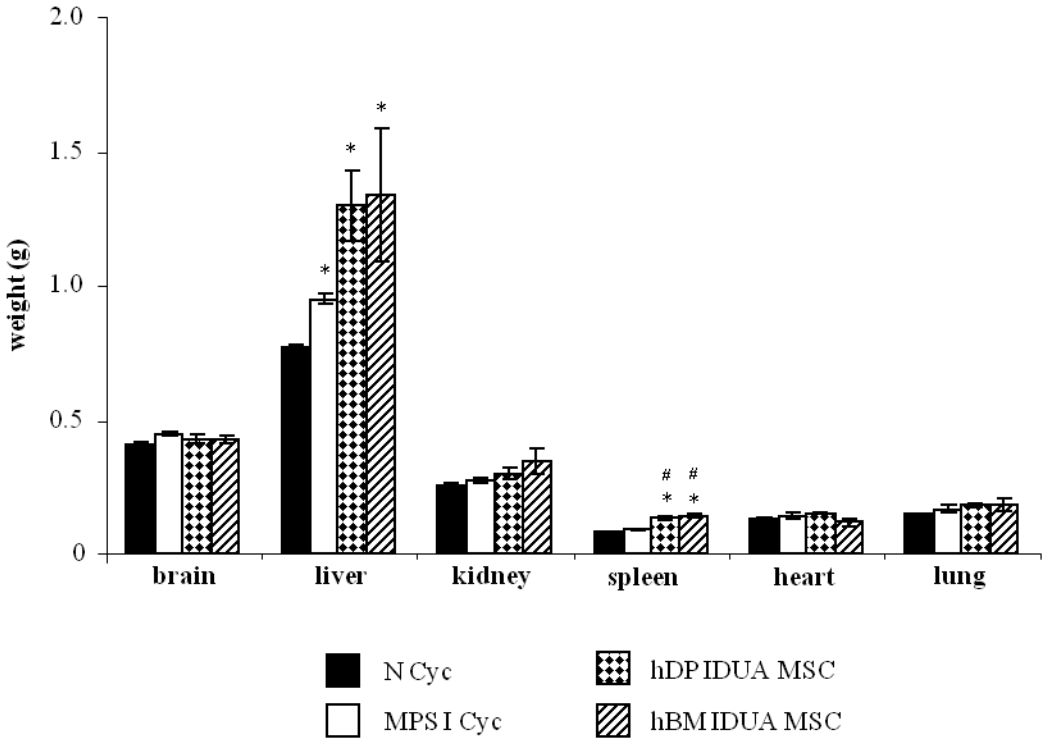


Figure 4.2: Tissue weight

Two months of age (two days post treatment) tissue weights. Mean ± SEM (n=3 animals) wet tissue weights are presented.

***** Indicates significant difference to N Cyc, **#** indicates significant difference to MPS I Cyc, $p<0.05$, one-way ANOVA, Tukey's HSD.

Table 4.1: Real-time PCR analysis of hMSCs in mouse tissues using huDS21 specific primers.

Treatment	Brain	Liver	Kidney	Spleen	Heart	Lung
hDP IDUA MSCs	<1 (12%)	<1 (57%)	ND (0%)	<1 (32%)	ND (0%)	ND (0%)
	<1 (4%)	<1 (15%)	2.8 (72%)	<1 (2%)	ND (0%)	<1 (7%)
	<1 (1%)	ND (0%)	<1 (5%)	<1 (6%)	ND (0%)	2.4 (88%)
Combined total proportion	**3%**	**13%**	**47%**	**5%**	**0%**	**32%**
hBM IDUA MSCs	<1 (42%)	ND (0%)	ND (0%)	<1 (58%)	ND (0%)	ND (0%)
	61 (68%)	1.33 (3%)	24 (18%)	19.4 (7%)	9.6 (2%)	3.3 (1%)
	ND (0%)	<1 (2%)	8.9 (53%)	16.7 (45%)	ND (0%)	ND (0%)
Combined total proportion	**60%**	**3%**	**22%**	**11%**	**2%**	**1%**

Real-time PCR performed with specific primer pairs complementary to the human Down syndrome genomic DNA sequence on chromosome 21 (huDS21). Purified tissue (20mg) genomic DNA from each individual animal was determined against a standard curve generated by serially diluted human MSC genomic DNA into murine genomic DNA. Results expressed as total cells detected per 10^4 cells, followed by the proportion of cells detected within that organ in relation to total cells detected per individual animal in brackets. ND = no cells detected in 20mg proportion of tissue converted to genomic DNA for analysis using the Wizard SV genomic DNA purification system (Promega).

IDUA MSCs were detected in the brain (3/3 animals; 3% total cells detected), liver (2/3 animals; 13% total cells detected), kidney (2/3 animals; 47% total cells detected), spleen (3/3 animals; 5% total cells detected) and lung (2/3 animals; 32% total cells detected) of treated MPS I mice, however, no cells were detected within the heart (0/3 animals; Table 4.1). Similarly, hBM IDUA MSCs were detected in the brain (2/3 animals; 60% total cells detected), liver (2/3 animals; 3% total cells detected), kidney (2/3 animals; 22% total cells detected), spleen (3/3 animals; 11% total cells detected), heart (1/3 animals; 2% total cells detected) and lung (1/3 animals; 1% total cells detected) of treated MPS I mice. However, less than 1% of originally injected cells were detected by RT-PCR two days post administration for each individual animal.

4.3.2 Human specific immunohistochemistry

Human specific B2MG staining confirmed RT-PCR results (Section 4.3.1); with the highest staining intensity present within the brain of hBM IDUA MSC treated mice and minimal, punctuated staining present in other somatic organs (Figure 4.3). Positive staining was highest within the cerebellum and frontal lobe, with minimal staining present within the temporal, parietal and occipital regions of the brain. Positive huB2MG staining was noted around blood vessels, supporting the *i.v.* delivery and distribution of hMSCs (Figure 4.3). Positive staining was confirmed against human MSCs, which were embedded in OCT and underwent the same experimental procedure (Figure 4.3).

4.4 Biochemical analysis

Biochemical analysis was performed on all tissues two days post *i.v.* MSC administration to determine if any biochemical parameters were changed with hMSC treatment. The typical spectrum of pathology associated with MPS I is a decrease in IDUA enzyme activity (Chapter

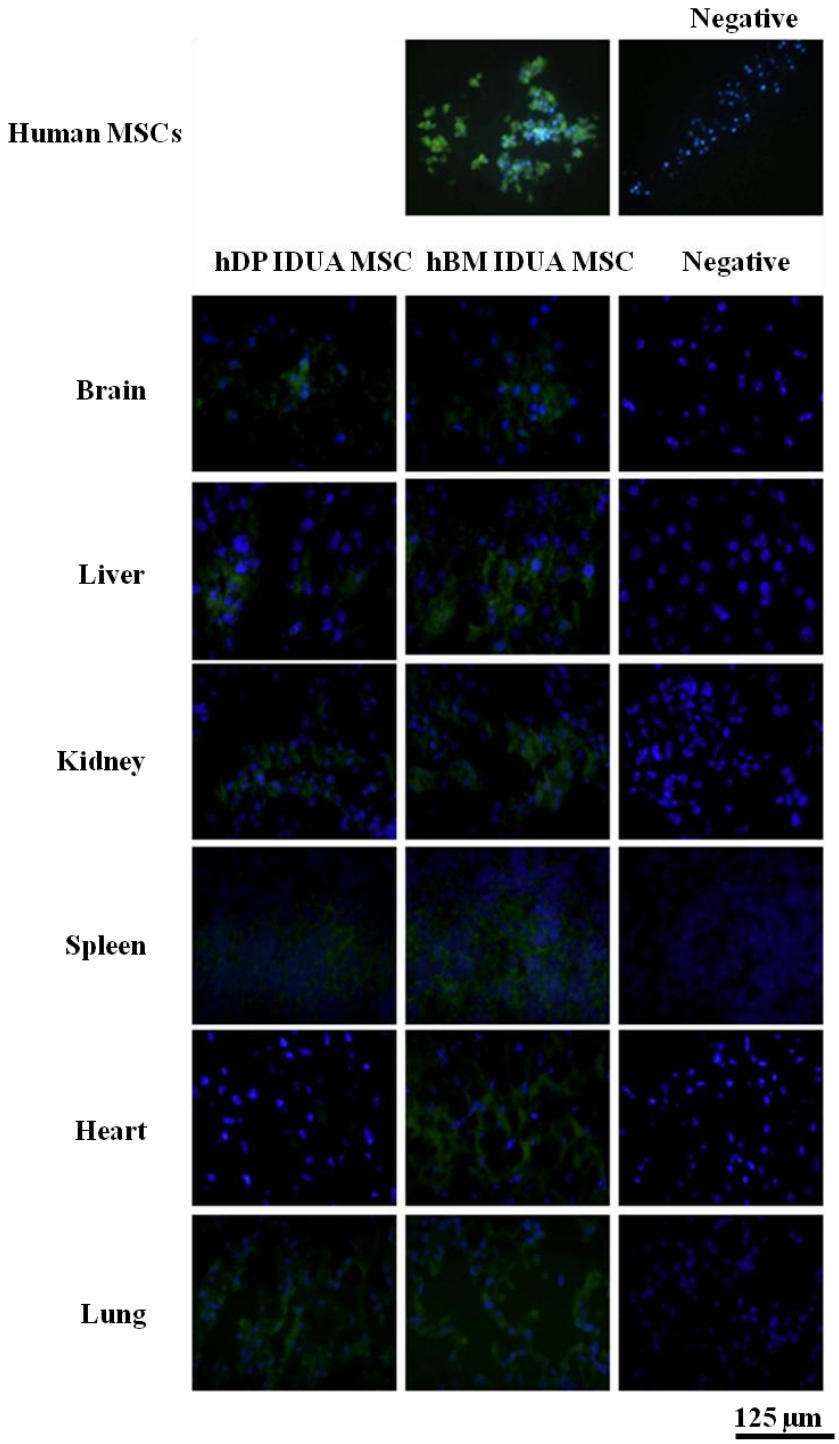


Figure 4.3: Human specific beta-2-microglobulin staining.

Localisation of human MSCs was determined on frozen sections post fixed with formalin using a primary antibody specific to human beta-2-microglobulin and a FITC conjugated secondary antibody. Nuclei were stained with DAPI. Target organs for each treatment group are shown, next to their negative control at 100x magnification. Human MSCs were used as a positive control.

2.5.4), an increase in β-hexosaminidase enzyme activity (Chapter 2.5.5) and an increase in gag storage; as shown through uronic acid (Chapter 2.5.6) and toluidine blue staining (Chapter 2.8).

4.4.1 α-L-iduronidase assay

As expected, MPS I cyclosporin treated mice had significantly reduced IDUA enzyme activity in all tissues analysed when compared to normal cyclosporin treated controls (one-way ANOVA, Tukey's HSD; Figure 4.4). Similarly, mice that received either hDP IDUA MSC or hBM IDUA MSCs also had significantly reduced IDUA enzyme levels in all tissues analysed when compared to normal cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 4.4). However, brain IDUA enzyme levels were slightly elevated in two mice receiving hBM IDUA MSCs (149% and 238% of MPS I cyclosporin; Figure 4.4A). While, hDP IDUA MSC and hBM IDUA MSC serum IDUA levels were also 215% and 210% higher than MPS I cyclosporin controls respectively (not significant; Figure 4.4G).

4.4.2 β-hexosaminidase assay

β-hexosaminidase is a secondary enzyme elevated in MPS and is often used as a biomarker of disease (Chung, Ma et al. 2007; Ma, Liu et al. 2007). As expected, β-hexosaminidase enzyme activity was significantly elevated in all MPS I cyclosporin tissues analysed when compared to normal cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 4.5). Similarly, mice that received either hDP IDUA or hBM IDUA MSCs also had significantly elevated β-hexosaminidase enzyme levels in all tissues analysed when compared to normal cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 4.5). In addition, brain β-hexosaminidase enzyme levels of hBM IDUA MSC treated mice was further elevated above MPS I cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 4.5A). However, β-

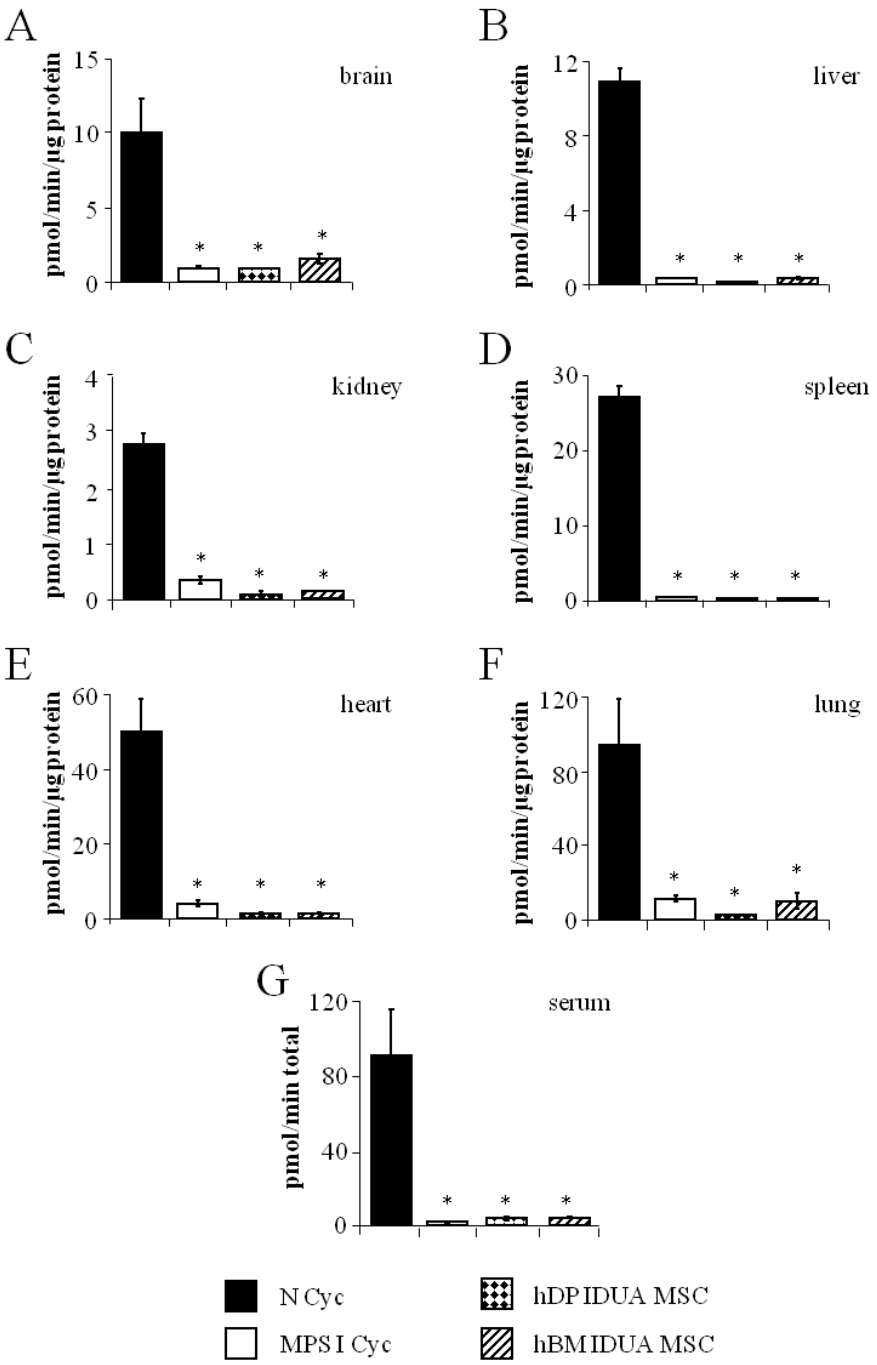


Figure 4.4: Tissue IDUA enzyme levels

Tissue IDUA enzyme levels were measured two days post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 4-umbelliferyl-α-*L*-iduronide substrate for four hours at 37ºC. Results were normalised to protein (not serum) and mean ± SEM (n=3 animals) IDUA enzyme levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E),** lung **(F)** and serum **(G)**.

***** Indicates significant difference to N Cyc, $p<0.05$, one-way ANOVA, Tukey's HSD.

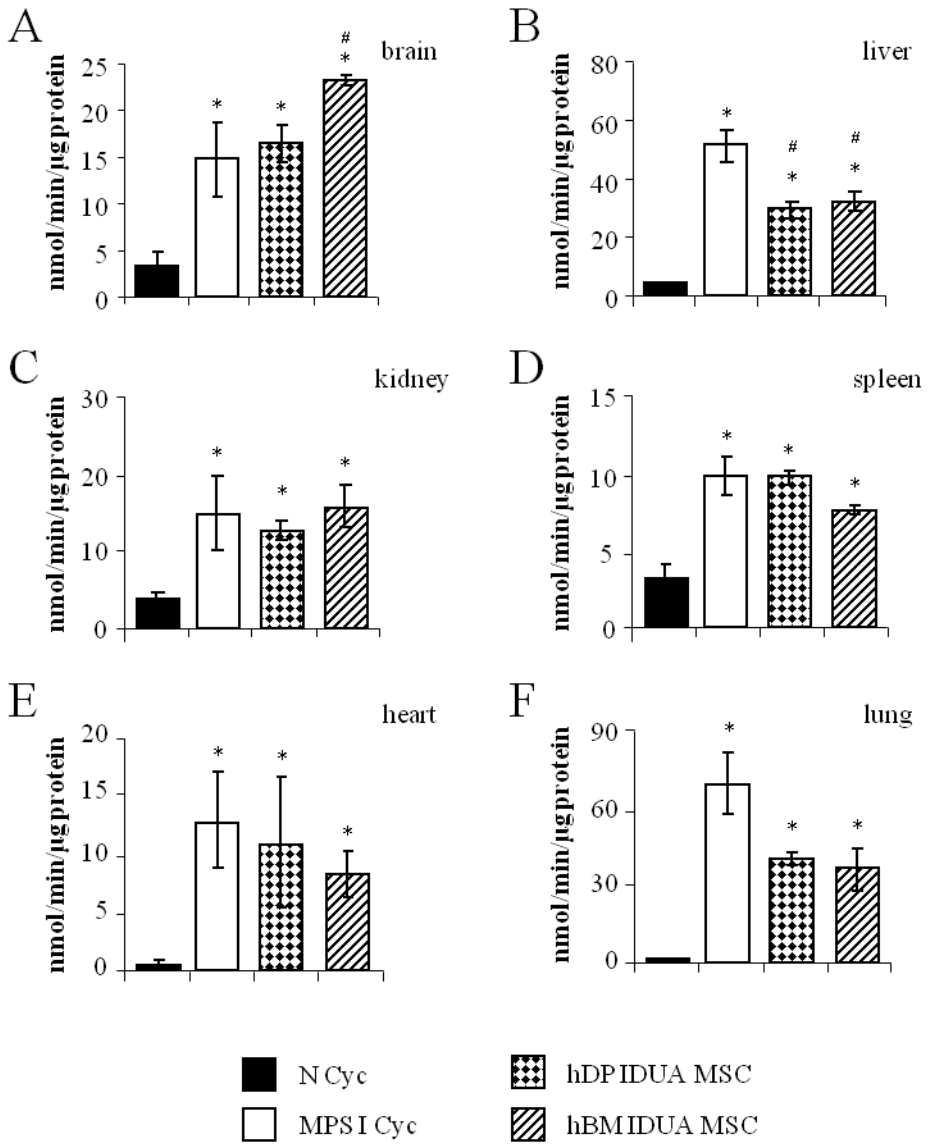


Figure 4.5: Tissue b-hexosaminidase enzyme levels

Tissue b-hexosaminidase enzyme levels were measured two days post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 4-methylumbellifryl-2-acetamindo-2-deoxy-b-D-glucopyranoside substrate for thirty minutes at 37ºC. Results were normalised to protein and mean ± SEM (n=3 animals) b-hexosaminidase enzyme levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E)** and lung **(F)**.

***** Indicates significant difference to N Cyc, **#** indicates significant difference to MPS I Cyc, $p<0.05$, one-way ANOVA, Tukey's HSD.

hexosaminidase levels within the liver of both hDP IDUA MSC and hBM IDUA MSC treated mice were significantly reduced when compared to MPS I cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 4.5B), with values also nearing significance in the lung (p=0.066 and 0.063 respectively; Figure 4.5F).

4.4.3 Uronic acid assay

As anticipated, MPS I cyclosporin mice had elevated uronic acid levels in all tissues analysed when compared to normal cyclosporin controls, reaching significance in all tissues except the brain (one-way ANOVA, Tukey's HSD; Figure 4.6). Mice treated with hDP IDUA MSCs had significantly elevated uronic acid levels in the liver, kidney, spleen, and lung (one-way ANOVA, Tukey's HSD; Figure 4.6B, C, D and F respectively), while mice treated with hBM IDUA MSCs had significantly elevated uronic acid levels in the brain, liver, kidney and lung (one-way ANOVA, Tukey's HSD; Figure 4.6A, B, C and F respectively) when compared to normal cyclosporin controls. Both hDP IDUA MSC and hBM IDUA MSC treated mice had significantly reduced uronic acid levels in the liver when compared to MPS I cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 4.6B).

4.4.4 Toluidine blue staining

Visualisation of gag storage on resin embedded tissue sections confirmed uronic acid results (Section 4.4.3), where no undegraded gag storage was detected in normal cyclosporin controls, but was abundant in MPS I cyclosporin tissues as white, foamy vacuoles (Figure 4.7). Lysosomal storage was evident in neurons (N), glial cells (G), perivascular macrophages, endothelial cells and Purkinje cells of the brain, kupffer cells (K) and hepatocytes (H) of the liver, proximal convoluted tubules (PCT) and podocytes (P) of the kidney, parenchyma and macrophages (M) of the spleen, laminar fibrosa and endothelium (E)

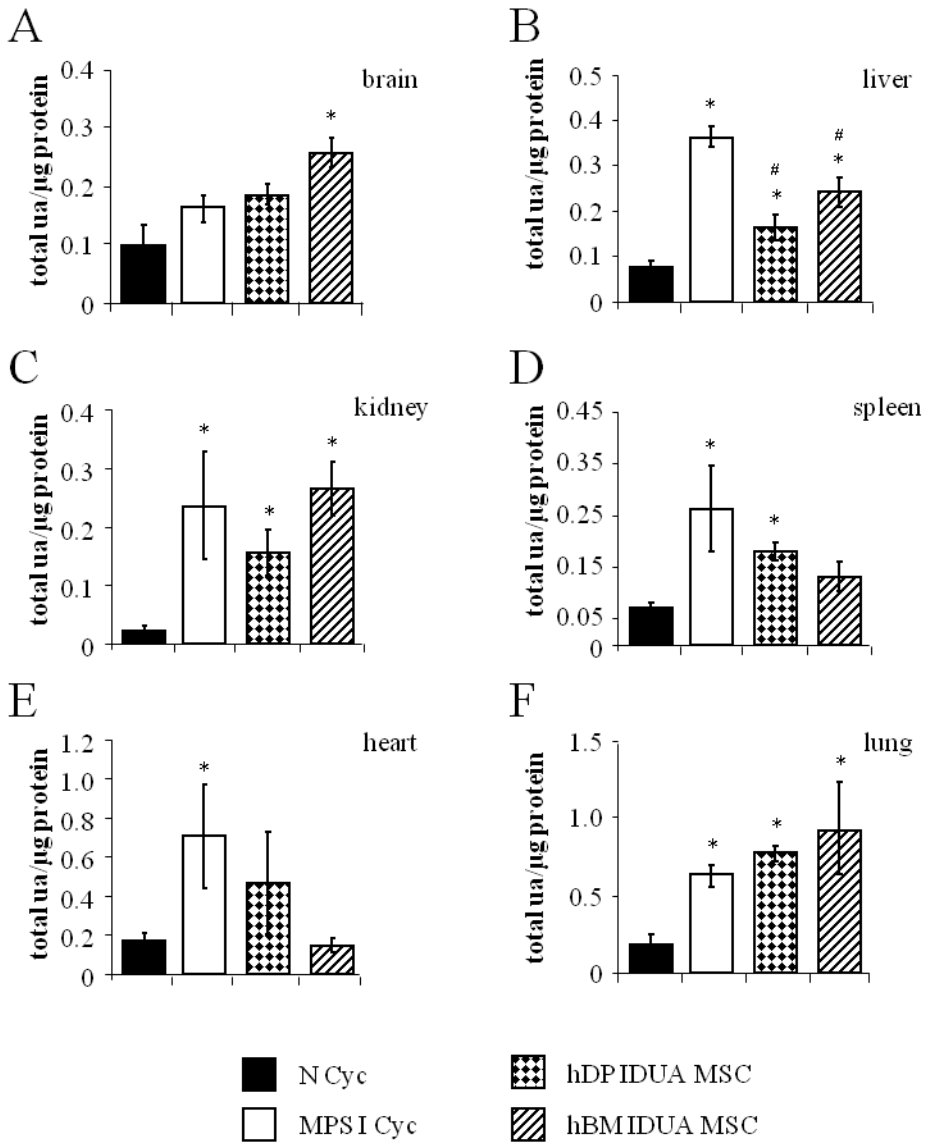


Figure 4.6: Tissue uronic acid levels

Tissue gag levels were measured two days post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 2.5 volumes 0.1% (w/v) cetylpridinium chloride in 0.054M citrate buffer, pH 4.8, for thirty minutes at 37ºC and measured for uronic acid. Results were normalised to protein and mean ± SEM (n=3 animals) uronic acid levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E)** and lung **(F)**.

***** Indicates significant difference to N Cyc, # indicates significant difference to MPS I Cyc, $p<0.05$, one-way ANOVA, Tukey's HSD.

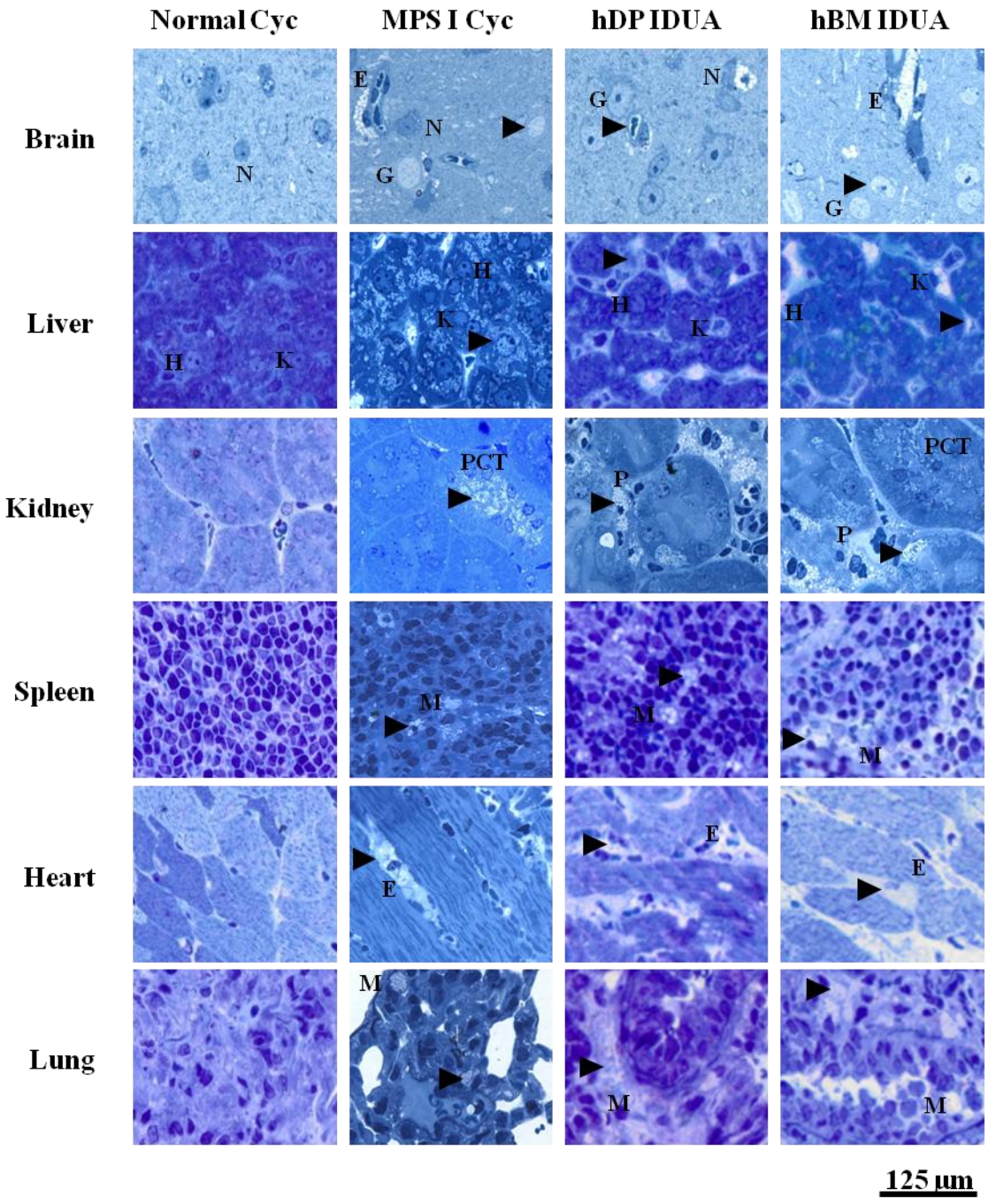


Figure 4.7: Tissue lysosomal storage

EM fixed (1.25% (v/v) glutaraldehyde, 4% (w/v) sucrose and 4% (w/v) paraformaldehyde in PBS) resin embedded 1µm tissue sections were stained with toluidine blue and visualised for gag storage (arrow head) by light microscopy at 100x magnification. **N** neuron, **G** glia, **H** hepatocyte, **K** kupffer cell, **PCT** proximal convoluted tubule, **P** podocyte, **M** macrophages and **E** endothelium.

of the heart, and phagocytes and pneumocytes (macrophages, M) of the lungs (Figure 4.7). Similarly, gag storage was still present in all tissues for both hDP IDUA and hBM IDUA MSC treated mice. However, in support of uronic acid results, gag storage was less evident in the liver of both treated groups, with hepatocytes (H) generally free of gag storage (Figure 4.7).

4.5 Discussion

MPS I is a multi-tissue and organ disease that available therapies are failing to adequately address. While HSCs can halt or slow the progression of MPS I disease, ongoing neurological and skeletal support is often necessary, even after total engraftment (Boelens 2006; Church, Tylee et al. 2007; Yoshida, Hayakawa et al. 2008). For MSCs to be effective at combating the multi-system pathology associated with MPS I, their *in vivo* distribution and engraftment would need to be widespread to allow replacement of damaged cells, or engrafted cells would need to secrete high levels of circulating enzyme to facilitate extensive cross-correction. MSCs are capable of differentiating into both bone and neural precursor cells *in vitro* (Chapter Three), and produce and secrete significantly higher levels of multiple lysosomal enzymes in both an endogenous and over-expressing system than HSCs *in vitro* (Chapter Three). The *i.v.* biodistribution of hDP IDUA MSCs and hBM IDUA MSCs was therefore investigated in this chapter to determine if MSCs were capable of diffusing to multiple organs, including the brain, in MPS I affected adult mice.

4.5.1 MSCs evade the lung following vasodilation

The lungs are the first organ that MSCs pass through when injected *i.v.* (Wagner and Henschler 2013), and more than 80% of cells have been found to form emboli within the long vessels of the lungs within a few minutes of administration (Lee, Fang et al. 2009; Lee, Pulin

et al. 2009; Wagner and Henschler 2013). Pre-treatment with a strong vasodilator, sodium nitroprusside, not only enlarges the diameter of pulmonary capillaries but also prevents the aggregation and adhesion of platelets (Tinker and Michenfelder 1976; Tinker and Cucchiara 1978; Krossnes, Mella et al. 1996; Krossnes, Mella et al. 1996; Krossnes, Geisler et al. 1998; Gao, Dennis et al. 2001). By administering sodium nitroprusside prior to stem cell infusion, Gao *et al* (2001) achieved a 15% decrease in cells trapped within the lung, and a resulting 10-50% increase in cells found in other organs; notably the liver and kidney (Gao, Dennis et al. 2001).

huDS21 gene expression demonstrated the effectiveness of vasodilator pre-treatment, with 68% of detected hDP IDUA MSCs and 99% of detected hBM IDUA MSCs evading the lung vasculature. However, cell distribution was limited in one hDP IDUA MSC treated mouse, as 88% of detected cells remained trapped within the lungs. In line with their improved lung evasion, a higher proportion of hBM IDUA MSCs were detected within other organs compared to hDP IDUA MSCs, however, both cell types were able to distribute widely and to multiple MPS I affected organs. In agreement with previous biodistribution reports in normal mice, MSCs were found to home primarily to the kidney, liver and spleen of MPS I mice (Gao, Dennis et al. 2001; Allers, Sierralta et al. 2004), suggesting that MPS I associated pathology did not notably influence the biodistribution of hMSCs. However, in contrast to normal biodistribution patterning, human MSCs were detected within the brain of five out of six MPS I mice (3/3 hDP IDUA MSC and 2/3 hBM IDUA MSC), confirming their ability to cross the BBB following *i.v.* administration, and their potential application for homing to, and combating neurological disease associated with MPS I. Previous studies have observed impairments in the BBB of MPS patients and animal models of disease, with damaged BBB endothelium and tight junctions possibly allowing increased influx of MSCs (Wang,

Cambray-Forker et al. 2009; Garbuzova-Davis, Louis et al. 2011; Garbuzova-Davis, Mirtyl et al. 2013; Ou, Herzog et al. 2014).

In confirmation of RT-PCR gene expression, huB2MG staining was present in the same organs that were positive for huDS21 gene expression, with higher intensity staining present within organs of hBM IDUA MSC treated animals. In agreement with previous reports (Francois, Bensidhoum et al. 2006; Francois, Usunier et al. 2014), huB2MG staining was not evenly distributed within each organ, but were confined to high intensity clusters and integrated around vascular endothelium, supporting *i.v.* administration and circulation.

While hMSCs were detected in multiple organs two days post administration, less than 1% of total infused hMSCs were detected by RT-PCR for each individual animal. Previous studies have also found that less than 0.01% of *i.v.* injected MSCs actually engraft and persist within the tissue after four days (Wu, Chen et al. 2007; Sasaki, Abe et al. 2008; Kidd, Spaeth et al. 2009; Lee, Pulin et al. 2009), often due to either poor oxygenation and nutrient supply, loss of cell-to-cell contact, retardation of trans-endothelial migration, or inflammation (Reinecke, Zhang et al. 1999; Bentzon, Stenderup et al. 2005; Murry, Field et al. 2005; Crisan, Yap et al. 2008; Kedziorek, Solaiyappan et al. 2013). However, *in vitro* (Chapter Three) results indicate that even with only 1% of total infused hDP IDUA MSCs or hBM IDUA MSCs engrafting, the long-term IDUA over-expression capacity of MSCs still greatly exceeds that of HSCs within the cell layer (93 and 58 fold increase respectively) and medium (55 fold and 25 fold increase respectively), if total HSC engraftment was to occur *in vivo*.

4.5.2 Short-term somatic improvements following MSC administration

While no significant increases of IDUA were detected in any somatic organ analysed, a small increase in brain (164% of MPS I cyclosporin; hBM IDUA MSC treated mice only) and serum (215% and 210% of MPS I cyclosporin respectively) enzyme levels were detected for hDP IDUA MSC and hBM IDUA MSC treated mice, suggesting that IDUA enzyme expression may only be slightly indicative of hMSCs biodistribution. However, it must be noted that hDP MSC and hBM MSCs *in vitro* over-expression capacity was relatively low two days post transduction, when compared to three weeks post transduction within the cell layer (115 and 75 fold increase respectively) and media (41 fold and 33 fold respectively); suggesting that two days may not have been a long enough period to assess elevations in tissue IDUA enzyme activity.

Despite no corresponding increase in IDUA levels, significant reduction in both β-hexosaminidase and gag levels were noted in the liver of both hDP IDUA MSC and hBM IDUA MSC treated mice. The liver is one of the body's largest organs that receives 25% of the circulating blood. Serum IDUA levels reached 5% of normal cyclosporin controls for both hDP IDUA MSC and hBM IDUA MSC treated mice, suggesting that hMSCs may still be in circulation, rather than residing within an organ two days post administration. The liver is considered an easy-to-treat organ, and often responds first to treatment, with previous ERT studies, in both MPS patients and animal models of disease, showing reduced liver size and gag storage soon after treatment is commenced (Shull, Munger et al. 1982; Kakkis, McEntee et al. 1996; Kakkis, Muenzer et al. 2001; Kakkis, Schuchman et al. 2001; Ponder 2008). However, in spite of the reduction in liver biomarkers of disease (β-hexosaminidase activity and lysosomal gag storage), no reduction in hepatosplenomegaly was observed in either treatment group.

Contrastingly, when MPS I cyclosporin treated mice received either hDP IDUA MSCs or hBM IDUA MSCs the size of the spleen was significantly increased above both normal cyclosporin and MPS I cyclosporin control mice. However, the significant increase in spleen size is consistent with the concept that MSCs support haematopoiesis (La Pushin and Trentin 1977; Pittenger, Mackay et al. 1999). MSCs home to the spleen and associate with haematopoietic colonies (La Pushin and Trentin 1977; Allers, Sierralta et al. 2004), where they accumulate (Wagner and Henschler 2013) and recruit macrophages to help with reducing inflammation and tissue damage (Chaturvedi, Gilkes et al. 2014). Previous studies have also found that MSCs home and reside within the spleen (Pereira, Halford et al. 1995; Gao, Dennis et al. 2001; Allers, Sierralta et al. 2004), which is further supported by huDS21 gene expression detected in the spleens of all hDP IDUA and hBM IDUA MSC treated animals.

4.5.3 Chapter conclusions and future directions

Collectively, this chapter has demonstrated that both hDP IDUA MSC and hBM IDUA MSCs are capable of evading the lungs following sodium nitroprusside pre-treatment and diffusing to multiple organs, including the brain, in MPS I affected mice. Neurological disease associated with MPS I is inadequately addressed by current treatment options. Laronidase, the recombinant enzyme used in ERT, is too large to cross the BBB and is therefore ineffective at combating neurological decline in MPS I Hurler patients. HSCs are only able to differentiate into cells of the blood lineage, and neurological improvement therefore relies solely on their ability to produce and secrete the deficient lysosomal enzyme; this was shown to be significantly less than both hDP and hBM MSCs *in vitro* (Chapter Three). In contrast, MSCs have the ability to form functional neurons *in vitro* (Arthur, Rychkov et al. 2008) while over-expressing a MPS deficient lysosomal enzyme (Chapter Three), and are also able to express trophic factors to promote neuronal survival, proliferation, differentiation and migration (Nosrat, Widenfalk et al. 2001; Sakai, Yamamoto et al. 2012). This biodistribution study

warrants the further investigation of hMSCs as a prospective multi-tissue and organ treatment in MPS I affected mice over a longer period of time *in vivo*.

While minimal differences were noted between hDP IDUA MSC and hBM IDUA MSC treatment, more hBM IDUA MSCs were detected by RT-PCR in the brain, kidney, spleen and heart of treated mice. hBM IDUA MSCs ability to produce and secrete IDUA *in vitro* was also superior to that of hDP IDUA MSCs (Chapter Three). Most importantly, their secretion of IDUA into the surrounding media was 2.64 fold that of hDP IDUA MSCs, suggesting a greater potential for cross-correction in the clinical setting. In addition to this, Koc *et al.* (2002) demonstrated that hBM MSCs infused into MPS I Hurler patients were well tolerated, with no related toxicities or GvHD, and minor improvements were noted in disease pathology (Koc, Day et al. 2002). As a result, hBM MSCs were chosen for short (two months post administration) and long-term (six months post administration) treatment, comparing hBM MSCs infused alone (comparable to current HSC transplantation) and hBM IDUA MSCs into two month old cyclosporin treated MPS I mice, elucidating any functional (Chapter Five) or biochemical (Chapter Six) benefits associated with either treatment.

Chapter Five: The effect of MSC treatment on MPS I mouse behaviour

5.1 Introduction

I.v. administered MSCs were found to distribute widely and were detected in multiple MPS I affected organs, including the brain (Chapter Four). As minor biochemical changes were noted within two days of treatment, an *in vivo* trial was initiated to assess their efficacy in altering the progression of MPS I pathology after two and six months of treatment. This chapter focuses on behavioural testing, which unlike biochemical analysis is assessed throughout the treatment period allowing analysis of both neurological and skeletal response to treatment, correlating to disease status and treatment efficacy.

Animal models of MPS disease exist in a range of species, however only murine models offer the advantage that; (i) large numbers can be generated over a short period, (ii) less genetic variation is observed between generations, and (iii) a range of well characterised and documented behaviour tests can be performed to provide a comprehensive analysis of functional deficits. The murine model of MPS I has been shown to be an accurate model of human disease (Rogers, Fisher et al. 1997; Neufeld and Muenzer 2001; Rogers, Peters et al. 2001), with both skeletal and neurological deficits (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998; Reolon, Braga et al. 2006; Garcia-Rivera, Colvin-Wanshura et al. 2007; Pan, Sciascia et al. 2008; Baldo, Mayer et al. 2012). Standardised behaviour tests used routinely in our laboratory to assess neuromuscular strength, motor control, balance, anxiety, activity and spatial learning (Roberts, Rees et al. 2007; Macsai, Derrick-Roberts et al. 2012; Derrick-Roberts, Pyragius et al. 2014) allowed qualitative and quantitative evaluation of functional disease progression, prior to biochemical analysis (Chapter Six).

Understanding the functional deficits that arise due to neurological and skeletal disease associated with MPS I is fundamental for developing and determining the efficacy of a

therapy. Baseline behavioural measurements between normal and MPS I mice in our laboratory (unpublished data) and previous murine studies found that functional deficits associated with MPS I pathology arise between two and four months of age (Reolon, Braga et al. 2006; Garcia-Rivera, Colvin-Wanshura et al. 2007; Pan, Sciascia et al. 2008; Baldo, Mayer et al. 2012; Nan, Shekels et al. 2012). While the average life span of a normal mouse is two years, the average life span of an MPS I affected mouse is only nine to ten months of age. Therefore treatment was initiated at two months of age, and behavioural analysis was determined monthly for the duration of six months.

This chapter therefore aims to determine if short-term (two months post administration; four months of age) or long-term (six months post administration; eight months of age) treatment with hBM MSC or hBM IDUA MSCs was able to prevent the progression of MPS I associated behavioural pathology. MPS I mice were treated with hBM MSCs to assess whether MSCs could be transplanted without manipulation, similar to the currently implemented BMT, due to their higher endogenous production of IDUA than HSCs *in vitro* (Chapter Three). As previous studies have noted a correlation between enzyme levels and disease correction in HSCs (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012), hBM IDUA MSCs were included to determine if IDUA over-expression led to improved functional outcomes.

5.2 Treatment efficacy in reducing behavioural abnormalities

Baseline behavioural analysis was determined at two months of age, after daily immunosuppression or saline injections had been initiated, but prior to hBM MSC or hBM IDUA MSC treatment (Figure 5.1). Inverted grid, rotarod and open field performance were then assessed at monthly intervals, while the water cross maze was only assessed prior to

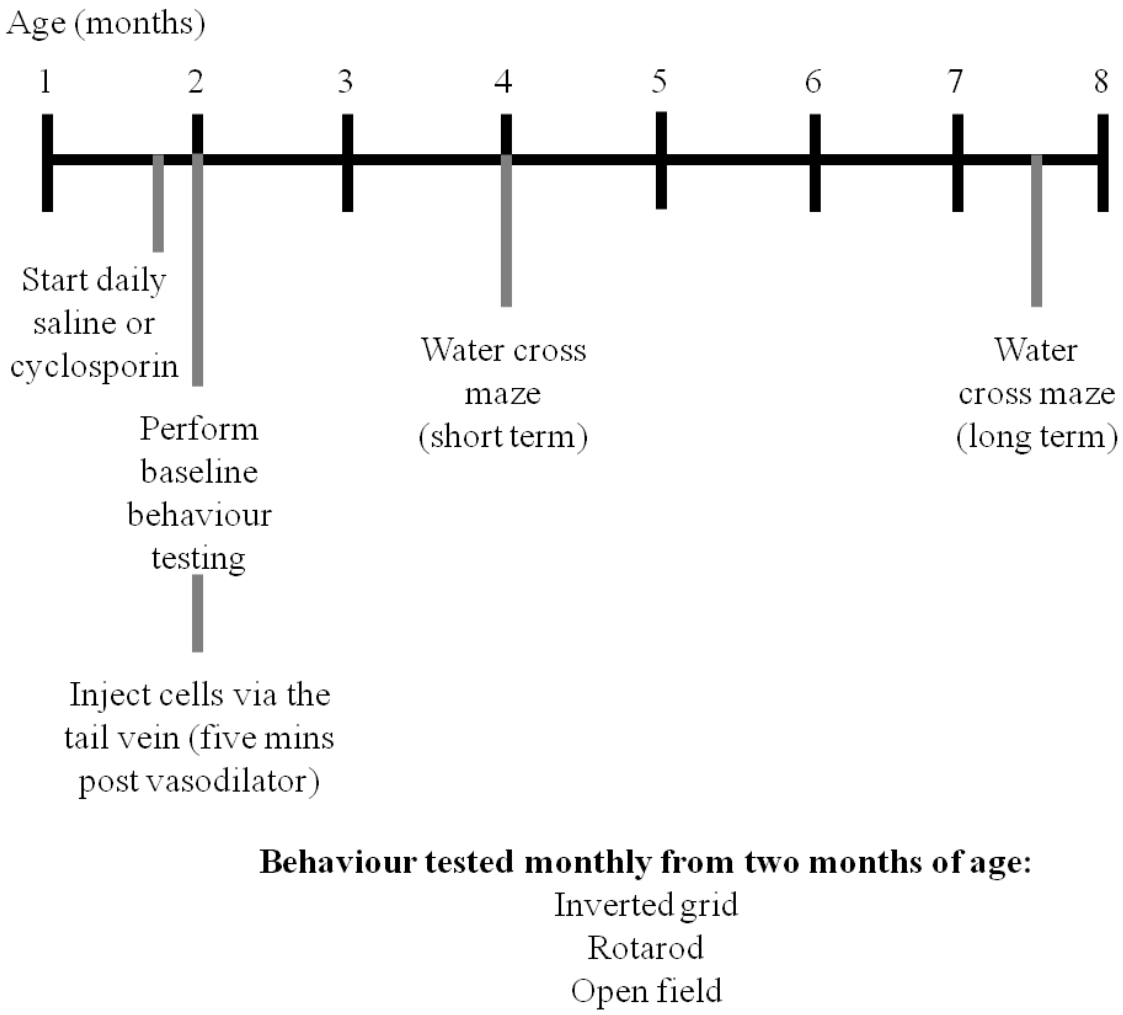


Figure 5.1: Timeline of treatment initiation and behavioural analysis.

Mice were treated from seven weeks of age with either daily saline or cyclosporin subcutaneous injections, depending on group classification (Table 2.1). After one week of daily injections, mice allocated to hBM MSCs or hBM IDUA MSC treatment groups (Table 2.1) received a tail vein injection of a vasodilator (1mg/kg nitroprusside). Five minutes post vasodilation, treated mice received a once off injection of $1x10^6$ hBM MSCs or hBM IDUA MSCs also via the tail vein. Inverted grid, rotarod and open field behaviour testing was initiated at two months of age (baseline), after initiation of daily saline or cyclosporin injections, but before stem cell administration, and repeated monthly. The water cross maze was only performed at four (two months post – short-term treatment) and seven and a half (six months post – long-term treatment) months of age prior to groups being humanely killed.

cessation of behavioural treatment analysis two (short-term) and six (long-term) months post treatment (Figure 5.1). Short-term (two to four months of age) and long-term (two to eight months of age) groups were combined for behavioural testing, generating a timeline of functional data from two to eight months of age. Animal numbers evaluated at each time point are illustrated in Table 5.1.

MPS I mice receiving MSCs were injected daily with cyclosporin starting one week prior to receiving MSCs (Figure 5.1). Untreated MPS I and normal controls were also injected daily with cyclosporin to control for any changes the administration of an immunosuppressive drug may have on behaviour (Chapter Five) and organ biochemistry (Chapter Six). Saline injected MPS I and normal mice were included to provide a baseline of typical behaviour for both genotypes, while controlling for any variations in performance due to daily handling and injections.

5.2.1 Animal health status

Cyclosporin is a wide-acting immunosuppressive drug that reduces the activity of the immune system (Epstein 1996; Byun, Kim et al. 2012), increasing susceptibility to opportunistic fungal and viral infections. Due to this, general health, as assessed by weight, body temperature, appearance and activity, was monitored daily.

From three months of age, the general health of MPS I cyclosporin treated mice was seen to decline, with two unexpected deaths occurring (Figure 5.2). Histopathology findings later identified acute inflammation and bacterial infection within the heart and lungs, with pulmonary oedema and severe pulmonary vasculopathy most likely related to MSCs entrapment. All mice were placed on a ten day on, ten day off prophylactic antibiotic regime

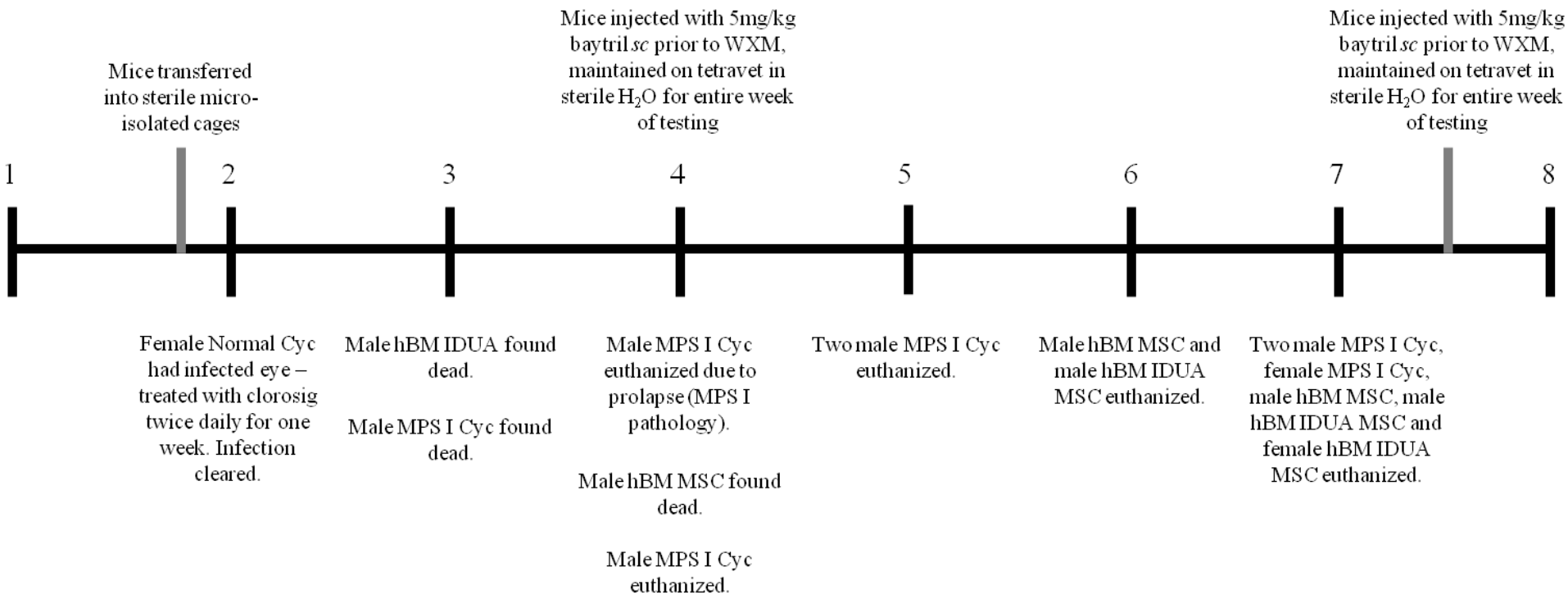
Table 5.1: Number of animals behaviour tested at each time point.

	Normal saline	MPS I Saline	Normal cyclosporin	MPS I cyclosporin	hBM MSC	hBM IDUA MSC
2 months	16 (8M, 8F)	16 (8M, 8F)	17 (8M, 9F)	52 (27M, 25F)		
3 months	16 (8M, 8F)	16 (8M, 8F)	17 (8M, 9F)	18 (8M, 10F)	16 (8M, 8F)	18 (11M, 7 F)
4 months	16 (8M, 8F)	16 (8M, 8F)	17 (8M, 9F)	18 (8M, 10F)	16 (8M, 8F)	18 (11M, 7 F)
4 month (WXM)	8 (4M, 4F)	8 (4M, 4F)	8 (4M, 4F)	8 (3M, 5F)	8 (4M, 4F)	9 (6M, 3F)
5 months	8 (4M, 4F)	8 (4M, 4F)	9 (4M, 5F)	7 (2M, 5F)	7 (3M, 4F)	8 (4M, 4F)
6 months	8 (4M, 4F)	8 (4M, 4F)	9 (4M, 5F)	7 (2M, 5F)	5 (1M, 4F)	8 (4M, 4F)
7 ½ months	8 (4M, 4F)	8 (4M, 4F)	9 (4M, 5F)	4 (0M, 4F)	5 (1M, 4F)	5 (2M, 3F)

Total numbers of animal tested at each time point, followed by sexes in brackets. M = male, F = female, WXM = water cross maze. Behavioural data from 2-4 months of age is a combination of short and long term treated mice, while 5-7 ½ month time points include only long term treated mice.

Figure 5.2: Health status timeline.

Mice were weighed and monitored daily for any signs of infection or deteriorating health due to either immunosuppression or MPS I disease pathology. When any weight loss or change in behaviour was noted, mice were monitored twice daily and fed mushy food (soaked normal chow). If animal health deteriorated further, mice were placed in a humidity crib, receiving a once off 5mg/kg baytril subcutaneous (*sc*) injection, and alternating 4% glucose and saline *sc* injections to prevent dehydration. If weight loss or poor health persisted, mice were humanely euthanized. Due to two animal deaths, from three months of age all mice were placed on a ten day on - ten day off prophylactic antibiotic regime (6.75mg/mL tetracycline (Tetravet) in sterile water, refreshed daily). Animal care staff were closely involved in all health assessment, monitoring and treatment. Final behavioural assessment was brought forward two weeks due to reduced animals numbers and deteriorating general health of MPS I cyclosporin treated mice.


Mice transferred into sterile micro-isolated cages
Mice injected with 5mg/kg baytril *sc* prior to WXM, maintained on tetravet in sterile H_2O for entire week of testing
Mice injected with 5mg/kg baytril *sc* prior to WXM, maintained on tetravet in sterile H_2O for entire week of testing
1
2
3
4
5
6
7
8
Female Normal Cyc had infected eye – treated with clorosig twice daily for one week. Infection cleared.
Male hBM IDUA found dead.
Male MPS I Cyc found dead.
Male MPS I Cyc euthanized due to prolapse (MPS I pathology).
Male hBM MSC found dead.
Male MPS I Cyc euthanized.
Two male MPS I Cyc euthanized.
Male hBM MSC and male hBM IDUA MSC euthanized.
Two male MPS I Cyc, female MPS I Cyc, male hBM MSC, male hBM IDUA MSC and female hBM IDUA MSC euthanized.
Post-mortem analysis revealed pulmonary embolism and heart damage.
Post-mortem analysis revealed opportunistic infection, particularly within the lungs, heart and spleen.

to aid in reducing susceptibility to infection. However, in spite of this, additional MPS I cyclosporin treated mice had to be humanely killed over the duration of the study, with post-mortem analysis revealing opportunistic infections, particularly within the lungs, heart and spleen. In total, twelve male and two female MPS I cyclosporin treated mice did not complete the study, conferring with the more noticeable physical manifestations of MPS I disease pathology in males than their female counterparts. While no normal cyclosporin treated controls had to be humanely killed over the treatment period, small, individual fluctuations in weight were noted, which never varied by more than 1-2%.

5.2.2 Neuromuscular strength

The inverted grid (Chapter 2.4.2) was performed monthly to quantitatively assess the neuromuscular strength of MPS I mice treated with hBM MSCs, with or without over-expression of IDUA, versus untreated MPS I mice and their age matched normal controls.

Both saline and cyclosporin treated MPS I mice spent significantly less time on the inverted grid at all ages when compared to respective normal saline and cyclosporin controls (two-way ANOVA, Tukey's HSD; Figure 5.3A). Typically, the administration of cyclosporin did not affect the neuromuscular strength of normal controls, except at seven months of age where normal cyclosporin treated mice only spent 77% of the time of normal saline controls (two-way ANOVA, Tukey's HSD; Figure 5.3A), which could be attributed to general health and immune status (Section 5.2.1). Time spent on the inverted grid by MPS I saline and cyclosporin treated animals declined with age, achieving 70% and 60% of normal respective controls at two months, but only 40% and 30% of respective normal controls at six months of age (two-way ANOVA, Tukey's HSD; Figure 5.3A), indicating the progressive nature of MPS I pathology. In addition, MPS I cyclosporin treated mice consistently spent less time on

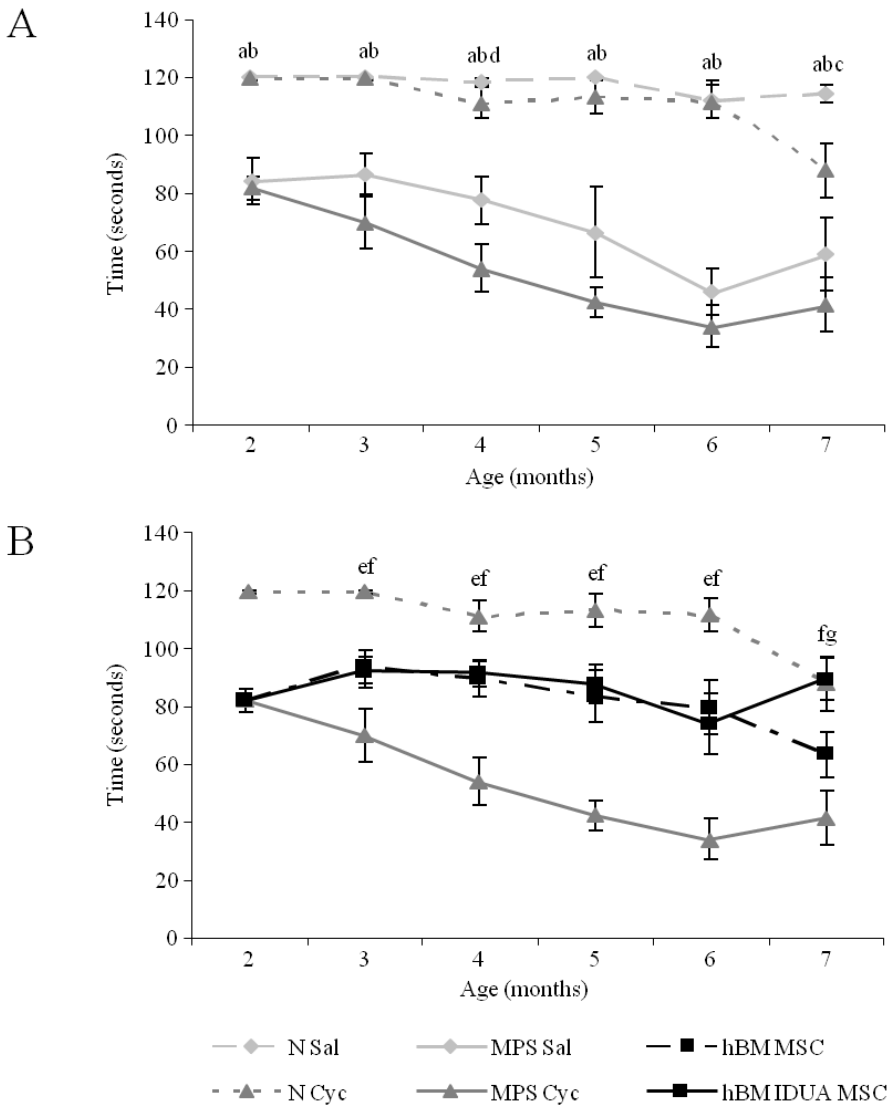


Figure 5.3: Inverted grid time course

Normal and MPS I saline and cyclosporin treated controls **(A)** and hBM MSC and hBM IDUA MSC treated mice versus cyclosporin treated controls **(B)** were placed on a wire grid which was inverted over a cushioned surface. Mean (± SEM; n = 4-17) latency to fall to a maximum of 120 seconds was recorded.

a indicates significance between N Sal and MPS Sal, **b** indicates significance between N Cyc and MPS Cyc, **c** indicates significance between N Sal and N Cyc, **d** indicates significance between MPS Sal and MPS Cyc, **e** indicates significance between hBM MSC and MPS Cyc, **f** **i**ndicates significance between hBM IDUA MSC and MPS Cyc and **g** indicates significance between hBM MSC and hBM IDUA MSC, $p<0.05$ (two-way ANOVA, Tukey's HSD).

the inverted grid than their saline treated counterparts at all time points (97%, 81%, 70%, 64%, 74% and 70% respectively), reaching significance at four months of age (two-way ANOVA, Tukey's HSD; Figure 5.3A), suggesting an adverse effect of cyclosporin administration on MPS I pathology.

The time spent on the inverted grid for both MSC treatment groups was significantly elevated above MPS I cyclosporin treated mice from three to six months of age (one to four months post treatment) for hBM MSC treated mice, and at all time points for hBM IDUA MSC treated MPS I mice (two-way ANOVA, Tukey's HSD; Figure 5.3B), indicating that both MSC treatments prevented the progressive decline of neuromuscular strength seen in MPS I untreated controls (Figure 5.3A). In addition, hBM IDUA MSC mice spent significantly longer on the inverted grid than hBM MSC treated mice at seven months of age (five months post treatment; 130% of hBM MSCs; two-way ANOVA, Tukey's HSD; Figure 5.3B), which could be attributed to enzyme over-expression or a decline in health and animal number noted at later time points (Figure 5.2 and Table 5.1 respectively).

5.2.3 Motor coordination and balance

The rotarod (Chapter 2.4.3) was performed to assess the effect of MSC treatment on the motor coordination and balance of untreated MPS I mice versus their normal age matched controls.

MPS I saline and cyclosporin treated animals achieved lower speeds on the accelerating rotarod than their respective age matched normal controls at all time points; which reached significance at two and three months of age (81% and 88% of normal respectively, saline group) and three and four months of age (86% of normal at both time points, cyclosporin

group; two-way ANOVA, Tukey's HSD; Figure 5.4A). From four months of age the performance of all cyclosporin treated animals declined with age, with greater differences seen between immune status rather than genotype at seven months of age, however, this did not reach significance.

The speed achieved on the rotarod for both MSC treatment groups was elevated above MPS I cyclosporin controls from three months of age (one month post treatment), reaching significance at all time points for hBM IDUA MSC treated mice, but only from five months of age (three months post treatment) for hBM MSC treated mice (two-way ANOVA, Tukey's HSD; Figure 5.4B). Both treatment groups also showed a trend towards achieving higher speeds than normal cyclosporin treated controls from five months of age (three months post treatment; 12%, 12% and 27% for hBM MSCs respectively and 22%, 19% and 41% for hBM IDUA MSC respectively), however, neither treatment group reached significance. Whilst, both treatment groups achieved similar speeds on the rotarod at all time points, hBM IDUA MSC treated mice achieved slightly higher speeds than hBM MSC treated mice from four months of age (two months post treatment; 5%, 8%, 6% and 10% higher speeds respectively; Figure 5.4B), suggesting that IDUA over-expression lead to slightly improved motor control and coordination. Performance on the rotarod indicates that while both MSC treatment groups prevented the decline in motor control and coordination, long-term treatment was required to see significant improvements in hBM MSC treated mice.

5.2.4 Activity and anxiety levels

The open field (Chapter 2.4.4) was performed to assess the activity (total distance travelled) and anxiety levels (rear events) of MPS I affected mice versus their age matched normal controls, with and without MSC treatment.

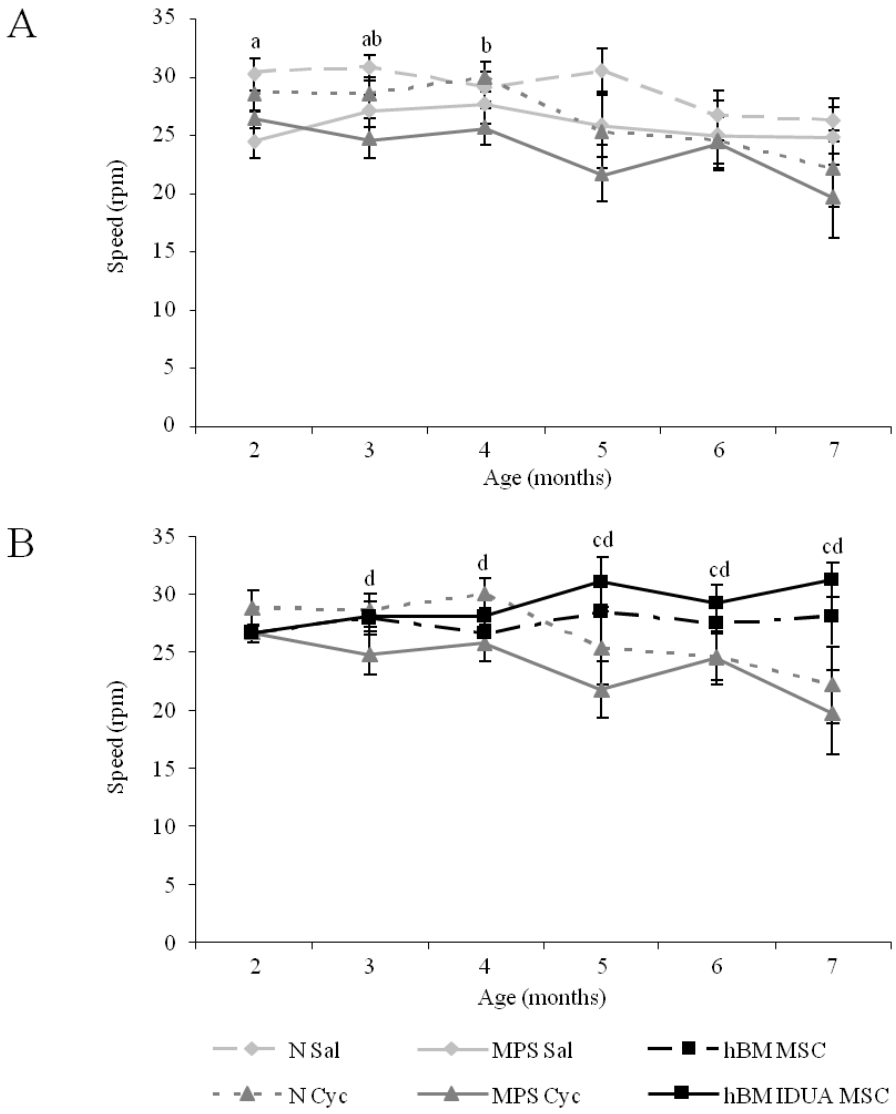


Figure 5.4: Rotarod time course

Normal and MPS I saline and cyclosporin treated control mice **(A)** and hBM MSC and hBM IDUA MSC treated mice versus cyclosporin controls **(B)** were placed on a rotating drum which accelerated from five to 35rpm over two minutes, before maintaining 35rpm for a further minute. Mean (± SEM; n = 4-17) latency to fall to a maximum of three minutes was recorded.

a indicates significance between N Sal and MPS Sal, **b** indicates significance between N Cyc and MPS Cyc, **c** indicates significance between hBM MSC and MPS Cyc and **d** indicates significance between hBM IDUA MSC and MPS Cyc, $p<0.05$ (two-way ANOVA, Tukey's HSD).

5.2.4.1 Activity levels

The total distance travelled for all untreated mice decreased with age, regardless of genotype or immune status (Figure 5.5A). MPS I mice travelled similar distances to their respective normal controls at all time points, however, cyclosporin mediated immunosuppression resulted in reduced activity levels in both genotypes, which progressed with age. MPS I cyclosporin treated mice travelled less distance than MPS I saline controls from two months of age, travelling 11%, 18%, 26%, 51%, 42% and 55% less distance, respectively, reaching significance at four, five and seven months of age (two-way ANOVA, Tukey's HSD; Figure 5.5A). While normal cyclosporin treated mice travelled 2%, 10%, 29%, 24%, 37% and 43% less distance than normal saline controls from two months of age, respectively, this did not reach significance. These results suggest that immunosuppression, rather than phenotype, dictated the general activity of MPS I and normal controls in the open field.

Neither hBM MSC nor hBM IDUA MSC treatment was capable of increasing the general activity of MPS I cyclosporin mice, with total distance travelled also decreasing with increasing age. In contrast to previous behavioural tests, MSC treated mice had reduced activity levels when compared to MPS I cyclosporin controls at most time points, reaching significance at three months of age (one month post treatment) with hBM IDUA MSC treatment (two-way ANOVA, Tukey's HSD; Figure 5.5B). While total distance travelled of hBM MSC treated mice increased at six months of age (four months post treatment) above both MPS I and normal cyclosporin controls, this did not reach significance and the trend was not maintained with time.

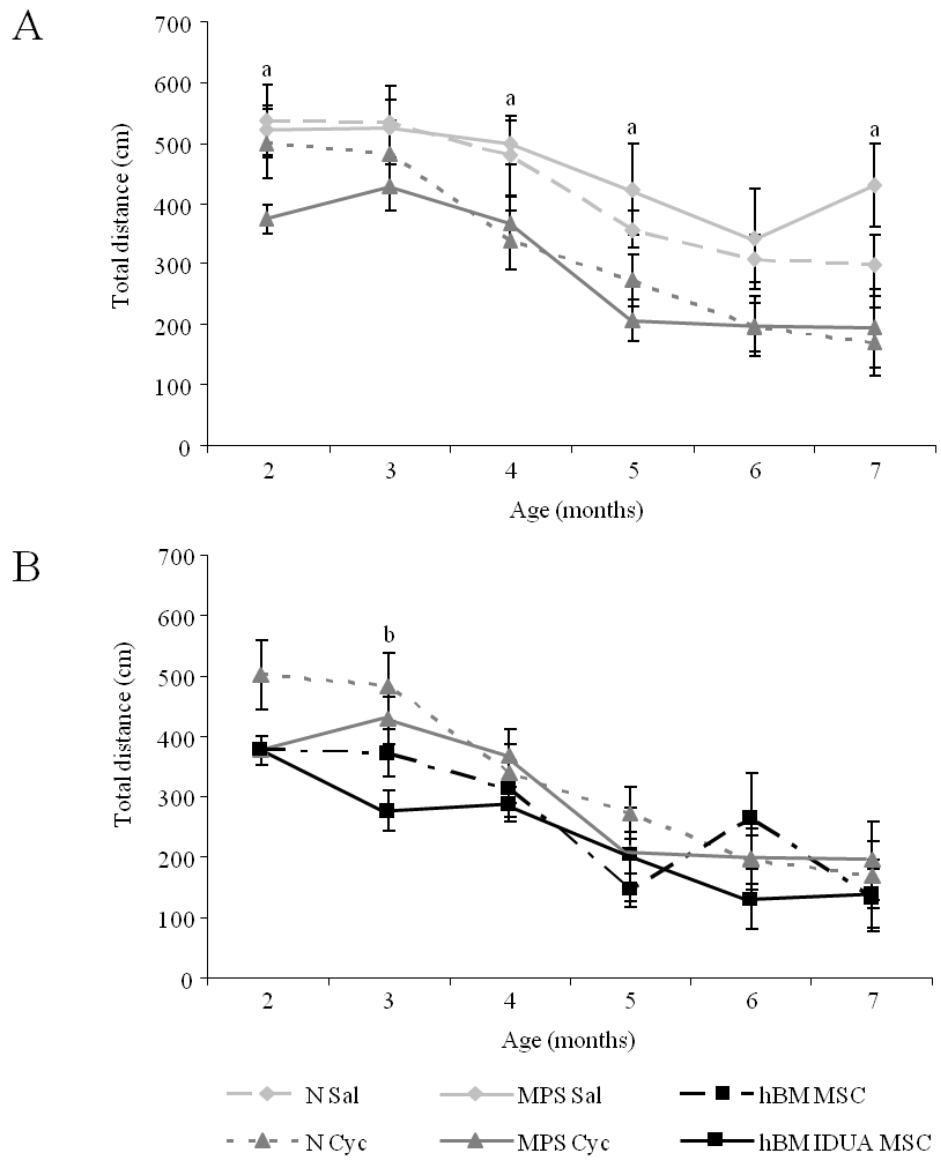


Figure 5.5: Time course of distance travelled in the open field

Normal and MPS I saline and cyclosporin treated control mice **(A)** and hBM MSC and hBM IDUA MSC treated mice versus cyclosporin controls **(B)** were placed in the front left hand corner of the apparatus. Mean (± SEM; n = 4-17) distance travelled (cm) over three minutes was recorded.

a indicates significance between MPS Sal and MPS Cyc and **b** indicates significance between hBM IDUA MSC and MPS Cyc, $p<0.05$ (two-way ANOVA, Tukey's HSD).

5.2.4.2 Anxiety levels

The number of rearing events that mice perform over the three minute testing period is an assessment of both the activity and anxiety levels of mice. Similar to total distance travelled (Section 5.2.4.1), the total number of rearing events also declined with age for all control groups, regardless of genotype and immune status (Figure 5.6A). Again, the number of rearing events for MPS I saline mice was similar to that of their age matched normal saline controls, which was above that of their cyclosporin treated counterparts; reaching significance from two to four months of age for normal mice and at five months of age for MPS I mice (two-way ANOVA, Tukey's HSD; Figure 5.6A). While the number of rearing events for MPS I and normal cyclosporin treated mice were similar from two to four months of age, between five and seven months of age MPS I cyclosporin treated mice had 46%, 36% and 58% less rearing events than normal cyclosporin controls (not significant; Figure 5.6A), suggesting that both immunosuppression and progression of phenotype are responsible for a decline in anxiety and activity levels.

Similar to total distance travelled, neither hBM MSC nor hBM IDUA MSC treatment improved the general activity and anxiety levels of MPS I cyclosporin mice, with a similar decline in rearing events noted with age (Figure 5.6B). At three and four months of age (one and two months post treatment) both MSC treatment groups had reduced rearing events when compared to MPS I cyclosporin controls, reaching significance at three months of age (one month post treatment; two-way ANOVA, Tukey's HSD; Figure 5.6B). Results indicate that a decline in general health (Figure 5.2) may be responsible for the decrease in general activity from five to seven months of age (five months post treatment) in MPS I mice receiving cyclosporin (Section 5.2.1).

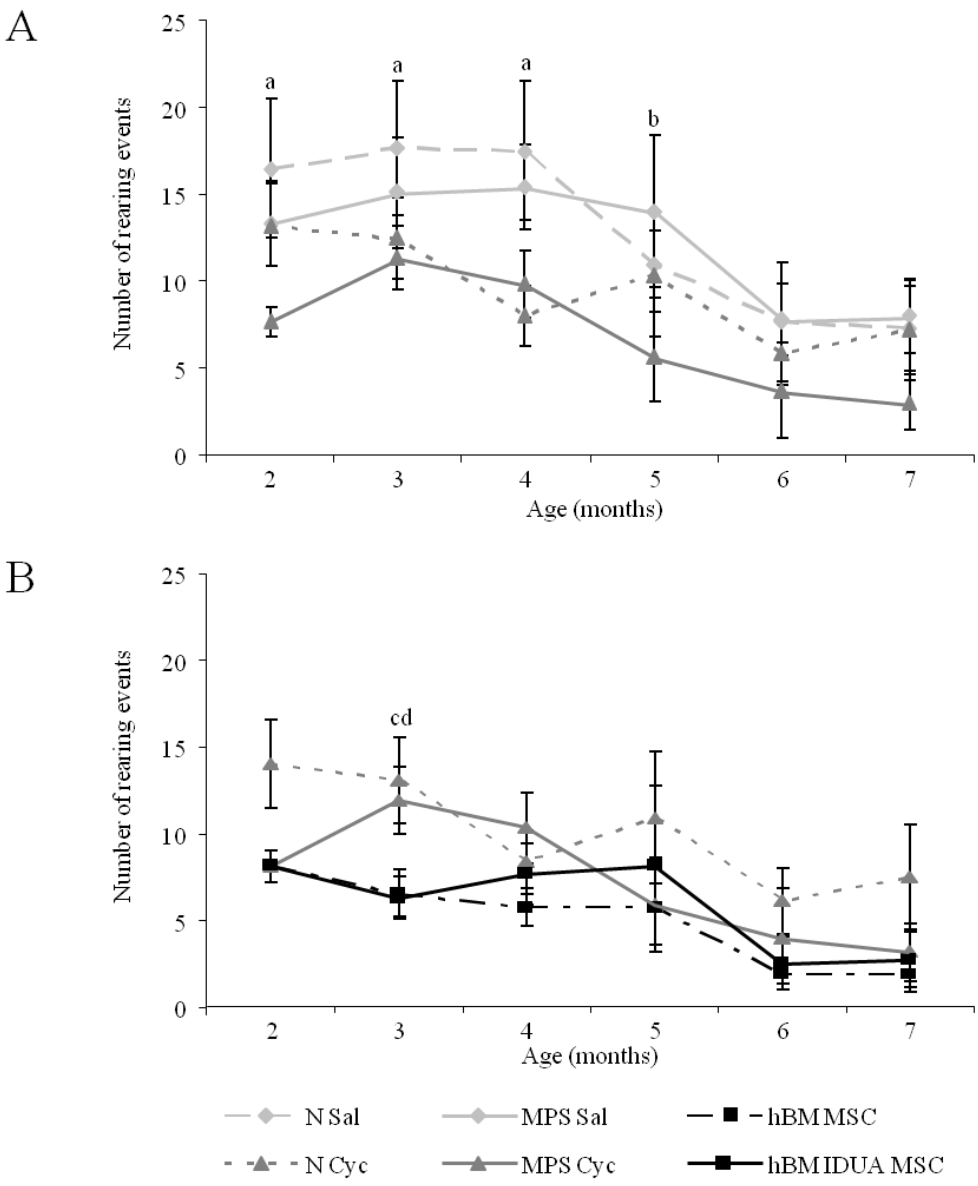


Figure 5.6: Time course of rearing events in the open field

Normal and MPS I saline and cyclosporin treated control mice **(A)** and hBM MSC and hBM IDUA MSC treated mice versus cyclosporin controls **(B)** were placed in the front left hand corner of the apparatus. Mean (± SEM; n = 4-17) number of rearing events over three minutes was recorded.

a indicates significance between N Sal and N Cyc, **b** indicates significance between MPS Sal and MPS Cyc, **c** indicates significance between hBM MSC and MPS Cyc and **d** indicates significance between hBM IDUA MSC and MPS Cyc, $p<0.05$ (two-way ANOVA, Tukey's HSD).

5.2.5 Learning

The water cross maze (Chapter 2.4.5) was performed over six consecutive days at four (two months post treatment) and eight (six months post treatment) months of age to quantitatively assess the learning ability of MPS I mice treated with hBM MSCs or hBM IDUA MSCs versus untreated MPS I mice and their age matched normal controls. Escape latency (time taken to locate the submerged platform), incorrect entries (number of entries or re-entries into cross arms which did not contain the submerged platform) and correct entries (when the animal located the submerged platform directly from the release point) were used for analysis of neurological function and learning. Day one versus day six data was used to establish that all mice were able to learn the location of the submerged platform, while day six data was used to assess treatment efficacy.

All mice, regardless of age, genotype or treatment group, were able to learn the task as shown by a significant reduction in the time taken to find the submerged platform (two-way ANOVA, Tukey's HSD; Figure 5.7A and 5.7C), a significant reduction in incorrect entries (two-way ANOVA, Tukey's HSD; Figure 5.8A and 5.8C) and a significant increase in correct entries (two-way ANOVA, Tukey's HSD; Figure 5.9A and 5.9C) over the six day protocol. By day four most mice had reached their maximum learning potential with no further reduction in incorrect entries, however normal saline and hBM IDUA MSC treated mice continued to improve over the six day protocol (Figure 5.8A and 5.8C).

5.2.5.1 Escape latency

MPS I saline controls took significantly longer to find the submerged platform at both four and eight months of age when compared to the normal saline controls (232% and 210% of normal respectively; two-way ANOVA, Tukey's HSD; Figure 5.7B and 5.7D respectively).

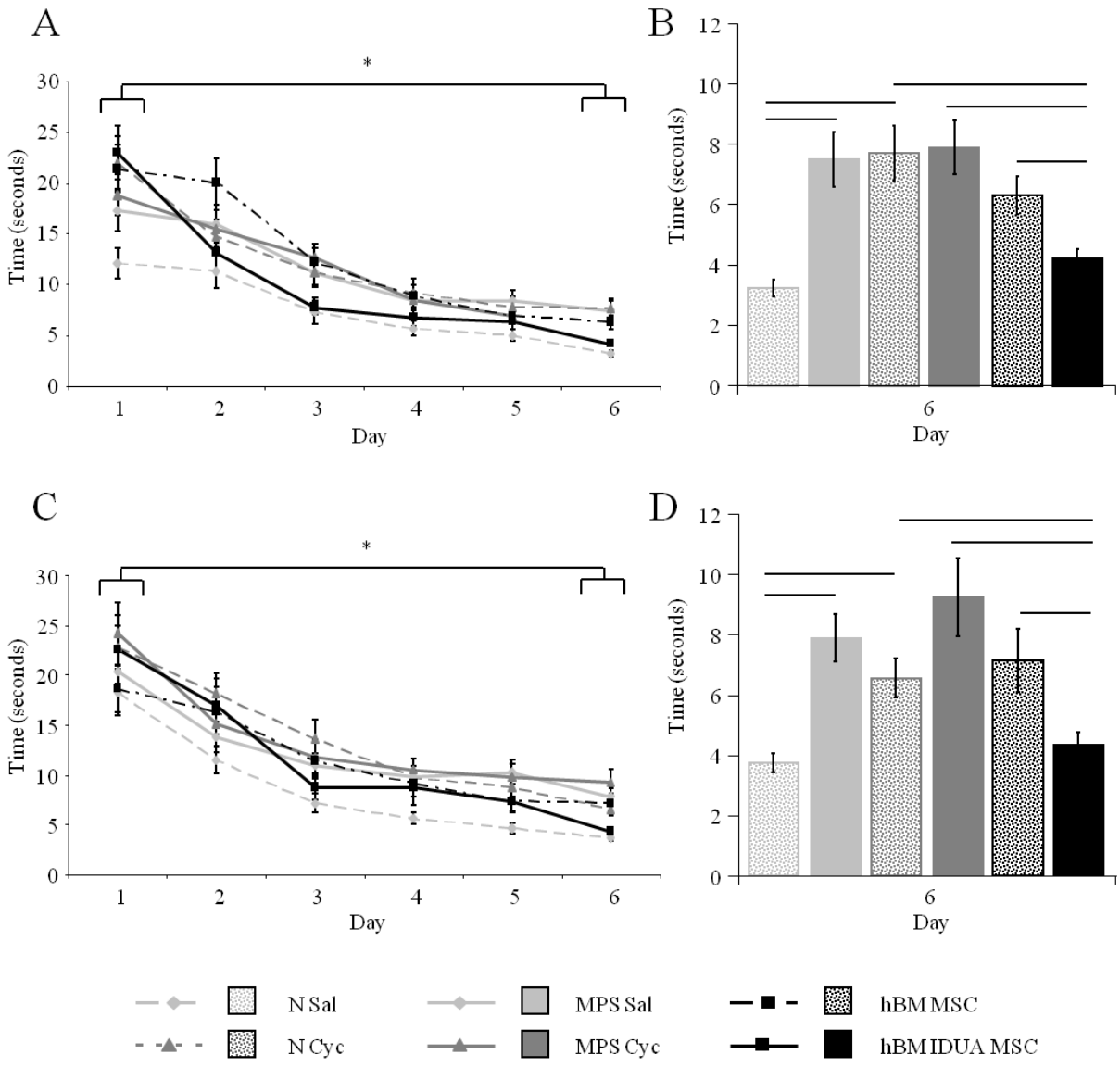


Figure 5.7: Escape latency

Mice at four **(A, B)** and eight **(C, D)** months of age were placed in a cross shaped pool filled with opaque water and required to find a submerged platform using constant visual cues, daily over a six day period. **A** and **C** show the learning trend of mice from day one to day six, **B** and **D** show day six data. Mean (± SEM; n = 24-102 trials) escape latency over a maximum of one minute was recorded.

***** Denotes significant difference between day one and day six (two-way ANOVA, Tukey's HSD), **—** indicates significant difference between groups, $p<0.05$ (one-way ANOVA, Tukey's HSD).

However, no significant difference was noted when comparing MPS I and normal cyclosporin treated mice at either age. Normal cyclosporin mice took 48% and 43% longer, respectively, to find the submerged platform than normal saline controls at four and eight months of age respectively (two-way ANOVA, Tukey's HSD; Figure 5.7B and 5.7D respectively), increasing their escape latency to that of MPS I untreated mice.

hBM MSC treated mice took 20% and 23% less time to find the submerged platform than MPS I cyclosporin treated mice at both four and eight months of age respectively (two and six months post treatment), but did not reach significance. However, the escape latency of hBM IDUA MSCs treated mice was significantly less than MPS I cyclosporin (47% and 53% respectively), normal cyclosporin (46% and 33% respectively) and hBM MSC treated (33% and 39% respectively) mice at both four and eight months of age (two and six months post treatment; two-way ANOVA, Tukey's HSD; Figure 5.7B and 5.7D respectively); indicating that IDUA over-expression leads to improved learning ability compared to hBM MSC treatment alone. Short-term hBM IDUA MSC treatment (Figure 5.7B) was effective at significantly reducing the escape latency of MPS I cyclosporin treated mice, which was maintained with long-term MSC treatment (Figure 5.7D), suggesting an early improvement and stabilisation of MPS I associated neurological or skeletal pathology.

5.2.5.2 Incorrect entries

MPS I saline controls had significantly higher incorrect entries than normal saline mice at both four and eight months of age (522% and 385% of normal respectively; two-way ANOVA, Tukey's HSD; Figure 5.8B and 5.8D). However, no significant difference was noted when comparing MPS I and normal cyclosporin treated mice at either age. Similarly to escape latency, normal cyclosporin mice had 811% and 410% more incorrect entries than

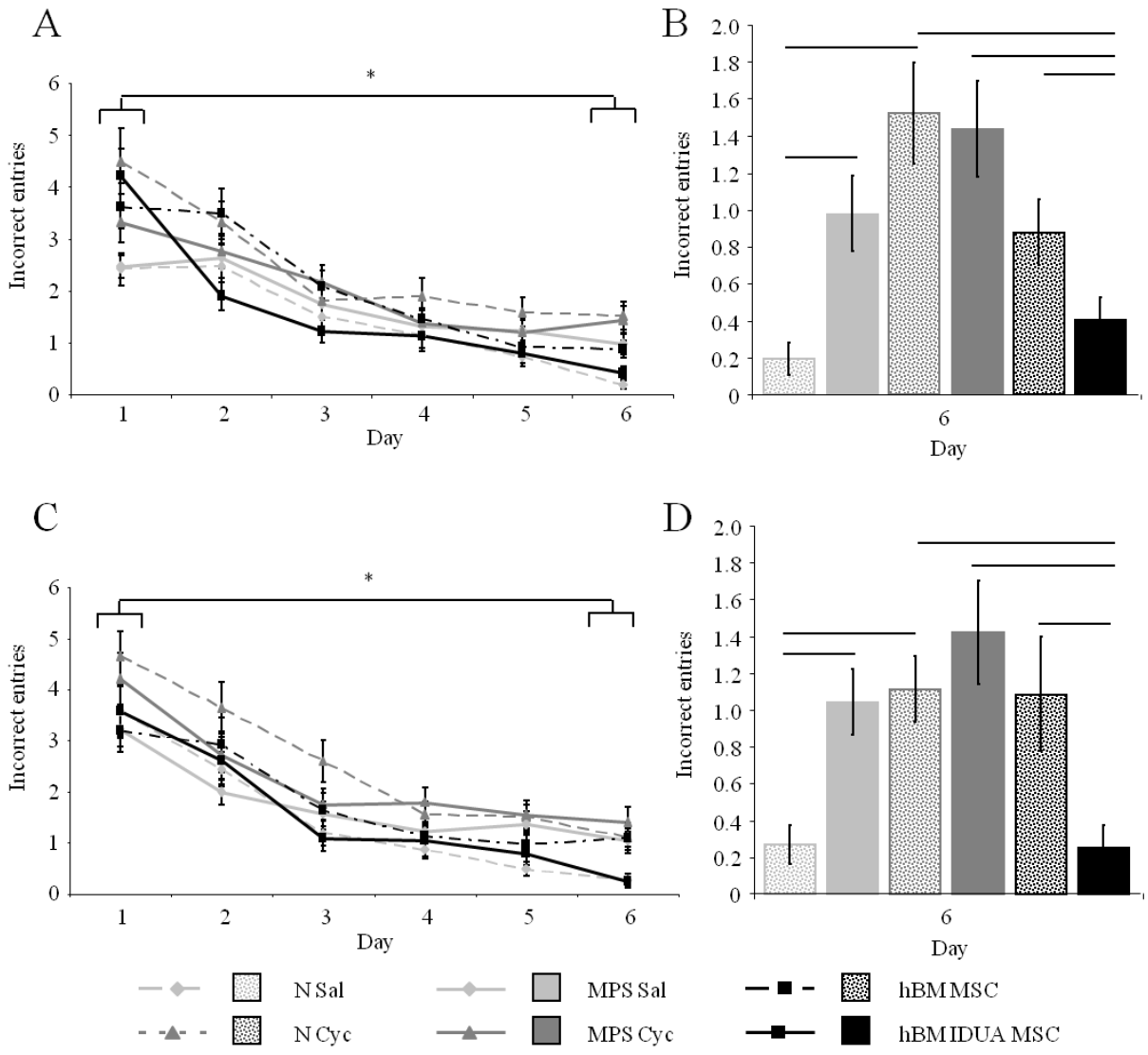


Figure 5.8: Incorrect entries

Mice at four **(A, B)** and eight **(C, D)** months of age were placed in a cross shaped pool filled with opaque water and required to find a submerged platform using constant visual cues over a six day period. **A** and **C** show the learning trend of mice from day one to day six, **B** and **D** show day six data. Mean (± SEM; n = 24-102 trials) incorrect entries over a maximum of one minute was recorded.

***** Denotes significant difference between day one and day six (two-way ANOVA, Tukey's HSD), **—** indicates significant difference between groups, $p<0.05$ (one-way ANOVA, Tukey's HSD).

normal saline controls at four and eight months of age respectively (two-way ANOVA, Tukey's HSD; Figure 5.8B and 5.8D respectively), increasing their incorrect entries to that of MPS I untreated mice.

hBM MSC treated mice had 40% and 24% fewer incorrect entries than MPS I cyclosporin mice at four and eight months of age (two and six months post treatment), respectively, neither reaching significance (Figure 5.8B and 5.8D respectively). However, hBM IDUA MSCs treated mice had significantly fewer incorrect entries at four and eight months of age (two and six months post treatment) when compared to MPS I cyclosporin (72% and 82% respectively), normal cyclosporin (73% and 77% respectively) and hBM MSC treated (53% and 77% respectively) mice (two-way ANOVA, Tukey's HSD; Figure 5.8B and 5.8D); indicating that IDUA over-expression lead to improved learning ability compared to hBM MSC treatment alone. Short-term hBM IDUA MSC treatment (Figure 5.8B) was effective at significantly reducing the number of incorrect entries of MPS I cyclosporin treated mice, which was maintained with long-term MSC treatment (Figure 5.8D), suggesting an early improvement and stabilisation of MPS I associated neurological pathology.

5.2.5.3 Correct entries

MPS I saline controls had significantly fewer correct entries than normal saline mice at both four and eight months of age (35% and 39% of normal respectively; two-way ANOVA, Tukey's HSD; Figure 5.9B and 5.9D), however, no significant difference was noted when comparing MPS I and normal cyclosporin mice at either age. Normal cyclosporin mice had 51% and 56% fewer correct entries than normal saline controls at four and eight months of age respectively (two-way ANOVA, Tukey's HSD; Figure 5.9B and 5.9D respectively), decreasing their correct entries to that of MPS I untreated mice.

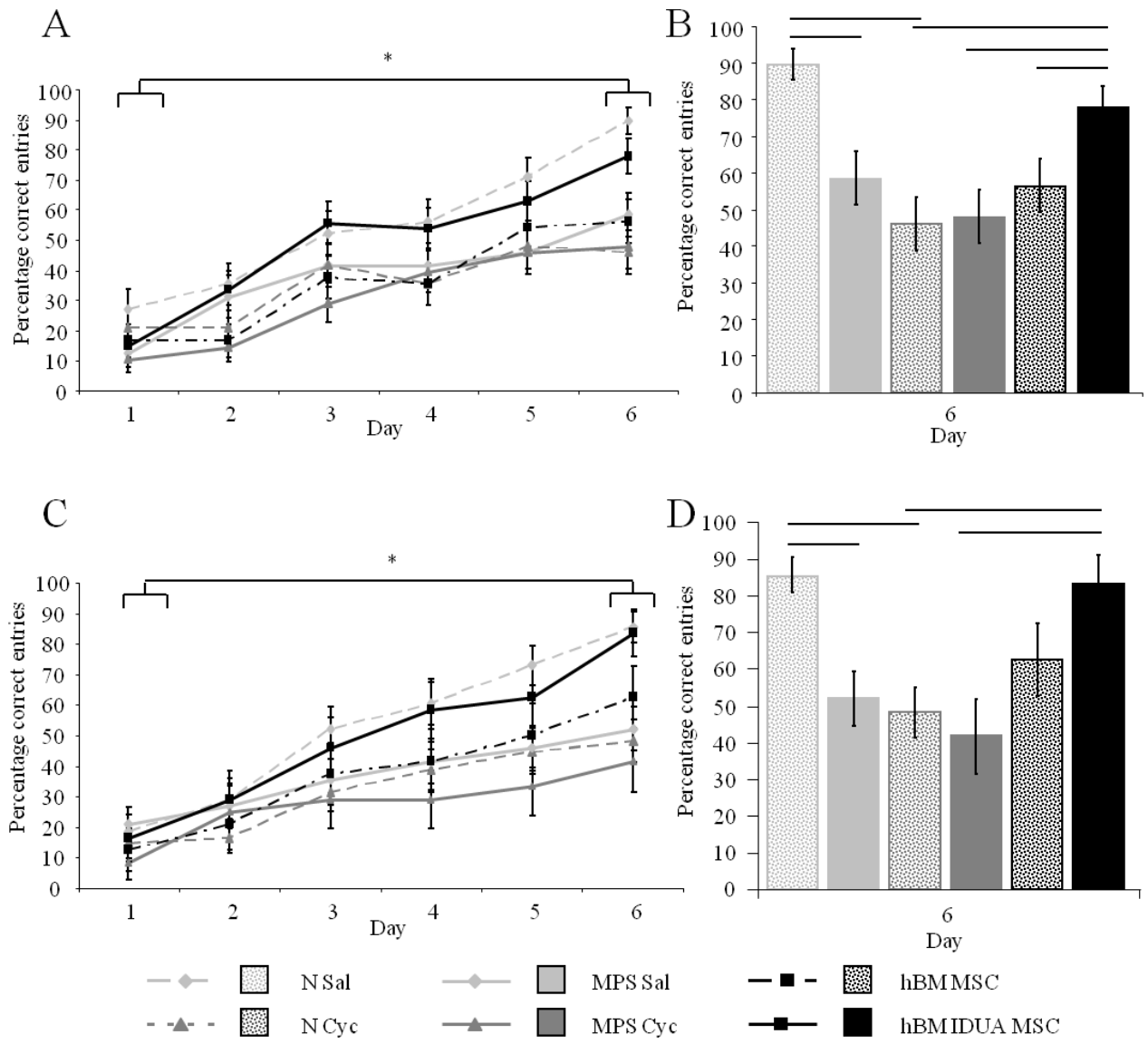


Figure 5.9: Correct entries

Mice at four **(A, B)** and eight **(C, D)** months of age were placed in a cross shaped pool filled with opaque water and required to find a submerged platform using constant visual cues over a six day period. **A** and **C** show the learning trend of mice from day one to day six, **B** and **D** show day six data. Mean (± SEM; n = 24-102 trials) correct entries (converted to percentage) over a maximum of one minute was recorded.

***** Denotes significant difference between day one and day six (two-way ANOVA, Tukey's HSD), **—** indicates significant difference between groups, $p<0.05$ (one-way ANOVA, Tukey's HSD).

hBM MSC treated mice showed minor improvements in the number of correct entries achieved when compared to either MPS I or normal cyclosporin treated mice at four (two months post treatment; 117% and 123% respectively) and eight (six months post treatment; 150% and 130% respectively) months of age, which failed to reach significance (Figure 5.9B and 5.9D). However, hBM IDUA MSC treated mice had significantly higher correct entries than MPS I cyclosporin (162% and 200% respectively), normal cyclosporin (170% and 173% respectively) and hBM MSC treated (138% and 133% respectively) mice at four and eight months of age (two and six months post treatment; two-way ANOVA, Tukey's HSD; Figure 5.9B and 5.9D); indicating that IDUA over-expression lead to improved learning ability compared to hBM MSC treatment alone. Short-term hBM IDUA MSC treatment (Figure 5.9B) was successful at significantly increasing the number of correct entries achieved by MPS I cyclosporin treated mice, which was further improved by 5% with long-term MSC treatment (Figure 5.9D), suggesting an early improvement and stabilisation of MPS I associated neurological pathology.

5.3 Discussion

MPS I is a debilitating childhood disease which presents with a chronic and progressive course, resulting in a decline or loss of neurological and physical skills with age (Neufeld and Muenzer 2001). Standardised behaviour testing allows high throughput analysis of neurological and skeletal pathology in murine models of disease, allowing functional assessment of treatment efficacy prior to biochemical investigation (Chapter Six). The degree to which *i.v.* administered hBM MSCs or hBM IDUA MSCs prevented the onset or decline in MPS I functional performance was evaluated for the first time in this study; assessing their neuromuscular strength, motor control and coordination, activity and anxiety levels and spatial learning ability.

5.3.1 Side-effects of cyclosporin-mediated immunosuppression

Cyclosporin is a wide-acting immunosuppressive agent that was implemented for the duration of the trial due to the xenogenic nature of the cell transplant. It acts by reducing the activity of the immune system by inactivating calcineurin and inhibiting the growth and activity of T cells and their soluble mediators (Epstein 1996; Byun, Kim et al. 2012), but has no effect on the proliferation and survival of hMSC populations *in vitro* (Gronthos and Arthur, unpublished observations). In recent traumatic brain injury studies, cyclosporin has been linked to improving behavioural outcomes as well as providing significant histological protection due to its ability to preserve mitochondrial integrity (Okonkwo, Buki et al. 1999; Okonkwo and Povlishock 1999; Riess, Bareyre et al. 2001; Alessandri, Rice et al. 2002; Mazzeo, Brophy et al. 2009; Mbye, Singh et al. 2009).

In contrast to previous studies (Okonkwo, Buki et al. 1999; Okonkwo and Povlishock 1999; Riess, Bareyre et al. 2001; Alessandri, Rice et al. 2002; Mazzeo, Brophy et al. 2009; Mbye, Singh et al. 2009), cyclosporin administration was found to reduced behavioural performance in both normal and MPS I treated mice (summarised in Table 5.2), suggesting that the influx of calcium and subsequent loss of mitochondrial membrane potential is unlikely to contribute to MPS I pathology/neuronal damage (Xiong, Gu et al. 1997; Alessandri, Rice et al. 2002; Starkov, Chinopoulos et al. 2004; Mazzeo, Brophy et al. 2009). The early decline in MPS I cyclosporin functional ability was more likely attributed to a decline in health status due to an altered immune system, which exacerbated pre-existing pathologies (Figure 5.2). While both short and long-term hBM MSC and hBM IDUA MSC treatment resulted in improved behavioural outcomes above that of MPS I cyclosporin controls (Table 5.2), the immunosuppressive state brought about by cyclosporin administration made these animals susceptible to opportunistic infections. This progressive decline in immune function and subsequent animal health from four months of age (two months post treatment; Figure 5.2)

Table 5.2: Overview of the effect of cyclosporin and treatment on normal and MPS I behaviour.

	Effect of cyclosporin		Effect of treatment		
	Normal	MPS I	hBM MSC	hBM IDUA MSC	IDUA O/E
Inverted Grid (neuromuscular strength)	↓ Late (7 ½ months)	↓ Early (from 3 months)	↑ Early (from 3 months)	↑ Early (from 3 months)	↑ Late (7 ½ months)
Rotarod (balance and coordination)	↓ Late (from 5 months)	↓ Early (from 3 months)	↑ Early (from 3 months)	↑ Early (from 3 months)	↑ Early (from 4 months)
Open field (total distance)	↓ Early (from 2 months)	↓ Early (from 2 months)	↓ Early (from 3 months)	↓ Early (from 3 months)	↓ Early (from 3 months)
Open field (rear events)	↓ Early (from 2 months)	↓ Early (from 2 months)	↓ Early (from 3 months)	↓ Early (from 3 months)	↑ Early (from 4 months)
WXM (correct entries)	↓ Early	↓ Early	↑ Early	↑ Early	↑ Early

Effect of cyclosporin compared normal and MPS I cyclosporin treated mice to respective saline treated controls. Effect of hBM MSC and hBM IDUA MSC treatment was determined against MPS I cyclosporin controls, while IDUA over-expression (O/E) compared hBM IDUA MSC treated mice versus hBM MSC treated mice. WXM = water cross maze (correct entries used for comparison, as all parameters showed similar trends).

potentially diminished the long-term therapeutic outcome of MSC treatment; supported by the stabilisation, but very minimal improvement in functional performance with prolonged treatment.

5.3.2 MSC administration resulted in improved functional performance

The neuromuscular strength of both saline and cyclosporin treated MPS I mice was significantly decreased when compared to their respective normal controls, with their latency to fall continuing to decline with age. This correlates with MPS I patients' reduced mobility, range of joint movement and decline in neurological function that occurs with age (Clarke and Heppner 2002). The administration of hBM MSCs was able to stabilise and prevent the decline in MPS I neuromuscular strength from one month post treatment (three months of age). No additional improvements were noted with long-term treatment or enzyme over-expression, suggesting that the introduction of hBM MSCs was enough to combat the progressive decline in MPS I neuromuscular strength. No previous MPS I studies using a murine model have investigated neuromuscular strength using the inverted grid. Novel findings of significantly reduced neuromuscular strength in MPS I and improvement with MSC treatment provides a quantitative measure for therapy effectiveness in reversal of both skeletal and neurological traits associated with MPS I.

The rotarod tests motor control and balance, requiring neuromuscular integrity (Nan, Shekels et al. 2012) and a high degree of sensorimotor coordination, with impairments associated with damage to the basal ganglia and cerebellum (Li, Chen et al. 2001; Fujimoto, Longhi et al. 2004; Garcia-Rivera, Colvin-Wanshura et al. 2007). These findings correlate with the deterioration of functional acuity and mobility in MPS I patients (Neufeld and Muenzer 2001; Clarke and Heppner 2002). The balance and motor control of MPS I mice was significantly

different from normal at early time-points (two to four months of age), however, while the trend that MPS I mice achieved lower speeds than their normal controls remained, the significance was lost due to a decline in the performance of normal controls. Previous studies in MPS I mouse models have shown that MPS I mice achieve significantly less time at higher speeds on the rotarod (Garcia-Rivera, Colvin-Wanshura et al. 2007; Nan, Shekels et al. 2012). While slight differences in protocol existed, both studies concluded that the higher speeds of rotation (15rpm to 35rpm) caused a significant reduction in MPS I balance, which was associated with neurological impairment (Garcia-Rivera, Colvin-Wanshura et al. 2007; Nan, Shekels et al. 2012).

hBM MSC treatment was again able to stabilise the decline in motor function noted in MPS I controls from four months of age (two months post treatment). Enzyme over-expression resulted in significant improvements in sensorimotor function from three months of age (one month post treatment) when compared to MPS I cyclosporin controls, conferring with previous reports relating treatment efficacy to enzyme over-expression (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012). Similar to the inverted grid, the rotarod requires skeletal, muscular and neurological integrity, therefore improved long-term performance by MSC treated mice cannot be directly attributed to an attenuation of one specific pathological condition or affected system.

The water cross maze is a reliable and robust tool that allows assessment of CNS function, without the number correct entries being strongly influenced by MPS I skeletal pathology (Roberts, Rees et al. 2007). As expected, MPS I saline mice took longer to locate the submerged platform (escape latency) than normal saline controls due to pre-existing skeletal pathology. However, their reduced ability to learn the location of the platform over the six day protocol (correct and incorrect entries) was a strong indicator of impaired neurological

function, suggesting possible hippocampal damage (Paylor, Morrison et al. 1992; Logue, Paylor et al. 1997) which is consistent with the developmental delay and mental retardation associated with Hurler patients (Martins, Dualibi et al. 2009).

Similarly, previous studies have also found that MPS I mice had significantly reduced cognitive function and spatial learning from four months of age (Pan, Sciascia et al. 2008; Wolf, Lenander et al. 2011; Ou, Herzog et al. 2014). This trend was not observed in cyclosporin treated mice, with both normal and MPS I cyclosporin treated mice achieving similar correct entries compared to MPS I saline controls, suggesting an interference with performance due to cyclosporin administration. A previous study in rats (Dantzer, Satinoff et al. 1987) showed that cyclosporin impeded normal thermoregulation, this coupled with the 23.5°C water temperature, repeat trialling (Chapter 2.4.5) and reduced immune status could account for the change in performance. In addition, a further 5-10% decline in performance was seen with age, corresponding with the progressive nature of MPS I pathology and reduced health state due to cyclosporin mediated immunosuppression.

Administration of hBM MSCs produced only slight improvements in spatial learning when compared to MPS I and normal cyclosporin mice at four months of age (two months post treatment). However, a 5% improvement in correct entries was noted at eight months of age (six months post treatment) suggesting stabilisation and possible prevention in further CNS deterioration. Conversely, hBM IDUA MSCs administration resulted in significant neurological improvements when compared to hBM MSC treated mice at both four and eight months of age (two and six months post treatment), suggesting an improved neurological outcome is associated with IDUA over-expression. In addition, hBM IDUA MSC treated mice also had significantly reduced escape latencies, suggesting possible improvements in neuromuscular and/or skeletal function. These results suggest that enzyme over-expression

may have added therapeutic benefits over hBM MSCs administration when combating the complex and progressive pathology associated with MPS I, supporting recent findings where improved disease outcome was correlated to enzyme over-expression in HSCs (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012).

5.3.3 Daily handling limited exploratory and anxiety behaviour

The open field determines exploratory behaviour by assessing total distance travelled (activity) and number of rearing events (activity and anxiety). In contrast to previous MPS I exploratory and anxiety deficits noted in our laboratory (unpublished data) from three months of age, no significant differences were noted between normal and MPS I mice in this study. Previous studies using MPS I knockout mice have also showed significant differences in the open field test relating to a significant decline in neurological function (Pan, Sciascia et al. 2008; Baldo, Mayer et al. 2012), where reduced activity could partially be attributed to the progressive skeletal disease associated with MPS I. Pan *et al.* (2008) showed hypoactivity from two months of age and altered anxiety from six months of age (Pan, Sciascia et al. 2008), while Baldo *et al.* (2012) demonstrated reduced line crosses (a crude measure of distance travelled) and rearing events from four months of age (Baldo, Mayer et al. 2012).

The lack of findings in the open field suggest that the natural anxiety and innate fear response and subsequent activity of mice may have been suppressed due to the daily handling and injection regime; with handling previously been found to reduce anxiety-like behaviour in rodents (Clausing, Mothes et al. 1997; Schmitt and Hiemke 1998; Moncek, Duncko et al. 2004; Pritchard, Van Kempen et al. 2013). In contrast to previous reports that have linked cyclosporin administration to improving behavioural outcomes in traumatic brain injury (Mazzeo, Brophy et al. 2009), the administration of cyclosporin in this study further

decreased the exploratory behaviour of normal and MPS I mice. Unlike previous behavioural tests, neither the administration of hBM MSC nor hBM IDUA MSC showed any improvement on MPS I cyclosporin exploratory behaviour, however, no direct assessment of treatment efficacy can be made due to the lack of difference between MPS I and normal controls.

5.3.4 Chapter conclusions and future directions

For the first time, this study has shown that *i.v.* administered hBM MSCs are capable of stabilising and preventing the progression of multiple behavioural deficits associated with MPS I, with additional behavioural improvements correlated with enzyme over-expression. MPS I patients suffer from widespread gag accumulation and inflammation, which is associated with organ enlargement and failure, CNS degeneration and skeletal malformations. Functional improvements noted in this study, including increased mobility, coordination, strength, range of movement and neurological function, would help greatly improve the quality of life for MPS I sufferers. Most importantly, improvements in CNS function suggest that *i.v.* administered MSCs are capable of combating the currently hard-to-treat neurological deterioration associated with MPS I disease. Whether the improvement in functional pathology was due to MSCs transient protection from widespread inflammation, or they permanently engrafted, trans-differentiated and secreted functional IDUA needs to be further elucidated. Further biochemical analysis of treatment efficacy by evaluating MSCs distribution, IDUA expression, gag accumulation, brain inflammation and bone morphology (Chapter Six) would help shed light on MSC's mode of action.

Chapter Six: The effect of MSC treatment on MPS I biochemistry, bone morphology and brain inflammation

6.1 Introduction

MSC treatment of MPS I mice from two to eight months of age demonstrated that *i.v.* administered hBM MSCs improved behavioural outcomes from three months of age (one month post treatment), with greater functional performance associated with IDUA over-expression from four months of age (two months post treatment; Chapter Five). These results indicate that systemically administered MSCs have the ability to stabilise and prevent the progressive neurological and skeletal pathology associated with MPS I, which are unaddressed by current therapeutic options (Muenzer, Wraith et al. 2009).

Whether MSCs engraft and trans-differentiate or exist only transiently to lower the inflammatory response to cellular damage is still largely debated (Reiser, Zhang et al. 2005; Chamberlain, Fox et al. 2007; Fox, Chamberlain et al. 2007; Semendo 2011). Biodistribution of MSCs two days post administration was widespread, with hBM IDUA MSCs detected within all organs analysed, including the brain (Chapter Four). Although less than 1% of the originally injected cells were detected via human specific RT-PCR (Chapter Four), the remaining cell population was enough to exert a functional affect on MPS I pathology (Chapter Five).

Thus, this chapter aims to investigate how *i.v.* administered MSCs function in an MPS I model of disease, two and six months post treatment, to help elucidate MSC's mode of action and long-term therapeutic use. MSC biodistribution, persistence *in vivo* and the effect of short (two months post treatment) and long-term (six months post treatment) treatment on MPS I associated pathology; body weight, organ size, biochemical parameters of disease (IDUA, β-hexosaminidase and gag levels), bone morphology and brain inflammation, were further characterised in this chapter (Figure 6.1).

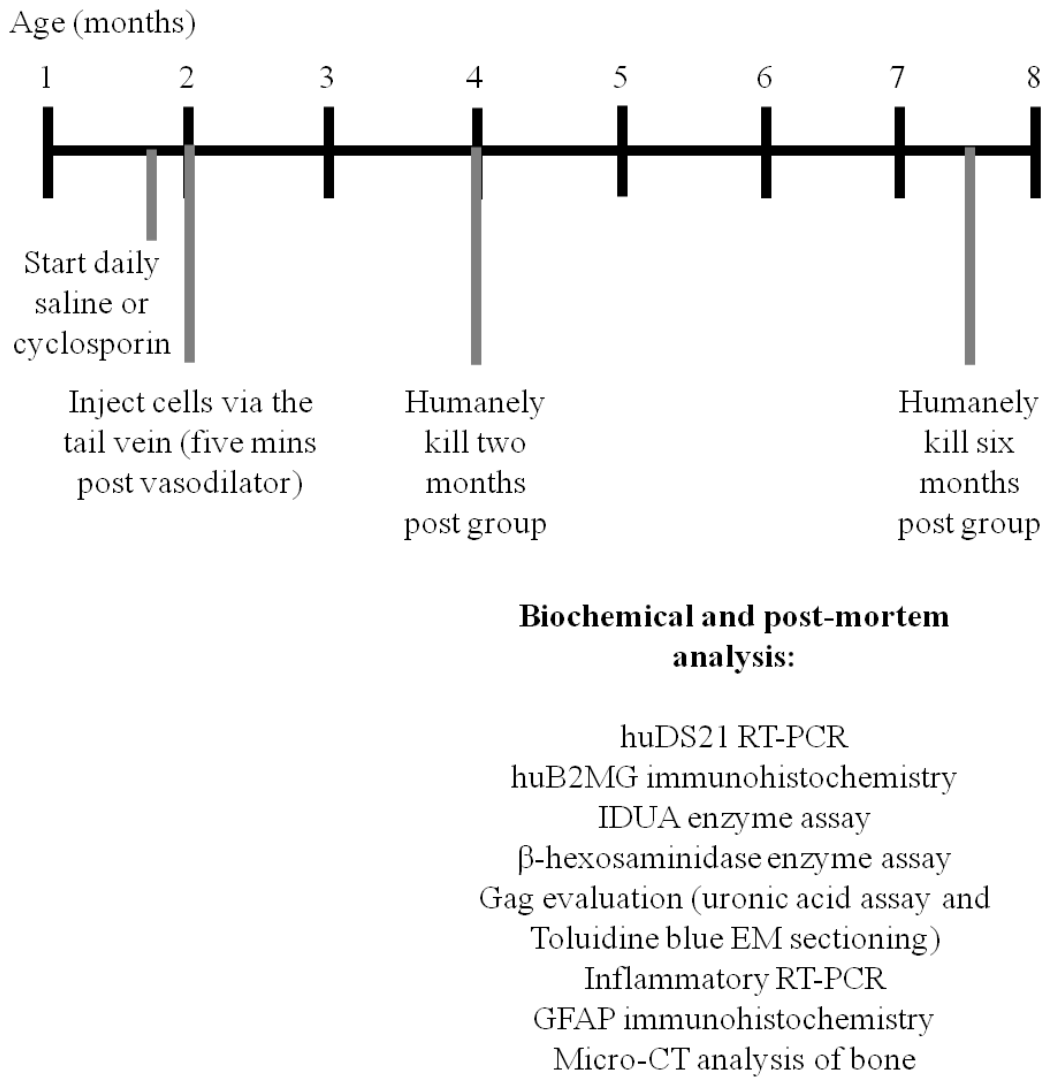


Figure 6.1: Timeline of treatment initiation and biochemical analysis.

Mice were treated from seven weeks of age with either daily saline or cyclosporin subcutaneous injections, depending on group classification (Table 2.1). After one week of daily injections, mice allocated to hBM MSCs, with or without IDUA over-expression, treatment groups (Table 2.1) received a tail vein injection of a vasodilator (1mg/kg nitroprusside). Five minutes post vasodilation, treated mice received a once off injection of $1x10^6$ hBM MSCs also via the tail vein.

Animals were humanely killed at either four months of age (two month post– short term treatment) or seven and a half months of age (six months post – long term treatment), after which dissected tissues underwent numerous biochemical and post-mortem testing.

6.2 Animal and tissue weights

MPS I patients are known to present with increased body weight and hepatosplenomegaly (Stevenson, Howell et al. 1976; Muenzer, Wraith et al. 2009). Body weight profiles and organ weights following short and long-term MSC treatment were therefore assessed to determine any effect of treatment on these established clinical observations.

6.2.1 Body weight

Body weights from two to four months of age were calculated using both short and long-term treated animals, however, from five to seven months of age body weights were determined using only the long-term treatment group (Table 5.1). Due to health reasons, some animals were humanely euthanized prior to the completion of the long-term study (Figure 5.2), therefore animal numbers in MPS I cyclosporin treated groups declined at six and seven months of age (Table 5.1).

The body weights of male and female MPS I and normal control mice significantly differed from one another and were therefore presented separately (Figure 6.2A and 6.2B respectively). However, male and female growth profiles were similar, with an early increase in body weight from two to five months of age, before reaching a plateau (Figure 6.2A and 6.2B respectively).

No significant differences in body weight were seen between groups in male mice at two months of age. MPS I saline and cyclosporin treated male mice were significantly heavier than their respective normal counterparts from five and three months of age respectively (two-way ANOVA, Tukey's HSD; Figure 6.2A). In addition to this, MPS I cyclosporin treated

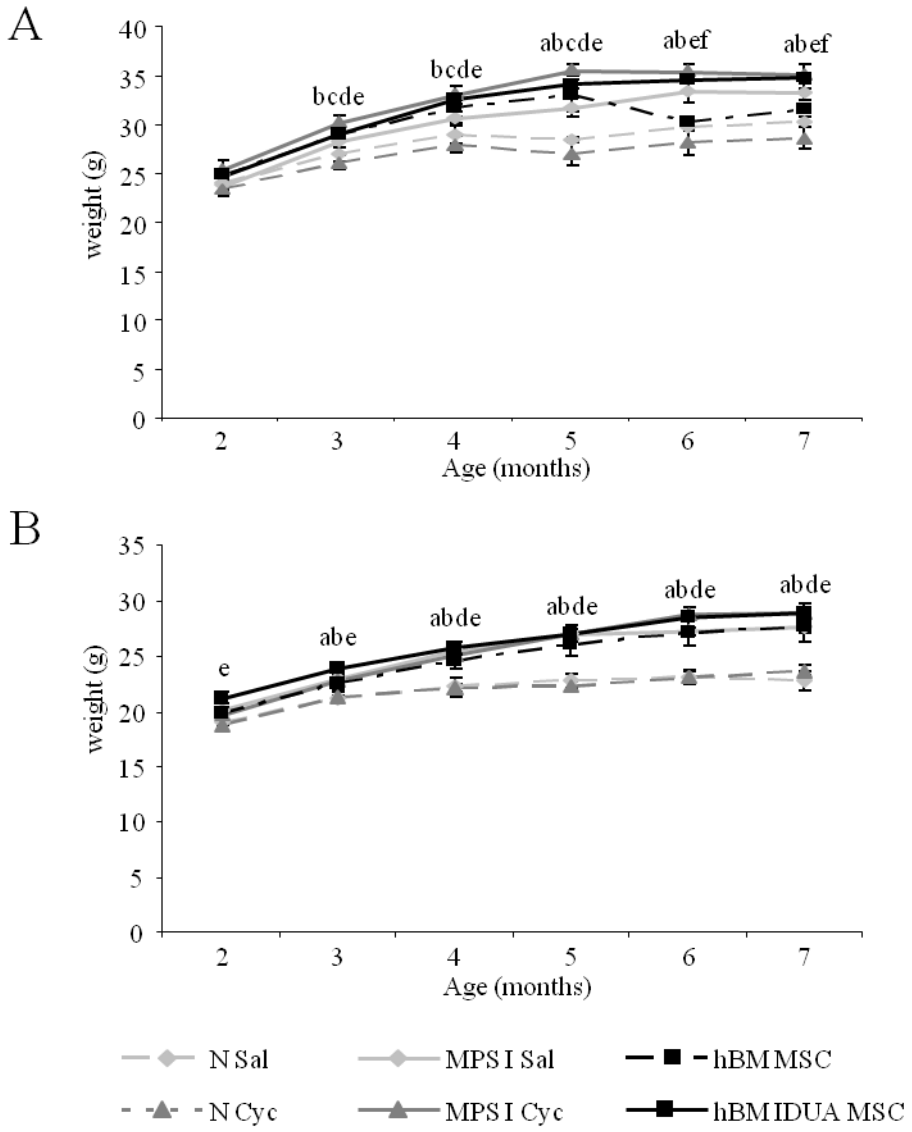


Figure 6.2: Body weight

Male **(A)** and female **(B)** mice were weighed daily. Mean (± SEM; n = 4-17) weights are presented at monthly intervals.

a indicates significance between N Sal and MPS Sal, **b** indicates significance between N Cyc and MPS Cyc, **c** indicates significance between MPS Sal and MPS Cyc, **d** indicates significance between hBM MSC and N Cyc, **e** indicates significance between hBM IDUA MSC and N Cyc and **f** indicates significance between hBM MSC and hBM IDUA MSC, $p<0.05$ (two-way ANOVA, Tukey's HSD).

male mice were significantly heavier than MPS I saline controls from three to five months of age (two-way ANOVA, Tukey's HSD; Figure 6.2A). hBM MSCs and hBM IDUA MSCs treated mice were significantly heavier than their normal cyclosporin controls from three to five months (one to three months post treatment) and three to seven months of age (one to five months post treatment), respectively (two-way ANOVA, Tukey's HSD; Figure 6.2A), indicating that treatment was not effective at reducing MPS I body weight. While, hBM MSC body weights were seen to decrease at six and seven months of age (four and five months post treatment), becoming significantly lighter than hBM IDUA MSC treated mice (two-way ANOVA, Tukey's HSD; Figure 6.2A), this was due to a deterioration of animal health and numbers (Figure 5.2 and Table 5.1 respectively), as well as lower starting body weights for several individual hBM MSC treated mice.

MPS I saline and cyclosporin treated female mice were both significantly heavier than their respective normal counterparts from three to seven months of age (two-way ANOVA, Tukey's HSD; Figure 6.2B). Female MPS I cyclosporin mice treated with either hBM MSCs or hBM IDUA MSCs were also significantly heavier than normal cyclosporin female controls from three (one month post treatment) and two months of age (following commencement of treatment), respectively (two-way ANOVA, Tukey's HSD; Figure 6.2B), indicating that treatment was not effective at reducing increased body weight of MPS I female mice.

6.2.2 Tissue weight

Regardless of genotype, immune-status or MSC treatment, the wet tissue weights of the brain, kidney and heart were unaltered two months post treatment (Figure 6.3A), while only the brain and kidney wet tissue weights remained unaltered six months post treatment (Figure 6.3B).

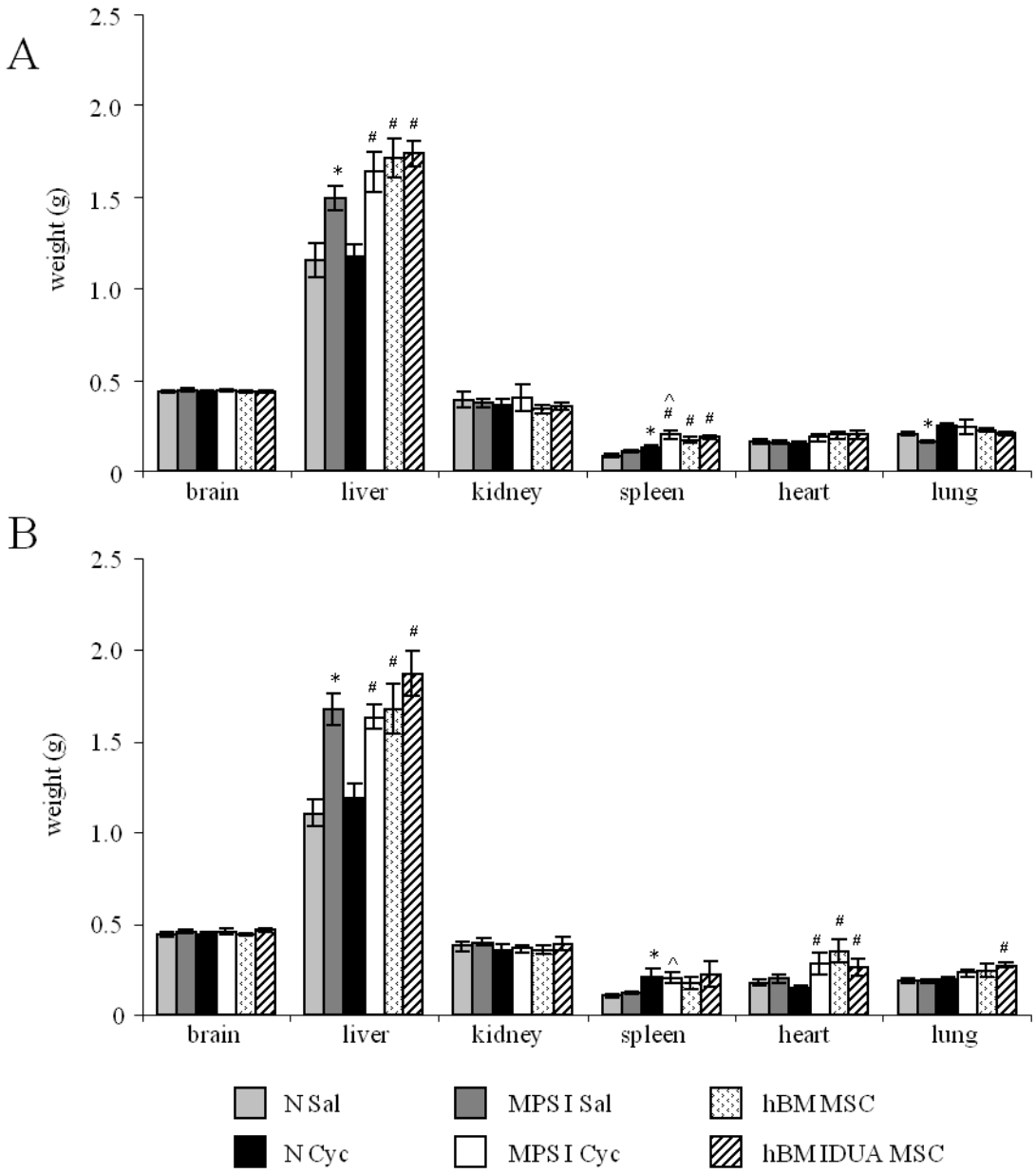


Figure 6.3: Tissue weight

Four months of age (two months post treatment) **(A)** and eight months of age (six months post treatment) **(B)** tissue weights. Mean (± SEM; n = 6-17) tissue weights are presented.

***** Indicates significant difference to N Sal, # indicates significant difference to N Cyc, ^ indicates significant difference to MPS I Sal $p<0.05$ (one-way ANOVA, Tukey's HSD).

As expected, the wet liver weights of MPS I saline (129% and 152% respectively) and MPS I cyclosporin (140% and 138% respectively) treated mice were significantly elevated when compared to their respective normal controls two and six months post (one-way ANOVA, Tukey's HSD; Figure 6.3A and 6.3B respectively). In contrast, spleen wet tissue weights were not significantly different when comparing MPS I and normal saline controls (Figure 6.3A and 6.3B respectively). However, normal (154% and 201% respectively) and MPS I cyclosporin (191% and 162% respectively) treated spleen weights were significantly elevated above respective saline controls at both time-points (one-way ANOVA, Tukey's HSD; Figure 6.3A and 6.3B respectively), indicating a significant effect of cyclosporin administration. While no changes were noted in the wet tissue weight of the heart two months post, MPS I cyclosporin mice had significantly enlarged hearts when compared to normal cyclosporin controls six months post treatment (186% of normal cyclosporin; one-way ANOVA, Tukey's HSD; Figure 6.3B), again suggesting an effect of cyclosporin mediated immunosuppression. Surprisingly, comparisons of wet lung weight two months post found a significant reduction in lung weight when comparing MPS I and normal saline controls (80% of normal saline; one-way ANOVA, Tukey's HSD; Figure 6.3A).

Similar to two days post treatment administration results (Chapter Four), the wet liver weights of hBM MSC (146% and 142% respectively) and hBM IDUA MSC (148% and 158% respectively) treated mice were significantly elevated when compared to their respective normal controls at both time-points (one-way ANOVA, Tukey's HSD; Figure 6.3A and 6.3B respectively). The wet spleen weights of hBM MSC (130%) and hBM IDUA MSC (141%) treated mice were also significantly elevated above normal cyclosporin controls two months post treatment (one-way ANOVA, Tukey's HSD; Figure 6.3A), however, this significant difference was not seen after six months of treatment due to an increase in normal cyclosporin

wet spleen weight (Figure 6.3B). These results suggest that MSC treatment did not ameliorate MPS I associated hepatosplenomegaly.

While no changes were noted in wet heart weight two months post, hBM MSC (229%) and hBM IDUA MSC (176%) treated mice all had significantly enlarged hearts when compared to normal cyclosporin controls six months post treatment (one-way ANOVA, Tukey's HSD; Figure 6.3B), suggestive of long-term cyclosporin mediated immunosuppression. Similarly, while no differences were noted in wet lung weight in either treatment group two months post (Figure 6.3A), a significant increase in lung weight was noted when comparing hBM IDUA MSC treated mice to normal cyclosporin controls six months post treatment (139%; one-way ANOVA, Tukey's HSD; Figure 6.3B).

6.3 Distribution of mesenchymal stem cells

Chapter Four indicated that hDP IDUA MSCs and hBM IDUA MSCs home to multiple organs when administered *i.v.* to MPS I cyclosporin treated mice, however, their ability to engraft and remain within the tissue over a longer duration *in vivo* is still largely debated (Semendo 2011; Singer and Caplan 2011). The biodistribution of human stem cells in each mouse organ was determined by human specific RT-PCR (huDS21) as per Chapter Four and Song *et al*. (2012) (Chapter 2.6.1). Similarly, immunohistochemical analysis of huB2MG was used to visualise and confirm real-time PCR biodistribution of human cells within the brain (Chapter 2.6.3).

6.3.1 Real-time PCR

Real-time PCR analysis of huDS21 gene expression (Chapter 2.6.1) indicated that hBM MSCs and hBM IDUA MSCs distributed to multiple MPS I affected organs, however, cell numbers detected within each organ varied between mice (Table 6.1). hBM MSCs were detected in the brain (8/8 animals; 9% total cells detected), liver (2/8 animals; 8% total cells detected), kidney (3/8 animals; <1% total cells detected), spleen (3/8 animals; 1% total cells detected), heart (3/8 animals; 1% total cells detected) and lung (4/8 animals; 81% total cells detected) of MPS I cyclosporin treated mice. Similarly, hBM IDUA MSCs were detected in the brain (9/9 animals; 79% total cells detected), liver (4/9 animals; 21% total cells detected), kidney (6/9 animals; <1% total cells detected), spleen (4/9 animals; <1% total cells detected), heart (2/9 animals; <1% total cells detected) and lung (4/9 animals; <1% total cells detected) of treated MPS I mice. These results are in accord with the low cell numbers detected two days post administration (Chapter Four), again with less than 1% of originally injected cells detected by RT-PCR two months post administration for each individual animal. However, 58,375 human cells per 10^4 total cells were detected within the spleen of one animal, which is equivalent to detecting 73% of originally injected cells, which was also associated with a significantly enlarged wet spleen weight.

6.3.2 Immunohistochemistry staining

To confirm and visualise RT-PCR results, huB2MG staining (Chapter 2.6.2) was carried out on multiple brain sections of hBM MSC and hBM IDUA MSC treated mice due to a high proportion of cells detected within the brain (Table 6.1) and associated neurological improvements (Chapter Five). Positive staining was confirmed against sections of the human tonsil.

*Real-time PCR performed with specific primer pairs complementary to the human Down syndrome genomic DNA sequence on chromosome 21 (huDS21). Purified tissue (20mg) genomic DNA from each individual animal was determined against a standard curve generated by serially diluted human MSC genomic DNA into murine genomic DNA. Results expressed as total cells detected per 10^4 cells, followed by the proportion of cells detected within that organ in relation to total cells detected per individual animal in brackets. ND = no cells detected in 20mg proportion of tissue converted to genomic DNA for analysis using the Wizard SV genomic DNA purification system (Promega). * denotes an outlier, and was therefore removed from total proportion calculations.*

Table 6.1: Real-time PCR analysis of hMSCs in mouse tissues using huDS21 specific primers

Treatment	Brain	Liver	Kidney	Spleen	Heart	Lung
hBM MSCs	1.76 (15%)	2.1 (85%)	ND (0%)	ND (0%)	ND (0%)	ND (0%)
	<1 (14%)	ND (0%)	<1 (67%)	<1 (10%)	<1 (3%)	<1 (7%)
	<1 (0%)	<1 (2%)	ND (0%)	<1 (0%)	3.35 (1%)	206 (97%)
	4.97 (96%)	ND (0%)	<1 (4%)	ND (0%)	<1 (0%)	ND (0%)
	<1 (32%)	ND (0%)	ND (0%)	ND (0%)	ND (0%)	<1 (68%)
	<1 (10%)	ND (0%)	<1 (52%)	ND (0%)	ND (0%)	<1 (38%)
	5.24 (1%)	ND (0%)	ND (0%)	58375 (100%) *	ND (0%)	ND (0%)
	<1 (100%)	ND (0%)	ND (0%)	ND (0%)	ND (0%)	ND (0%)
Combined total proportion	**9%**	**8%**	**<1%**	**1%**	**1%**	**81%**
hBM IDUA MSCs	<1 (0%)	24 (100%)	ND (0%)	<1 (0%)	ND (0%)	<1 (0%)
	4 (36%)	1.7 (62%)	<1 (2%)	ND (0%)	ND (0%)	<1 (0%)
	1.4 (72%)	ND (0%)	<1 (4%)	ND (0%)	<1 (24%)	ND (0%)
	<1 (5%)	1.8 (91%)	<1 (3%)	<1 (1%)	ND (0%)	ND (0%)
	31 (96%)	ND (0%)	ND (0%)	3.1 (4%)	ND (0%)	<1 (0%)
	4.6 (94%)	ND (0%)	<1 (1%)	<1 (5%)	<1 (0%)	<1 (0%)
	417 (100%)	ND (0%)	<1 (0%)	ND (0%)	ND (0%)	ND (0%)
	<1 (25%)	<1 (68%)	<1 (7%)	ND (0%)	ND (0%)	ND (0%)
	<1 (100%)	ND (0%)	ND (0%)	ND (0%)	ND (0%)	ND (0%)
Combined total proportion	**79%**	**21%**	**<1%**	**<1%**	**<1%**	**<1%**

Minimal non-specific background staining was present on brain sections for normal and MPS I saline and cyclosporin animals; with cyclosporin treated controls presented for comparison (Figure 6.4). Positive staining with similar intensity to that of the human tonsil was noted in high responding animals (Figure 6.4), representing mice where more than four human cells were detected per 10^4 total cells (Table 6.1). The majority of cells were found either within the frontal lobe or cerebellum, with neuron-like cells, perivascular endothelium and Purkinje cells staining positive for huB2MG (Figure 6.4), indicating a similar brain biodistribution to two days post hBM IDUA MSC administration (Chapter Four). Positive huB2MG staining was also noted around blood vessels, consistent with the *i.v.* delivery and distribution of hMSCs. Staining was clustered to areas of high and low intensity, and was not present within all brains analysed (data not shown), confirming the specificity of the human RT-PCR results (Table 6.1).

6.4 Biochemical analysis

The typical spectrum of pathology associated with MPS I is a decrease in IDUA enzyme activity (Chapter 2.5.4), a secondary elevation in β-hexosaminidase (Chapter 2.5.5) and an increase in gag storage; as shown through the quantification of uronic acid (Chapter 2.5.6) and toluidine blue staining (Chapter 2.8). Biochemical analysis was performed on all tissues collected two months (short-term) and six months post (long-term) *i.v.* MSC administration to determine whether lentiviral transduction of hBM MSCs had any additional advantages when assessing clearance or reversal of MPS I pathology.

6.4.1 α-L-iduronidase assay

As expected, MPS I saline and cyclosporin treated mice exhibited significantly reduced IDUA enzyme activity in all tissue analysed when compared to respective normal controls (one-way

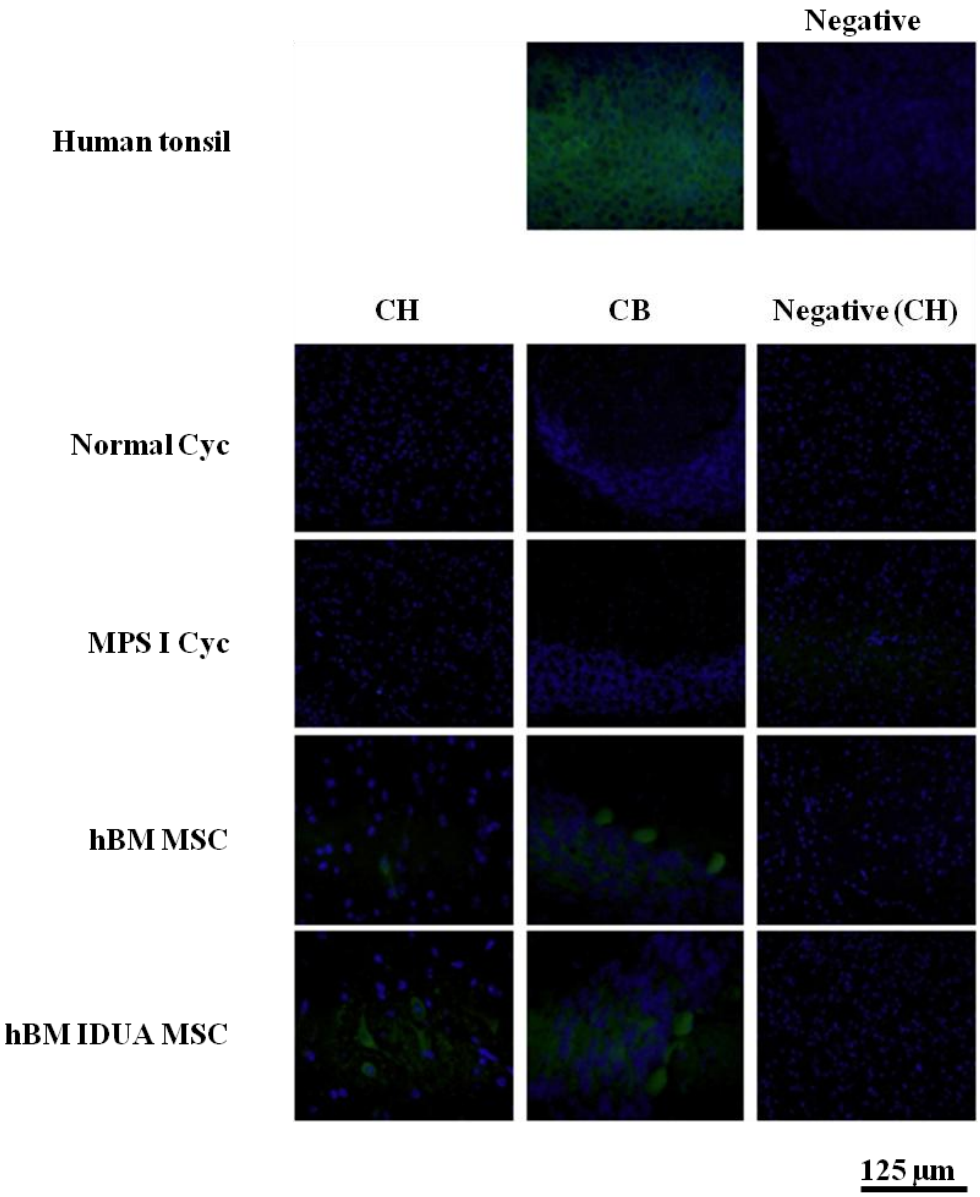


Figure 6.4: Human specific beta-2-microglobulin immunohistochemistry

Localisation of human MSCs within the brain was determined on formalin fixed, paraffin embedded sections using a primary antibody specific to human beta-2-microglobulin and a FITC conjugated secondary antibody. Nuclei were stained with DAPI. Representative pictures were taken within the cerebral hemisphere (CH) and cerebellum (CB) (not tonsil) at 100x magnification, with their negative controls shown for relative background staining. Normal and MPS I mice cyclosporin treated mice shown as controls, as no difference was noted between saline and cyclosporin treated sections. Human tonsil sections were used as a positive control.

ANOVA, Tukey's HSD; Figure 6.5 and 6.6 respectively). Similarly, mice that received either hBM MSC or hBM IDUA MSCs had significantly reduced IDUA enzyme levels in all tissues analysed when compared to normal cyclosporin controls two and six months post treatment (one-way ANOVA, Tukey's HSD; Figure 6.5 and 6.6 respectively). However, brain IDUA enzyme levels were elevated in hBM MSC and hBM IDUA MSC treated mice when compared to MPS I cyclosporin controls two months post treatment (824% and 1930% of MPS I cyclosporin respectively; Figure 6.5A), reaching significance for hBM IDUA MSC treated mice. Interestingly, one hBM IDUA MSC treated mouse had brain IDUA levels comparable to 80% of normal activity two months post treatment, which correlated to the highest number of human cells being detected within the brain (417 human cells per 10^4 cells; Table 6.1). However, elevations in brain IDUA activity did not persist six months post treatment (Figure 6.6A). In contrast, serum IDUA activity for hBM MSC and hBM IDUA MSC treated mice were elevated above MPS I cyclosporin controls six months post treatment (152% and 195% of MPS I cyclosporin; Figure 6.6G), reaching significance for hBM IDUA MSC treated mice.

6.4.2 β-hexosaminidase assay

Two and six months post treatment, β-hexosaminidase enzyme levels were significantly elevated in all MPS I saline and cyclosporin tissues analysed when compared to their respective normal controls (one-way ANOVA, Tukey's HSD; Figure 6.7 and 6.8 respectively). No significant changes in β-hexosaminidase levels were noted in any of the hBM MSC or hBM IDUA MSC treated tissues at either time-point (Figure 6.7 and 6.8 respectively), despite significantly elevated brain and circulating IDUA levels noted at two and six months post treatment, respectively (Figure 6.5A and 6.6G respectively), and in contrast to liver β-hexosaminidase reductions seen two days post stem cell administration (Chapter Four).

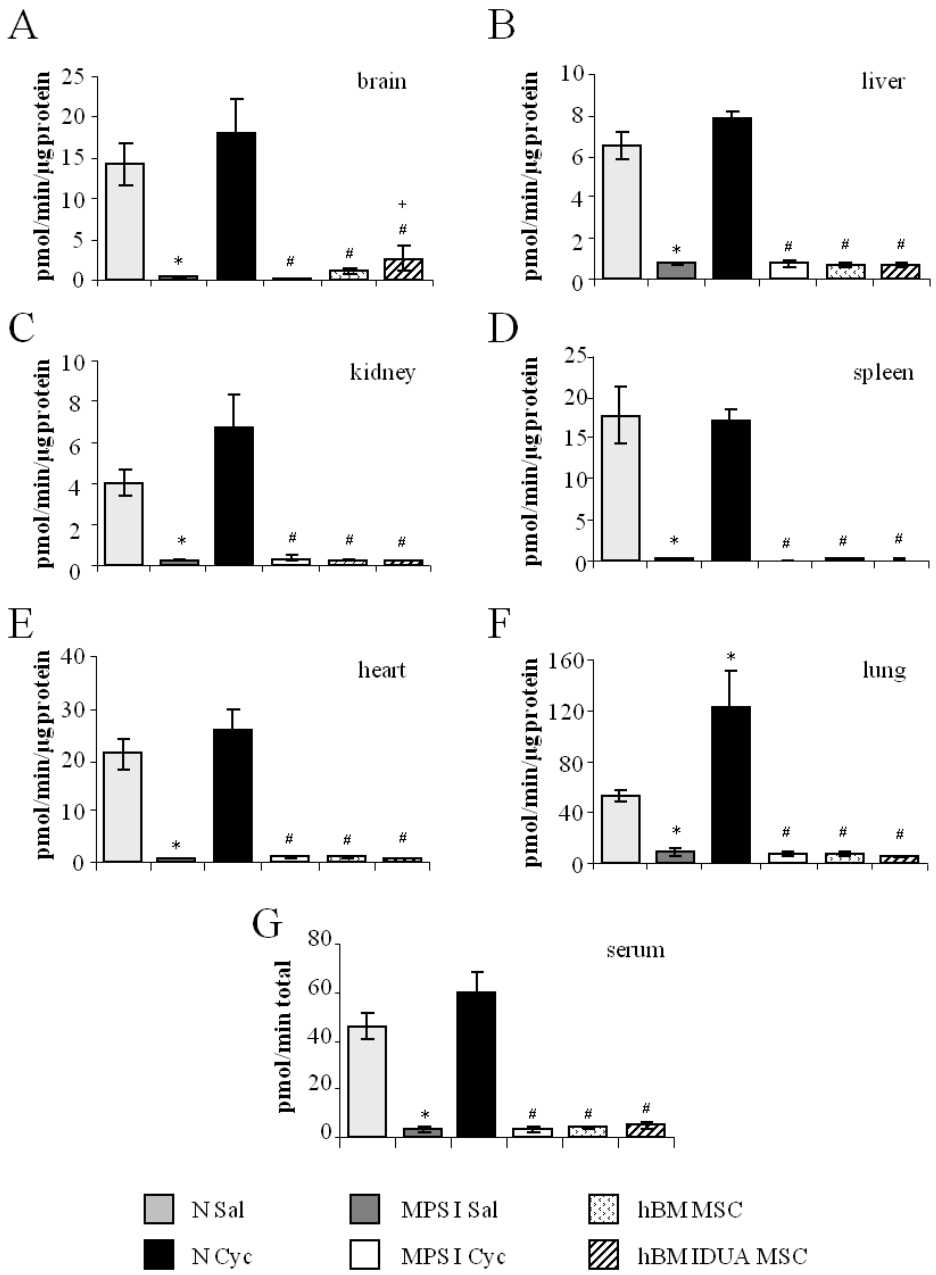


Figure 6.5: Short term tissue IDUA enzyme levels

Tissue IDUA enzyme levels were measured two months post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 4-umbelliferyl-α-L-iduronide substrate for four hours at 37ºC. Results were normalised to protein (excluding serum) and mean (± SEM; n = 8-9) IDUA enzyme levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E)**, lung **(F**) and serum **(G)**.

***** Indicates significant difference to N Sal, # indicates significant difference to N Cyc, + indicates significant difference to MPS I Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

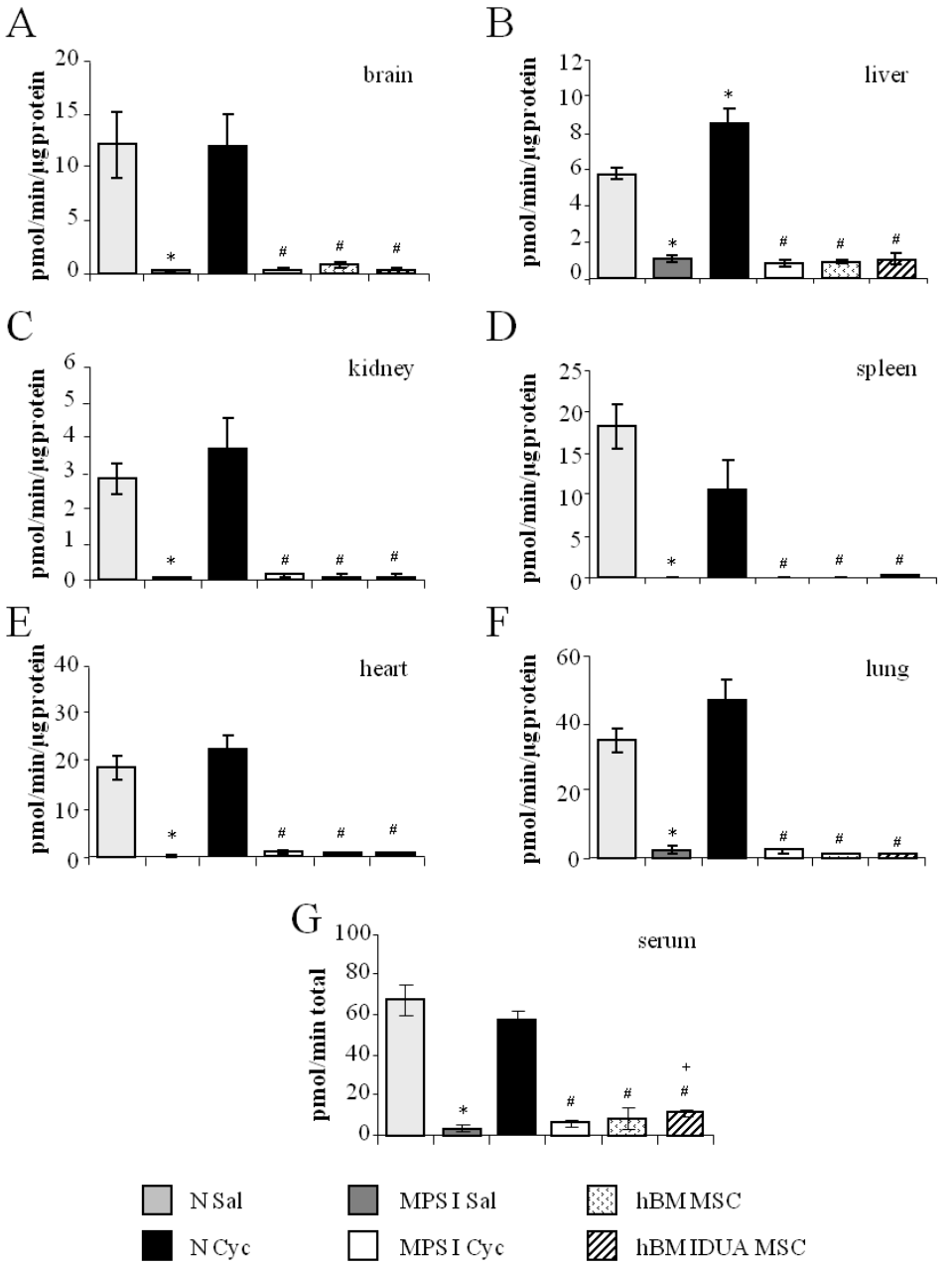


Figure 6.6: Long term tissue IDUA enzyme levels

Tissue IDUA enzyme levels were measured six months post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 4-umbelliferyl-α-L-iduronide substrate for four hours at 37ºC. Results were normalised to protein (excluding serum) and mean (± SEM; n = 6-9) IDUA enzyme levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E)**, lung **(F**) and serum **(G)**.

***** Indicates significant difference to N Sal, # indicates significant difference to N Cyc, + indicates significant difference to MPS I Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

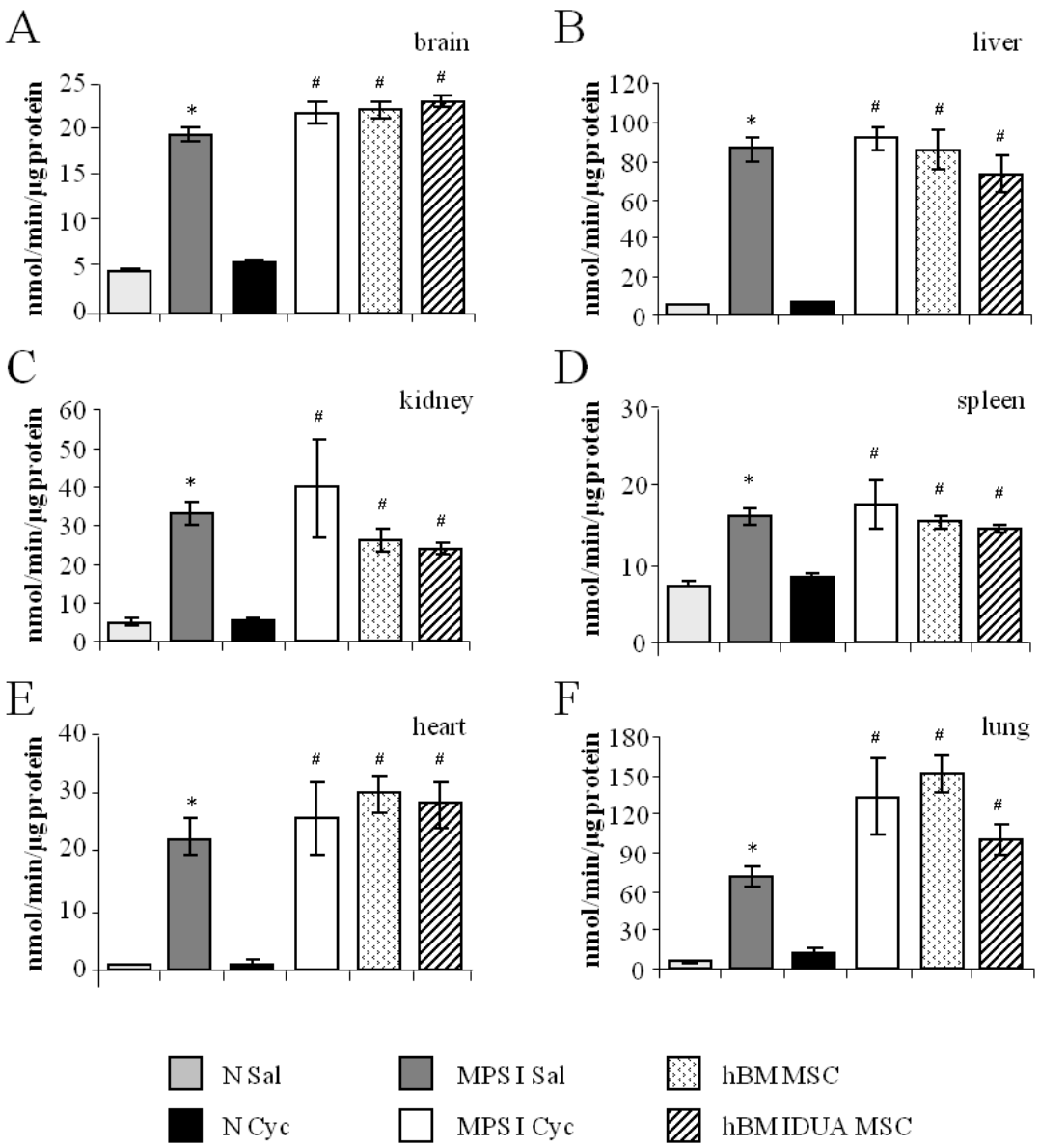


Figure 6.7: Short term tissue β-hexosaminidase enzyme levels

Tissue β-hexosaminidase enzyme levels were measured two months post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 4-methylumbellifryl-2-acetamindo-2-deoxy-b-D-glucopyranoside substrate for thirty minutes at 37ºC. Results were normalised to protein and mean (± SEM; n = 8-9) b-hexosaminidase enzyme levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E)** and lung **(F)**.

***** Indicates significant difference to N Sal, **#** indicates significant difference to N Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

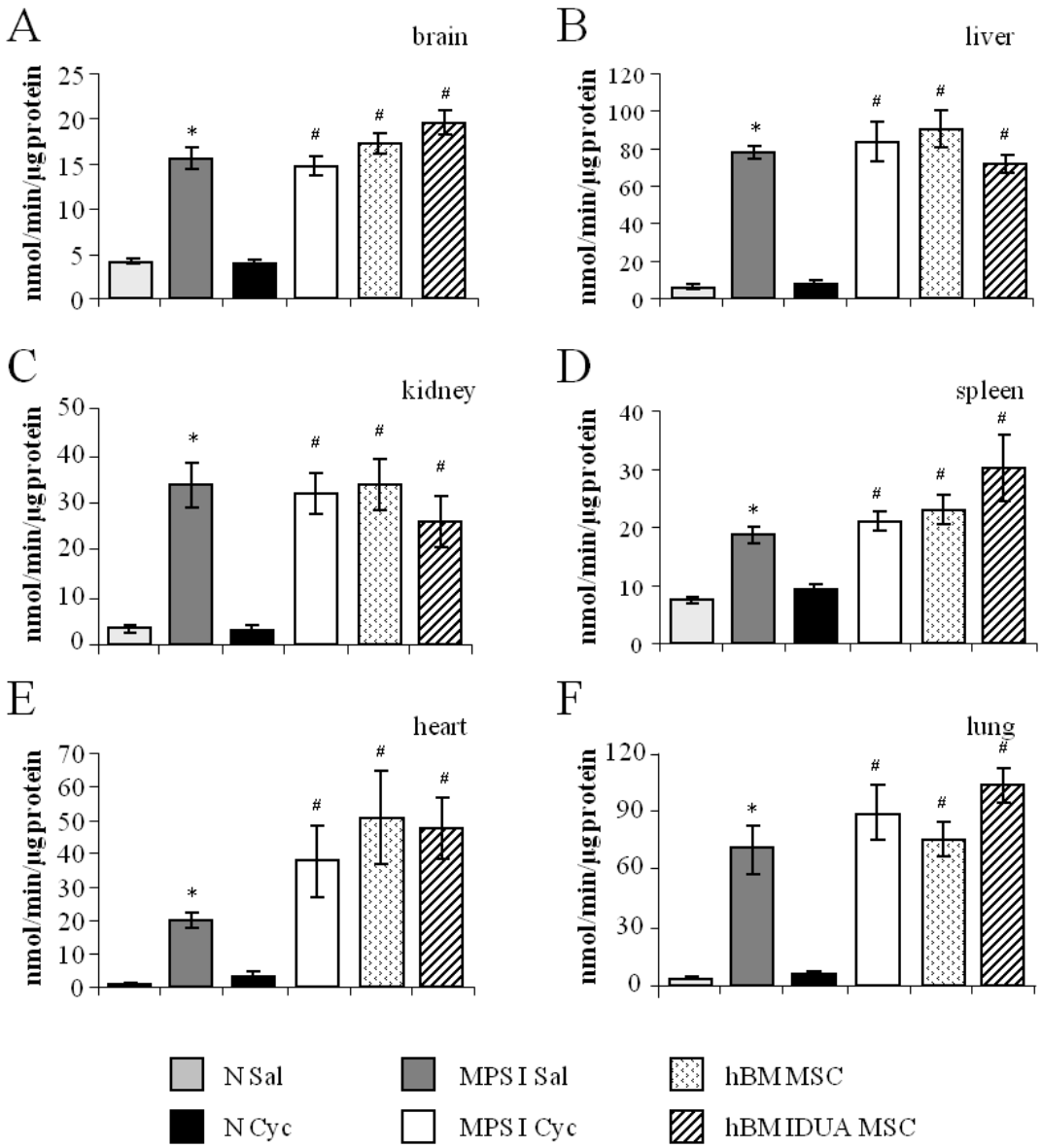


Figure 6.8: Long term tissue β-hexosaminidase enzyme levels

Tissue β-hexosaminidase enzyme levels were measured six months post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 4-methylumbellifryl-2-acetamindo-2-deoxy-b-D-glucopyranoside substrate for thirty minutes at 37ºC. Results were normalised to protein and mean (± SEM; n = 6-9) b-hexosaminidase enzyme levels are presented for the brain (A), liver (B), kidney (C), spleen (D), heart (E) and lung (F).

* Indicates significant difference to N Sal, # indicates significant difference to N Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

6.4.3 Gag storage

MPS I is characterised by the presence of undegraded HS and DS gags inside the lysosomes contained within nearly every cell and tissue (Clarke, Russell et al. 1997; Neufeld and Muenzer 2001; Muenzer, Wraith et al. 2009). Gag storage can be analysed by determining total uronic acid content of tissues (Section 6.4.3.1) or by visualising lysosomal distension in resin embedded, toluidine blue stained tissue sections (Section 6.4.3.2).

6.4.3.1 Uronic acid assay

Total uronic acid content of all MPS I saline and cyclosporin treated tissues analysed was significantly elevated two and six months post when compared to their respective normal controls (one-way ANOVA, Tukey's HSD; Figure 6.9 and 6.10 respectively). In addition, MPS I cyclosporin lung uronic acid levels were significantly elevated above MPS I saline controls two months post (one-way ANOVA, Tukey's HSD; Figure 6.9F), suggesting a relationship with cyclosporin mediated immunosuppression. Neither hBM MSCs nor hBM IDUA MSCs administration was able to significantly reduce gag content within any tissue analysed at either time-point (one-way ANOVA, Tukey's HSD; Figure 6.9 and 6.10 respectively). However, the administration of both hBM MSC and hBM IDUA MSCs resulted in further elevation of total spleen uronic acid when compared to MPS I cyclosporin controls two months post treatment (one-way ANOVA, Tukey's HSD; Figure 6.9D), with this elevation no longer being significant six months post treatment (Figure 6.10D).

6.4.3.2 Toluidine blue staining

As expected, lysosomal distension and undegraded gag accumulation was not evident in either normal saline or normal cyclosporin treated toluidine blue stained sections two months

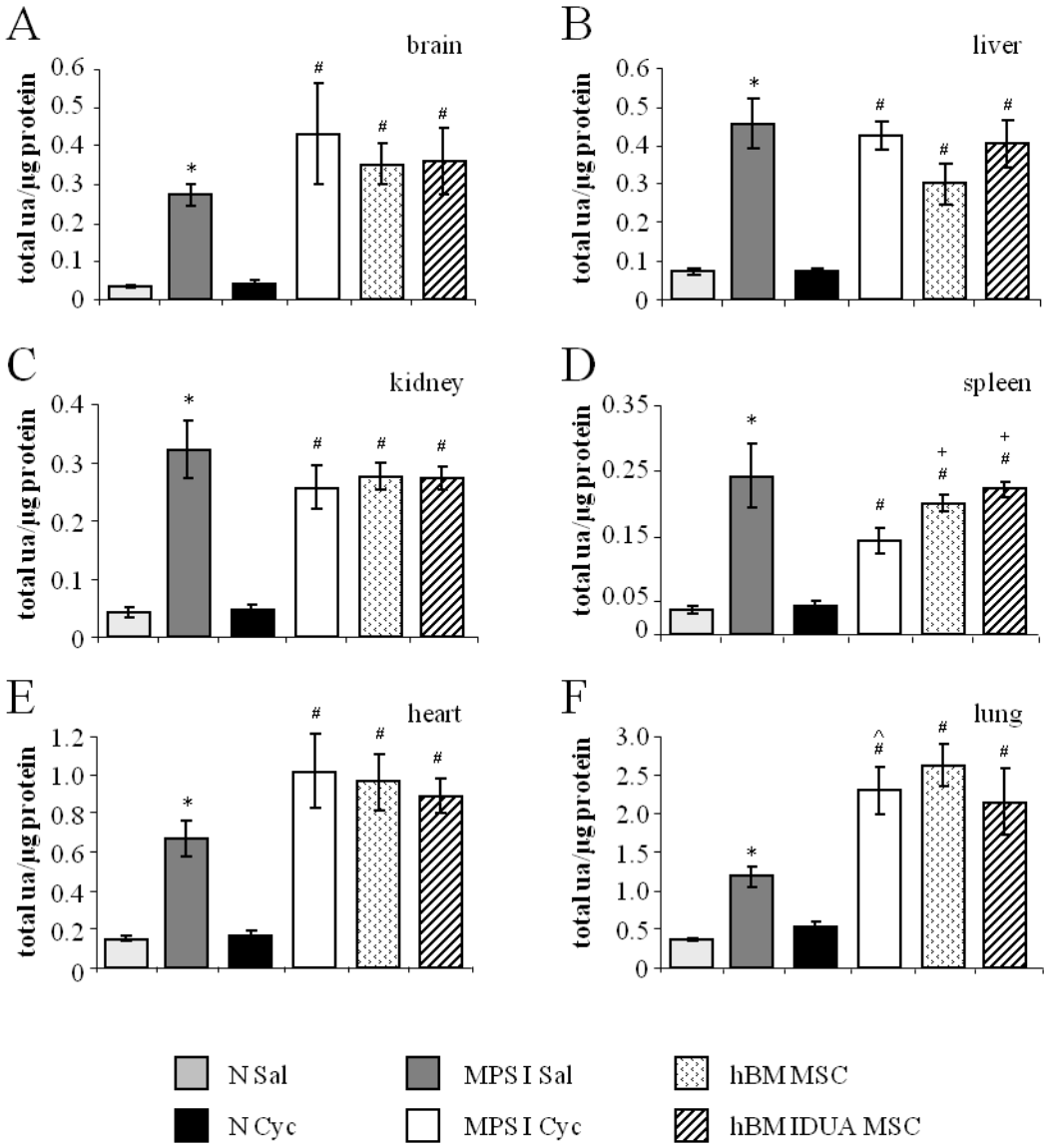


Figure 6.9: Short term tissue uronic acid levels

Tissue gag levels were measured two months post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 2.5 volumes 0.1% (w/v) cetylpridinium chloride in 0.054M citrate buffer, pH 4.8, for thirty minutes at 37ºC and measured for uronic acid. Results were normalised to protein and mean (± SEM; n = 8-9) uronic acid levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E)** and lung **(F)**.

***** Indicates significant difference to N Sal, **#** indicates significant difference to N Cyc, ^ indicates significant difference to MPS I Sal, + indicates significant difference to MPS I Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

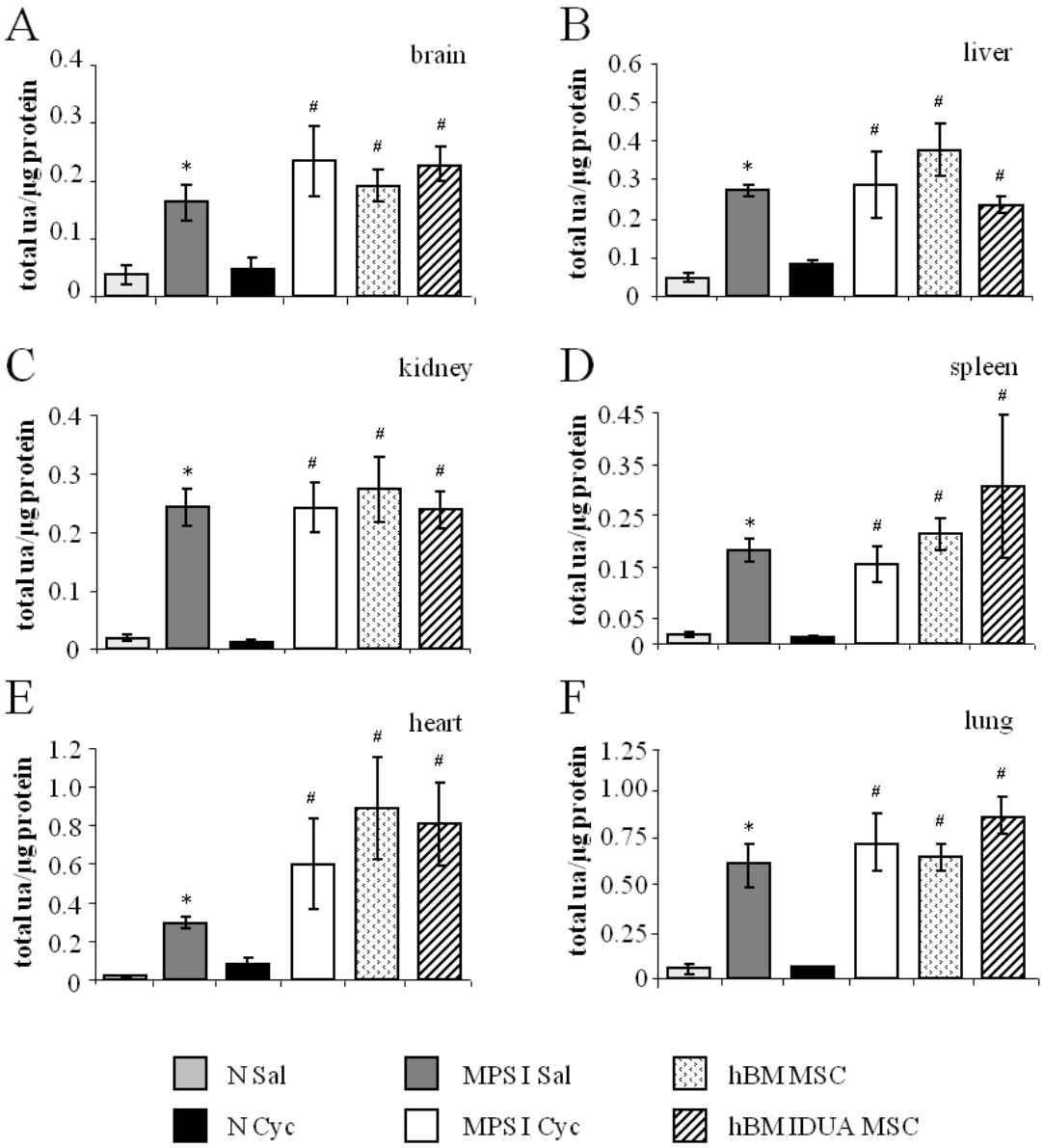


Figure 6.10: Long term tissue uronic acid levels

Tissue gag levels were measured six months post treatment on 0.1% (v/v) Triton X-100 extractions incubated with 2.5 volumes 0.1% (w/v) cetylpridinium chloride in 0.054M citrate buffer, pH 4.8, for thirty minutes at 37ºC and measured for uronic acid. Results were normalised to protein and mean (± SEM; n = 6-9) uronic acid levels are presented for the brain **(A)**, liver **(B)**, kidney **(C)**, spleen **(D)**, heart **(E)** and lung **(F)**.

***** Indicates significant difference to N Sal, **#** indicates significant difference to N Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

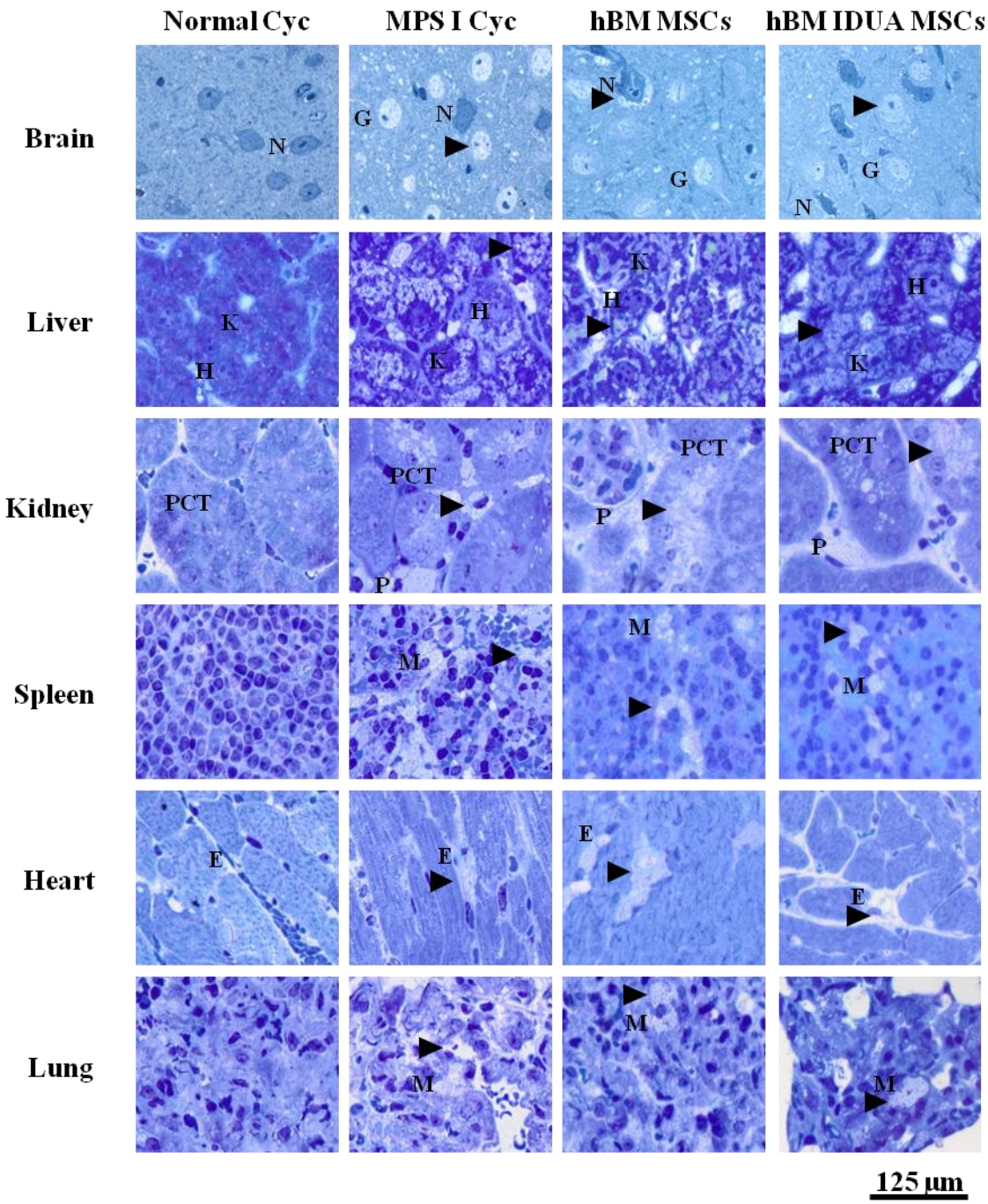


Figure 6.11: Short term tissue lysosomal storage

EM fixed (1.25% (v/v) glutaraldehyde, 4% (w/v) sucrose and 4% (w/v) paraformaldehyde in PBS) resin embedded 1µm tissue sections were stained with toluidine blue and visualised for gag storage (arrow head) by light microscopy at 100x magnification. **N** neuron, **G** glia, **H** hepatocyte, **K** kupffer cell, **PCT** proximal convoluted tubule, **P** podocyte, **M** macrophages and **E** endothelium.

post, however, gag storage was noted in all tissues analysed for both MPS I saline and MPS I cyclosporin treated controls (Figure 6.11). Since MPS I lysosomal storage did not differ visually between MPS I saline and cyclosporin treated mice, cyclosporin controls are presented for comparison (Figure 6.11).

Lysosomal distension and undegraded gag accumulation was present in the same cell types of MPS I, hBM MSC and hBM IDUA MSC animals, regardless of cyclosporin or MSC treatment. Undegraded gag accumulated within neurons (N), perivascular macrophages, endothelial cells, glial cells (G), and Purkinje cells of the brain, hepatocytes (H) and Kupffer cells (K) of the liver, proximal convoluted tubule (PCT), podocytes (P) and juxtaglomerular apparatus of the kidney, endothelium and parenchyma (M) of the spleen, laminar fibrosa and endothelium (E) of the heart, and within the phagocytes and pneumocytes (macrophages, M) of the lung (Figure 6.11). Neither hBM MSCs nor hBM IDUA MSCs administration resulted in the clearance or reduction of gag storage, consistent with the significantly elevated uronic acid levels two months post treatment (Section 6.4.3.1).

6.5 Bone (L5 vertebrae) morphology

Micro-CT analysis was performed on the fifth lumbar vertebrae two months post treatment to assess bone microarchitecture and determine if hBM MSCs or hBM IDUA MSC treatment improved MPS I associated skeletal pathology. Lumbar height and width were analysed to determine if any shortening or thickening of the vertebral body was evident. Bone mineral density and the rate of bone formation and resorption were defined as the ratio of segmented bone volume or surface to the total volume of the region of interest (Bouxsein, Boyd et al. 2010), while mean trabecular thickness, number and spacing are indicative of normal or altered bone remodelling that occurs over the lifetime of the mouse (Glatt, Canalis et al. 2007;

Bouxsein, Boyd et al. 2010). Taken together, these quantitative parameters allowed insight into complex skeletal pathology associated with MPS I.

MPS I skeletal disease was evident at four months of age (two months post), with MPS I saline mice having significantly increased vertebral height, bone volume to total volume, bone surface to total volume and trabecular number, in addition to significantly reduced trabecular thickness, when compared to normal saline controls (one-way ANOVA, Tukey's HSD; Figure 6.12A, C, D, F and G respectively). Similarly, these changes were also evident when comparing MPS I cyclosporin and normal cyclosporin treated mice. In addition, vertebral width and bone surface were also significantly elevated in MPS I cyclosporin treated mice, and trabecular spacing was also significantly reduced when compared to normal cyclosporin controls (one-way ANOVA, Tukey's HSD, Figure 6.12B, E and H respectively). Furthermore, normal cyclosporin vertebral width and bone surface were significantly decreased when compared to normal saline controls, but in contrast, these same parameters were significantly elevated in MPS I cyclosporin mice when compared to MPS I saline controls (one-way ANOVA, Tukey's HSD; Figure 6.12B and E respectively), suggestive of differing effects of cyclosporin mediated immunosuppression in normal and MPS I mice.

hBM MSC treatment had a minimal effect on MPS I associated skeletal changes, with all parameters remaining significantly different to normal cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 6.12). However, hBM IDUA MSC treated vertebral width was significantly reduced when compared to MPS I cyclosporin controls (1.24 ± 0.015mm versus 1.33 ± 0.045mm respectively, one-way ANOVA, Tukey's HSD; Figure 6.12B), suggestive of stabilisation or reversal of skeletal pathology.

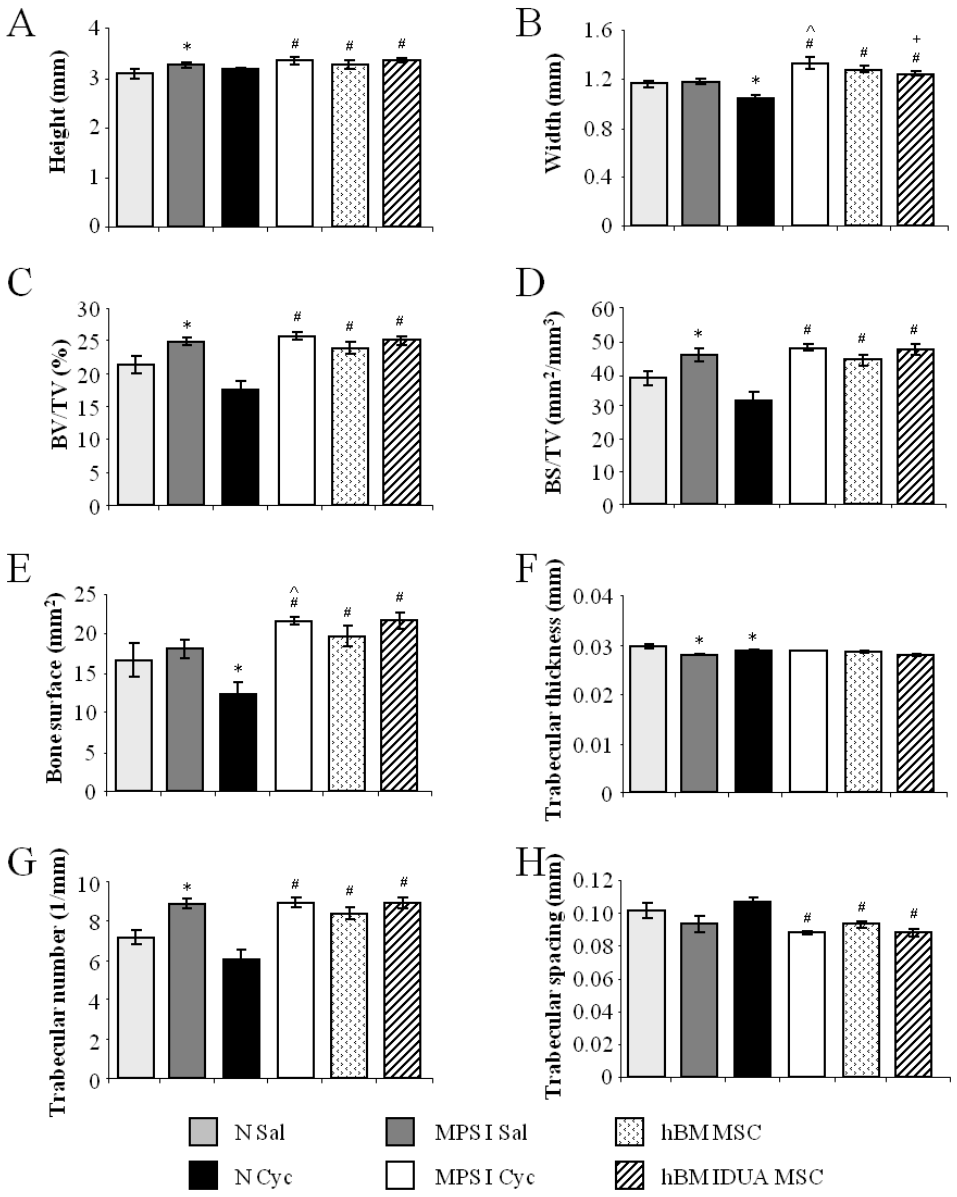


Figure 6.12: Micro-CT analysis of lumbar (L5) vertebrae

Lumbar vertebrae (L4-L6) were fixed in 10% (v/v) formalin overnight before L5 vertebra were scanned using a SkyScan 1174 (Belgium) machine and software (Dataviewer (SkyScan) and CTAn (SkyScan)). Results are expressed as mean (± SEM; n = 2-9) height **(A)**, width **(B)**, bone volume/total volume (BV/TV) **(C)**, bone surface/total volume (BS/TV) **(D)**, bone surface **(E),** trabecular thickness **(F**), trabecular number **(G**) and trabecular spacing **(H)**.

***** Indicates significant difference to N Sal, **#** indicates significant difference to N Cyc, ^ indicates significant difference to MPS I Sal, + indicates significant difference to MPS I Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

6.6 Brain inflammation

Brain inflammation has been previously explored in MPS disorders resulting from the disruption of both apoptotic and autophagy related pathways and astroglial activation due to lysosomal accumulation (Li, Zhao et al. 2002; Ohmi, Greenberg et al. 2003; Villani, Gargiulo et al. 2007; Arfi, Richard et al. 2011; Baldo, Mayer et al. 2012; Wilkinson, Holley et al. 2012; Archer, Langford-Smith et al. 2014). Microglial activation has been previously demonstrated to play a key role in MPS associated neuroinflammation (Ohmi, Greenberg et al. 2003; Wilkinson, Holley et al. 2012), which is related to T-cell recruitment (Olson and Miller 2004; Block, Zecca et al. 2007; Pais, Figueiredo et al. 2008).

MSCs are known to home to areas of inflammation, where they exert their immunomodulatory and anti-inflammatory effects by inhibiting macrophage pro-inflammatory cytokine production (TNFα, Il1β and Ccl3) and modulating T lymphocyte proliferation (Cd68, Tgfβ1 and IFNγ) (Semendo 2011; Singer and Caplan 2011; Prockop and Oh 2012; Rustad and Gurtner 2012), suggesting that MSCs may exhibit a positive role in reducing MPS associated inflammatory brain disease. Gene expression of inflammatory markers of disease were determined from sagittal sections of the brain two months post treatment to assess MPS I brain inflammation and any changes following MSC administration (Chapter 2.7.1).

6.6.1 Inflammatory gene expression

The gene expression profile of brain inflammatory markers did not show any overall pattern when comparing MPS I levels to normal controls, regardless of immunosuppression status or macrophage versus T-cell activity (Figure 6.13). Gene expression of *Ccl3* (4, 7.35, 7 and 9.3 fold respectively), *Cd68* (3.5, 5, 2.8 and 3 fold respectively) and *Tnf* (4, 3.8, 2.2 and 2.8 fold

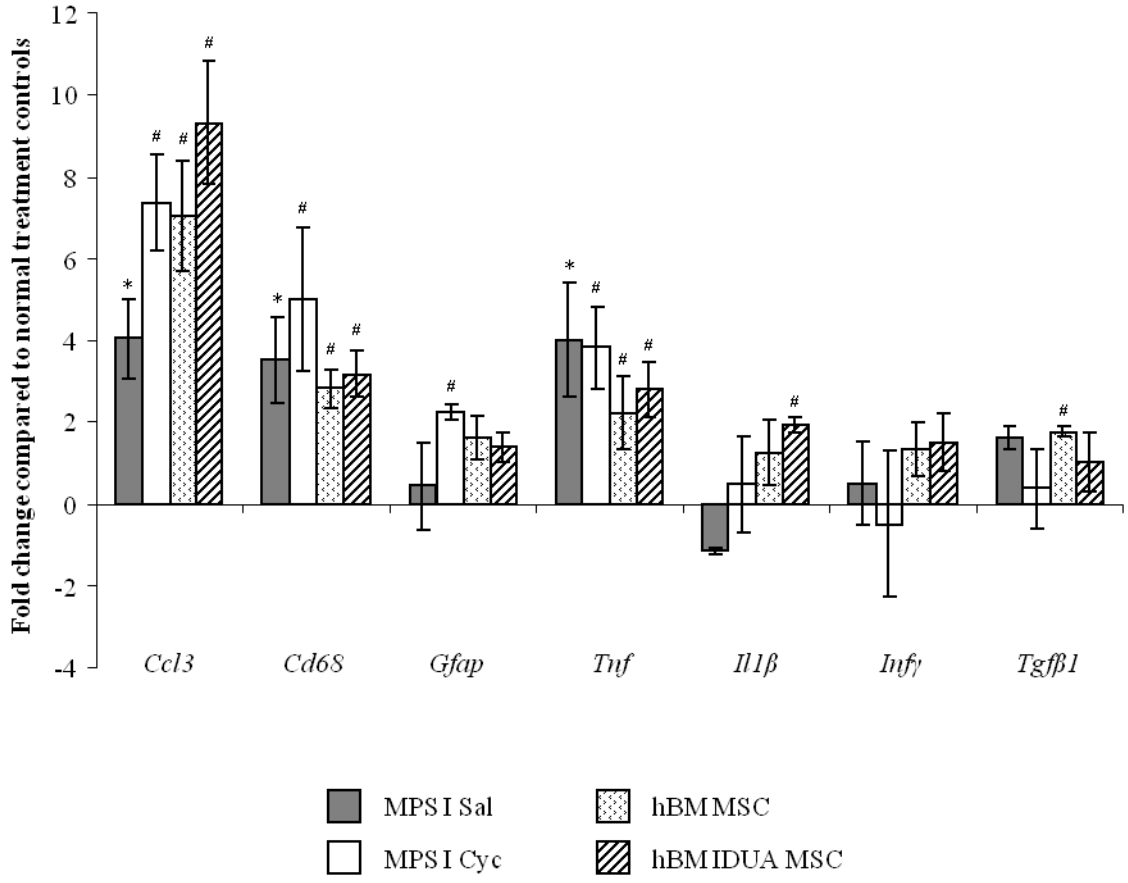


Figure 6.13: Inflammatory mediators of the brain

Total RNA was extracted from thin sagittal brain slices of N Sal, N Cyc, MPS I Sal, MPS I Cyc, hBM MSC and hBM IDUA MSC animals. cDNA was generated from 1µg RNA and 0.5µg equivalent was used to measure *Ccl3*, *Cd68*, *Gfap*, *Tnf*, *Il1β*, *Infg* and *Tgfβ1* gene expression, normalised to *CycpA* as a housekeeping gene. Fold change compared to respective normal (saline or cyclosporin treated) was calculated using the ΔΔCt method, and results expressed as mean (± SEM; n = 3-9).

***** Indicates significant difference to N Sal, **#** indicates significant difference to N Cyc, $p<0.05$ (one-way ANOVA, Tukey's HSD).

respectively) were significantly elevated above their respective normal controls (one-way ANOVA, Tukey's HSD; Figure 6.13) in MPS I saline, MPS I cyclosporin, hBM MSC treated and hBM IDUA MSC treated mice. Although not significant, *Tnf* gene expression was reduced with both hBM MSC and hBM IDUA MSC treatment (p=0.051; Figure 6.13). *Gfap* gene expression was unaltered in MPS I saline mice when compared to normal saline controls (0.4 fold), but was significantly elevated by 2.2 fold in MPS I cyclosporin treated mice (one-way ANOVA, Tukey's HSD; Figure 6.13). No significant elevations in *Gfap* were noted for either hBM MSC or hBM IDUA MSC treated mice (1.6 and 1.4 fold respectively; Figure 6.12). Gene expression of *Il1β* (-1.17 and 0.47 fold respectively), *Infγ* (0.49 and -0.5 fold respectively) and *Tgfβ1* (1.61 and 0.47 fold respectively) were not significantly altered from normal in either MPS I saline or cyclosporin controls (Figure 6.13). However, hBM IDUA MSC treated animals had significantly elevated *Il1β* expression (1.9 fold) and hBM MSC treated mice had significantly elevated *Tgfβ1* expression (1.75 fold) over normal cyclosporin controls (one-way ANOVA, Tukey's HSD; Figure 6.13). Within each treatment group there were high and low responders, demonstrated by the large error bars (Figure 6.13).

6.6.2 Astroglial activation immunohistochemistry

Gfap was the only inflammatory mediator that was significantly elevated in MPS I cyclosporin mice, but not significantly elevated above normal in either MSC treatment group. Astroglial activation was therefore determined on formalin fixed, paraffin embedded sagittal brain sections two months post treatment to confirm *Gfap* gene expression (Section 6.6.1).

Minimal GFAP staining was noted in the brains of normal saline controls, however, cyclosporin administration increased staining intensity within the frontal cortex of normal brains (Figure 6.14). Corresponding with *Gfap* gene expression (Section 6.6.1), more

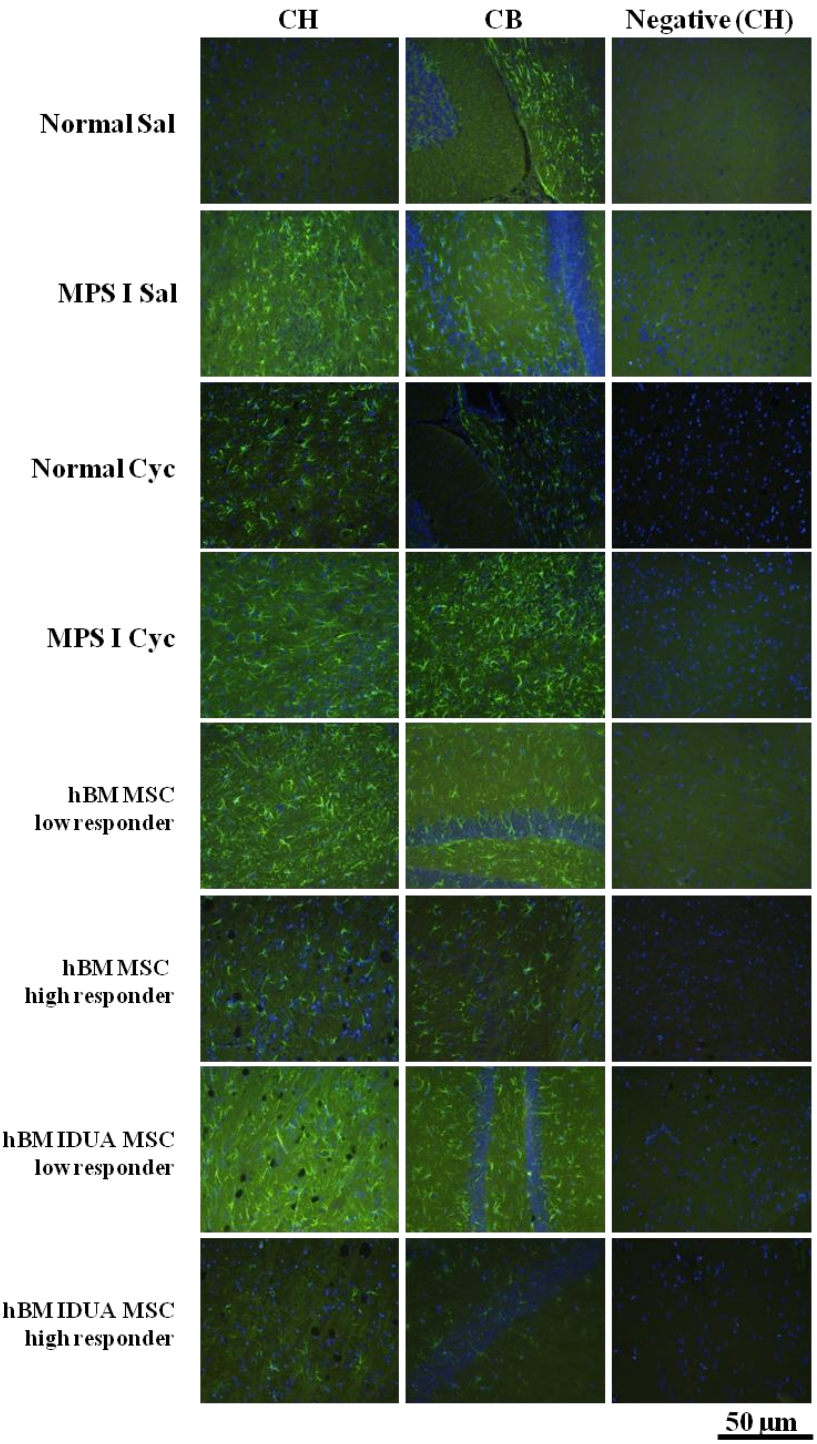


Figure 6.14: Astroglial activation in the brain

Localisation of astroglial activation within the brain was determined on formalin fixed, paraffin embedded sections using a primary antibody specific to GFAP and a FITC conjugated secondary antibody. Nuclei were stained with DAPI. Representative pictures were taken within the cerebral hemisphere (CH) and cerebellum (CB) at 40x magnification, with their negative controls shown for relative background staining.

Real-time PCR was performed using Gfap specific primers and cDNA generated from thin, sagittal brain sections. Fold change compared to respective normal controls was determined using the ΔΔCt method, with cyclophilin A as a housekeeping gene. Staining intensity and distribution was determined using a primary antibody specific to Gfap and a FITC conjugated secondary antibody on 5μM thick paraffin embedded sagittal brain sections. Sections were scored as low, moderate or high in comparison to normal and MPS I controls, with staining either present evenly throughout the section or punctated, with areas of minimal staining.

Table 6.2: Astroglial activation gene expression and immunohistochemistry

	Gene expression	Staining intensity	Staining distribution
MPS I saline	0.408 ± 1.06	Moderate	Throughout
MPS I cyclosporin	2.24 ± 0.18	High	Throughout
hBM MSCs	1.169	Low	Cleared areas
	1.641	High	Throughout
	1.371	High	Throughout
	3.000	Moderate	Cleared areas
	4.243	Moderate	Throughout
	1.596	Moderate	Cleared areas
	1.053	High	Throughout
	-1.106	Low	Throughout
hBM IDUA MSCs	2.540	High	Throughout
	1.500	Moderate	Throughout
	1.419	High	Throughout
	1.834	Low	Cleared areas
	1.399	Low	Cleared areas
	-1.315	Moderate	Cleared areas
	1.872	Moderate	Throughout
	1.409	Moderate	Throughout
	1.912	Moderate	Cleared areas

widespread staining was noted in MPS I mice, again with increased staining noted in cyclosporin treated mice (Figure 6.14). Both hBM MSC and hBM IDUA MSC treatment returned varying results, with some brains still having widespread, high intensity staining (low responders), while others had staining similar to that of normal controls (high responders; Figure 6.14). A reduction in GFAP staining was not always consistent across the entire brain, however, most notable changes were within the frontal cortex and cerebellum. The GFAP staining intensity and distribution in the brain of MSC treated mice was scored as low, moderate or high and compared back to *Gfap* gene expression (Section 6.6.1); where the majority of staining scores aligned with gene expression levels (Table 6.2).

6.7 Discussion

In MPS I patients there is a strong correlation between residual enzyme activity and severity of disease (Haley, Fragala Pinkham et al. 2006). Patients who have just 7% residual enzyme activity generally have milder manifestations of disease, known as Scheie syndrome, which does not present with changes in CNS function (Scott, Litjens et al. 1993; Bunge, Clements et al. 1998; Neufeld and Muenzer 2001). This genotype-phenotype correlation implies that providing even a small amount of functional enzyme activity can shift the disease spectrum from severe to attenuated in MPS I. MSCs were found to produce and secrete significantly higher amounts of MPS enzymes *in vitro* than currently used HSCs (Chapter Three), and are also known to home to areas of inflammation, where they reduce the response to inflammatory cues to allow regeneration of the injured tissue (Singer and Caplan 2011). This chapter therefore aimed to determine if the ability of MSCs to stabilise and repair functional deficits in MPS I mouse behaviour (Chapter Five) was due to their transient immunomodulation, or whether they trans-differentiated and secreted high levels of functional IDUA to shift the murine phenotype from severe to attenuated.

6.7.1 MSC persist within multiple MPS I affected organs

Biodistribution of hBM MSCs and hBM IDUA MSCs was determined two months post treatment, after significant behavioural changes were already evident in MSC treated mice (Chapter Five). Similar to two days post treatment (Chapter Four), the majority of hBM IDUA MSCs were found to evade the lungs, with the brain and liver being the two main target organs. In contrast, only 19% of detected hBM MSCs were found to evade the lungs, which could account for the limited behavioural improvements in comparison to hBM IDUA MSC treatment. Differences in lung evasion could be attributed to the success of vasodilation, as a previous study showed no difference in cell surface profiles between transduced and non-transduced MSCs (Choi, Ahn et al. 2011). MSCs are known to home to areas of inflammation and damage (Chamberlain, Fox et al. 2007; Fox, Chamberlain et al. 2007; Semendo 2011; Singer and Caplan 2011), suggesting that in the majority of cases MPS I associated brain and liver disease is forefront to their inflammatory pathology. However, over half of the originally injected hBM MSCs were detected within the spleen of one treated mouse (Table 6.1; hBM MSC treated animal 7), which is also consistent with MPS I associated hepatosplenomegaly (Neufeld and Muenzer 2001; Muenzer, Wraith et al. 2009).

Homing of MSCs to the brain was confirmed by positive huB2MG staining. Cells were found to be unevenly distributed within the brain of MPS I mice, located mainly within the frontal lobe and cerebellum. Positive staining around the perivascular endothelium, neuron-like cells and Purkinje cells was noted, consistent with recent reports showing that MSCs can undergo heterotypic fusion with Purkinje cells due to increased inflammation and cytokine release within the brain (Alvarez-Dolado, Pardal et al. 2003; Bae, Han et al. 2007; Alvarez-Dolado and Martinez-Losa 2011; Kemp, Gordon et al. 2011; Semendo 2011). Previous behavioural deficits have been linked to cerebellum (Li, Zhao et al. 2002; Fujimoto, Longhi et al. 2004; Garcia-Rivera, Colvin-Wanshura et al. 2007) and hippocampal damage (Paylor, Morrison et

al. 1992; Logue, Paylor et al. 1997), indicating that engraftment within these regions of the brain would promote and coincide with increased functional performance (Chapter Five).

Collectively, biodistribution findings suggest that many MSCs are capable of migrating to the brain following *i.v.* administration, where they survive, engraft and promote long-term functional improvements in MPS I associated behavioural deficits (Chapter Five). However, hBM IDUA MSC treatment resulted in additional neurological improvements from two months post treatment when compared to hBM MSC administration (Chapter Five), which may be attributed to the over-expression of IDUA enzyme.

6.7.2 MSCs influence on MPS I somatic pathology is limited

In support of the greater number of cells detected within the brain of hBM IDUA MSC mice, IDUA enzyme activity was significantly elevated in the brain two months post hBM IDUA MSC administration, with enzyme levels more than double that of hBM MSC administration alone. These findings confirm previous reports, which showed a correlation between behavioural improvements and enzyme over-expression (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012), and confer with the significantly higher enzyme production of MSCs following lentiviral transduction (Chapter Three). In addition, this finding supports the elevated brain IDUA levels observed two days post hBM IDUA MSC administration (Chapter Four), although, increased levels were not found to persist to six months post treatment, with brain enzyme activity returning to background. However, hBM IDUA MSC circulating IDUA levels were significantly elevated six months post treatment, which could account for the sustained behavioural performance with no additional neurological improvements from two months post treatment (four months of age; Chapter Five).

Elevated circulating IDUA following BMT is not usually detected in the serum of MPS I patients until three to four months post HSC transplantation (Hobbs, Hugh-Jones et al. 1981; Church, Tylee et al. 2007; Wynn, Wraith et al. 2009), however, MSC secretion capacity was shown to peak two to three weeks post transduction *in vitro* (Chapter Three). The significant increase in circulating IDUA, but not tissue IDUA, six months post treatment suggests that MSCs may be residing outside an organ analysed in this study, which could also account for the low number of cells that were detected by RT-PCR. MSC-like populations have been identified and isolated from a range of tissues (da Silva Meirelles, Chagastelles et al. 2006; Murray, West et al. 2014), including; adipose tissue (Zuk, Zhu et al. 2001), dental pulp (Gronthos, Mankani et al. 2000; Yamaza, Kentaro et al. 2010), connective tissue of the dermis and skeletal muscle (Jiang, Vaessen et al. 2002), gut and pancreas (Crisan, Yap et al. 2008) and tendon (Bi, Ehirchiou et al. 2007). These tissues and organs were not analysed in our study, however MSC engraftment and proliferation within these niches could account for the increase in circulating IDUA in long-term treated animals.

Despite widespread biodistribution of MSCs and significant elevations in IDUA, analysis of biochemical markers revealed minimal changes in MPS I associated pathology following short and long-term treatment, with no significant reductions in β-hexosaminidase activity or gag storage. Lentiviral induced over-expression of another lysosomal enzyme, β-*D*-glucuronidase, was shown to be stable in hBM MSCs during continuous and passaged culture, indicating that lentiviral transgene integration and over-expression persisted with cell proliferation (Chapter Three). In addition, hBM IDUA MSCs were found to produce functional IDUA, which could be internalised by MPS I deficient cells via the M6P receptor (Chapter Three). *In vitro* results suggest that cell biodistribution and enzyme production should have resulted in some normalisation of MPS I biochemical pathology, to further support the significant reduction in liver β-hexosaminidase and gag levels noted two days post

administration (Chapter Four). However, gag accumulation has been shown to take up to six months to plateau following HSC transplantation, even when full donor chimerism is achieved (Church, Tylee et al. 2007). Additionally, the introduction of functional IDUA via ERT in MPS I patients results in a reduction, but not total clearance of gag and β-hexosaminidase levels (Wraith, Beck et al. 2007; Clarke, Wraith et al. 2009; Jameson, Jones et al. 2013).

Collectively, the increased homing of hBM IDUA MSCs to the brain and subsequent significant increase in IDUA activity, with no associated reduction in MPS I associated pathology (β-hexosaminidase activity and gag accumulation), suggests that MSCs may be exerting their functional improvement in behaviour (Chapter Five) via an alternative mechanism, that does not involve the classical removal of stored gag. MSCs are known to modulate the host environment and lower the inflammatory contribution to cellular damage (Reiser, Zhang et al. 2005; Chamberlain, Fox et al. 2007; Fox, Chamberlain et al. 2007; Singer and Caplan 2011), inflammatory gene expression was therefore examined in sagittal brain sections of MSC treated mice and untreated controls.

6.7.3 MSCs anti-inflammatory role in MPS I brain disease

Ccl3, *Cd68*, *Gfap* and *Tnfα* were significantly up-regulated in MPS I cyclosporin mice when compared to normal controls, confirming neuroinflammation noted previously in the MPSs (Richard, Arfi et al. 2008; DiRosario, Divers et al. 2009; Arfi, Richard et al. 2011; Baldo, Mayer et al. 2012; Archer, Langford-Smith et al. 2014). In contrast to previous studies using immunosuppressive agents to treat MPS, the administration of cyclosporin did not significantly reduce the gene expression of any inflammatory associated genes, suggesting a different mode of action to prednisolone and aspirin treatment in MPS IIIA (DiRosario,

Divers et al. 2009; Arfi, Richard et al. 2011). While MSC administration lowered the gene expression of *Cd68*, *Gfap* and *Tnfα*, none of these reached significance. Previous studies have shown that multiple murine cytokines are not homologous to human cytokines, sharing only 58% homology (Hemmi, Peghini et al. 1989; Lembo, Ricciardi-Castagnoli et al. 1996). Therefore, human MSCs are not capable of fully suppressing the release of these pro-inflammatory cytokines from activated T-cells when administered into mice (Kim, Lee et al. 2013), supporting the reduction but not normalisation in brain inflammation with MSC treatment.

Astroglial activation was further characterised by immunohistochemistry, as *Gfap* was the only gene that was significantly elevated in MPS I cyclosporin mice, but not significantly elevated above normal in either treatment group. GFAP staining was consistent with gene expression data; increased staining was observed in MPS I cyclosporin mice when compared to normal controls, which was reduced in some, but not all MSC treated mice. Variations in staining distribution and intensity were noted with MSC treatment, suggesting that some animals responded better to treatment than others. However, this was not directly related to the number of MSCs detected within the brain of individual animals, but did correspond in the majority of cases to *Gfap* gene expression levels. Previous studies have also demonstrated that MSC treatment decreases GFAP staining and associated neuroinflammation (Bae, Furuya et al. 2005; Donega, Nijboer et al. 2014), confirming their immunomodulatory role.

Neuroinflammation is a significant component of MPS associated brain disease and has been previously attributed to behavioural deficits (Richard, Arfi et al. 2008; DiRosario, Divers et al. 2009; Arfi, Richard et al. 2011). Modulating the inflammatory process has been shown to target the secondary aspects of MPS disease, resulting in a deceleration of neurodegeneration, despite no change in enzyme activity levels (DiRosario, Divers et al. 2009; Arfi, Richard et al.

2011). However, previous studies have also shown improved behavioural outcomes correlate to enzyme activity (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012). Our results suggest a cohesive effect of MSC treatment, combining their ability to cross the BBB, immunomodulatory properties and enzyme production to result in improved behavioural outcomes with hBM IDUA MSC treatment (Chapter Five).

6.7.4 Skeletal phenotype of MPS I was partially attenuated by MSC treatment

In addition to profound neurological disease and widespread tissue and organ dysfunction, MPS I is also characterised by skeletal pathology (Neufeld and Muenzer 2001). Previous studies rely on radiographs to determine bone morphology changes that occur with disease progression (Clarke, Russell et al. 1997; Russell, Hendson et al. 1998), however, micro-CT provides in-depth quantitative analysis of bone and trabecular architecture (Bouxsein, Boyd et al. 2010). Skeletal pathology was analysed on the fifth lumbar vertebrae due to a greater bone volume with uniformed shape (Bouxsein, Boyd et al. 2010).

MPS I mice had significantly altered vertebral architecture when compared to normal controls, however vertebral width was the only parameter that was improved with hBM IDUA MSC treatment when compared to MPS I cyclosporin mice. In support of this, Visigalli *et al*. (2010) showed reductions in skull, femur and humerus width with systemically administered HSCs over-expressing IDUA; although, MPS I mice were pre-treated with irradiation, which increases homing and engraftment of stem cells within bone (Visigalli, Delai et al. 2010). Administration of MSCs into five MPS I patients resulted in stabilisation of bone mineral density (Koc, Day et al. 2002), however, this parameter was unchanged in MPS I treatment groups, conferring with the unchanged trabecular architecture. The minimal changes noted

following hBM MSC treatment support the complex nature of MPS I skeletal disease, and indicate a more direct approach may be required to stabilise and reverse skeletal disease.

In addition, MSC administration was not able to alter the growth profiles or hepatosplenomegaly of MPS I cyclosporin mice, suggesting the multi-system pathology associated with MPS I was not corrected, but actually further exacerbated by cyclosporin administration. Previous studies have demonstrated morphological changes and atrophy of the spleen following cyclosporin administration (Masri, Naiem et al. 1987; Masri, Naiem et al. 1988; Masri, Naiem et al. 1989; Masri 1995), with a previous study in rats also showing a significant increase in spleen weight at 10mg/kg dosage (Luke 1992), supporting the enlargement of both normal and MPS I cyclosporin treated spleens. An increase in heart weight was also noted in MPS I cyclosporin mice six months post treatment, which could be attributed to opportunistic infections associated with immunosuppression (Chapter Five). However, cyclosporin toxicity has been previously correlated with a predisposition to either liver, kidney or heart disease (Bennett and Norman 1986; Miller 1996; Gijtenbeek, van den Bent et al. 1999); with MPS I patients also suffering from severe cardiac and respiratory problems in addition to hepatosplenomegaly (Neufeld and Muenzer 2001; Clarke and Heppner 2002). The unimproved growth profiles and hepatosplenomegaly corresponds to the limited improvements noted in MPS I biochemical pathology, with significantly elevated β-hexosaminidase activity and gag storage still evident in all tissues.

6.7.5 Chapter conclusions and future directions

For the first time, this chapter has investigated biochemical changes due to *i.v.* administered MSCs in MPS I mice to account for the functional changes that were noted in Chapter Five. In support of the neurological and skeletal improvements noted in Chapter Five, hBM IDUA MSCs were found to cross the BBB and engraft within the brain, where they exhibited both

their anti-inflammatory properties and secreted significantly elevated IDUA. In addition, vertebral width was significantly reduced with hBM IDUA MSC treatment when compared to MPS I cyclosporin mice, suggesting that systemically administered MSCs have the ability to stabilise at least one component of the progressive, hard-to-treat skeletal disease associated with MPS I. While less than 1% of originally injected cells were detected two days post administration (Chapter Four), remaining MSCs were found to persist and secrete IDUA into circulation up to six months post administration. Taken together, this data suggests that IDUA over-expressing MSCs have the capacity to stabilise the multi-faceted pathology associated with MPS I by lowering MPS I associated brain inflammation and trans-differentiating and secreting IDUA. However, further work needs to be done to separate the effect of cyclosporin and determine the exact role and function of human MSCs in MPS I inflammatory disease.

Chapter Seven: Discussion

7.1 MSC treatment for MPS

Collectively, MPS affects 1 in 22,500 Australian children, and to date the associated progressive neurological and skeletal pathology is not adequately addressed by clinically available therapies, such as ERT or BMT (Meikle, Hopwood et al. 1999; Neufeld and Muenzer 2001; Muenzer, Wraith et al. 2009; Noh and Lee 2014).

As MSCs have the ability to differentiate into many of the cells that are affected in MPS, they have the potential to provide a significant improvement in the way MPS children are treated. The goals of this thesis were to determine the ability of MSCs to produce and over-express MPS enzymes and the effect of over-expression on MSC differentiation, and to evaluate their efficacy in altering the progression of MPS pathology using the MPS I mouse model.

7.1.2 MSCs hold a greater potential than HSCs for combating the multi-system pathology of MPS

HSCs are the current treatment of choice for MPS I patients presenting with neurological involvement (Peters, Balthazor et al. 1996; Peters, Shapiro et al. 1998; Boelens 2006; Wynn, Wraith et al. 2009; Langereis, Borgo et al. 2013), however, CNS stabilisation is only observed when treatment is initiated before the onset of significant neurological impairment (Boelens 2006; Church, Tylee et al. 2007; Kingma, Langereis et al. 2013). In addition, HSC trials in other MPS subtypes have failed to show improvements in neurological function (Shapiro, Lockman et al. 1995; Sivakumur and Wraith 1999; Prasad, Mendizabal et al. 2008; Lau, Hannouche et al. 2010; Langford-Smith, Wilkinson et al. 2012), highlighting the complex nature of MPS disease. ERT is employed for multiple MPS subtypes, which results in attenuated somatic pathology, with minor improvements in mobility and flexibility observed

(Neufeld and Muenzer 2001; Sifuentes, Doroshow et al. 2007; Clarke, Wraith et al. 2009; Muenzer, Wraith et al. 2009). However, the recombinant enzyme is too large to cross the BBB (Barton and Neufeld 1971), limiting its effect on improving cognitive or CNS function in patients. MSCs have the ability to encompass multiple sites of MPS pathology in one single treatment, warranting their further investigation.

Previous animal studies have demonstrated that enzyme production by HSC derived cells of lymphoid and myeloid lineages is not sufficient to combat the multi-tissue symptoms associated with MPS, but that improved efficacy can be achieved by transducing HSCs with viral vectors to over-produce the enzymes deficient in the MPSs (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012), indicating that the amount of enzyme produced is rate limiting. In line with these studies, this thesis has demonstrated that MSCs produce and secrete significantly higher levels of a series of gag degrading enzymes compared to HSCs, both naturally and when engineered to over-produce MPS enzymes (Chapter Three). This greater lysosomal enzyme secretion by MSCs would allow for a more widespread clearance of MPS associated pathology, through cross-correction, than is observed with HSCs.

Markedly different levels of up-regulation and over-expression of enzymes deficient in the MPSs were observed *in vitro*, suggesting that each lysosomal enzyme may be under different regulation or cellular control. In particular, the levels of IDUA and β-*D*-glucuronidase exceeded that of 4-sulphatase and sulphamidase (Chapter Three). Previous studies have demonstrated that the sulphatases, deficient in MPS II, III, IV and VI, undergo post-translational modification of a highly conserved cysteine residue to an α-formylglycine in the active site of the enzyme by SUMF1 (Schmidt, Selmer et al. 1995; Miech, Dierks et al. 1998). Over-expression of sulphatases can thus be further manipulated by concurrent over-expression of SUMF1 (McIntyre, Byers et al. 2010) or by employing a secretory signal in the

vector construct (Sorrentino, D'Orsi et al. 2013). Even though the sulphatase enzyme levels were not elevated to the same extent as the other gag degrading enzymes, levels achieved by MSCs still greatly surpassed that of HSCs (Chapter Three). Sulphamidase over-expressing HSCs have previously resulted in disease correction *in vivo*, therefore superior levels reached by transduced MSCs, without further manipulation, should also be therapeutic (Langford-Smith, Wilkinson et al. 2012). As previous studies have noted a correlation between enzyme levels and disease correction (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012), Chapter Three findings indicate that the administration of MSCs should lead to more widespread clearance of stored material in the MPSs than could be expected with HSC treatment.

In addition to their superior lysosomal enzyme production and secretion capacity, MSCs can differentiate into multiple cell types; including bone, cartilage and neuronal-like cells, potentially encompassing the tissues that are more difficult to treat with current MPS therapy. Differentiation down these lineages was unaffected by lentiviral transduction and enzyme over-expression, boosting their therapeutic potential (Chapter Three). However, HSCs can only give rise to cells of the blood, and a successful transplant therefore relies on the migration of transplant-derived leukocytes into the MPS-affected organs to lower gag storage and produce functional lysosomal enzymes (Krivit, Sung et al. 1995; Koc, Day et al. 2002).

Based on the *in vitro* data, an *in vivo* therapy trial was initiated using bone marrow derived MSCs in the MPS I mouse. MPS I is considered the prototypical MPS disorder and is characterised by profound intellectual deterioration and skeletal disease, in addition to widespread somatic pathology (Neufeld and Muenzer 2001). The murine model of MPS I is well characterised and mimics the severe form of MPS I (Clarke, Russell et al. 1997).

7.2 MSC over-expressing α-*L*-iduronidase improve multiple facets of MPS I pathology

MSCs were capable of widespread biodistribution to multiple organs, and were capable of crossing the BBB and engrafting within the brain of MPS I affected mice (Chapter Four), with MSCs still present in multiple organs at least two months post administration (Chapter Six). Improvement of MPS associated liver pathology was observed within two days of MSC administration (Chapter Four), however, this did not persist past this time-point. Despite there being no further resolution in lysosomal gag storage or β-hexosaminidase activity, increases in IDUA activity were noted within the brain and serum two and six months post administration, respectively (Chapter Six), which coincided with improved behavioural outcomes (Chapter Five). Investigation into MSCs ability to suppress T-cell activation and pro-inflammatory cytokine release suggested that MSCs were partially suppressing, but not resolving, MPS associated brain inflammation (Chapter Six). Many murine cytokines are not homologous to human cytokines, sharing only 58% homology (Hemmi, Peghini et al. 1989; Lembo, Ricciardi-Castagnoli et al. 1996), therefore a complete resolution of inflammation using human derived MSCs was unlikely (Kim, Lee et al. 2013).

MSCs have been shown to home to areas of inflammation and damage, where they suppress T-cell proliferation and aid in host-tissue repair (Chamberlain, Fox et al. 2007; Fox, Chamberlain et al. 2007; Singer and Caplan 2011). However, the exact mechanism behind this mode of action is currently unknown (Singer and Caplan 2011). Some studies suggest that MSCs exert their influence transiently, by modulating the host environment, releasing trophic factors and anti-apoptotic molecules, which allows the host cells to repair and recover (Uccelli, Moretta et al. 2008; Scuteri, Miloso et al. 2011; Zhang, Liu et al. 2013). It is believed that injury, inflammation and foreign cells lead to the activation and proliferation of T cells, however, MSCs also act by inhibiting T cell proliferation by reducing the gene

expression of pro-inflammatory cytokines (Le Blanc 2003; Semendo 2011; Singer and Caplan 2011; De Miguel, Fuentes-Julian et al. 2012; Zhang, Liu et al. 2013). Another potential mechanism is that MSCs migrate to the site of injury, trans-differentiate to replace the damaged cells, and provide growth factors to promote long-term repair (Chopp and Li 2002; Parr, Tator et al. 2007; Si, Zhao et al. 2011; Zhang, Liu et al. 2013).

Neuroinflammation is a significant component of MPS associated brain disease and has been previously attributed to behavioural deficits (Richard, Arfi et al. 2008; DiRosario, Divers et al. 2009; Arfi, Richard et al. 2011). Modulating the inflammatory process has been shown to result in a deceleration of neurodegeneration in murine models of MPS, despite no change in enzyme activity levels (DiRosario, Divers et al. 2009; Arfi, Richard et al. 2011). However, in line with previous studies (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012), a correlation was observed between functional improvements and IDUA over-expression (Chapter Five), suggesting that an enzyme threshold may exist before biochemical changes are evident (Chapter Six).

It is known that there is a 12-24 month lag in disease resolution following HSC transplantation (Rovelli 2008), suggesting that engraftment of cell populations and subsequent proliferation are vital to combat the progressive nature of MPS disease. It is unknown whether a similar lag occurs after MSC administration. Stabilisation of bone mineral density was observed within six months of MSC administration in MPS I patients, but no comparative evaluation of biochemical pathology was performed (Koc, Day et al. 2002). Due to the small percentage of cells detected by RT-PCR (Chapter Six), a comparable lag interval to HSCs may exist before remaining MSC populations have proliferated to an extent that overcomes the suggested enzyme threshold, and resolution of somatic pathology occurs. Circulating IDUA levels were significantly elevated six months post treatment, with no associated

increase in tissue IDUA, suggesting that MSCs may be residing, and proliferating, outside an organ analysed in this study (Chapter Six). The extent of disease resolution may have been more extensive if murine derived MSCs were administered into the murine model of MPS I, eliminating the need for immunosuppression, warranting further investigation.

7.2.1 MPS I treatments currently in clinical use

Clinically available MPS therapies lack the ability to stabilise the progressive neurological aspect of MPS I, unless BMT treatment is initiated before two years of age (Boelens 2006; Church, Tylee et al. 2007; Kingma, Langereis et al. 2013). Supplementary to HSCs abilities, MSCs possess the ability to cross the BBB and differentiate into neuronal like cells (Chapter Three and Six) and hold added immunological benefits (Chapter Six), which resulted in functional CNS improvements (Chapter Five). However, unlike ERT treatment, minimal resolution in MPS I somatic pathology was noted (Chapter Four and Six), suggesting these symptoms may persist, or take longer to resolve, following MSC administration.

Previous pre-clinical studies implementing transduced HSCs have shown diminution of MPS I associated pathology in two month old MPS I mice (Visigalli, Delai et al. 2010). A drawback of HSC administration is the need for irradiation prior to HSC administration, which has been shown to improve the engraftment and promote proliferation of donor HSCs (Goebel, Yoder et al. 2002; Zhong, Zhan et al. 2002; Goebel, Pech et al. 2004; Barese, Pech et al. 2007; Sadat, Dirscherl et al. 2009). However, normalisation of MPS I pathology was not noted, unless IDUA over-expression exceeded 20nmol/mg/hr within the brain (Visigalli, Delai et al. 2010), also suggesting that an enzyme activity threshold is present.

Due to their added immunological benefits, MSCs have been previously administered to five MPS I patients following successful HSC transplantation. While minimal disease parameters were tested following administration, no adverse toxicities were noted (Koc, Day et al. 2002). More recently, da Silva *et al*. (2012) injected IDUA over-expressing MSCs directly into the left ventricle of MPS I adult mice, which resulted in improved neurological outcomes (da Silva, Pereira et al. 2012). However, IDUA levels returned to basal levels after six weeks *in vitro* and *in vivo*, which could have been related to gene silencing or using MPS I derived MSCs, rather than healthy donor MSCs. In addition, this mode of delivery is invasive and would require stringent regulation if implemented in the clinic. MPS I is also characterised by widespread somatic pathology, and it is likely that somatic pathology would be unaffected by direct CNS administration, however, this was not assessed in the da Silva *et al*. (2012) study (da Silva, Pereira et al. 2012).

For the first time, this thesis has demonstrated that *i.v.* administered hBM MSCs are capable of stabilising and preventing the progression of multiple behavioural deficits associated with MPS I (Chapter Five). In line with previous HSC studies (Visigalli, Delai et al. 2010; Langford-Smith, Wilkinson et al. 2012), over-expression of IDUA correlated with improved functional outcomes, most notably, significantly improved spatial learning above hBM MSC treated mice (Chapter Five). This thesis also established that IDUA over-expressing MSCs have the capacity to stabilise the multi-faceted pathology associated with MPS I by lowering MPS I associated brain inflammation and trans-differentiating and secreting IDUA (Chapter Six). Minimal changes in somatic and skeletal parameters of MPS I disease were noted (Chapter Six), therefore the use of MSCs as a stand-alone treatment is still limited. However, the administration of MSCs in combination with ERT, which resolves MPS associated somatic pathology and improves skeletal flexion and mobility (Neufeld and Muenzer 2001; Sifuentes, Doroshow et al. 2007; Clarke, Wraith et al. 2009; Muenzer, Wraith et al. 2009),

would provide additional clinical benefits to MPS I children. Patients would also be treated soon after diagnosis, as they would not be required to undergo any pre-treatment associated with HSC transplantation (Church, Tylee et al. 2007), and an allogeneic donor is suitable due to MSCs immunomodulatory properties (Chamberlain, Fox et al. 2007; Fox, Chamberlain et al. 2007).

7.3 Future directions

This thesis has demonstrated that MSCs possess enzyme secretion, anti-inflammatory and lineage commitment qualities that have the potential to improve patient response to current treatment options. However, further work is required to fully understand how MSCs alter MPS pathology prior to translation to the clinic. Due to the use of human derived MSCs, an immunosuppressive agent was employed, which resulted in the exacerbation of pre-existing MPS I health conditions and limited MSCs suppression of pro-inflammatory cytokines (Chapter Five and Six). A repeat study using murine derived MSCs would separate, and further elucidate, the effect of MSCs immunomodulation and cyclosporin's immunosuppression.

As MSCs were found to over-express multiple gag degrading enzymes in vitro (Chapter Three), this treatment strategy could be beneficial in treating multiple MPS subtypes, warranting their further investigation *in vivo*. MPS is a very debilitating childhood disease, which often leaves sufferers with reduced mobility or wheelchair bound in their early teens, with limited speech and cognitive function (Neufeld and Muenzer 2001; Valayannopoulos and Wijburg 2011). Results obtained from this thesis suggest that with further investigation, MSC treatment, either stand-alone or in combination, could improve functional aspect of MPS disease, helping to improve patient's quality of life.

Appendix

Appendix One: Materials list

3-phenylphenol	Sigma-Aldrich Pty. Ltd., NSW, Australia
4-MU standard	Sigma-Aldrich Pty. Ltd., NSW, Australia
4-methylumbelliferyl-β-*D*-glucuronide hydrate	Sigma-Aldrich Pty. Ltd., NSW, Australia
4-methylumbelliferyl-α-*D*-N-sulphoglucosaminide	Moscerdam Substrates, Netherlands
4-methylumbelliferyl-α-*L*-iduronide	Moscerdam Substrates, Netherlands
4-methylumbelliferyl sulphate	Sigma-Aldrich Pty. Ltd., NSW, Australia
α-*L*-iduronidase mouse codon optimised plasmid	Genscript, Piscataway, USA
α-MEM	Life Technologies Pty. Ltd., VIC, Australia
Acetone	BDH (Merck) Pty. Ltd., VIC, Australia
Acetic Acid	Ajax Finechem Pty. Ltd., Seven Hills, NSW
Agarose, DNA grade	Progen Biosciences, QLD, Australia
Alcian blue	Sigma-Aldrich Pty. Ltd., NSW, Australia
Alkaline phosphatise calf intestinal enzyme	New England Biolabs Inc., USA
Anti-rabbit FITC conjugated	Silenus Labs, Australia
Anti-beta-2-microglobulin	Leica Biosystems Pty. Ltd., Australia
Araldite 502	ProSciTech, QLD, Australia
Ascorbate-2-phosphate	WAKO Chemicals, USA
β-hexosaminidase substrate	Sigma-Aldrich Pty. Ltd., NSW, Australia
Bradford Reagent	Bio-rad laboratories, California, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich Pty. Ltd., NSW, Australia

Bromophenol blue	BDH Chemicals Ltd., England
BstBI	New England Biolabs Inc., USA
Citrate	Sigma-Aldrich Pty. Ltd., NSW, Australia
Citric acid	Ajax Finechem Pty. Ltd., Seven Hills, NSW
Cetylpyridinium chloride (CPC)	Sigma-Aldrich Pty. Ltd., NSW, Australia
Chloramphenicol	Sigma-Aldrich Pty. Ltd., NSW, Australia
Cyclosporin (250mg/5mL)	Novartis Pharma Pty. Ltd., North Ryde, NSW
D.D.S.A (dodecenyl succinic anhydride)	ProSciTech, QLD, Australia
D-glucuronolactone standard	Sigma-Aldrich Pty. Ltd., NSW, Australia
Di-sodium hydrogen phosphate	Ajax Finechem Pty. Ltd., Seven Hills, NSW
DMEM	Life Technologies Pty. Ltd., VIC, Australia
DMP-30 (2,4,6-tridimethylaminomethyl phenol)	ProSciTech, QLD, Australia
dNTPs	Roche Diagnostics, Mannheim, Germany
Dulbecco's Phosphate Buffered Saline (PBS)	JRH Biosciences Pty. Ltd., VIC, Australia
Endofree plasmid mega/giga kit	Qiagen Company, Netherlands
Ethanol	BDH (Merck) Pty. Ltd., VIC, Australia
Ethidium Bromide	Sigma-Aldrich Pty. Ltd., NSW, Australia
Fetal calf serum (FCS)	Life Technologies Pty. Ltd., VIC, Australia
Ficoll 400	Sigma-Aldrich Pty. Ltd., NSW, Australia
Neutral buffered formalin	ACE Chemical Company, Australia
Genopure plasmid midi kit	Roche Diagnostics, Mannheim, Germany
Gentamycin	Life Technologies Pty. Ltd., VIC, Australia
Glutamine	Sigma-Aldrich Pty. Ltd., NSW, Australia
Glutaraldehyde	Sigma-Aldrich Pty. Ltd., NSW, Australia
Glycine	Ajax Finechem Pty. Ltd., Seven Hills, NSW

HEPES	Sigma-Aldrich Pty. Ltd., NSW, Australia
HIV-1 p24 ELISA kit	XpressBio, Australia
Hi-Yield Gel/PCR DNA extraction kit	RBC Biosciences Corp., Taiwan
Immunocal	Decal Corporation, NY, USA
Insulin syringe (0.5mL, 0.33mm, 29G)	Becton Dickinson Pty. Ltd., North Ryde, NSW
Isoflurane	Abbott Australasia Pty. Ltd.
Isopropanol	Ajax Finechem Pty. Ltd., Seven Hills, NSW
ITS+ premix stock	BD Biosciences, San Jose, CA
KpnI	New England Biolabs Inc., USA
Laminin	Sigma-Aldrich Pty. Ltd., NSW, Australia
Lithium Chloride (LiCl)	Sigma-Aldrich Pty. Ltd., NSW, Australia
Magnesium Chloride ($MgCl_{2)}$	Roche Diagnostics, Mannheim, Germany
Mannose-6-phosphate	Sigma-Aldrich Pty. Ltd., NSW, Australia
Metabolic Cages	Hatteras Instruments Inc., Cary, North Carolina
Methyl Green 0.5% w/v	ProSciTech, Thuringowa, QLD
Milk powder	Fonterra Brands Pty. Ltd., VIC, Australia
Milli-Q	
N-butanol	Chem Supply, Gillman, SA
NdeI	New England Biolabs Inc., USA
NEB4 buffer	New England Biolabs Inc., USA
OCT	Sakura Finetek USA Inc., USA
Opti-Pro SFM	Life Technologies Pty. Ltd., VIC, Australia
Osmium tetroxide	
Paraformaldehyde	MP Biomedicals, Ohio, USA
Penicillin/Streptomycin	Sigma-Aldrich Pty. Ltd., NSW, Australia

Phosphate buffered saline (tissue culture)	Life Technologies Pty. Ltd., VIC, Australia
PCR Lysis Reagent	Viagen Biotech Inc., Los Angeles, CA
PCR reaction buffer	Roche Diagnostics, Mannheim, Germany
Phenol	Invitrogen Australia Pty. Ltd., VIC, Australia
Phenol/Chloroform/TN	Sigma-Aldrich Pty. Ltd., NSW, Australia
Polybrene	Sigma-Aldrich Pty. Ltd., NSW, Australia
Poly-L-ornithine	Sigma-Aldrich Pty. Ltd., NSW, Australia
Primers and Oligos	GeneWorks, SA, Australia
Procure 812 (epon-S12 substitute)	ProSciTech, QLD, Australia
ProLong Gold antifade reagent with DAPI	Invitrogen Australia Pty. Ltd., VIC, Australia
Propylene oxide	Sigma-Aldrich Pty. Ltd., NSW, Australia
Proteinase K	Roche Diagnostics, Mannheim, Germany
pUC19/HpaII DNA markers	Geneworks, SA, Australia
QuantiTect reverse transcription kit	Qiagen Company, Netherlands
Rabbit-anti GFAP primary antibody	DAKO, Denmark
SDS	Ajax Chemicals, Sydney, NSW
Sodium Acetate	BDH (Merck) Pty. Ltd., VIC, Australia
Sodium Carbonate (Na_2CO_3)	Ajax Finechem Pty. Ltd., Seven Hills, NSW
Sodium Chloride (NaCl)	Ajax Finechem Pty. Ltd., Seven Hills, NS
Sodium Citrate (Na_3citrate)	Ajax Finechem Pty. Ltd., Seven Hills, NSW
Sodium Ethylenediaminetetraacetic acid (Na_2EDTA)	BDH (Merck) Pty. Ltd., VIC, Australia
Sodium Hydroxide (NaOH)	Ajax Finechem Pty. Ltd., Seven Hills, NSW
Sodium Nitroprusside (Nitropress; 50mg/2mL)	Hospira, Inc., Lake Forest, IL, USA
Sodium Pyruvate	Sigma-Aldrich Pty. Ltd., NSW, Australia

SPPI molecular weight marker	Geneworks, SA, Australia
Sucrose	Ajax Chemicals, Sydney, NSW
Sulphuric acid	Ajax Chemicals, Sydney, NSW
StemSpan	Stem Cell Technologies, Canada
SYBRGreen PCR master mix	Applied Biosystems, California, USA
T4 DNA ligase	Roche Diagnostics, Mannheim, Germany
T4 ligase buffer	Roche Diagnostics, Mannheim, Germany
Taq DNA polymerase	Roche Diagnostics, Mannheim, Germany
Tetraborate	Sigma-Aldrich Pty. Ltd., NSW, Australia
TGFβ3	Prospec-TanyTechnoGene Ltd., USA
Tris-acetate	Roche Diagnostics, Mannheim, Germany
Triton X-100	Sigma-Aldrich Pty. Ltd., NSW, Australia
Trizol Reagent	Invitrogen Australia Pty. Ltd., VIC, Australia
Trypan blue	Invitrogen Australia Pty. Ltd., VIC, Australia
Trypsin	Sigma-Aldrich Pty. Ltd., NSW, Australia
Wizard SV genomic DNA purification system	Promega Corporation, Madison, USA
Xylene	Ajax Finechem Pty. Ltd., Seven Hills, NSW

All other reagents used in this study were of analytical reagent grade and were obtained from:

Ajax Finechem Pty. Ltd., Seven Hills, NSW

BDH (Merck) Pty. Ltd., VIC, Australia

Sigma-Aldrich Pty. Ltd., NSW, Australia

Appendix Two: Publications

Publications arising from this work:

M.R. Jackson, A.L.K. Derrick Roberts, E.M. Martin, N.B. Rout-Pitt, S. Gronthos and S. Byers, 2015, 'Mucopolysaccharidosis enzyme production by bone marrow and dental pulp derived human Mesenchymal stem cells', *Molecular Genetics and Metabolism*, vol. 114, pp. 584-593.

Publications arising from collaborative work:

A.L.K. Derrick Roberts, C.E. Pyragius, X.M. Kaidonis, **M.R. Jackson**, D.S. Anson and S. Byers, 2014, 'Lentiviral-mediated gene therapy results in sustained expression of β-glucuronidase for up to 12 months in $Gus^{mps/mps}$ and up to 18 months in the $Gus^{tm(L175F)Sly}$ mouse models of MPS VII', *Human Gene Therapy*, vol. 25, is. 9, pp. 798-810.

Abstracts arising from this work:

Jackson MR, Derrick Roberts ALK, Gronthos S, Byers S., 'α-L-iduronidase transduced mesenchymal stem cells improve the behavioural deficits in Mucopolysaccharidosis I mice' Proceedings for the WORLD Symposium 2015, Orlando, Florida. *oral presentation*

Jackson MR, Derrick Roberts ALK, Gronthos S, Byers S., 'α-L-iduronidase transduced mesenchymal stem cells improve the behavioural deficits in Mucopolysaccharidosis I mice' Proceedings for the Australasian Gene and Cell Therapy Society 2015, Melbourne, Australia. *oral presentation*

Jackson MR, Derrick Roberts ALK, Gronthos S, Byers S., 'Iduronidase transduced MSCs improve the behavioural deficits in MPS I mice' Proceedings for the MBSANZ 2013, Victor Harbour, Australia. *poster presentation*

Jackson MR, Kang SL, Gronthos S, Byers S., 'Engineering mesenchymal stem cells for MPS therapy.' Proceedings for the Australian Society for Medical Research 2011, Adelaide, Australia. *oral presentation*

Jackson MR, Kang SL, Gronthos S, Byers S., 'The potential benefits of engineering mesenchymal stem cells for MPS therapy' Proceedings for the 7th International Proteoglycan conference 2011, Sydney, Australia. *poster presentation*

Jackson MR, Kang SL, Gronthos S, Byers S., 'The potential benefits of engineering mesenchymal stem cells for MPS therapy' Proceedings for the Australasian Society for Stem Cell Research 2011, Blue Mountains, Australia. *poster presentation*

Jackson MR, Kang SL, Gronthos S, Byers S., 'Mesenchymal stem cells: to divide and conquer MPS.' And **Jackson MR**, Derrick Roberts A, Macsai CE, Byers S., 'Skeletal disease in mucopolysaccharidosis type I.' Proceedings for the Australia Health and Medical Research Congress 2010, Melbourne, Australia. *poster presentation*

References

Abdallah, B. M., P. Boissy, et al. (2007). "dlk1/FA1 regulates the function of human bone marrow mesenchymal stem cells by modulating gene expression of pro-inflammatory cytokines and immune response-related factors." J Biol Chem **282**(10): 7339-7351.

Abdel Aziz, M. T., H. M. Atta, et al. (2007). "Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis." Clin Biochem **40**(12): 893-899.

Akiyama, K., C. Chen, et al. (2012). "Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis." Cell Stem Cell **10**(5): 544-555.

Albensi, B. C., P. G. Sullivan, et al. (2000). "Cyclosporin ameliorates traumatic brain-injury-induced alterations of hippocampal synaptic plasticity." Exp Neurol **162**(2): 385-389.

Alessandri, B., A. C. Rice, et al. (2002). "Cyclosporin A improves brain tissue oxygen consumption and learning/memory performance after lateral fluid percussion injury in rats." J Neurotrauma **19**(7): 829-841.

Allers, C., W. D. Sierralta, et al. (2004). "Dynamic of distribution of human bone marrow-derived mesenchymal stem cells after transplantation into adult unconditioned mice." Transplantation **78**(4): 503-508.

Alvarez-Dolado, M. and M. Martinez-Losa (2011). "Cell fusion and tissue regeneration." Adv Exp Med Biol **713**: 161-175.

Alvarez-Dolado, M., R. Pardal, et al. (2003). "Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes." Nature **425**(6961): 968-973.

Amado, L. C., A. P. Saliaris, et al. (2005). "Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction." Proc Natl Acad Sci U S A **102**(32): 11474-11479.

Archer, L. D., K. J. Langford-Smith, et al. (2014). "Mucopolysaccharide diseases: a complex interplay between neuroinflammation, microglial activation and adaptive immunity." J Inherit Metab Dis **37**(1): 1-12.

Arfi, A., M. Richard, et al. (2011). "Neuroinflammatory and oxidative stress phenomena in MPS IIIA mouse model: the positive effect of long-term aspirin treatment." Mol Genet Metab **103**(1): 18-25.

Arthur, A., G. Rychkov, et al. (2008). "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues." Stem Cells **26**(7): 1787-1795.

Arthur, A., S. Shi, et al. (2009). "Implanted adult human dental pulp stem cells induce endogenous axon guidance." Stem Cells **27**(9): 2229-2237.

Augello, A., R. Tasso, et al. (2007). "Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis." Arthritis Rheum **56**(4): 1175-1186.

Bach, G., R. Friedman, et al. (1972). "The defect in the Hurler and Scheie syndromes: deficiency of -L-iduronidase." Proc Natl Acad Sci U S A **69**(8): 2048-2051.

Baddoo, M., K. Hill, et al. (2003). "Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection." J Cell Biochem **89**(6): 1235-1249.

Bae, J. S., S. Furuya, et al. (2005). "Neuroglial activation in Niemann-Pick Type C mice is suppressed by intracerebral transplantation of bone marrow-derived mesenchymal stem cells." Neurosci Lett **381**(3): 234-236.

Bae, J. S., H. S. Han, et al. (2007). "Bone marrow-derived mesenchymal stem cells promote neuronal networks with functional synaptic transmission after transplantation into mice with neurodegeneration." Stem Cells **25**(5): 1307-1316.

Baldo, G., F. Q. Mayer, et al. (2012). "Evidence of a progressive motor dysfunction in Mucopolysaccharidosis type I mice." Behav Brain Res **233**(1): 169-175.

Bang, O. Y., J. S. Lee, et al. (2005). "Autologous mesenchymal stem cell transplantation in stroke patients." Ann Neurol **57**(6): 874-882.

Barbash, I. M., P. Chouraqui, et al. (2003). "Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution." Circulation **108**(7): 863-868.

Barese, C., N. Pech, et al. (2007). "Granulocyte colony-stimulating factor prior to nonmyeloablative irradiation decreases murine host hematopoietic stem cell function and increases engraftment of donor marrow cells." Stem Cells **25**(6): 1578-1585.

Barton, R. W. and E. F. Neufeld (1971). "The Hurler corrective factor. Purification and some properties." J Biol Chem **246**(24): 7773-7779.

Bastedo, L., M. S. Sands, et al. (1994). "Behavioral consequences of bone marrow transplantation in the treatment of murine mucopolysaccharidosis type VII." J Clin Invest **94**(3): 1180-1186.

Bennett, W. M. and D. J. Norman (1986). "Action and toxicity of cyclosporine." Annu Rev Med **37**: 215-224.

Bentzon, J. F., K. Stenderup, et al. (2005). "Tissue distribution and engraftment of human mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene." Biochem Biophys Res Commun **330**(3): 633-640.

Beutler, E. (2006). "Lysosomal storage diseases: natural history and ethical and economic aspects." Mol Genet Metab **88**(3): 208-215.

Bhansali, A., V. Upreti, et al. (2009). "Efficacy of autologous bone marrow-derived stem cell transplantation in patients with type 2 diabetes mellitus." Stem Cells Dev **18**(10): 1407-1416.

Bi, Y., D. Ehirchiou, et al. (2007). "Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche." Nat Med **13**(10): 1219-1227.

Bieback, K., S. Kern, et al. (2004). "Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood." Stem Cells **22**(4): 625-634.

Bielicki, J., C. McIntyre, et al. (2010). "Comparison of ventricular and intravenous lentiviral-mediated gene therapy for murine MPS VII." Mol Genet Metab **101**(4): 370-382.

Biswas, S. and S. M. LeVine (2002). "Substrate-reduction therapy enhances the benefits of bone marrow transplantation in young mice with globoid cell leukodystrophy." Pediatr Res **51**(1): 40-47.

Block, M. L., L. Zecca, et al. (2007). "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms." Nat Rev Neurosci **8**(1): 57-69.

Blumenkrantz, N. and G. Asboe-Hansen (1973). "New method for quantitative determination of uronic acids." Anal Biochem **54**(2): 484-489.

Boelens, J. J. (2006). "Trends in haematopoietic cell transplantation for inborn errors of metabolism." J Inherit Metab Dis **29**(2-3): 413-420.

Bonab, M. M., M. A. Sahraian, et al. (2012). "Autologous mesenchymal stem cell therapy in progressive multiple sclerosis: an open label study." Curr Stem Cell Res Ther **7**(6): 407-414.

Bouxsein, M. L., S. K. Boyd, et al. (2010). "Guidelines for assessment of bone microstructure in rodents using micro-computed tomography." J Bone Miner Res **25**(7): 1468-1486.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-254.

Brooks, D. A. (2002). "Alpha-L-iduronidase and enzyme replacement therapy for mucopolysaccharidosis I." Expert Opin Biol Ther **2**(8): 967-976.

Brooks, D. A., S. Fabrega, et al. (2001). "Glycosidase active site mutations in human alpha-L-iduronidase." Glycobiology **11**(9): 741-750.

Bunge, S., P. R. Clements, et al. (1998). "Genotype-phenotype correlations in mucopolysaccharidosis type I using enzyme kinetics, immunoquantification and in vitro turnover studies." Biochim Biophys Acta **1407**(3): 249-256.

Byun, Y. K., K. H. Kim, et al. (2012). "Effects of immunosuppressants, FK506 and cyclosporin A, on the osteogenic differentiation of rat mesenchymal stem cells." J Periodontal Implant Sci **42**(3): 73-80.

Calkoen, F. G., C. Vervat, et al. (2014). "Mesenchymal Stromal Cell Therapy Is Associated With Increased Adenovirus-Associated but not Cytomegalovirus-Associated Mortality in Children With Severe Acute Graft-Versus-Host Disease." Stem Cells Transl Med.

Cao, F. J. and S. Q. Feng (2009). "Human umbilical cord mesenchymal stem cells and the treatment of spinal cord injury." Chin Med J (Engl) **122**(2): 225-231.

Castro-Malaspina, H., R. E. Gay, et al. (1980). "Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny." Blood **56**(2): 289-301.

Chai, Y., X. Jiang, et al. (2000). "Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis." Development **127**(8): 1671-1679.

Challita, P. M. and D. B. Kohn (1994). "Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo." Proc Natl Acad Sci U S A **91**(7): 2567-2571.

Chamberlain, G., J. Fox, et al. (2007). "Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing." Stem Cells **25**(11): 2739-2749.

Chaturvedi, P., D. M. Gilkes, et al. (2014). "Hypoxia-inducible factor-dependent signaling between triple-negative breast cancer cells and mesenchymal stem cells promotes macrophage recruitment." Proc Natl Acad Sci U S A **111**(20): E2120-2129.

Chen, J., Y. Li, et al. (2001). "Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats." Stroke **32**(4): 1005-1011.

Chen, L. B., X. B. Jiang, et al. (2004). "Differentiation of rat marrow mesenchymal stem cells into pancreatic islet beta-cells." World J Gastroenterol **10**(20): 3016-3020.

Chen, S., Z. Liu, et al. (2006). "Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery." J Invasive Cardiol **18**(11): 552-556.

Chen, S. L., W. W. Fang, et al. (2004). "Improvement of cardiac function after transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction." Chin Med J (Engl) **117**(10): 1443-1448.

Choi, K. S., S. Y. Ahn, et al. (2011). "Characterization and biodistribution of human mesenchymal stem cells transduced with lentiviral-mediated BMP2." Arch Pharm Res **34**(4): 599-606.

Chopp, M. and Y. Li (2002). "Treatment of neural injury with marrow stromal cells." Lancet Neurol **1**(2): 92-100.

Chopp, M., X. H. Zhang, et al. (2000). "Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation." Neuroreport **11**(13): 3001-3005.

Chung, S., X. Ma, et al. (2007). "Effect of neonatal administration of a retroviral vector expressing alpha-L-iduronidase upon lysosomal storage in brain and other organs in mucopolysaccharidosis I mice." Mol Genet Metab **90**(2): 181-192.

Church, H., K. Tylee, et al. (2007). "Biochemical monitoring after haemopoietic stem cell transplant for Hurler syndrome (MPSIH): implications for functional outcome after transplant in metabolic disease." Bone Marrow Transplant **39**(4): 207-210.

Ciron, C., N. Desmaris, et al. (2006). "Gene therapy of the brain in the dog model of Hurler's syndrome." Ann Neurol **60**(2): 204-213.

Clarke, L. A. and J. Heppner (2002). Mucopolysaccharidosis Type I. GeneReviews [Internet]. R. A. Pagon, M. P. Adam, H. H. Ardinger and e. al. Seattle, WA, University of Washington, Seattle.

Clarke, L. A., C. S. Russell, et al. (1997). "Murine mucopolysaccharidosis type I: targeted disruption of the murine alpha-L-iduronidase gene." Hum Mol Genet **6**(4): 503-511.

Clarke, L. A., J. E. Wraith, et al. (2009). "Long-term efficacy and safety of laronidase in the treatment of mucopolysaccharidosis I." Pediatrics **123**(1): 229-240.

Clausing, P., H. K. Mothes, et al. (1997). "Differential effects of communal rearing and preweaning handling on open-field behavior and hot-plate latencies in mice." Behav Brain Res **82**(2): 179-184.

Constantopoulos, G. and A. S. Dekaban (1978). "Neurochemistry of the mucopolysaccharidoses: brain lipids and lysosomal enzymes in patients with four types of mucopolysaccharidosis and in normal controls." J Neurochem **30**(5): 965-973.

Constantopoulos, G., K. Iqbal, et al. (1980). "Mucopolysaccharidosis types IH, IS, II, and IIIA: glycosaminoglycans and lipids of isolated brain cells and other fractions from autopsied tissues." J Neurochem **34**(6): 1399-1411.

Constantopoulos, G., R. D. McComb, et al. (1976). "Neurochemistry of the mucopolysaccharidoses: brain glycosaminoglycans in normals and four types of mucopolysaccharidoses." J Neurochem **26**(5): 901-908.

Crawley, A. C., B. L. Gliddon, et al. (2006). "Characterization of a C57BL/6 congenic mouse strain of mucopolysaccharidosis type IIIA." Brain Res **1104**(1): 1-17.

Crisan, M., S. Yap, et al. (2008). "A perivascular origin for mesenchymal stem cells in multiple human organs." Cell Stem Cell **3**(3): 301-313.

da Silva, F. H., V. G. Pereira, et al. (2012). "Treatment of adult MPSI mouse brains with IDUA-expressing mesenchymal stem cells decreases GAG deposition and improves exploratory behavior." Genet Vaccines Ther **10**(1): 2.

da Silva Meirelles, L., P. C. Chagastelles, et al. (2006). "Mesenchymal stem cells reside in virtually all post-natal organs and tissues." J Cell Sci **119**(Pt 11): 2204-2213.

Dai, G., X. Liu, et al. (2013). "Transplantation of autologous bone marrow mesenchymal stem cells in the treatment of complete and chronic cervical spinal cord injury." Brain Res **1533**: 73-79.

Dantzer, R., E. Satinoff, et al. (1987). "Cyclosporine and alpha-interferon do not attenuate morphine withdrawal in rats but do impair thermoregulation." Physiol Behav **39**(5): 593-598.

De Miguel, M. P., S. Fuentes-Julian, et al. (2012). "Immunosuppressive properties of mesenchymal stem cells: advances and applications." Curr Mol Med **12**(5): 574-591.

Dekaban, A. S. and G. Constantopoulos (1977). "Mucopolysaccharidosis type I, II, IIIA and V. Pathological and biochemical abnormalities in the neural and mesenchymal elements of the brain." Acta Neuropathol **39**(1): 1-7.

Derrick-Roberts, A., C. E. Pyragius, et al. (2014). "Lentiviral-mediated gene therapy results in sustained expression of beta-glucuronidase for up to 12 months in the Gusmps/mps and up to 18 months in the Gustm(L175F)Sly mouse models of MPS VII." Hum Gene Ther.

Devine, S. M., C. Cobbs, et al. (2003). "Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates." Blood **101**(8): 2999-3001.

Di Ferrante, N., P. V. Donnelly, et al. (1971). "Metabolism of glycosaminoglycans in cultured normal and abnormal human fibroblasts." Biochem Med **5**(3): 269-278.

Di Natale, P., C. Di Domenico, et al. (2002). "In vitro gene therapy of mucopolysaccharidosis type I by lentiviral vectors." Eur J Biochem **269**(11): 2764-2771.

Dierenfeld, A. D., M. F. McEntee, et al. (2010). "Replacing the enzyme alpha-L-iduronidase at birth ameliorates symptoms in the brain and periphery of dogs with mucopolysaccharidosis type I." Sci Transl Med **2**(60): 60ra89.

Digirolamo, C. M., D. Stokes, et al. (1999). "Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate." Br J Haematol **107**(2): 275-281.

DiRosario, J., E. Divers, et al. (2009). "Innate and adaptive immune activation in the brain of MPS IIIB mouse model." J Neurosci Res **87**(4): 978-990.

Ditschkowski, M., H. Einsele, et al. (2003). "Improvement of inflammatory bowel disease after allogeneic stem-cell transplantation." Transplantation **75**(10): 1745-1747.

Dominici, M., K. Le Blanc, et al. (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.

Donega, V., C. H. Nijboer, et al. (2014). "Intranasally administered mesenchymal stem cells promote a regenerative niche for repair of neonatal ischemic brain injury." Exp Neurol **261C**: 53-64.

Dunham, N. W. and T. S. Miya (1957). "A note on a simple apparatus for detecting neurological deficit in rats and mice." J Am Pharm Assoc Am Pharm Assoc (Baltim) **46**(3): 208-209.

Ellis, J. (2005). "Silencing and variegation of gammaretrovirus and lentivirus vectors." Hum Gene Ther **16**(11): 1241-1246.

Epstein, S. (1996). "Post-transplantation bone disease: the role of immunosuppressive agents and the skeleton." J Bone Miner Res **11**(1): 1-7.

Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." Nature **292**(5819): 154-156.

Fang, B., M. Shi, et al. (2004). "Systemic infusion of FLK1(+) mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice." Transplantation **78**(1): 83-88.

Fazekasova, H., R. Lechler, et al. (2010). "Placenta-derived MSCs are partially immunogenic and less immunomodulatory than bone marrow-derived MSCs." J Tissue Eng Regen Med.

Fehrer, C., G. Laschober, et al. (2006). "Aging of murine mesenchymal stem cells." Ann N Y Acad Sci **1067**: 235-242.

Field, R. E., J. A. Buchanan, et al. (1994). "Bone-marrow transplantation in Hurler's syndrome. Effect on skeletal development." J Bone Joint Surg Br **76**(6): 975-981.

Fischer, U. M., M. T. Harting, et al. (2009). "Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect." Stem Cells Dev **18**(5): 683-692.

Fox, J. M., G. Chamberlain, et al. (2007). "Recent advances into the understanding of mesenchymal stem cell trafficking." Br J Haematol **137**(6): 491-502.

Francois, S., M. Bensidhoum, et al. (2006). "Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage." Stem Cells **24**(4): 1020-1029.

Francois, S., B. Usunier, et al. (2014). "Long-Term Quantitative Biodistribution and Side Effects of Human Mesenchymal Stem Cells (hMSCs) Engraftment in NOD/SCID Mice following Irradiation." Stem Cells Int **2014**: 939275.

Fratantoni, J. C., C. W. Hall, et al. (1968). "The defect in Hurler's and Hunter's syndromes: faulty degradation of mucopolysaccharide." Proc Natl Acad Sci U S A **60**(2): 699-706.

Fratantoni, J. C., C. W. Hall, et al. (1968). "Hurler and Hunter syndromes: mutual correction of the defect in cultured fibroblasts." Science **162**(853): 570-572.

Frecha, C., C. Costa, et al. (2012). "A novel lentiviral vector targets gene transfer into human hematopoietic stem cells in marrow from patients with bone marrow failure syndrome and in vivo in humanized mice." Blood **119**(5): 1139-1150.

Friedenstein, A. J., R. K. Chailakhyan, et al. (1974). "Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo." Transplantation **17**(4): 331-340.

Frisella, W. A., L. H. O'Connor, et al. (2001). "Intracranial injection of recombinant adeno-associated virus improves cognitive function in a murine model of mucopolysaccharidosis type VII." Mol Ther **3**(3): 351-358.

Frohbergh, M., Y. Ge, et al. (2014). "Dose Responsive Effects of Subcutaneous Pentosan Polysulfate Injection in Mucopolysaccharidosis Type VI Rats and Comparison to Oral Treatment." PLoS One **9**(6): e100882.

Fujimoto, S. T., L. Longhi, et al. (2004). "Motor and cognitive function evaluation following experimental traumatic brain injury." Neurosci Biobehav Rev **28**(4): 365-378.

Gao, J., J. E. Dennis, et al. (2001). "The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion." Cells Tissues Organs **169**(1): 12-20.

Garbuzova-Davis, S., M. K. Louis, et al. (2011). "Blood-brain barrier impairment in an animal model of MPS III B." PLoS One **6**(3): e16601.

Garbuzova-Davis, S., S. Mirtyl, et al. (2013). "Blood-brain barrier impairment in MPS III patients." BMC Neurol **13**: 174.

Garcia-Olmo, D., M. Garcia-Arranz, et al. (2005). "A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation." Dis Colon Rectum **48**(7): 1416-1423.

Garcia-Olmo, D., D. Herreros, et al. (2009). "Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial." Dis Colon Rectum **52**(1): 79-86.

Garcia-Rivera, M. F., L. E. Colvin-Wanshura, et al. (2007). "Characterization of an immunodeficient mouse model of mucopolysaccharidosis type I suitable for preclinical testing of human stem cell and gene therapy." Brain Res Bull **74**(6): 429-438.

Ghaedi, M., M. Soleimani, et al. (2011). "Mesenchymal stem cells as vehicles for targeted delivery of antiangiogenic protein to solid tumors." J Gene Med.

Gijtenbeek, J. M., M. J. van den Bent, et al. (1999). "Cyclosporine neurotoxicity: a review." J Neurol **246**(5): 339-346.

Glatt, V., E. Canalis, et al. (2007). "Age-related changes in trabecular architecture differ in female and male C57BL/6J mice." J Bone Miner Res **22**(8): 1197-1207.

Gliddon, B. L. and J. J. Hopwood (2004). "Enzyme-replacement therapy from birth delays the development of behavior and learning problems in mucopolysaccharidosis type IIIA mice." Pediatr Res **56**(1): 65-72.

Goebel, W. S., N. K. Pech, et al. (2004). "Stable long-term gene correction with low-dose radiation conditioning in murine X-linked chronic granulomatous disease." Blood Cells Mol Dis **33**(3): 365-371.

Goebel, W. S., M. C. Yoder, et al. (2002). "Donor chimerism and stem cell function in a murine congenic transplantation model after low-dose radiation conditioning: effects of a retroviral-mediated gene transfer protocol and implications for gene therapy." Exp Hematol **30**(11): 1324-1332.

Gonzalez, M. A., E. Gonzalez-Rey, et al. (2009). "Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses." Gastroenterology **136**(3): 978-989.

Goring, S. M., A. R. Levy, et al. (2014). "A network meta-analysis of the efficacy of belatacept, cyclosporine and tacrolimus for immunosuppression therapy in adult renal transplant recipients." Curr Med Res Opin.

Gronthos, S., J. Brahim, et al. (2002). "Stem cell properties of human dental pulp stem cells." J Dent Res **81**(8): 531-535.

Gronthos, S., S. E. Graves, et al. (1994). "The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors." Blood **84**(12): 4164-4173.

Gronthos, S., M. Mankani, et al. (2000). "Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo." Proc Natl Acad Sci U S A **97**(25): 13625-13630.

Gronthos, S., R. McCarty, et al. (2009). "Heat shock protein-90 beta is expressed at the surface of multipotential mesenchymal precursor cells: generation of a novel monoclonal antibody, STRO-4, with specificity for mesenchymal precursor cells from human and ovine tissues." Stem Cells Dev **18**(9): 1253-1262.

Gronthos, S. and A. C. Zannettino (2011). "Methods for the purification and characterization of human adipose-derived stem cells." Methods Mol Biol **702**: 109-120.

Gronthos, S., A. C. Zannettino, et al. (2003). "Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow." J Cell Sci **116**(Pt 9): 1827-1835.

Guillot, P. V., O. Abass, et al. (2008). "Intrauterine transplantation of human fetal mesenchymal stem cells from first-trimester blood repairs bone and reduces fractures in osteogenesis imperfecta mice." Blood **111**(3): 1717-1725.

Hale, S. L., W. Dai, et al. (2008). "Mesenchymal stem cell administration at coronary artery reperfusion in the rat by two delivery routes: a quantitative assessment." Life Sci **83**(13-14): 511-515.

Haley, S. M., M. A. Fragala Pinkham, et al. (2006). "A physical performance measure for individuals with mucopolysaccharidosis type I." Dev Med Child Neurol **48**(7): 576-581.

Han, H., S. K. Chang, et al. (2011). "Intrathecal injection of human umbilical cord blood-derived mesenchymal stem cells for the treatment of basilar artery dissection: a case report." J Med Case Rep **5**: 562.

Hare, J. M., J. H. Traverse, et al. (2009). "A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction." J Am Coll Cardiol **54**(24): 2277-2286.

Haskins, M. E., P. F. Jezyk, et al. (1979). "Alpha-L-iduronidase deficiency in a cat: a model of mucopolysaccharidosis I." Pediatr Res **13**(11): 1294-1297.

Heldermon, C. D., A. K. Hennig, et al. (2007). "Development of sensory, motor and behavioral deficits in the murine model of Sanfilippo syndrome type B." PLoS One **2**(8): e772.

Hemmi, S., P. Peghini, et al. (1989). "Cloning of murine interferon gamma receptor cDNA: expression in human cells mediates high-affinity binding but is not sufficient to confer sensitivity to murine interferon gamma." Proc Natl Acad Sci U S A **86**(24): 9901-9905.

Hemsley, K. M. and J. J. Hopwood (2005). "Development of motor deficits in a murine model of mucopolysaccharidosis type IIIA (MPS-IIIA)." Behav Brain Res **158**(2): 191-199.

Herati, R. S., X. Ma, et al. (2008). "Improved retroviral vector design results in sustained expression after adult gene therapy in mucopolysaccharidosis I mice." J Gene Med **10**(9): 972-982.

Herrmann, R. P. and M. J. Sturm (2014). "Adult human mesenchymal stromal cells and the treatment of graft versus host disease." Stem Cells Cloning **7**: 45-52.

Hobbs, J. R., K. Hugh-Jones, et al. (1981). "Reversal of clinical features of Hurler's disease and biochemical improvement after treatment by bone-marrow transplantation." Lancet **2**(8249): 709-712.

Hofling, A. A., S. Devine, et al. (2004). "Human CD34+ hematopoietic progenitor cell-directed lentiviral-mediated gene therapy in a xenotransplantation model of lysosomal storage disease." Mol Ther **9**(6): 856-865.

Honma, T., O. Honmou, et al. (2006). "Intravenous infusion of immortalized human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat." Exp Neurol **199**(1): 56-66.

Hopwood, J. J. (1979). "alpha-L-iduronidase, beta-D-glucuronidase, and 2-sulfo-L-iduronate 2-sulfatase: preparation and characterization of radioactive substrates from heparin." Carbohydr Res **69**: 203-216.

Hopwood, J. J. and H. Elliott (1983). "Detection of Morquio A syndrome using radiolabelled substrates derived from keratan sulphate for the estimation of galactose 6-sulphate sulphatase." Clin Sci (Lond) **65**(3): 325-331.

Hopwood, J. J., H. Elliott, et al. (1986). "Diagnosis of Maroteaux-Lamy syndrome by the use of radiolabelled oligosaccharides as substrates for the determination of arylsulphatase B activity." Biochem J **234**(3): 507-514.

Hopwood, J. J., V. Muller, et al. (1979). "A fluorometric assay using 4-methylumbelliferyl alpha-L-iduronide for the estimation of alpha-L-iduronidase activity and the detection of Hurler and Scheie syndromes." Clin Chim Acta **92**(2): 257-265.

Horwitz, E. M. (2001). "Marrow mesenchymal cell transplantation for genetic disorders of bone." Cytotherapy **3**(5): 399-401.

Horwitz, E. M., P. L. Gordon, et al. (2002). "Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone." Proc Natl Acad Sci U S A **99**(13): 8932-8937.
Horwitz, E. M., D. J. Prockop, et al. (1999). "Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta." Nat Med **5**(3): 309-313.

Horwitz, E. M., D. J. Prockop, et al. (2001). "Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta." Blood **97**(5): 1227-1231.

Hou, Z. L., Y. Liu, et al. (2013). "Transplantation of umbilical cord and bone marrow-derived mesenchymal stem cells in a patient with relapsing-remitting multiple sclerosis." Cell Adh Migr **7**(5): 404-407.

Itescu, S., M. D. Schuster, et al. (2003). "New directions in strategies using cell therapy for heart disease." J Mol Med **81**(5): 288-296.

Ito, T., A. Suzuki, et al. (2001). "Bone marrow is a reservoir of repopulating mesangial cells during glomerular remodeling." J Am Soc Nephrol **12**(12): 2625-2635.

Jakobkiewicz-Banecka, J., A. Wegrzyn, et al. (2007). "Substrate deprivation therapy: a new hope for patients suffering from neuronopathic forms of inherited lysosomal storage diseases." J Appl Genet **48**(4): 383-388.

Jameson, E., S. Jones, et al. (2013). "Enzyme replacement therapy with laronidase (Aldurazyme) for treating mucopolysaccharidosis type I." Cochrane Database Syst Rev **9**: CD009354.

Jang, M. J., H. S. Kim, et al. (2013). "Placenta-derived mesenchymal stem cells have an immunomodulatory effect that can control acute graft-versus-host disease in mice." Acta Haematol **129**(4): 197-206.

Janssens, S., C. Dubois, et al. (2006). "Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial." Lancet **367**(9505): 113-121.

Jeevanantham, V., M. R. Afzal, et al. (2013). "Clinical trials of cardiac repair with adult bone marrow- derived cells." Methods Mol Biol **1036**: 179-205.

Jessop, H. L., B. S. Noble, et al. (1994). "The differentiation of a potential mesenchymal stem cell population within ovine bone marrow." Biochem Soc Trans **22**(3): 248S.

Jeyakumar, M., T. D. Butters, et al. (1999). "Delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with N-butyldeoxynojirimycin." Proc Natl Acad Sci U S A **96**(11): 6388-6393.

Jiang, M., L. Lv, et al. (2011). "Induction of pluripotent stem cells transplantation therapy for ischemic stroke." Mol Cell Biochem.

Jiang, R., Z. Han, et al. (2011). "Transplantation of placenta-derived mesenchymal stem cells in type 2 diabetes: a pilot study." Front Med **5**(1): 94-100.

Jiang, X., P. C. Cui, et al. (2003). "[In vivo chondrogenesis of induced human marrow mesenchymal stem cells in nude mice]." Di Yi Jun Yi Da Xue Xue Bao **23**(8): 766-769, 773.

Jiang, Y., B. N. Jahagirdar, et al. (2002). "Pluripotency of mesenchymal stem cells derived from adult marrow." Nature **418**(6893): 41-49.

Jiang, Y., B. Vaessen, et al. (2002). "Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain." Exp Hematol **30**(8): 896-904.

Jones, G. N., D. Moschidou, et al. (2012). "Upregulating CXCR4 in human fetal mesenchymal stem cells enhances engraftment and bone mechanics in a mouse model of osteogenesis imperfecta." Stem Cells Transl Med **1**(1): 70-78.

Kadiyala, S., R. G. Young, et al. (1997). "Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro." Cell Transplant **6**(2): 125-134.

Kakkis, E. D., M. F. McEntee, et al. (1996). "Long-term and high-dose trials of enzyme replacement therapy in the canine model of mucopolysaccharidosis I." Biochem Mol Med **58**(2): 156-167.

Kakkis, E. D., J. Muenzer, et al. (2001). "Enzyme-replacement therapy in mucopolysaccharidosis I." N Engl J Med **344**(3): 182-188.

Kakkis, E. D., E. Schuchman, et al. (2001). "Enzyme replacement therapy in feline mucopolysaccharidosis I." Mol Genet Metab **72**(3): 199-208.

Kale, S., A. Karihaloo, et al. (2003). "Bone marrow stem cells contribute to repair of the ischemically injured renal tubule." J Clin Invest **112**(1): 42-49.

Karamouzian, S., S. N. Nematollahi-Mahani, et al. (2012). "Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients." Clin Neurol Neurosurg **114**(7): 935-939.

Karpova, E. A., V. Voznyi Ya, et al. (1996). "A fluorimetric enzyme assay for the diagnosis of Sanfilippo disease type A (MPS IIIA)." J Inherit Metab Dis **19**(3): 278-285.

Kebriaei, P. and S. Robinson (2011). "Treatment of graft-versus-host-disease with mesenchymal stromal cells." Cytotherapy **13**(3): 262-268.

Kedziorek, D. A., M. Solaiyappan, et al. (2013). "Using C-arm x-ray imaging to guide local reporter probe delivery for tracking stem cell engraftment." Theranostics **3**(11): 916-926.

Kemp, K., D. Gordon, et al. (2011). "Fusion between human mesenchymal stem cells and rodent cerebellar Purkinje cells." Neuropathol Appl Neurobiol **37**(2): 166-178.

Kidd, S., E. Spaeth, et al. (2009). "Direct evidence of mesenchymal stem cell tropism for tumor and wounding microenvironments using in vivo bioluminescent imaging." Stem Cells **27**(10): 2614-2623.

Kim, J. H., Y. T. Lee, et al. (2013). "Suppression of in vitro murine T cell proliferation by human adipose tissue-derived mesenchymal stem cells is dependent mainly on cyclooxygenase-2 expression." Anat Cell Biol **46**(4): 262-271.

Kingma, S. D., E. J. Langereis, et al. (2013). "An algorithm to predict phenotypic severity in mucopolysaccharidosis type I in the first month of life." Orphanet J Rare Dis **8**(1): 99.
Kobayashi, H., D. Carbonaro, et al. (2005). "Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector." Mol Ther **11**(5): 776-789.

Koc, O. N., J. Day, et al. (2002). "Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH)." Bone Marrow Transplant **30**(4): 215-222.

Koc, O. N., S. L. Gerson, et al. (2000). "Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy." J Clin Oncol **18**(2): 307-316.

Koc, O. N., C. Peters, et al. (1999). "Bone marrow-derived mesenchymal stem cells remain host-derived despite successful hematopoietic engraftment after allogeneic transplantation in patients with lysosomal and peroxisomal storage diseases." Exp Hematol **27**(11): 1675-1681.

Kornfeld, S. (1992). "Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors." Annu Rev Biochem **61**: 307-330.

Krivit, W., J. H. Sung, et al. (1995). "Microglia: the effector cell for reconstitution of the central nervous system following bone marrow transplantation for lysosomal and peroxisomal storage diseases." Cell Transplant **4**(4): 385-392.

Krossnes, B. K., S. Geisler, et al. (1998). "Intravenous infusion of glucose and the vasodilator sodium nitroprusside in combination with local hyperthermia: effects on tumour pH and tumour response in the BT4An rat tumour model." Int J Hyperthermia **14**(4): 403-416.

Krossnes, B. K., O. Mella, et al. (1996). "Use of the vasodilator sodium nitroprusside during local hyperthermia: effects on tumor temperature and tumor response in a rat tumor model." Int J Radiat Oncol Biol Phys **36**(2): 403-415.

Krossnes, B. K., O. Mella, et al. (1996). "Effect of sodium nitroprusside-induced hypotension on the blood flow in subcutaneous and intramuscular BT4AN tumors and normal tissues in rats." Int J Radiat Oncol Biol Phys **36**(2): 393-401.

Kuznetsov, S. A., M. H. Mankani, et al. (2001). "Circulating skeletal stem cells." J Cell Biol **153**(5): 1133-1140.

La Pushin, R. W. and J. J. Trentin (1977). "Identification of distinctive stromal elements in erythroid and neutrophil granuloid spleen colonies: light and electron microscopic study." Exp Hematol **5**(6): 505-522.

Langereis, E. J., A. Borgo, et al. (2013). "Treatment of hip dysplasia in patients with mucopolysaccharidosis type I after hematopoietic stem cell transplantation: results of an international consensus procedure." Orphanet J Rare Dis **8**: 155.

Langford-Smith, A., K. J. Langford-Smith, et al. (2011). "Female mucopolysaccharidosis IIIA mice exhibit hyperactivity and a reduced sense of danger in the open field test." PLoS One **6**(10): e25717.

Langford-Smith, A., M. Malinowska, et al. (2011). "Hyperactive behaviour in the mouse model of mucopolysaccharidosis IIIB in the open field and home cage environments." Genes Brain Behav **10**(6): 673-682.

Langford-Smith, A., F. L. Wilkinson, et al. (2012). "Hematopoietic stem cell and gene therapy corrects primary neuropathology and behavior in mucopolysaccharidosis IIIA mice." Mol Ther **20**(8): 1610-1621.

Lau, A. A., H. Hannouche, et al. (2010). "Allogeneic stem cell transplantation does not improve neurological deficits in mucopolysaccharidosis type IIIA mice." Exp Neurol **225**(2): 445-454.

Lazarus, H. M., S. E. Haynesworth, et al. (1995). "Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use." Bone Marrow Transplant **16**(4): 557-564.

Le Blanc, K. (2003). "Immunomodulatory effects of fetal and adult mesenchymal stem cells." Cytotherapy **5**(6): 485-489.

Le Blanc, K., F. Frassoni, et al. (2008). "Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study." Lancet **371**(9624): 1579-1586.

Le Blanc, K., C. Gotherstrom, et al. (2005). "Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta." Transplantation **79**(11): 1607-1614.

Le Blanc, K., I. Rasmusson, et al. (2004). "Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells." Lancet **363**(9419): 1439-1441.

Leaback, D. H. and P. G. Walker (1961). "Studies on glucosaminidase. 4. The fluorimetric assay of N-acetyl-beta-glucosaminidase." Biochem J **78**: 151-156.

Lee, J. S., J. M. Hong, et al. (2010). "A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke." Stem Cells **28**(6): 1099-1106.

Lee, J. W., X. Fang, et al. (2009). "Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury in the ex vivo perfused human lung." Proc Natl Acad Sci U S A **106**(38): 16357-16362.

Lee, R. H., A. A. Pulin, et al. (2009). "Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6." Cell Stem Cell **5**(1): 54-63.

Lembo, D., P. Ricciardi-Castagnoli, et al. (1996). "Mouse macrophages carrying both subunits of the human interferon-gamma (IFN-gamma) receptor respond to human IFN-gamma but do not acquire full protection against viral cytopathic effect." J Biol Chem **271**(51): 32659-32666.

Leong, W. K., T. L. Henshall, et al. (2012). "Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms." Stem Cells Transl Med **1**(3): 177-187.

Leuci, V., G. Mesiano, et al. (2011). "Transient proteasome inhibition as a strategy to enhance lentiviral transduction of hematopoietic CD34(+) cells and T lymphocytes: implications for the use of low viral doses and large-size vectors." J Biotechnol **156**(3): 218-226.

Levy, G., F. G. Villamil, et al. (2014). "REFINE: a randomized trial comparing cyclosporine A and tacrolimus on fibrosis after liver transplantation for hepatitis C." Am J Transplant **14**(3): 635-646.

Li, H. H., H. Z. Zhao, et al. (2002). "Attenuated plasticity in neurons and astrocytes in the mouse model of Sanfilippo syndrome type B." J Neurosci Res **69**(1): 30-38.

Li, J., M. B. Ezzelarab, et al. (2012). "Do mesenchymal stem cells function across species barriers? Relevance for xenotransplantation." Xenotransplantation **19**(5): 273-285.

Li, Y., J. Chen, et al. (2001). "Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease." Neurosci Lett **316**(2): 67-70.

Li, Z. H., W. Liao, et al. (2011). "Intravenous transplantation of allogeneic bone marrow mesenchymal stem cells and its directional migration to the necrotic femoral head." Int J Med Sci **8**(1): 74-83.

Lin, G., G. Wang, et al. (2009). "Treatment of type 1 diabetes with adipose tissue-derived stem cells expressing pancreatic duodenal homeobox 1." Stem Cells Dev **18**(10): 1399-1406.

Lin, H., Z. Zhang, et al. (2012). "[Prospective controlled trial of safety of human umbilical cord derived-mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis]." Zhonghua Gan Zang Bing Za Zhi **20**(7): 487-491.

Lin, Y. F., D. R. Lairson, et al. (2011). "Children with acute leukemia: a comparison of outcomes from allogeneic blood stem cell and bone marrow transplantation." Pediatr Blood Cancer **56**(1): 143-151.

Lin, Y. T., Y. Chern, et al. (2011). "Human mesenchymal stem cells prolong survival and ameliorate motor deficit through trophic support in Huntington's disease mouse models." PLoS One **6**(8): e22924.

Liu, Z. J., Y. Zhuge, et al. (2009). "Trafficking and differentiation of mesenchymal stem cells." J Cell Biochem **106**(6): 984-991.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Logue, S. F., R. Paylor, et al. (1997). "Hippocampal lesions cause learning deficits in inbred mice in the Morris water maze and conditioned-fear task." Behav Neurosci **111**(1): 104-113.

Love, Z., F. Wang, et al. (2007). "Imaging of mesenchymal stem cell transplant by bioluminescence and PET." J Nucl Med **48**(12): 2011-2020.
Luke, D. R. (1992). "Immunosuppressive effect of cyclosporine in the hyperlipidemic rat model." Biopharm Drug Dispos **13**(9): 635-645.

Ma, X., Y. Liu, et al. (2007). "Improvements in mucopolysaccharidosis I mice after adult retroviral vector-mediated gene therapy with immunomodulation." Mol Ther **15**(5): 889-902.

Macsai, C. E., A. L. Derrick-Roberts, et al. (2012). "Skeletal response to lentiviral mediated gene therapy in a mouse model of MPS VII." Mol Genet Metab **106**(2): 202-213.

Majhail, N. S. (2010). "Late complications in blood and marrow transplant survivors." Minn Med **93**(10): 45-49.

Majumdar, M. K., M. A. Thiede, et al. (2000). "Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when

differentiated toward stromal and osteogenic lineages." J Hematother Stem Cell Res **9**(6): 841-848.

Martin, D. R., N. R. Cox, et al. (2002). "Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow." Exp Hematol **30**(8): 879-886.

Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." Proc Natl Acad Sci U S A **78**(12): 7634-7638.

Martin, P. K., R. S. Stilhano, et al. (2014). "Mesenchymal stem cells do not prevent antibody responses against human alpha-L-iduronidase when used to treat mucopolysaccharidosis type I." PLoS One **9**(3): e92420.

Martins, A. M., A. P. Dualibi, et al. (2009). "Guidelines for the management of mucopolysaccharidosis type I." J Pediatr **155**(4 Suppl): S32-46.

Masri, M., M. Naiem, et al. (1987). "Age and dose dependence of cyclosporine G nephrotoxicity in BALB/c mice." Transplant Proc **19**(1 Pt 2): 1212-1213.

Masri, M. A. (1995). "Comparative study of toxicity and efficacy of cyclosporine, cyclosporine G (OG37), FK 506, and rapamycin in BALB/c mice with fitted skin grafts." Transplant Proc **27**(1): 366.

Masri, M. A., M. Naiem, et al. (1989). "Immunopathological changes in the spleen of BALB/c mice treated with either cyclosporine A or cyclosporine G." Transplant Proc **21**(1 Pt 1): 952-953.

Masri, M. A., M. Naiem, et al. (1988). "Cyclosporine A versus cyclosporine G: a comparative study of survival, hepatotoxicity, nephrotoxicity, and splenic atrophy in BALB/c mice." Transpl Int **1**(1): 13-18.

Masterson, E. L., P. G. Murphy, et al. (1996). "Hip dysplasia in Hurler's syndrome: orthopaedic management after bone marrow transplantation." J Pediatr Orthop **16**(6): 731-733.

Matalon, R. and A. Dorfman (1972). "Hurler's syndrome, an -L-iduronidase deficiency." Biochem Biophys Res Commun **47**(4): 959-964.

Mazzeo, A. T., G. M. Brophy, et al. (2009). "Safety and tolerability of cyclosporin a in severe traumatic brain injury patients: results from a prospective randomized trial." J Neurotrauma **26**(12): 2195-2206.

Mbye, L. H., I. N. Singh, et al. (2009). "Comparative neuroprotective effects of cyclosporin A and NIM811, a nonimmunosuppressive cyclosporin A analog, following traumatic brain injury." J Cereb Blood Flow Metab **29**(1): 87-97.

McGinley, L., J. McMahon, et al. (2011). "Lentiviral vector mediated modification of mesenchymal stem cells & enhanced survival in an in vitro model of ischaemia." Stem Cell Res Ther **2**(2): 12.

McIntyre, C., S. Byers, et al. (2010). "Correction of mucopolysaccharidosis type IIIA somatic and central nervous system pathology by lentiviral-mediated gene transfer." J Gene Med **12**(9): 717-728.

McKusick, V. A., R. R. Howell, et al. (1972). "Allelism, non-allelism, and genetic compounds among the mucopolysaccharidoses." Lancet **1**(7758): 993-996.

McMahon, J. M., S. Conroy, et al. (2006). "Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors." Stem Cells Dev **15**(1): 87-96.

Mead, B., A. Logan, et al. (2013). "Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury." Invest Ophthalmol Vis Sci **54**(12): 7544-7556.

Meikle, P. J., J. J. Hopwood, et al. (1999). "Prevalence of lysosomal storage disorders." Jama **281**(3): 249-254.

Meyerrose, T. E., M. Roberts, et al. (2008). "Lentiviral-transduced human mesenchymal stem cells persistently express therapeutic levels of enzyme in a xenotransplantation model of human disease." Stem Cells **26**(7): 1713-1722.

Mezey, E., K. J. Chandross, et al. (2000). "Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow." Science **290**(5497): 1779-1782.

Miao, C. H., H. Nakai, et al. (2000). "Nonrandom transduction of recombinant adeno-associated virus vectors in mouse hepatocytes in vivo: cell cycling does not influence hepatocyte transduction." J Virol **74**(8): 3793-3803.

Miech, C., T. Dierks, et al. (1998). "Arylsulfatase from Klebsiella pneumoniae carries a formylglycine generated from a serine." J Biol Chem **273**(9): 4835-4837.

Miller, L. W. (1996). "Cyclosporine-associated neurotoxicity. The need for a better guide for immunosuppressive therapy." Circulation **94**(6): 1209-1211.

Miura, M., S. Gronthos, et al. (2003). "SHED: stem cells from human exfoliated deciduous teeth." Proc Natl Acad Sci U S A **100**(10): 5807-5812.

Mohamadnejad, M., K. Alimoghaddam, et al. (2013). "Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis." Liver Int **33**(10): 1490-1496.

Mohammed, E. E., E. M. Snella, et al. (2012). "Accelerated clinical disease and pathology in mucopolysaccharidosis type IIIB and GalNAc transferase double knockout mice." Mol Genet Metab **107**(1-2): 129-135.

Moncek, F., R. Duncko, et al. (2004). "Effect of environmental enrichment on stress related systems in rats." J Neuroendocrinol **16**(5): 423-431.

Muenzer, J., J. E. Wraith, et al. (2009). "Mucopolysaccharidosis I: management and treatment guidelines." Pediatrics **123**(1): 19-29.

Murray, I. R., C. C. West, et al. (2014). "Natural history of mesenchymal stem cells, from vessel walls to culture vessels." Cell Mol Life Sci **71**(8): 1353-1374.

Murry, C. E., L. J. Field, et al. (2005). "Cell-based cardiac repair: reflections at the 10-year point." Circulation **112**(20): 3174-3183.

Nadri, S. and M. Soleimani (2007). "Isolation murine mesenchymal stem cells by positive selection." In Vitro Cell Dev Biol Anim **43**(8-9): 276-282.

Naldini, L., U. Blomer, et al. (1996). "Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector." Proc Natl Acad Sci U S A **93**(21): 11382-11388.

Naldini, L., U. Blomer, et al. (1996). "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector." Science **272**(5259): 263-267.

Nan, Z., L. Shekels, et al. (2012). "Intracerebroventricular transplantation of human bone marrow-derived multipotent progenitor cells in an immunodeficient mouse model of mucopolysaccharidosis type I (MPS-I)." Cell Transplant **21**(7): 1577-1593.

Neufeld, E. F. and J. Muenzer (2001). The Mucopolysaccharidoses. The Metabolic and Molecular Bases of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle. New York, McGraw-Hill Companies Inc. **3:** 3421-3452.

Niemeyer, P., J. Vohrer, et al. (2008). "Survival of human mesenchymal stromal cells from bone marrow and adipose tissue after xenogenic transplantation in immunocompetent mice." Cytotherapy **10**(8): 784-795.

Noh, H. and J. I. Lee (2014). "Current and potential therapeutic strategies for mucopolysaccharidoses." J Clin Pharm Ther **39**(3): 215-224.

Nosrat, I. V., J. Widenfalk, et al. (2001). "Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury." Dev Biol **238**(1): 120-132.

O'Connor, L. H., L. C. Erway, et al. (1998). "Enzyme replacement therapy for murine mucopolysaccharidosis type VII leads to improvements in behavior and auditory function." J Clin Invest **101**(7): 1394-1400.

Oh, S. H., T. M. Muzzonigro, et al. (2004). "Adult bone marrow-derived cells trans-differentiating into insulin-producing cells for the treatment of type I diabetes." Lab Invest **84**(5): 607-617.

Ohmi, K., D. S. Greenberg, et al. (2003). "Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB." Proc Natl Acad Sci U S A **100**(4): 1902-1907.

Okonkwo, D. O., A. Buki, et al. (1999). "Cyclosporin A limits calcium-induced axonal damage following traumatic brain injury." Neuroreport **10**(2): 353-358.

Okonkwo, D. O. and J. T. Povlishock (1999). "An intrathecal bolus of cyclosporin A before injury preserves mitochondrial integrity and attenuates axonal disruption in traumatic brain injury." J Cereb Blood Flow Metab **19**(4): 443-451.

Olson, J. K. and S. D. Miller (2004). "Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs." J Immunol **173**(6): 3916-3924.

Olson, L. J., F. C. Peterson, et al. (2010). "Structural basis for recognition of phosphodiester-containing lysosomal enzymes by the cation-independent mannose 6-phosphate receptor." Proc Natl Acad Sci U S A **107**(28): 12493-12498.

Omori, Y., O. Honmou, et al. (2008). "Optimization of a therapeutic protocol for intravenous injection of human mesenchymal stem cells after cerebral ischemia in adult rats." Brain Res **1236**: 30-38.

Orozco, L., A. Munar, et al. (2013). "Treatment of knee osteoarthritis with autologous mesenchymal stem cells: a pilot study." Transplantation **95**(12): 1535-1541.

Ou, L., T. Herzog, et al. (2014). "High-dose enzyme replacement therapy in murine Hurler syndrome." Mol Genet Metab **111**(2): 116-122.

Pais, T. F., C. Figueiredo, et al. (2008). "Necrotic neurons enhance microglial neurotoxicity through induction of glutaminase by a MyD88-dependent pathway." J Neuroinflammation **5**: 43.

Palmucci, S., G. Attina, et al. (2013). "Imaging findings of mucopolysaccharidoses: a pictorial review." Insights Imaging **4**(4): 443-459.

Pan, D., A. Sciascia, 2nd, et al. (2008). "Progression of multiple behavioral deficits with various ages of onset in a murine model of Hurler syndrome." Brain Res **1188**: 241-253.

Parr, A. M., C. H. Tator, et al. (2007). "Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury." Bone Marrow Transplant **40**(7): 609-619.

Paylor, R., S. K. Morrison, et al. (1992). "Brief exposure to an enriched environment improves performance on the Morris water task and increases hippocampal cytosolic protein kinase C activity in young rats." Behav Brain Res **52**(1): 49-59.

Pereira, R. F., K. W. Halford, et al. (1995). "Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice." Proc Natl Acad Sci U S A **92**(11): 4857-4861.

Pereira, R. F., M. D. O'Hara, et al. (1998). "Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta." Proc Natl Acad Sci U S A **95**(3): 1142-1147.

Perez-Simon, J. A., O. Lopez-Villar, et al. (2011). "Mesenchymal stem cells expanded in vitro with human serum for the treatment of acute and chronic graft-versus-host disease: results of a phase I/II clinical trial." Haematologica **96**(7): 1072-1076.

Peters, C., M. Balthazor, et al. (1996). "Outcome of unrelated donor bone marrow transplantation in 40 children with Hurler syndrome." Blood **87**(11): 4894-4902.

Peters, C., E. G. Shapiro, et al. (1998). "Hurler syndrome: II. Outcome of HLA-genotypically identical sibling and HLA-haploidentical related donor bone marrow transplantation in fifty-four children. The Storage Disease Collaborative Study Group." Blood **91**(7): 2601-2608.

Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-147.

Polgreen, L. E., J. Tolar, et al. (2008). "Growth and endocrine function in patients with Hurler syndrome after hematopoietic stem cell transplantation." Bone Marrow Transplant **41**(12): 1005-1011.

Polito, V. A., S. Abbondante, et al. (2010). "Correction of CNS defects in the MPSII mouse model via systemic enzyme replacement therapy." Hum Mol Genet **19**(24): 4871-4885.

Ponder, K. P. (2008). "Immune response hinders therapy for lysosomal storage diseases." J Clin Invest **118**(8): 2686-2689.

Prasad, V. K., A. Mendizabal, et al. (2008). "Unrelated donor umbilical cord blood transplantation for inherited metabolic disorders in 159 pediatric patients from a single center: influence of cellular composition of the graft on transplantation outcomes." Blood **112**(7): 2979-2989.

Pritchard, L. M., T. A. Van Kempen, et al. (2013). "Behavioral effects of repeated handling differ in rats reared in social isolation and environmental enrichment." Neurosci Lett **536**: 47-51.

Prockop, D. J. and J. Y. Oh (2012). "Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation." Mol Ther **20**(1): 14-20.

Qian, H., X. Zhang, et al. (2010). "Lentivirus-modified human umbilical cord mesenchymal stem cells maintain their pluripotency." Biotechnol Appl Biochem **55**(1): 53-62.

Ramezani, A., T. S. Hawley, et al. (2003). "Performance- and safety-enhanced lentiviral vectors containing the human interferon-beta scaffold attachment region and the chicken beta-globin insulator." Blood **101**(12): 4717-4724.

Reinecke, H., M. Zhang, et al. (1999). "Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts." Circulation **100**(2): 193-202.

Reiser, J., X. Y. Zhang, et al. (2005). "Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases." Expert Opin Biol Ther **5**(12): 1571-1584.

Reolon, G. K., L. M. Braga, et al. (2006). "Long-term memory for aversive training is impaired in Idua(-/-) mice, a genetic model of mucopolysaccharidosis type I." Brain Res **1076**(1): 225-230.

Richard, M., A. Arfi, et al. (2008). "Identification of new markers for neurodegeneration process in the mouse model of Sly disease as revealed by expression profiling of selected genes." J Neurosci Res **86**(15): 3285-3294.

Riess, P., F. M. Bareyre, et al. (2001). "Effects of chronic, post-injury Cyclosporin A administration on motor and sensorimotor function following severe, experimental traumatic brain injury." Restor Neurol Neurosci **18**(1): 1-8.

Ringe, J., C. Kaps, et al. (2002). "Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages." Cell Tissue Res **307**(3): 321-327.

Roberts, A. L., M. H. Rees, et al. (2007). "Improvement in behaviour after substrate deprivation therapy with rhodamine B in a mouse model of MPS IIIA." Mol Genet Metab **92**(1-2): 115-121.

Roberts, D. L., D. J. Weix, et al. (1998). "Molecular basis of lysosomal enzyme recognition: three-dimensional structure of the cation-dependent mannose 6-phosphate receptor." Cell **93**(4): 639-648.

Robinson, A. J., G. Zhao, et al. (2010). "Embryonic stem cell-derived glial precursors as a vehicle for sulfamidase production in the MPS-IIIA mouse brain." Cell Transplant **19**(8): 985-998.

Rodrigo, S. F., J. van Ramshorst, et al. (2013). "Intramyocardial injection of autologous bone marrow-derived ex vivo expanded mesenchymal stem cells in acute myocardial infarction patients is feasible and safe up to 5 years of follow-up." J Cardiovasc Transl Res **6**(5): 816-825.

Rogers, D. C., E. M. Fisher, et al. (1997). "Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment." Mamm Genome **8**(10): 711-713.

Rogers, D. C., J. Peters, et al. (2001). "SHIRPA, a protocol for behavioral assessment: validation for longitudinal study of neurological dysfunction in mice." Neurosci Lett **306**(1-2): 89-92.

Rovelli, A. M. (2008). "The controversial and changing role of haematopoietic cell transplantation for lysosomal storage disorders: an update." Bone Marrow Transplant **41 Suppl 2**: S87-89.

Russell, C., G. Hendson, et al. (1998). "Murine MPS I: insights into the pathogenesis of Hurler syndrome." Clin Genet **53**(5): 349-361.

Rustad, K. C. and G. C. Gurtner (2012). "Mesenchymal Stem Cells Home to Sites of Injury and Inflammation." Adv Wound Care (New Rochelle) **1**(4): 147-152.

Sadat, M. A., S. Dirscherl, et al. (2009). "Retroviral vector integration in post-transplant hematopoiesis in mice conditioned with either submyeloablative or ablative irradiation." Gene Ther **16**(12): 1452-1464.

Sakaguchi, Y., I. Sekiya, et al. (2005). "Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source." Arthritis Rheum **52**(8): 2521-2529.

Sakai, K., A. Yamamoto, et al. (2012). "Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms." J Clin Invest **122**(1): 80-90.

Sakurai, K., S. Iizuka, et al. (2004). "Brain transplantation of genetically modified bone marrow stromal cells corrects CNS pathology and cognitive function in MPS VII mice." Gene Ther **11**(19): 1475-1481.

Sands, M. S. and B. L. Davidson (2006). "Gene therapy for lysosomal storage diseases." Mol Ther **13**(5): 839-849.

Sasaki, M., R. Abe, et al. (2008). "Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type." J Immunol **180**(4): 2581-2587.

Sato, K., K. Ozaki, et al. (2010). "Mesenchymal stromal cells for graft-versus-host disease : basic aspects and clinical outcomes." J Clin Exp Hematop **50**(2): 79-89.

Scherer, H. U., M. van Pel, et al. (2010). "Mesenchymal stem cells in autoimmune diseases: hype or hope?" Arthritis Res Ther **12**(3): 126.

Schmidt, B., T. Selmer, et al. (1995). "A novel amino acid modification in sulfatases that is defective in multiple sulfatase deficiency." Cell **82**(2): 271-278.

Schmidt, H., K. Ullrich, et al. (1987). "Radiological findings in patients with mucopolysaccharidosis I H/S (Hurler-Scheie syndrome)." Pediatr Radiol **17**(5): 409-414.

Schmitt, U. and C. Hiemke (1998). "Strain differences in open-field and elevated plus-maze behavior of rats without and with pretest handling." Pharmacol Biochem Behav **59**(4): 807-811.

Schrepfer, S., T. Deuse, et al. (2007). "Stem cell transplantation: the lung barrier." Transplant Proc **39**(2): 573-576.

Schuchman, E. H., N. A. Guzman, et al. (1984). "Human alpha-L-iduronidase. I. Purification and properties of the high uptake (higher molecular weight) and the low uptake (processed) forms." J Biol Chem **259**(5): 3132-3140.

Scott, H. S., S. Bunge, et al. (1995). "Molecular genetics of mucopolysaccharidosis type I: diagnostic, clinical, and biological implications." Hum Mutat **6**(4): 288-302.

Scott, H. S., X. H. Guo, et al. (1992). "Structure and sequence of the human alpha-L-iduronidase gene." Genomics **13**(4): 1311-1313.

Scott, H. S., T. Litjens, et al. (1993). "Identification of mutations in the alpha-L-iduronidase gene (IDUA) that cause Hurler and Scheie syndromes." Am J Hum Genet **53**(5): 973-986.

Scuteri, A., M. Miloso, et al. (2011). "Mesenchymal stem cells neuronal differentiation ability: a real perspective for nervous system repair?" Curr Stem Cell Res Ther **6**(2): 82-92.

Semendo, P., Burgos-Silva, M., Donizetti-Oliveira, C. and Camara, N.O.S. (2011). How do Mesenchymal Stem Cells Repair? Stem Cells in Clinic and research. D. A. Gholamrezanezhad. Brazil, InTech**:** 804.

Senju, S., M. Haruta, et al. (2011). "Generation of dendritic cells and macrophages from human induced pluripotent stem cells aiming at cell therapy." Gene Ther.

Seo, B. M., M. Miura, et al. (2004). "Investigation of multipotent postnatal stem cells from human periodontal ligament." Lancet **364**(9429): 149-155.

Shao, X., J. C. Goh, et al. (2006). "Repair of large articular osteochondral defects using hybrid scaffolds and bone marrow-derived mesenchymal stem cells in a rabbit model." Tissue Eng **12**(6): 1539-1551.

Shapiro, E. G., L. A. Lockman, et al. (1995). "Neuropsychological outcomes of several storage diseases with and without bone marrow transplantation." J Inherit Metab Dis **18**(4): 413-429.

Shapiro, L. J., C. W. Hall, et al. (1976). "The relationship of alpha-L-iduronidase and Hurler corrective factor." Arch Biochem Biophys **172**(1): 156-161.

Shi, M., Z. Zhang, et al. (2012). "Human mesenchymal stem cell transfusion is safe and improves liver function in acute-on-chronic liver failure patients." Stem Cells Transl Med **1**(10): 725-731.

Shi, S. and S. Gronthos (2003). "Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp." J Bone Miner Res **18**(4): 696-704.

Shi, S., S. Gronthos, et al. (2002). "Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression." Nat Biotechnol **20**(6): 587-591.

Shull, R. M., R. J. Munger, et al. (1982). "Canine alpha-L-iduronidase deficiency. A model of mucopolysaccharidosis I." Am J Pathol **109**(2): 244-248.

Si, Y. L., Y. L. Zhao, et al. (2011). "MSCs: Biological characteristics, clinical applications and their outstanding concerns." Ageing Res Rev **10**(1): 93-103.

Sifuentes, M., R. Doroshow, et al. (2007). "A follow-up study of MPS I patients treated with laronidase enzyme replacement therapy for 6 years." Mol Genet Metab **90**(2): 171-180.

Silva, H. T., Jr., H. C. Yang, et al. (2014). "Long-term follow-up of a phase III clinical trial comparing tacrolimus extended-release/MMF, tacrolimus/MMF, and cyclosporine/MMF in de novo kidney transplant recipients." Transplantation **97**(6): 636-641.

Singer, N. G. and A. I. Caplan (2011). "Mesenchymal stem cells: mechanisms of inflammation." Annu Rev Pathol **6**: 457-478.

Sivakumur, P. and J. E. Wraith (1999). "Bone marrow transplantation in mucopolysaccharidosis type IIIA: a comparison of an early treated patient with his untreated sibling." J Inherit Metab Dis **22**(7): 849-850.

Song, P., Z. Xie, et al. (2012). "Human genome-specific real-time PCR method for sensitive detection and reproducible quantitation of human cells in mice." Stem Cell Rev **8**(4): 1155-1162.

Sorrentino, N. C., L. D'Orsi, et al. (2013). "A highly secreted sulphamidase engineered to cross the blood-brain barrier corrects brain lesions of mice with mucopolysaccharidoses type IIIA." EMBO Mol Med **5**(5): 675-690.

Sotiropoulou, P. A., S. A. Perez, et al. (2006). "Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells." Stem Cells **24**(2): 462-471.

Souillet, G., N. Guffon, et al. (2003). "Outcome of 27 patients with Hurler's syndrome transplanted from either related or unrelated haematopoietic stem cell sources." Bone Marrow Transplant **31**(12): 1105-1117.

Spellacy, E., R. M. Shull, et al. (1983). "A canine model of human alpha-L-iduronidase deficiency." Proc Natl Acad Sci U S A **80**(19): 6091-6095.

Starkov, A. A., C. Chinopoulos, et al. (2004). "Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury." Cell Calcium **36**(3-4): 257-264.

Stenson, P. D., D. N. Cooper, et al. (2009). "The Human Genome Mutation Database." IDUA Retrieved 24th March 2011, from http://www.hgmd.cf.ac.uk/ac/gene.php?gene=IDUA.

Stevenson, R. E., R. R. Howell, et al. (1976). "The iduronidase-deficient mucopolysaccharidoses: clinical and roentgenorgraphic features." Pediatrics **57**(1): 111-122.

Sullivan, P. G., A. G. Rabchevsky, et al. (2000). "Dose-response curve and optimal dosing regimen of cyclosporin A after traumatic brain injury in rats." Neuroscience **101**(2): 289-295.

Sullivan, P. G., M. Thompson, et al. (2000). "Continuous infusion of cyclosporin A postinjury significantly ameliorates cortical damage following traumatic brain injury." Exp Neurol **161**(2): 631-637.

Tandon, V., J. B. Williamson, et al. (1996). "Spinal problems in mucopolysaccharidosis I (Hurler syndrome)." J Bone Joint Surg Br **78**(6): 938-944.

Tang, D. Q., L. Z. Cao, et al. (2004). "In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow." Diabetes **53**(7): 1721-1732.

Tang, Q. Q., T. C. Otto, et al. (2003). "CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis." Proc Natl Acad Sci U S A **100**(3): 850-855.

Thomas, J. A., S. Jacobs, et al. (2006). "Outcome after three years of laronidase enzyme replacement therapy in a patient with Hurler syndrome." J Inherit Metab Dis **29**(6): 762.

Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." Science **282**(5391): 1145-1147.

Tinker, J. H. and R. F. Cucchiara (1978). "Use of sodium nitroprusside during anesthesia and surgery." Int Anesthesiol Clin **16**(2): 89-112.

Tinker, J. H. and J. D. Michenfelder (1976). "Sodium nitroprusside: pharmacology, toxicology and therapeutics." Anesthesiology **45**(3): 340-354.

Tobin, L. M., M. E. Healy, et al. (2013). "Human mesenchymal stem cells suppress donor CD4(+) T cell proliferation and reduce pathology in a humanized mouse model of acute graft-versus-host disease." Clin Exp Immunol **172**(2): 333-348.

Togel, F., Y. Yang, et al. (2008). "Bioluminescence imaging to monitor the in vivo distribution of administered mesenchymal stem cells in acute kidney injury." Am J Physiol Renal Physiol **295**(1): F315-321.

Tzouvelekis, A., V. Paspaliaris, et al. (2013). "A prospective, non-randomized, no placebo-controlled, phase Ib clinical trial to study the safety of the adipose derived stromal cells-stromal vascular fraction in idiopathic pulmonary fibrosis." J Transl Med **11**: 171.

Uccelli, A., L. Moretta, et al. (2008). "Mesenchymal stem cells in health and disease." Nat Rev Immunol **8**(9): 726-736.

Valayannopoulos, V. and F. A. Wijburg (2011). "Therapy for the mucopolysaccharidoses." Rheumatology (Oxford) **50 Suppl 5**: v49-59.

van Buul, G. M., M. Siebelt, et al. (2014). "Mesenchymal stem cells reduce pain but not degenerative changes in a mono-iodoacetate rat model of osteoarthritis." J Orthop Res.

Van Damme, A., L. Thorrez, et al. (2006). "Efficient lentiviral transduction and improved engraftment of human bone marrow mesenchymal cells." Stem Cells **24**(4): 896-907.

Vangsness, C. T., Jr., J. Farr, 2nd, et al. (2014). "Adult human mesenchymal stem cells delivered via intra-articular injection to the knee following partial medial meniscectomy: a randomized, double-blind, controlled study." J Bone Joint Surg Am **96**(2): 90-98.

Varma, N. R., B. Janic, et al. (2011). "Lentiviral Based Gene Transduction and Promoter Studies in Human Hematopoietic Stem Cells (hHSCs)." J Stem Cells Regen Med **7**(1): 41-53.

Vellodi, A., E. P. Young, et al. (1997). "Bone marrow transplantation for mucopolysaccharidosis type I: experience of two British centres." Arch Dis Child **76**(2): 92-99.

Venturi, N., A. Rovelli, et al. (2002). "Molecular analysis of 30 mucopolysaccharidosis type I patients: evaluation of the mutational spectrum in Italian population and identification of 13 novel mutations." Hum Mutat **20**(3): 231.

Villani, G. R., N. Gargiulo, et al. (2007). "Cytokines, neurotrophins, and oxidative stress in brain disease from mucopolysaccharidosis IIIB." J Neurosci Res **85**(3): 612-622.

Visigalli, I., S. Delai, et al. (2010). "Gene therapy augments the efficacy of hematopoietic cell transplantation and fully corrects mucopolysaccharidosis type I phenotype in the mouse model." Blood **116**(24): 5130-5139.

Wada, N., D. Menicanin, et al. (2009). "Immunomodulatory properties of human periodontal ligament stem cells." J Cell Physiol **219**(3): 667-676.

Wada, N., B. Wang, et al. (2011). "Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts." J Periodontal Res.

Wagner, B. and R. Henschler (2013). "Fate of intravenously injected mesenchymal stem cells and significance for clinical application." Adv Biochem Eng Biotechnol **130**: 19-37.

Wagner, W. and A. D. Ho (2007). "Mesenchymal stem cell preparations--comparing apples and oranges." Stem Cell Rev **3**(4): 239-248.

Walczak, P., J. Zhang, et al. (2008). "Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia." Stroke **39**(5): 1569-1574.

Walkley, S. U. (2004). "Secondary accumulation of gangliosides in lysosomal storage disorders." Semin Cell Dev Biol **15**(4): 433-444.

Walkley, S. U., M. Zervas, et al. (2000). "Gangliosides as modulators of dendritogenesis in normal and storage disease-affected pyramidal neurons." Cereb Cortex **10**(10): 1028-1037.

Wang, F., J. E. Dennis, et al. (2009). "Transcriptional profiling of human mesenchymal stem cells transduced with reporter genes for imaging." Physiol Genomics **37**(1): 23-34.

Wang, L., J. Li, et al. (2013). "Pilot study of umbilical cord-derived mesenchymal stem cell transfusion in patients with primary biliary cirrhosis." J Gastroenterol Hepatol **28 Suppl 1**: 85-92.

Wang, R. Y., E. J. Cambray-Forker, et al. (2009). "Treatment reduces or stabilizes brain imaging abnormalities in patients with MPS I and II." Mol Genet Metab **98**(4): 406-411.

Wang, S., X. Qu, et al. (2012). "Clinical applications of mesenchymal stem cells." J Hematol Oncol **5**: 19.

Weiss, D. J., R. Casaburi, et al. (2013). "A placebo-controlled, randomized trial of mesenchymal stem cells in COPD." Chest **143**(6): 1590-1598.

Wilkinson, F. L., R. J. Holley, et al. (2012). "Neuropathology in mouse models of mucopolysaccharidosis type I, IIIA and IIIB." PLoS One **7**(4): e35787.

Wolf, D. A., A. W. Lenander, et al. (2011). "Direct gene transfer to the CNS prevents emergence of neurologic disease in a murine model of mucopolysaccharidosis type I." Neurobiol Dis **43**(1): 123-133.

Wolfe, J. H. and M. S. Sands (1996). Murine mucopolysaccharidosis type VII: A model system for somatic gene therapy of the central nervous system. Protocols for Gene Transfer in Neuroscience: Towards Gene Therapy of Neurological Disorders. P. Lowenstein and L. Enquist. New York, Wiley.

Wong, K. L., K. B. Lee, et al. (2013). "Injectable cultured bone marrow-derived mesenchymal stem cells in varus knees with cartilage defects undergoing high tibial osteotomy: a prospective, randomized controlled clinical trial with 2 years' follow-up." Arthroscopy **29**(12): 2020-2028.

Wraith, J. E., M. Beck, et al. (2007). "Enzyme replacement therapy in patients who have mucopolysaccharidosis I and are younger than 5 years: results of a multinational study of recombinant human alpha-L-iduronidase (laronidase)." Pediatrics **120**(1): e37-46.

Wu, Y., L. Chen, et al. (2007). "Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis." Stem Cells **25**(10): 2648-2659.

Wynn, R. F., J. E. Wraith, et al. (2009). "Improved metabolic correction in patients with lysosomal storage disease treated with hematopoietic stem cell transplant compared with enzyme replacement therapy." J Pediatr **154**(4): 609-611.

Xia, X., Y. Zhang, et al. (2007). "Transgenes delivered by lentiviral vector are suppressed in human embryonic stem cells in a promoter-dependent manner." Stem Cells Dev **16**(1): 167-176.

Xiong, Y., Q. Gu, et al. (1997). "Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury." J Neurotrauma **14**(1): 23-34.

Xu, S., A. De Becker, et al. (2010). "An improved harvest and in vitro expansion protocol for murine bone marrow-derived mesenchymal stem cells." J Biomed Biotechnol **2010**: 105940.

Yamaza, T., A. Kentaro, et al. (2010). "Immunomodulatory properties of stem cells from human exfoliated deciduous teeth." Stem Cell Res Ther **1**(1): 5.

Yao, S., T. Sukonnik, et al. (2004). "Retrovirus silencing, variegation, extinction, and memory are controlled by a dynamic interplay of multiple epigenetic modifications." Mol Ther **10**(1): 27-36.

Yoshida, S., K. Hayakawa, et al. (2008). "The central nervous system complications of bone marrow transplantation in children." Eur Radiol **18**(10): 2048-2059.

Young, G. F., H. J. Wolfe, et al. (1966). "Mental subnormality in Hunter-Hurler syndrome (gargoylism): a suggested biochemical cause." Dev Med Child Neurol **8**(1): 37-44.

Zannettino, A. C., S. Paton, et al. (2007). "Human mulipotential mesenchymal/stromal stem cells are derived from a discrete subpopulation of STRO-1bright/CD34 /CD45(-)/glycophorin-A-bone marrow cells." Haematologica **92**(12): 1707-1708.

Zhang, L. S., Q. F. Liu, et al. (2009). "[Mesenchymal stem cells for treatment of steroid-resistant chronic graft-versus-host disease]." Zhonghua Nei Ke Za Zhi **48**(7): 542-546.

Zhang, R., Y. Liu, et al. (2013). "Anti-inflammatory and immunomodulatory mechanisms of mesenchymal stem cell transplantation in experimental traumatic brain injury." J Neuroinflammation **10**(1): 106.

Zhang, X. Y., V. F. La Russa, et al. (2002). "Lentiviral vectors for sustained transgene expression in human bone marrow-derived stromal cells." Mol Ther **5**(5 Pt 1): 555-565.
Zhang, Z., H. Lin, et al. (2012). "Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients." J Gastroenterol Hepatol **27 Suppl 2**: 112-120.

Zhong, J. F., Y. Zhan, et al. (2002). "Murine hematopoietic stem cell distribution and proliferation in ablated and nonablated bone marrow transplantation." Blood **100**(10): 3521-3526.

Zuk, P. A., M. Zhu, et al. (2001). "Multilineage cells from human adipose tissue: implications for cell-based therapies." Tissue Eng **7**(2): 211-228.