GENE THERAPY FOR CANINE MUCOPOLYSACCHARIDOSIS TYPE I

by

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This thesis is dedicated to my parents who have provided lifelong encouragement and support for me to pursue my goals.

Abstract

Gene Therapy for Mucopolysaccharidosis type I (MPS I)
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Hematopoietic stem cell (HSC) gene transfer is being developed as a treatment for genetic disease. This thesis evaluated the therapeutic potential of HSC gene therapy in a canine model of MPS I which results from a deficiency of α-L-iduronidase. The first experiment demonstrated the maintenance of 5-10% (87/1220) of hematopoietic progenitors (CFU) and ≤1% of leukocytes provirus positive for 2 years post-infusion in normal recipients. Specific antibodies against culture media components were detected but did not abrogate engraftment with potentially therapeutic levels of provirally marked cells. To evaluate the therapeutic potential of HSC gene therapy, marrow from 5 MPS I dogs was transduced with iduronidase containing recombinant retroviruses. Following adoptive transfer into autologous unconditioned recipients, proviral DNA was detected in 2-10% CFU (51/1288) and ≤1% of leukocytes for 1-3 years post-infusion. However, proviral iduronidase expression was not detected in any dog and all dogs had continued disease progression. Humoral and cellular immune responses against iduronidase protein and autologous cells expressing iduronidase were identified, which possibly abrogated the therapeutic benefits of gene therapy. To evaluate gene therapy in the absence of confounding anti-transgene immune responses, iduronidase transduced MPS I marrow cells were adoptively transferred into fetal pups. 17 fetal pups were injected with iduronidase transduced MPS I marrow cells. Nine normal and 3 MPS I fetal recipients survived and proviral DNA was detected in 2-12% (76/1790) CFU and ~1% of leukocytes for 1year. Proviral iduronidase activity was detected in 1.5% (6/404) CFU but not mature leukocytes from MPS I pups. Specific humoral

immune responses against iduronidase were not detected in MPS recipients, even after boosting with iduronidase transduced marrow cells, consistent with the induction of tolerance to iduronidase in these pups. All MPS I dogs died at 8-10 months with no evidence for disease amelioration. These results show that iduronidase transduced progenitors can engraft in adult, juvenile and fetal recipients, and contribute to hematopoiesis in normal and MPS I dogs. However, the therapeutic potential of HSC gene transfer in canine MPS I appears to be limited by poor maintenance of proviral iduronidase expression and low levels of genetically corrected circulating leukocytes.

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Table of Contents

Dedication ii

Abstract iii

Acknowledgements v

Table of Contents vi

List of Tables xii

List of Figures xiv

List of Abbreviations xvi

CHAPTER 1: Literature Review 1

1.1 Gene Transfer into Hematopoietic Stem Cells **2**

1.1.1 Hematopoiesis 2

Hematopoietic stem cells 4

Clonality of hematopoiesis 5

Restricted hematopoietic progenitors 5

Assays for HSCs 6

1.1.2 *In Vitro* Characteristics of HSCs 8

Cell surface molecule expression on human HSCs 8

Physiological properties of HSCs 9

Culture of hematopoietic progenitors and HSCs 10

Culturing HSCs 11

HSCs as targets for gene transfer 11

1.1.3 Gene Delivery Systems 12

Retroviral gene transfer systems 12

Retroviral vectors 13

Packaging cell lines 16

Safety of retroviral gene transfer systems 16

Adenoviral gene transfer systems 17

Adeno-associated viral gene transfer systems 17

Physical methods of gene transfer 18

1.1.4 Transgene Expression 18

Viral promoters 19

Cellular promoters 19
Regulatable promoters 20
Vector sequences affecting proviral gene expression 20
1.1.5 HSC Gene Marking....22
Murine studies 22
Large animal studies 22
Gene marking in patients 24
1.1.6 HSC Gene Transfer for Genetic Disease....26
Adenosine deaminase deficiency 26
Chronic granulomatous disease 27
Gaucher disease 29
1.1.7 Strategies to Improve HSC Gene Therapy....29
Inducing HSCs to cycle 31
Cell-virus contact 32
Pseudotyped recombinant retroviruses 33
Gene delivery by lentiviral vectors 34
Positive selection of transduced cells 35
1.1.8 Conclusions....36
1.2 Biochemical, Genetic and Clinical Features of MPS I....37
1.2.1 The Mucopolysaccharidoses....37
Classification of the mucopolysaccharidoses 38
1.2.2 Lysosomal Enzymes....40
Biosynthesis and protein maturation 40
Enzyme targeting to lysosomes 41
1.2.3 Glycosaminoglycans....42
Degradation of heparan and dermatan sulfates 43
1.2.4 Clinical Presentation of MPS I....46
Severe MPS I: Hurler syndrome 46
Intermediate and mild MPS I: Hurler-Scheie and Scheie syndromes 46
1.2.5 Pathology of MPS I....47
Neurologic pathology 47
Skeletal abnormalities 47

Cardiovascular pathology 48
Spleen, lymph nodes and liver pathology 48
1.2.6 The Iduronidase Gene (IDUA) 48
Genetic mutations in MPS I 52
Biochemical diagnosis of MPS I 52
1.2.7 Treatment of MPS I....53
1.2.8 Canine α-L-iduronidase Deficiency....55
Canine α-L-iduronidase gene 56
Genetic mutation in canine MPS I 56
Bone marrow transplantation for canine MPS I 56
Enzyme replacement therapy 57
1.2.9 Conclusion....57
1.3 Rationale....58
1.3.1 Hypotheses....59
1.3.2 Objectives....59
1.4 References....61

CHAPTER 2: Materials and Methods 99
2.1 Veterinary Procedures....100
2.1.1 Animal Husbandry and Pathology....100
2.1.2 Marrow Harvests....100
2.1.3 Infusion of Autologous LTMC Cells....102
2.1.4 *In Utero* Adoptive Transfer and Parturition....102
2.2 Primary Hematopoietic Cell Culture....103
2.2.1 Primary Hematopoietic Cell Processing....103
2.2.2 Long Term Marrow Cultures and Transductions....103
2.2.3 Secretion of Provirally Derived Iduronidase....104
2.2.4 Hematopoietic Progenitor Assays....105
G418 resistance assays 105
2.3 Retroviral Producer Cell Lines....106
2.3.1 Retroviral Producer Cells....106
2.3.2 Replication Competent Retrovirus Testing....108

2.4 Molecular Biology and Genetic Assays....108
2.4.1 α-L-Iduronidase Genotyping....108
2.4.2 *In Vitro* Assays of Gene Transfer....109
2.4.3 Southern Blot Analysis....110
PCR product blots 110
Genomic Southern blots 110
2.4.4 Cytogenetic Analysis....111
2.4.5 *In Situ* PCR Analysis....111
In situ PCR validation....112
2.5 Assays for Proviral Iduronidase Expression....115
2.5.1 Iduronidase Activity Assay....115
2.5.2 Reverse Transcriptase PCR (RT-PCR)....115
2.6 Immunoassays....117
2.6.1 Enzyme Linked Immunosorbent Assay (ELISA)....117
2.6.2 Cell Proliferation Assay....118
2.8 References....119

CHAPTER 3: Adoptive Transfer of Genetically Modified Hematopoietic Stem Cells to Autologous Unconditioned Canine Recipients 121
3.1 Summary....122
3.2 Introduction....123
3.3 Experimental Design....125
3.4 Results....125
3.4.1 Marrow Harvests and LTMCs....125
3.4.2 *In Vitro* Assays of Gene Transfer....128
3.4.3 Autografts....129
3.4.4 Gene Transfer into I*n Vivo* Repopulating Cells....132
Proviral marking of hematopoietic colonies 132
PCR and Southern blot analysis 134
Proviral *in situ* PCR analysis 135
Summary of Engraftment Data 136
3.4.5 Immune Responses Against Graft Antigens....136
3.5 Discussion....138

3.5 References.....143

CHAPTER 4: Genetically Corrected Autologous Stem Cells Engraft, But Host Immune Responses and Low Transgene Expression Limit Their Utility for Canine Iduronidase Deficiency. 149

4.1 Summary.....150

4.2 Introduction.....151

4.3 Results.....153

4.3.1 Mating and Genotype Analysis 153

4.3.2 Marrow Harvest and LTMCs.....156

4.3.3 Exposure to Retroviral Containing Supernatant During LTMC.....157

4.3.4 Gene Transfer and Proviral Expression in LTMC Cells.....157

4.3.5 Autografts.....161

4.3.6 Gene transfer into in-vivo repopulating cells.....162

4.3.7 Proviral Iduronidase Expression *In Vivo*.....167

4.3.8 Immune response assays.....169

4.3.9 Pathology.....173

4.4 Discussion.....175

4.5 References.....181

CHAPTER 5: Adoptive Transfer of Genetically corrected Hematopoietic Progenitors to Fetal MPS I Pups Results in Engraftment but Not Amelioration of Disease 187

5.1 Summary.....188

5.2 Introduction.....189

5.3 Experimental Design.....192

5.4 Results.....192

5.4.1 Marrow Harvests, LTMC and Transductions.....192

5.4.2 Gene Transfer in LTMC Allografts.....193

5.4.3 Adoptive Transfer of LTMC Cells into Fetal Canine Recipients.....194

5.4.4 Engraftment of LTMC Cells in Normal Fetal Recipients.....197

5.4.5 Engraftment of LTMC Cells in Iduronidase Deficient Pups.....203

5.4.6 Proviral Iduronidase Expression.....205

Comparison of proviral marking and expression in CFU-GM 205
5.4.7 Adoptive Transfer of Transduced LTMC Cells to MPS I Recipients.....207
5.4.8 Evaluation of Immune Responses.....209
5.4.9 Pathology.....210
5.5 Discussion.....211
5.6 References.....217
Summary and Future Directions 222

List of Tables

Table 1.1: Summary of commonly used gene delivery systems

Table 1.2: Summary of reported results from gene marking trials

Table 1.3: Gene therapy trials for ADA deficiency

Table 1.4: Strategies for optimisation of gene transfer into HSCs

Table 1.5: Classification of the Mucopolysaccharidoses

Table 2.1: Summary of dogs used in gene transfer studies

Table 2.2: Comparison of the frequency of scored *in situ* PCR samples which vary from the actual percentage of positive cells in the sample

Table 3.1: Summary of gene transfer efficiency in LTMC derived hematopoietic colonies measured by resistance to 2.0mg/mL G418

Table 3.2: Detection of proviral sequences in marrow derived hematopoietic colonies by PCR analysis in months post-infusion

Table 3.3: Detection of proviral sequences by PCR amplification of neo^r in blood (PB) and marrow (BM) leukocytes post-infusion

Table 4.1: Iduronidase genotype analysis on pups resulting from the mating of iduronidase heterozygotes

Table 4.2: Evaluation of gene transfer and proviral gene expression into canine MPS I LTMC adherent layers and individual CFU-GM derived from transduced LTMCs

Table 4.3: Detection of iduronidase activity in individual hematopoietic colonies from normal and MPS I marrow samples

Table 4.4: The percentage of cells positive for proviral neo^r sequences by neo^r *in situ* PCR in peripheral blood and bone marrow leukocytes from M2 and M6 post-infusion

Table 4.5: Detection of proviral specific sequences in marrow derived hematopoietic colonies from MPS I dogs at time points post-infusion

Table 4.6: Iduronidase activity in peripheral blood and bone marrow leukocytes and individual CFU-GM from M2 and M5 post-infusion

Table 4.7: Serum anti-iduronidase IgG titre detected by ELISA in serum samples from MPS I dogs post-infusion

Table 5.1: Summary of donor allograft marrow harvests, LTMCs and transductions

Table 5.2: Gene transfer and proviral iduronidase expression in transduced LTMC cells and LTMC derived CFU-GM

Table 5.3: Adoptive transfer of allogeneic LTMC cells to mid-gestation normal and iduronidase deficient fetal recipients

Table 5.4: Detection of proviral neo^r sequences in hematopoietic colonies of normal neonatal pups by PCR analysis

Table 5.5: Long-term engraftment of provirus positive hematopoietic colonies in pups injected with transduced LTMC cells *in utero*

Table 5.6: Detection of opposite sex donor karyotypes from blood and marrow leukocytes in normal and MPS I fetal recipient pups

Table 5.7: Number (%) of hematopoietic colonies positive for proviral sequences (PCR) and iduronidase activity (ID) at 10, 20 and 30 weeks post-partum

Table 5.8: Proviral iduronidase expression in marrow autografts from *in utero* recipient MPS I dogs at marrow harvest, after transduction with M48ID in LTMC and 1 month post-infusion

Table 5.9: Summary of IgG titres detected by ELISA in serum samples from MPS I dogs before and after infusion of a transduced LTMC autograft

List of Figures

Figure 1.1: Schematic diagram of the hematopoietic system

Figure 1.2: Schematic diagrams of the wildtype Moloney leukemia virus and commonly used retroviral vectors

Figure 1.3: Stepwise degradation of dermatan sulfate

Figure 1.4: Stepwise degradation of heparan sulfate

Figure 1.5: Ideogram of human chromosome 4, at standard ISCN resolution of 400 bands

Figure 1.6: Schematic representation of the human and canine α-iduronidase genes

Figure 2.1: Schematic diagram of LTMC co-culture experiment

Figure 2.2: Illustration of retroviral vectors

Figure 2.3: Schematic diagram of PCR based genotype analysis for iduronidase alleles.

Figure 2.4: Comparison of actual and scored percentages of positive cells by *in situ* PCR in mixing experiments

Figure 3.1: Average cell recoveries from marrow harvest and following 21 days of LTMC for each normal dogs

Figure 3.2: Photomicrograph of a day 21 long-term marrow culture from dog N1

Figure 3.3: Resistance of normal untransduced canine progenitors to G418

Figure 3.4: Neo^r PCR amplification of G418 resistant CFU from transduced LTMCs

Figure 3.5: Summary of LTMC cells infused into normal dogs. Infusions were separated by one week

Figure 3.6: Neo^r PCR amplification of marrow derived CFU from N1 at 12 months post-infusion

Figure 3.7: Detection of neo^r sequences in blood and marrow leukocytes from normal dogs by *in situ* PCR

Figure 3.8: Detection of serum IgG specific for iduronidase, horse serum, fetal bovine serum and bovine serum albumin in N1 by ELISA

Figure 4.1: Iduronidase genotype PCR analysis of M6 and littermates

Figure 4.2: Average mononuclear cell (MNC) and hematopoietic progenitors (CFU) yields from marrow harvests (harvested) and recovered at the end of 21 days of LTMC from 8 marrow harvests from MPS I dogs

Figure 4.3: PCR amplification of endogenous dystrophin and proviral iduronidase sequences from day 21 LTMC cells

Figure 4.4: Iduronidase enzyme activity in untransduced and transduced MPS I LTMCs co-cultured with transduced MPS I LTMCs

Figure 4.5: Summary of LTMC cells infused per kg into MPS I dogs

Figure 4.6: PCR amplification and Southern blot analysis of dystrophin and proviral iduronidase and neo^r sequences from M2

Figure 4.7: Percentage provirus positive progenitors in five MPS I (M2-M6) dogs followed for 1-2 years post-infusion

Figure 4.8: Proviral iduronidase expression evaluated in leukocytes from M2 post-infusion by reverse transcriptase PCR

Figure 4.9: Detection of serum IgG specific for iduronidase, horse serum, fetal bovine serum and bovine serum albumin in dog M2 by ELISA

Figure 4.10: Peripheral blood mononuclear cell proliferative response to autologous LCIDSN transduced stroma in dog M2

Figure 4.11: Histopathologic photomicrographs of tissues from dog M6 at the time of euthanasia

Figure 5.1: Iduronidase genotype PCR analysis on experiment 2 pups

Figure 5.2: Detection of neo^r sequences by PCR amplification of genomic DNA from marrow mononuclear cells from pups at 5 weeks of age

Figure 5.3: Percentage neo^r positive cells by *in situ* PCR on blood and marrow leukocytes in experiment 2 pups

Figure 5.4: Summary of engraftment in dog N7

Figure 5.5: PCR amplification and Southern blot analysis of 10 marrow derived CFU-GM from dog M8 at 20 weeks

Figure 5.6: Expression of proviral iduronidase transcripts detected by RT-PCR analysis on marrow from dogs M7 and M8

List of Abbreviations

4MU	4-methylumbelliferide
4MUαID	4-methylumbelliferyliduronide
β-gal	β-galactosidase
ψ	Murine leukemia virus packaging signal
AAV	Adeno-associated virus
ADA	Adenosine deaminase
AR	Autosomal recessive
BFU	Burst forming unit
BFU-E	Burst forming unit, erythrocyte
BM	Bone marrow
BNX	Beige nude, X-linked immunodeficiency
BSA	Bovine serum albumin
CD	Clustered determinant; cluster of differentiation
CD-MPR	Cation dependent MPR
CD34	Cell marker hematopoietic progenitors and vascular endothelium.
CFU	Colony forming unit
CFU-GEMM	Colony forming unit, granulocyte, erythrocyte, monocyte, megakaryocyte
CFU-GM	Colony forming unit-granulocyte, monocyte
CFU-S	Colony forming unit, spleen
cIDUA	Canine iduronidase gene
cpm	Counts per minute
DHFR	Dihydrofolate reducatse
DNA	Deoxyribonucleic acid
DS	Dermatan sulfate
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
env	Retrovirus envelope
FBS	Fetal bovine serum
Flt-3L	Flt-3 ligand
G6PD	Glucose-6-phosphate dehydrogenase
GAG	Glycosaminoglycan

gag	Group associated antigen
GALV	Gibbon ape leukemia virus
GAVLR	Gibbon ape leukemia virus receptor
HLA	Human leukocyte antigen
HoS	Horse serum
HSC	Pluripotent hematopoietic stem cell
IDUA	Iduronidase gene
iduronidase	α-L-iduronidase
Ig	Immunoglobulin
IL-	Interleukin, eg, IL-6, Interleukin 6
I-MDM	Iscove's modified Dulbecco's media
IRES	Internal ribosomal entry site
Lin^{neg}	Refers to cells which do not express standard lineage markers
LTMC	Long-term marrow culture
LTR	Long terminal repeat
MLV	Murine leukemia virus
MMLV	Moloney murine leukemia virus
MPSV	Murine myeloproliferative sarcoma virus
MPB	Mobilized peripheral blood
MPR	Mannose 6-phosphate receptor
MPS	Mucopolysaccharidosis
MPS I	Mucopolysaccharidosis type I
NCR	Negative control region
N.I.H.	National Institutes of Health
NAc	N-acetyl-
neo^r	Neomycin phosphotransferase
NOD-SCID	Non-obese diabetic, severe combined immunodeficiency
PB	Peripheral blood
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PHA	Phytohemagglutanin
Ph+	Philadelphia chromosome positive CML

pol	Reverse transcriptase polymerase gene
R	Receptor
Ram	Receptor, amphotropic
RCR	Replication competent retrovirus
RT	Reverse transcriptase
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SRC	SCID repopulating cell
SP	Side population
tTA	Tetracycline transactivator
TPO	Thrombopoietin
VSV-G	Vesicular stomatitis virus, G protein

CHAPTER 1

Literature Review

1.1 Gene Transfer into Hematopoietic Stem Cells

Genetically modified human hematopoietic stem cells may offer new treatment options for patients with inherited metabolic disease by producing and delivering gene products *in vivo*. The potential of hematopoietic stem cells as vehicles for gene delivery in human genetic disease was first demonstrated thirty years ago by allogeneic bone marrow transplantation to correct immune deficiencies. The potential of stem cell gene therapy has been illustrated by the reconstitution of multilineage hematopoiesis with vector transduced bone marrow in lethally irradiated mice. In contrast, gene transfer into hematopoietic stem cells from large animals has proved more difficult. Indeed, clinically relevant levels of gene transfer into large animal and human hematopoietic stem cells has not been widely achieved. Improved gene delivery systems and better understanding of the biology of hematopoietic stem cell gene transfer continue to fuel intense research activity. Preliminary results from human stem cell gene marking and therapy trials currently underway are encouraging. This chapter will begin with an outline of the biology of hematopoietic stem cells and commonly used gene delivery systems and follow with a discussion of the results from large animals and human trials of gene transfer. Finally, new strategies for optimised stem cell based gene therapy will be discussed.

1.1.1 Hematopoiesis

Hematopoiesis is a dynamic process through which the cells of the blood forming system are produced. It is maintained by a pool of pluripotent hematopoietic stem cells (HSCs), each with the capacity to contribute to all hematopoietic lineages for long periods of time. HSCs have capacities for self-renewal. The differentiating progeny of HSCs give rise to myeloid and lymphoid lineage restricted progenitors, lineage committed progenitors and mature effector cells with successive generations. As hematopoietic progenitors and stem cells become more differentiated there is decreased self-renewal and increased proliferation. A current model of hematopoiesis is shown in Figure 1.1.

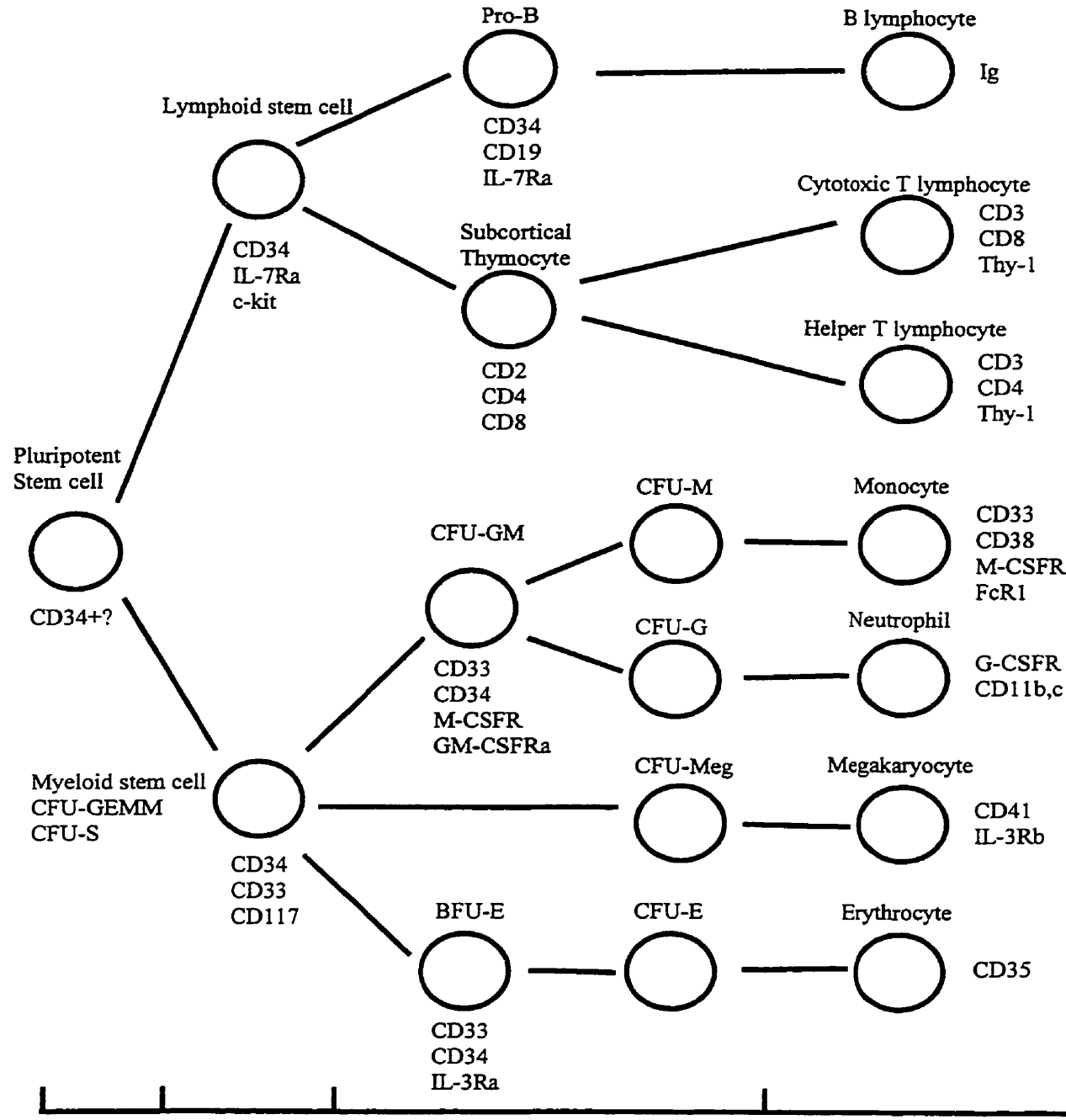

Figure 1.1: A schematic diagram of the hematopoietic system. The expression of some common lineage or differentiation markers are shown for each cell type. These markings are not exclusive nor inclusive of cell type and are typically combined with other markers for the isolation of each cell population. A) Lists the renewal capacity of each cell population. B) Describes the renewal capacity of each type of cell and progenitor. Abbreviation: CD, clustered determinant; Ig, immunoglobulin.

Hematopoietic stem cells

The existence of a cell or cells capable of repopulating multilineage hematopoiesis was hypothesised in the 1950's to account for the hematopoietic rescue of lethally irradiated mice with peripheral blood cells from a normal mouse[1]. However, it was not until 1961 that a hematopoietic progenitor capable of producing cells of multiple hematopoietic lineages was identified and characterised[2]. This hematopoietic progenitor was able to give rise to a discrete spleen colony containing both erythrocytes and granulocytes upon transfer to lethally irradiated or congenitally anaemic mice. The progenitor was named a colony forming unit-spleen (CFU-S)[2,3]. The existence of a common myeloid and lymphoid stem cell was demonstrated by genetic marking studies. The principle of this type of study is that a genetically marked cell will transmit the genetic marker to all of its progeny[3-5]. In a study by Siminovitch and colleagues, unique chromosomal markers were induced in hematopoietic progenitors by exposing mice to radiation[4]. Following transplantation of the marked marrow to recipients, the same chromosomal abnormality was identified in both myeloid and lymphoid tissues. The results of this and other studies indicated that both lineages of hematopoietic cells have a common progenitor, at least in the mouse[4,6,7].

HSCs can also be genetically marked by the random integration of retroviral genomes. Each recombinant replication-incompetent retrovirus gives rise to a single, random integration in a target cell. In a study by Jordan and Lemishka[8], murine marrow exposed to recombinant retroviruses was transplanted into lethally irradiated syngeneic recipients. Individually marked long lived HSCs that gave rise to both myeloid and lymphoid differentiated progeny for at least one year were identified[8]. This study confirmed the existence of pluripotent HSCs that could contribute to long-term multilineage hematopoiesis for the lifespan of a mouse[8].

Pluripotent human HSCs capable of contributing to both lymphopoiesis and myelopoiesis have also been identified. Detection of unique cytogenetic markers or retroviral integration sites in both lymphoid and myeloid cells in atomic bomb survivors or clinical stem cell marking studies has demonstrated the existence of a common lymphoid and myeloid progenitor in humans[9-13]. These studies have also identified restricted stem cells that contribute to either lymphopoiesis or myelopoiesis for months or years *in vivo*[7-10].

Clonality of hematopoiesis

The genetic marking studies have also provided insight into the clonality of hematopoiesis through analysis of the proportion of cells carrying the genetic marker over time. Collectively, these studies have indicated that hematopoiesis is oligoclonal with a small pool of HSCs actively contributing to hematopoiesis at a given time. For example, as few as one HSC can maintain long-term hematopoiesis in mice[7,8]. Abkowitz and colleagues have performed detailed analysis of hematopoiesis in female cats, heterozygous for the X chromosome gene glucose-6-phosphate dehydrogenase (G6PD)[14,15]. Due to random X chromosome inactivation during embryogenesis, these cats are cellular mosaics with cells expressing one of the two electrophoretically distinct G6PD enzymes. Hematopoietic clonality has been evaluated by determining the ratio of hematopoietic progenitors expressing each G6PD allele, and the change in the ratio over time. These studies have demonstrated that long-term hematopoiesis could be maintained by one or a few HSCs, indicating that individual HSCs having vast capacities for self-renewal and proliferation[14,15]. Clonality of human hematopoiesis has been evaluated in human atomic bomb survivors with unique cytogenetic markers. These studies have indicated that a single marked human HSC can produce 2-10% of myeloid and lymphoid cells[9,10]. Collectively, these data suggest that hematopoiesis is oligoclonal with a small pool of HSCs contributing to hematopoiesis. The corollary to this is that the vast majority of HSCs are quiescent and not contributing to hematopoiesis at a given time. This concept of stem cell quiescence is supported by HSC survival following exposure to either cycle specific chemotherapeutics or radioactive thymidine[16,17].

Restricted hematopoietic progenitors

In the 1960's, hematopoietic progenitors that give rise to colonies of hematopoietic cells *in vitro* when plated with hematopoietic growth and differentiation factors, called colony-forming units (CFUs) were identified[18,19]. Colonies of ~1,000-10,000 granulocytes, erythroblasts, megakaryocytes and macrophages arise from multipotent myeloid progenitors[20,21]. *In vitro* characterisation of the multipotent murine and human progenitors called burst-forming units (BFU) or CFU-granulocyte, erythrocyte, macrophage and megakaryocyte (CFU-GEMM) indicated that these progenitors have extensive proliferative and limited self-renewal capacities[21,22]. Injection of murine burst colonies into irradiated

recipients resulted in approximately one CFU-S per burst colony[23]. Replating of multipotent hematopoietic colonies results in the formation of lineage restricted colonies such as CFU-granulocyte-macrophage (CFU-GM) *in vitro* and a limited number of multilineage colonies[22]. Lineage restricted progenitors such as CFU-GM have less capacity for proliferation than their multilineage precursors and produce colonies of ~50-500 single or bi-lineage cells. They have little, if any, self-renewal capacity.

Assays for HSCs

Murine HSCs are assayed by their ability to repopulate multilineage hematopoiesis in lethally irradiated or otherwise myeloablated syngeneic mice[24]. A common assay for murine HSCs is a competitive repopulation assay in which two test populations of hematopoietic cells with a quantifiable allelic variant are transplanted into a lethally irradiated recipient[24]. Comparison of the contribution of each donor population to long-term multilineage hematopoiesis enables an evaluation of stem cell phenotype and function in each population. Limiting dilution competitive repopulation studies demonstrated that murine HSCs are rare, occurring at a frequency of approximately 1 in 10^5 steady-state murine marrow mononuclear cells[25].

In vivo assays for human HSCs have used xenogeneic hosts for ethical reasons. One xenogeneic model system for humans HSCs is the *in utero* sheep transplantation system. Populations of human hematopoietic progenitor cells are adoptively transferred to the peritoneal cavities of mid-gestation pre-immune fetal sheep[26]. Human hematopoietic progenitors and HSCs can engraft, proliferate and to some extent differentiate in the sheep. Neonatal sheep are then monitored for the contribution of human cells to hematopoiesis. Unfortunately, there is high fetal mortality of injected fetuses in this system and only about half the surviving lambs are chimaeric. A fetal transplant model in which adoptive transfer of human hematopoietic progenitors to the yolk sacs of pre-immune fetal dogs results in chimaeric neonatal pups is being developed at this Institution[27]. An advantage to using a canine model system is the larger litter size and therefore larger number of evaluable pups for each experiment. In a preliminary experiment, seven of eight fetal pups infused with human cells survived to term. Six pups were monitored for the engraftment of human hematopoietic cells and all were chimaeric. Further experiments are needed to determine the consistency and levels of chimaerism achievable with this technique[27].

Another xenogeneic host used for assaying human hematopoietic progenitors are immune deficient mice such as non-obese diabetic-severe combine immunodeficient (NOD-SCID) or beige-nude XID (BNX) mice[12,28-30]. The human cells are not rejected in these mice due to multiple immune defects resulting in a lack of functional lymphoid cells, and reduced natural killer activity in NOD-SCID mice[31], and severely compromised T-cell, B-cell, natural killer and lymphokine-activated killer cell responses in BNX mice[32]. Human hematopoietic progenitor cells can proliferate and to some extent differentiate in the immune-deficient murine hematopoietic systems if animals are treated with recombinant human growth factors[12,28] or co-transplanted with stromal cells expressing human growth factors[30]. Dick and colleagues have used NOD-SCID mice to characterise a primitive human hematopoietic cell with multilineage repopulating ability, called a SCID repopulating cell (SRC)[12]. The frequency of human SRC was estimated at 1 in 10^6 marrow cells in these studies[33], approximately one log lower than the 1 in 10^5 observed for murine HSCs in competitive repopulation studies[25]. The differences in these frequencies may reflect biologic differences in the stem cell populations and assays. Alternatively, the proportions of murine and human stem cells may be similar, however the full capacities of human HSCs may not be realised in a murine environment because specific growth factor or other micro-environmental needs may not be met in the mouse.

Of the two xenogeneic assays for human hematopoiesis the mouse model is more widely applicable as sterile environments for maintaining immune-deficient mice exist in most research facilities, while fetal transplants and maintenance of large animals requires specialised technical staff, equipment and facilities. Assay of human progenitors in mice enables the analysis of many samples with multiple replicates and can provide a clear demonstration of the presence of HSCs in the test population. Unfortunately, the *in vivo* murine environment may not reflect the normal physiologic demand on human HSCs in human hosts. Analysis of human hematopoiesis in a chimaeric large animal may more closely reflect the normal physiologic demand on hematopoietic progenitors and HSCs. However, both *in vivo* xenogeneic models of human hematopoiesis have provided insight into the biology of human hematopoietic progenitors and HSCs.

1.1.2 *In Vitro* Characteristics of HSCs

There are a wide variety of surface markers on hematopoietic cells. Some markers such as CD45 are found on most hematopoietic cells, while others are lineage restricted such as CD4 on T lymphocytes and CD33 on myeloid cells (see Figure 1.1). Murine HSCs can be greatly enriched based on their cell surface phenotype. For example, as few as one hundred murine hematopoietic cells with the cell surface marker profile Thy1.1^{lo}lin^{neg}Sca-1^{+} cells[34] or one mCD34$^{neg/lo}$c-kit^{+}Sca-1^{+}lin^{neg} cell[35] can successfully engraft and maintain long-term murine hematopoiesis.

Cell surface molecule expression on human HSCs

Populations of human hematopoietic cells can be enriched for HSCs, however HSC purification has not been achieved. Characterisation of the cell surface phenotype of human HSCs has been achieved through sorting cell populations by one or more markers, and assaying for stem cell activity in xenogeneic hosts as discussed above[12,36]. CD34 is a cell surface molecule expressed on 1-2% of marrow mononuclear cells and most *in vitro* hematopoietic colony forming cells[37]. CD34^{+} cells can reconstitute hematopoiesis in ablated baboons[38], dogs[39], and patients[40]. Infusion of human hematopoietic cells sorted exclusively by CD34 expression in NOD-SCID mice demonstrated that chimaeric hematopoiesis is maintained in the CD34^{+} fraction and not with comparable or two logs higher numbers of CD34^{neg} cells[12]. Similar results were demonstrated in xenogeneic fetal sheep transplants[36,41]. Collectively these experiments indicate that the CD34^{+} sub-population of hematopoietic cells contains most of the long-term repopulating activity.

Further characterisation of the cell surface molecule profile on HSCs based on high or low/negative expression of lineage markers has recently been performed. Data from these studies suggest that HSCs express low levels of Thy-1 and are lineage negative (lin^{neg}) as they do not express lineage or differentiation antigens such as HLA-DR, CD38, CD4, CD8[42-44]. Fractionating lin^{neg} cells by CD34 expression has indicated that there is a population of human HSCs which are CD34$^{neg/lo}$lin^{neg}. For example, transplantation of either the human CD34$^{neg/lo}$lin^{neg} or CD34^{+}lin^{neg} fractions of cells into fetal sheep results in engraftment of human hematopoietic cells in primary and secondary recipients[45]. Significantly higher numbers of human CD34^{+} cells were produced in recipients of the human CD34$^{neg/lo}$ graft. In

secondary recipients, higher levels of chimaerism were observed in recipients of $CD34^{neg/lo}lin^{neg}$ grafts. These data suggest that $CD34^{neg/lo}lin^{neg}$ HSCs may be more primitive than $CD34^{+}$ HSCs[45].

Similar results have also been demonstrated in NOD-SCID mouse studies[46]. There has been some general discussion about whether contamination of the $CD34^{neg/lo}$ cells with $CD34^{+}$ cells could account for the observed engraftment. This is possible, but unlikely because the contamination of $CD34^{+}$ cells in the $CD34^{neg/lo}lin^{neg}$ grafts does not exceed the number of $CD34^{+}$ cells in the $CD34^{+}$ graft in these studies. One interpretation of the CD34 repopulation data is that there are two populations of HSCs capable of contributing to hematopoiesis, those that express CD34 and those that do not. The relationship between these two populations of cells is not clear, although the data suggest that $CD34^{neg/lo}$ HSCs may be more primitive and give rise to $CD34^{+}$ HSCs. Further investigation is needed to characterise the biology of these two populations of HSCs.

Physiological properties of HSCs

HSCs can be characterised by biologic characteristics such as an inability to uptake Rhodamine-123 vital dye[42,43], low expression of proliferation markers such as the transferrin receptor (CD71)[36] or dye efflux[47]. For example, Goodell and colleagues have isolated a population of murine hematopoietic cells which efflux fluorescent DNA-binding Hoechst 33342 dye. These dye effluxing cells have been called side population (SP) cells after their appearance following flow sorting as a small side streak of cells[47]. Similar hematopoietic populations which efflux Hoechst dye have been identified in human, rhesus and miniature swine marrow[48]. Human SP cells do not express lineage specific antigens such as CD3, 4, 5, 8, 33, or 71. SP cells do not express CD34, however they convert to $CD34^{+}$ if cultured *in vitro*[48]. Murine SP cells are highly enriched for long-term repopulating cells. It is possible that human SP cells are also highly enriched for HSCs because they have similar cell surface markers to murine and human HSCs[48]. It is also likely that physiological properties such as Hoechst dye efflux are conserved across species. Evaluation of marrow repopulating capacity of human SP cells is necessary to determine whether this population contains human HSCs.

Culture of hematopoietic progenitors and HSCs

Hematopoietic progenitor assays in the 1960's and 70's utilised a variety of different sources of colony stimulating factors which provided different colony numbers, types and plating efficiencies, likely due to the different growth factors present in each source and batch of colony stimulating factors. The most reliable sources of colony stimulating activity were pokeweed-mitogen stimulated splenocyte conditioned medium for murine[49,50] and phytohemagglutinin stimulated leukocyte conditioned medium for human progenitors[20,51]. The identification and purification of the specific colony stimulating factors has facilitated analysis of hematopoietic progenitor content in test samples. Some factors are lineage restricted, such as erythropoietin (EPO) which exclusively stimulates the formation of erythroid colonies[52-55]. Factors such as IL-3 are necessary (in combination with other factors) for the formation of a variety of colony types such as CFU-GM, BFU-E and CFU-GEMM colonies[56]. Other growth factors such as IL-6 and IL-1 individually have little proliferative effect on progenitor cell growth, but act synergistically with other colony stimulating factors to produce larger colonies[57]. The earliest *in vitro* assayable progenitors can be maintained in culture in the presence of stem cell factor (SCF), Flt-3 ligand (Flt-3L) and/or thrombopoietin (TPO)[58]. These factors alone do not induce significant proliferation of hematopoietic progenitors, however they enhance other colony stimulating and synergistic factors such as G-CSF, IL-3, IL-6, and IL-11[59-63].

Many approaches for maintaining and expanding HSCs *in vitro* have been evaluated. The first success was by Dexter and colleagues who developed a specialised murine long-term marrow culture (LTMC) system[64]. LTMCs for human, non-human primate and canine cells have also been described[65-68]. In the LTMC system, total marrow cells are plated at high density in serum enriched medium and maintained by weekly demi-depopulation and feeding. Over the course of a few weeks a stromal layer with macrophages, adipocytes, and myofibroblastic cells develops. The heterogeneous stromal layer provides the appropriate marrow microenvironment for the survival, self-renewal, proliferation and differentiation of early hematopoietic progenitors and HSCs[64]. The progenitors are found in association with the stromal layer in "hematopoietic islands" or "cobblestone areas" while their mature progeny are released into the culture medium. Hematopoiesis can be maintained in LTMCs

for several months, as evidence by the continued production of lineage restricted progenitors[64,65,69] and CFU-S[70] for months.

The maintenance of HSCs in LTMCs has been evaluated by transplanting patients with autologous LTMC cells following marrow ablation. In two studies, marrow from patients with Philadelphia chromosome positive (Ph^+) myeloid leukemia was cultured in LTMC to purge Ph^+ leukemic cells. The LTMC cells were then infused following high-dose chemotherapy[71,72]. In general, patients had at least short term engraftment with the LTMC cells. However, since the grafts were autologous it is unclear whether the long-term hematopoietic reconstitution was from the LTMC graft or the recovery of endogenous HSCs[71,72]. Retroviral gene marking in canine and human LTMCs have demonstrated that HSCs can be maintained in LTMCs[73-75]. In these studies, infusion of retrovirally transduced LTMC cells results in the maintenance of proviral marking in up to 5% of myeloid progenitors and lymphoid cells for at least 2 years post-infusion[73-75].

Culturing HSCs

It is difficult to maintain HSCs *in vitro* without causing terminal differentiation. Combinations of hematopoietic cytokines that enhance maintenance and proliferation of primitive hematopoietic progenitors and stem cells include SCF, Flt-3L and TPO[76-78]. For example, culturing of $CD34^+Thy\text{-}1^+Lin^{neg}$ cells in the presence of TPO, SCF, and Flt-3L resulted in virtually all cells dividing with an overall 3-fold expansion in the number of SRC[58]. Combining early acting factors SCF, Flt-3L and TPO with colony stimulating and synergistic factors such as G-SCF, IL-3, and/or IL-6 has resulted in $2x10^6$ fold expansion of $CD34^+$ cells over 20 weeks of culture although the repopulating capacity of these expanded cells has not yet been evaluated[79].

HSCs as targets for gene transfer

Allogeneic bone marrow transplantation has been used as a therapeutic strategy for a variety of diseases. In successful marrow transplants, donor derived HSCs engraft and contribute to or maintain long-term hematopoiesis in the recipient, thus providing a self-renewing population which produces large numbers of differentiated hematopoietic cells. The normal differentiated donor cells produce the therapeutic gene product *in vivo*. Limitations to marrow

transplantation include a limited donor pool, transplant-related mortality from myeloablative conditioning regimens, long-term immunosuppression in engrafted recipients and graft-versus host disease. Genetic modification of autologous HSCs to carry and express a therapeutic gene could result in the benefits of marrow transplantation without problems associated with allogeneic transplant. In theory, a relatively small number of genetically modified HSCs could generate large numbers of differentiated hematopoietic cells carrying and expressing an exogenous gene for long periods of time, possibly the lifespan of the recipient. The next section will review some of the most commonly used systems for the delivery of genes to HSCs.

1.1.3 Gene Delivery Systems

Viral and physical methods have been used to transfer genes to HSCs. Viral methods take advantage of the normal viral life cycle in which genetic material is transferred into the host cell. In viral gene transfer vectors, the viral genome is modified by deleting viral genes and inserting therapeutic and/or marker genes in their place. The most commonly used viral backbones are based on the murine leukemia retroviruses. Adenoviral and adeno-associated viral (AAV) systems have also been used as gene transfer vehicles. Non-viral or physical gene transfer methods such as electroporation, direct DNA transfer and liposome mediated transfer have also been used. Each system has features that offer some advantages for hematopoietic cell gene transfer applications. A summary of the main features of viral and physical gene transfer systems used for hematopoietic cells and HSCs is presented in Table 1.1 and will be discussed in this section.

Retroviral gene transfer systems

Retroviruses are single stranded RNA viruses[80]. The viral particle consists of an RNA genome, group associated antigen proteins (*gag*) which form the viral core, and *pol* protein which is a reverse transcript polymerase (RT). The viral particle is coated with a host cell membrane and is studded with viral envelope glycoproteins (*env*). Following interaction of the viral *env* protein with the appropriate host cell surface receptor, the viral particle is internalised, uncoated and the viral core is released into the cytoplasm. The RNA transcript is reverse transcribed into double stranded DNA, which subsequently moves into the nucleus

and integrates into the genome of dividing cells[81-84]. Retroviral vectors derived from the Moloney murine leukemia virus (MMLV) and murine myeloproliferative sarcoma virus (MPSV) have been most commonly used for gene transfer into hematopoietic cells (reviewed in[85]). New retroviral gene transfer systems based on lentiviruses and foamy viruses are currently being developed and will be discussed in Section 1.1.7.

Retroviral vectors

Retroviral gene transfer vectors are engineered from wild type retroviral genomes by deleting the *gag*, *pol* and *env* genes which makes 6-8kb available for the insertion of desired genes (Figure 1.2A)[86-90]. These modifications render the retroviral vectors replication incompetent. The packaging signal (ψ) and long terminal repeats (LTRs), necessary for viral packaging and integration are retained in the retroviral vector[90,91].

Gene transfer vectors generally carry one or two genes. One gene may encode a selectable marker gene, such as neomycin phosphotransferase (neo^r), which facilitates *in vitro* assays of gene transfer and expression. Commonly used basic vector designs are shown in Figure 1.2. Transgenes may utilise the promoter in the retroviral LTR (Figure 1.2B), or another internal promoter such as the CMV or SV40 promoter for expression (Figure 1.2C)[92,93]. In bicistronic vectors one gene may be expressed from the LTR and the second from an internal promoter (Figure 1.2D)[92,93]. Alternatively, both genes may be transcribed as a single transcript from the LTR, with an internal ribosomal entry site (IRES) between the genes to facilitate translation of the second gene (Figure 1.2E)[94].

Table 1.1: Summary of commonly used gene delivery systems.

Delivery System	Advantages	Disadvantages
MMLV based retroviral vectors	• can stably integrate into target cell genome • transferred gene passed on to daughter cells • wide host ranges	• no gene transfer to quiescent cells • risk of replication competent retrovirus production requires extensive safety testing • limited insert size • insertional mutagenesis
Adenoviral vectors	• infects quiescent and dividing cells efficiently • high levels of gene expression	• vectors do not integrate and are lost in cycling cells • strong immune responses with repeat administration
Adeno-associated viral vectors	• efficiently transduce stationary and cycling cells • non-pathogenic virus	• vectors rarely integrate • maximum 4kb insert
Physical methods	• no viral sequences in vector • unlimited size and number of genes can be transferred	• high cell mortality • low gene transfer efficiency • majority of transferred DNA remains episomal

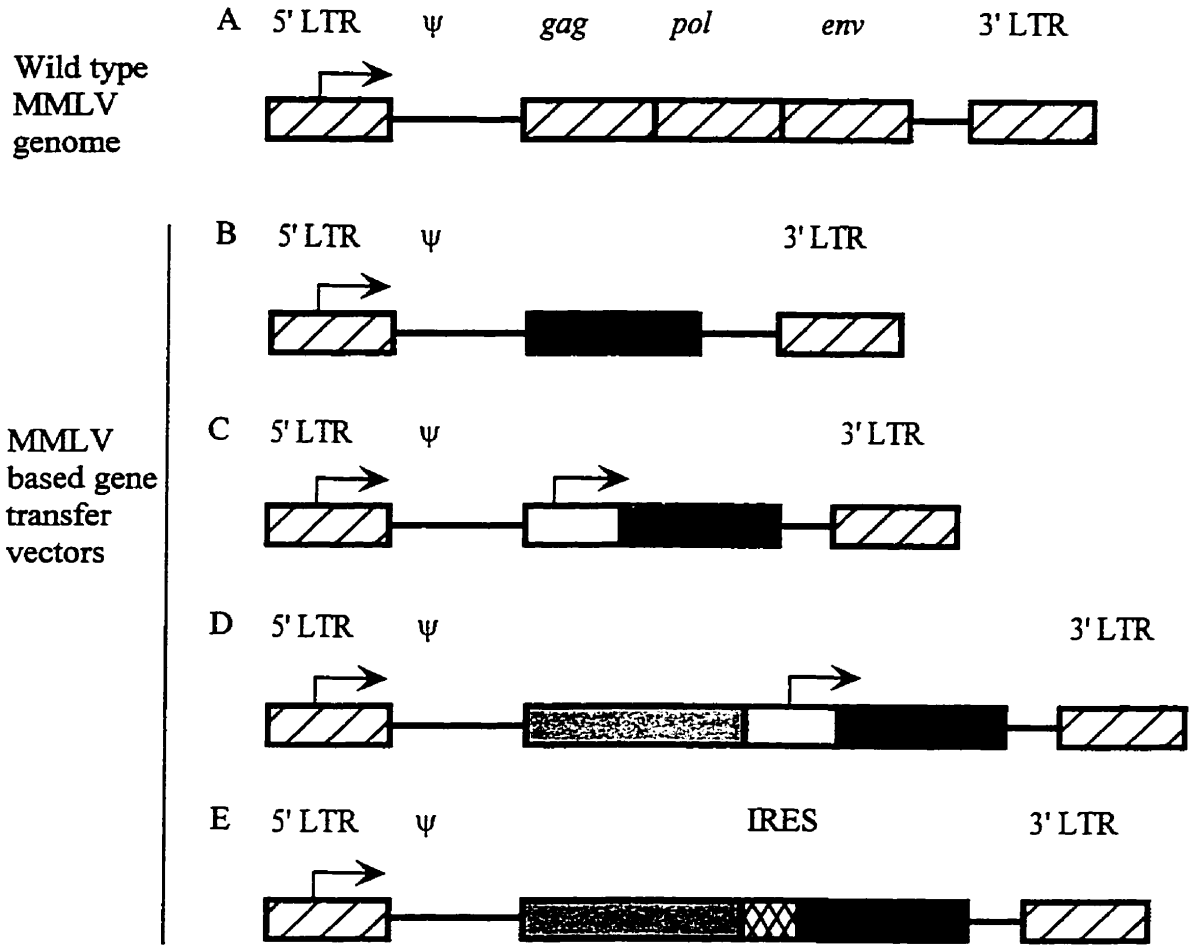

Figure 1.2: Schematic diagrams of the wildtype Moloney murine leukemia virus (MMLV) and commonly used retroviral vectors as described in Section 1.1.3. Legend: retroviral genes; promoter; internal ribosomal entry site; gene A; gene B; ψ, packaging signal.

Packaging cell lines

The production of biologically active retroviruses requires the presence of the retroviral vector transcript and the *gag*, *pol* and *env* proteins. The *gag*, *pol* and *env* protein products are provided *in trans* by packaging cell lines transfected with plasmids carrying these retroviral genes. Packaging plasmids are based on the wild type retroviral genome but have extensive deletions of viral sequences, such as the packaging signal and 3' LTR, to prevent them from being packaged into a virion[95]. As an added safety feature the genes encoding essential viral packaging proteins can be on two separate plasmids[96-98]. These modifications minimise recombination-prone homologous sequences between the gene transfer vector and the packaging plasmids, thereby significantly reducing the chances of recombinations leading to replication competent retrovirus.

A variety of retrovector packaging cell lines that produce recombinant retroviral particles with different envelope proteins have been developed. Modifications to the envelope protein can lead to differential host and target cell ranges. Retroviral particles with gibbon-ape leukemia viral or amphotropic envelope proteins transduce a wide variety of species and cell types which express the cellular receptors gibbon-ape leukemia virus (GLVR or Pit-1) and amphotropic receptor (Ram or Pit-2) respectively[87,95].

Safety of retroviral gene transfer systems

Following the transfection of a retroviral vector into packaging cells, replication incompetent retroviral particles are produced and secreted into the culture supernatant. Replication incompetent viruses are safe for clinical applications because they transduce target cells only once and are unable to cause a secondary infection due to the absence of viral genes. Primate experiments at the National Institutes of Health (N.I.H.) confirmed the safety of modern retroviral gene transfer systems[99-101]. In addition to these safety features all viral supernatants, and in some cases patient samples which have been exposed to retroviral supernatants, are extensively tested for the presence of replication competent retrovirus as clinical trial requirements[102].

Adenoviral gene transfer systems

Recombinant adenoviral gene transfer systems are commonly used in some applications. Adenoviruses can infect both cycling and quiescent cells, and transduce cells efficiently *in vitro* and *in vivo* with high levels of transgene expression[103]. Adenoviral vectors have been used to efficiently transfer marker genes into hematopoietic progenitor cells[104-106]. For example, in one study up to 45% of human $CD34^+$ cells were transduced with adenoviral vectors[104].

Adenoviral vectors may induce potent immune responses to viral genes encoded on the vectors and/or contaminating helper virus. This results in the elimination of transduced cells, and prevents successful re-administration of adenoviral vectors[107]. However, new modifications to adenoviral vectors have reduced immune responses by retaining endogenous immunosuppressive viral sequences in the E3 region[108], eliminating all viral sequences except the necessary inverted terminal repeats[109] or developing a helper dependent system with low levels of contaminating helper virus[110]. Adenoviruses are ideal gene transfer vehicles if immune responses or short term high transgene expression are desired. However, they are not suitable for long-term HSC gene therapies since the vector would be lost as HSCs divide to produce differentiated progeny[103].

Adeno-associated viral gene transfer systems

Adeno-associated virus (AAV), a human parvovirus, is also being developed as a vehicle for gene transfer. AAV is non-pathogenic to humans and establishes a latent infection in the absence of adenovirus[111-113]. Wildtype AAV integrates with high efficiency into human chromosome 19q13.3-qter, which appears to be a benign genomic site[114,115]. All AAV sequences except the inverted terminal repeats can be removed in recombinant AAV vectors, allowing ~4kb for inserts[111]. However, both the efficiency and specificity of integration in chromosome 19 are lost along with the wild type genes[111]. A number of studies are focused on determining the sequences responsible for the specific integration site, and preliminary results suggest that the AAV *rep* protein is necessary for chromosome 19 specific integration[116]. Recombinant AAV have titres of $\sim 10^6$/ml, and can be concentrated up to 10^9/ml. Viral stocks must be free of contaminating vector encoding proteins to prevent

protein transfer and artificial gene transfer results[117]. Recombinant AAV particles can integrate into both quiescent and cycling cells albeit inefficiently[111,118].

The potential of AAV for HSC gene transfer is illustrated by gene transfer of a recombinant AAV vector carrying the human β-globin gene into murine[119] and primate HSCs[120]. Rhesus $CD34^+$ cells were transduced *ex vivo* and transplanted into autologous irradiated recipients. Vector-transduced peripheral blood mononuclear cells and granulocytes were present in the circulation for more than 15 months after transplantation. Vector DNA was detected in approximately 1 in 10^5 circulating cells, including both myeloid and lymphoid cells. This study demonstrates the ability of recombinant AAV vectors to transfer genes to HSCs of non-human primates[120].

Physical methods of gene transfer

Physical methods of gene transfer to hematopoietic cells are also being developed. In these gene transfer systems, bacterial plasmids carrying the gene of interest are introduced into the culture media. In electroporation, DNA uptake can be enhanced by introducing pores or holes in the plasma membrane by pulsing cells with an electric current. There is high cell mortality associated with electroporation[121]. Another approach is to coat the DNA with lipid complexes which can fuse with the plasma membrane[122]. The advantages of these systems are that genes are transferred without viral sequences that may affect the biology of the target cell and that large or multiple genes can be transferred regardless of the proliferative status and cell surface receptor profile of the target cell. One preliminary report of liposome mediated gene transfer into hematopoietic cells demonstrated gene transfer to 0.1-13% hematopoietic progenitors[123]. However, genes rarely integrate into host cell genome in physical gene transfer methods and the vectors are lost in dividing cells. Physical methods of gene transfer are generally of little value in gene transfer applications requiring long-term stable gene expression (Table 1.1).

1.1.4 Transgene Expression

Long-term or highly regulated expression of transgenes is a requirement for many gene therapy applications. This section will review some of the promoters commonly used for

transgene expression and end with a discussion of other vector sequences affecting transgene expression.

Viral promoters

Early generation recombinant retroviral vectors generally utilised the endogenous MMLV retroviral promoter for transgene expression. However, it was discovered in the early 1980's that expression from MMLV based vectors was unreliable[124,125]. Furthermore, it has since been demonstrated that transgene expression from MMLV based vectors is consistently suppressed in hematopoietic cells. In one study, only 10% of the secondary CFU-S which carried the MMLV provirus expressed the transgene[126,127]. In double gene vectors, expression was not detected from either gene in secondary CFU-S despite continued gene expression *in vitro*[126].

Improved *in vivo* gene expression has been demonstrated using MPSV or murine embryonic stem cell virus LTRs[126,128]. Vectors carrying these modifications, such as the MSCV[93] or MND[129] vectors, have increased long-term *in vivo* expression in hematopoietic cells. In one murine marrow gene transfer experiment, for example, proviral gene expression was maintained in 28% and 90% of secondary CFU-S carrying provirus in MMLV and modified MND vectors respectively[130].

Cellular promoters

Transgenes may also be expressed by the promoters of normal cellular genes. This allows for cell enhanced or specific expression. For example, using the cellular CD18 promoter, which is normally expressed in myeloid cells, to express transgenes in a viral vector results in myeloid enhanced expression[131-133].

HSC gene therapy for the hemoglobinopathies requires highly regulated expression of β-globin in erythrocytes but not other cells. Erythroid specific transgene expression has been achieved with inclusion of β-globin locus control region sequences in the vector[134-136]. For example, retroviral or adenoviral vector transduction of erythroleukemia cells and fibroblasts with vectors carrying these locus control region sequences resulted in exclusive transgene expression in erythroleukemia cells[134,137].

Regulatable promoters

Regulated transgene expression is desirable for many gene transfer applications. Inducible promoters that allow strict regulation of gene expression have recently been incorporated in gene transfer vectors. In the tetracycline regulatable system, addition of tetracycline inhibits dependent reporter gene expression. This system works following the transfer of a Tetracycline responsive/VP16 transactivator (tTA) and a tTA dependent reporter gene, either on the same or different vectors results in the expression of the reporter gene[138]. Jordan and colleagues have used the tetracycline system to regulate transgene expression in hematopoietic cells[104]. In this study, $CD34^+$ cells were simultaneously transduced with two adenoviral vectors, one carrying the tTA and the second carrying a tTA-dependent β-gal reporter gene. β-gal was expressed in cells infected with both adenoviruses in the absence of tetracycline. Upon addition of tetracycline, β-gal expression was inhibited in a dose dependent manner, demonstrating that regulated gene expression can be achieved in hematopoietic cells at least *in vitro*[104].

Another inducible system is based on the insect steroid hormone ecdysone and its nuclear receptor. The ecdysone receptor expression system allows modulation of gene expression in target mammalian cells with higher inducibility and lower basal activity than the tetracycline system[139]. High β-gal expression from a promoter carrying ecdysone response elements was observed in cell lines when transduced with a second retrovirus carrying an ecdysone receptor and treated with an ecdysone analog[140]. There is no human homologue for the ecdysone receptor, and ecdysone administration has no apparent effect on mammals[141,142]. The ecdysone expression system has demonstrated highly regulated expression *in vitro* and *in vivo* in transgenic mice[139] and may enable regulated gene expression in gene therapy applications.

Vector sequences affecting proviral gene expression

A number of factors are involved in the down-regulation of proviral expression from MMLV based vectors in hematopoietic cells. Primarily, each randomly integrated retroviral vector is affected by the surrounding chromosomal sequences leading to unpredictable transgene expression or positional variegated expression[143]. Decreased positional variegated transgene expression in cell lines has been achieved through inclusion of insulators[144] or scaffold/matrix attachment regions[145]. However, the long-term effects of these sequences on

retroviral transgene expression in primary hematopoietic progenitor and differentiated cells has not yet been reported. In addition to the surrounding chromosomal environment, vector sequences also contribute to proviral transgene silencing in hematopoietic and embryonic stem cells. For example, the MMLV LTR enhancers do not function in embryonic or hematopoietic stem cells[146]. However, the MPSV LTR, which is transcriptionally active in hematopoietic and embryonic stem cells, differs from the MMLV LTR by a single point mutation which introduces an Sp1 factor binding site[126,147]. The primer binding site in the MMLV LTR also contains cis-inhibitory sequences[148,149]. Replacement of the MMLV primer binding site with that from an endogenous murine retrovirus (dl587rev) has resulted in significantly improved expression in embryonic stem cells[128] and hematopoietic cells *in vivo*[150].

In addition to the above changes, Kohn and colleagues have constructed a series of vectors with further modifications which have resulted in consistent, reliable vector transgene expression in hematopoietic cells. For example, deleting the "negative control region" (NCR)[151] from the 5'LTR of the MND vector removes a Yin Yang-1 (YY-1) binding site[130]. YY-1 is a transcription factor that organises the topology of the transcription complex and can mediate transcription activation or repression[152]. Removal of the NCR in the MND vector resulted in very high, reliable transgene expression in embryonic stem cells and hematopoietic cells *in vivo*[130]. DNA methylation has long been associated with gene expression and including proviral gene silencing[127,153]. Inclusion of a hypomethylation sequence from the murine Thy-1 gene in the LTR of the MTD vector resulted in significantly decreased LTR methylation in and embryonic stem cells and CFU-S and significantly higher levels of transgene expression[130]. The long-term transgene expression in hematopoietic cells from these and other modified vectors needs to evaluated in large animal and human cells to determine their potential for clinical gene therapy applications.

1.1.5 HSC Gene Marking

Murine studies

Retroviral mediated gene transfer into murine hematopoietic progenitors and stem cells was first achieved in the early 1980's through co-culture of marrow cells with retroviral producer cells. Gene transfer into up to 20% of primary and secondary CFU-S following a 48 hour *in vitro* co-culture gene transfer protocol was reported[154,155]. Subsequently, three groups demonstrated successful engraftment of lethally irradiated recipients with marrow transduced with a retrovirus carrying the neo^r reporter gene[156-158]. In one study, steady-state marrow was transduced with or without IL-3 resulting in gene transfer to 47-100% and 39-78% of CFU-S respectively. In this study mice receiving transduced marrow maintained low levels of neo^r expressing cells for at least 4 months[157]. In the other two studies, donors were pre-treated with 5-FU to recruit HSCs into cycle and IL-3 was included during transduction. Transduced cells were pre-selected in liquid culture with G418 prior to transplant. Using this protocol, 70-100% of CFU-S were positive for neo^r. In the recipient mice up to 20% of hematopoietic cells were provirally marked for at least 4 months post-infusion[156,158]. Spleen, thymus and marrow from some mice carried common retroviral integrants, suggesting retroviral marking of HSC[156,158].

These murine studies demonstrated that HSCs could be transduced with retroviral vectors and subsequently contribute to multilineage hematopoiesis. They also demonstrated higher levels of HSC gene transfer when donors were ablated prior to marrow collection, cultured with hematopoietic cytokines, and pre-selected for proviral gene expression prior to transplantation.

Large animal studies

Despite the early success of gene transfer into murine HSCs, efficient gene transfer into HSCs from large animals and humans has proved much more difficult. Early studies utilised transduction protocols similar to those used in early murine studies.

For example, steady-state canine marrow was co-cultured with producer cells for 12-24 hours. This resulted in transfer of dihydrofolate reductase (DHFR; confers resistance to methotrexate) to 3-13% of CFU-GM[159]. However, there was no evidence for gene marked

cells following transplantation into lethally irradiated autologous recipients. Following treatment of recipients with methotrexate 0.03-0.1% of CFU-GM were resistant to methotrexate at 3-5 weeks post-transplant[159].

A number of different approaches were designed to increase HSC gene transfer efficiency by stimulating HSCs into cycle. For example, in one study, donors were treated with G-CSF or cyclophosphamide prior to marrow harvest [160]. Marrow was subsequently transduced by 24 hour co-culture with retroviral producer cells[160]. However, following transplant into ablated recipients, <1% of CFU-GM were provirally marked for up to two years post-transplant[160]. Although these studies demonstrated increased gene transfer to hematopoietic progenitors, higher levels of gene transfer and safer cell free supernatant protocols were needed for initiation of human HSC gene therapy applications.

Other approaches to increasing HSC cycling included culturing HSCs *ex vivo* on stromal layers and adding recombinant cytokines prior to and during transduction[11,12,161-163]. Increased target cell to virus ration through CD34+ selection[11,12,161-163] was also employed in many of these studies. For example, marrow $CD34^+$ cells from 5-FU pre-treated monkeys were transduced on stromal cells expressing membrane bound SCF, with the addition of soluble recombinant IL-6 and SCF. Proviral marking was detected in 2% of circulating hematopoietic cells up to one year post-infusion[161]. Gene transfer to canine HSCs during LTMC resulted in the maintenance of 5-10% retrovirally marked hematopoietic progenitors and cells in autologous recipients for up to 2 years post-infusion[73,74]. However the levels of provirally marked mature hematopoietic cells was less than 1%. This approach took advantage of the normal cell cycle kinetics of progenitors and stem cells in LTMC, where hematopoietic progenitors and possibly stem cells cycle 24-48 hours after weekly demi-depopulation and media change[164,165].

Collectively, large animal studies undertaken in the early 1990's demonstrated increased gene transfer in up to 100% of hematopoietic progenitors. However, levels of gene transfer into HSCs assessed by *in vivo* reconstitution experiments have been disappointing with less than 2% of hematopoietic cells carrying proviral sequences by one year post-transplant[11-13,73,74,161-163,166,167].

Promising results have been demonstrated in two recent primate HSC gene transfer studies. In both studies 10-20% of circulating leukocytes carried provirus for 4-6 months after transplantation of provirally marked cells[168,169]. In one protocol, HSCs were transduced over 4 days of *in vitro* culture with IL-3, IL-6, SCF, Flt-3L and autologous stromal layers[168]. In the second protocol, HSCs were transduced in the presence of IL-6, SCF, Flt-3L, TPO and fibronectin[169]. The engraftment of high levels of provirally marked cells in these studies indicates that clinically relevant levels of HSC gene transfer are achievable in non-human primates. Further study is needed to determine whether the efficient transduction of HSCs reported in these studies can also be achieved in human HSCs and maintained for longer periods of time.

Gene marking in patients

The first human gene marking studies used gene transfer protocols similar to those discussed above for large animals. Not surprisingly, the data from the clinical trials are similar to that discussed above for large animal studies. A summary of the results from three human gene marking trials is presented in Table 1.2 and will be discussed in this section.

The first human clinical HSC gene transfer study was undertaken at St. Jude's Hospital in Memphis by Brenner and colleagues. In this protocol, a portion of the marrow harvested for pediatric cancer patients undergoing high-dose chemotherapy and autologous marrow transplantation was exposed to neo^r containing retroviral supernatant for 6 hours. At the end of the transduction period 5-20% of the CFU were resistant to G418. At one year post-transplant between 2-15% of CFU were resistant to G418. The marker gene was detectable for up to four years in 0.1-1% of blood derived myeloid and lymphoid cells and marrow derived CFU[170-172]. It has been hypothesised that the surprisingly high level of HSC gene transfer with this simple protocol was due to an endogenously high level of HSC cycling in the marrow harvested from these patients. The increased HSC cycling could be due to previous myeloablation or endogenously higher proportions of HSC cycling in juvenile marrow.

Table 1.2: Summary of reported results from gene marking trials*.

Investigator	Cells	Protocol	CFU gene transfer	*In vivo* results
Brenner et al.	BM	1 exp 6 hours	5-20%	• 2-15% CFU-1 year • 0.1-1% blood positive for 6 years
Dunbar et al.	MPB $CD34^+$ BM $CD34^+$	3 exp 3 days +GF	21%	• 0.01-0.001% blood positive >18 months
Stewart et al.	BM	2 exp 21 days stroma	37%	• 3%CFU positive -2 years • 0.02% blood positive 2 years

Legend: * See text Section 1.1.6 for details. MPB, mobilised peripheral blood; BM, bone marrow; exp, exposure(s) to retrovirus; +GF, addition of IL-3, IL-6 and SCF.

The results from adult gene marking studies reported to date are less encouraging. In a study by Dunbar and colleagues at the N.I.H., marrow and mobilised blood $CD34^+$ cells from myeloma and breast cancer patients were marked with neo^r containing retroviral vectors and infused into autologous recipients[173]. An average of 21% of CFUs were transduced during a 3 day culture with SCF, IL-3 and IL-6 and retroviral supernatant. Vector sequences were detected in 0.01-0.001% of myeloid and lymphoid cells of patients for up to 12 months with little or no long-term transgene expression observed[173]. The overall results of this study are comparable to those of primate studies from this group using a similar gene transfer protocol[161].

The LTMC based gene transfer protocol developed and evaluated in dogs was utilised in patients with multiple myeloma undergoing autologous marrow transplantation[75]. At the end

of the LTMC/transduction protocol up to 60% hematopoietic colonies carried the neo^r containing provirus. During the first 3 months post-transplant there was a high level of gene marked hematopoietic progenitors (CFU-GM) in patients, with a mean of 20%. However, by 2 years post-infusion the level dropped to 3% and <1% gene marked progenitors and circulating blood cells respecitvely[75]. These results are very similar to gene transfer into canine HSCs, where approximately 5% of marrow derived hematopoietic progenitors were resistant to G418 and <1% blood and marrow leukocytes carried provirus at 2 years post-infusion[74].

The results from the large animal and clinical gene marking studies illustrate several points: i) CFU-GM gene transfer efficiency does not reliably predict gene transfer into the HSC population; ii) increasing the multiplicity of infection through $CD34^+$ enrichment of hematopoietic cells using existing gene transfer protocols has not increased the level of gene transfer into HSCs; iii) the gene transfer efficiencies into HSCs from large animal and primates are indicative of those achievable in human HSCs; and iv) inclusion of TPO, SCF, Flt-3L with stromal layers improves gene transfer efficiency into hematopoietic progenitors and possibly HSCs.

1.1.6 HSC Gene Transfer for Genetic Disease

Gene transfer into HSCs has been proposed for the treatment of inherited single gene disorders amenable to treatment through blood or marrow based therapy[174]. Genetic diseases which are most likely to benefit from HSC gene transfer strategies will probably have the following characteristics: the disorder is a single gene defect for which the cDNA is cloned; there is a wide range of enzyme/protein levels that are compatible with a normal or mild phenotype; and matched and related bone marrow transplantation is of known clinical benefit in affected patients. This section will review the results of human clinical trials of gene therapy for three genetic disorders, adenosine deaminase (ADA) deficiency, Gaucher disease and chronic granulomatous disease.

Adenosine deaminase deficiency

The first human clinical gene transfer trials for single gene disorders were in patients with ADA-deficiency, which causes severe combined immunodeficiency (SCID). T-lymphoid

cells expressing the normal ADA gene product are expected to have a selective growth and survival advantage over ADA-deficient cells, even though patients are maintained on recombinant enzyme therapy[175]. Recombinant retroviruses containing the normal human ADA cDNA were transferred into peripheral blood T-lymphocytes, marrow or cord blood cells from ADA deficient patients[175-180]. The results from 5 ADA gene transfer trials have been reported and are summarised in Table 1.3.

In 2 studies, patients received multiple infusions of ADA-transduced autologous T cells. Proviral ADA has been detected in up to 20% of circulating T cells in these patients[175,180]. Increased serum ADA and immune function was observed in 1 of 3 treated patients[181]. In 3 trials, patients received autologous marrow or cord blood cells transduced with ADA containing retroviruses. In one marrow study, provirally marked cells were not maintained for greater than 6 months[179], while in a second marrow gene transfer study marked cells were found for greater than 1 year post-infusion[176]. In the cord blood study, provirally marked cells have been detected for over 4 years[178]. There was evidence for a selective growth advantage in T cells carrying proviral ADA, because higher proportions T lymphocytes (1-10%) carried proviral ADA than myeloid cells (0.01-0.1%). Patients in this study were maintained on progressively lower doses of PEG-ADA enzyme therapy. Withdrawal of enzyme replacement therapy from one patient resulted in an increase in the proportion of T cells carrying the provirus to >30% however, there was an overall decline in the absolute number of T cells and immune function. The patient subsequently resumed PEG-ADA treatment[178].

Chronic granulomatous disease

Chronic granulomatous disease is a rare genetic disorder in which phagocytic hematopoietic cells fail to produce superoxide resulting in recurrent infections. The mutations are in one of three genes encoding the gp91, p47 or p22 subnits of the phagocyte NADPH oxidase (phox). Gene transfer of the p47phox gene into p47phox deficient hematopoietic cells results in the correction of superoxide production *in vitro*[182]. Following infusion of autologous p47phox transduced hematopoietic progenitors into patients resulted in phenotypic correction of 0.004-0.05% of total peripheral blood granulocytes at 3-6 weeks post-infusion[183]. Cells able to produce superoxide were detected for up to 6 months post-infusion. The recipients in this study were unconditioned, demonstrating that prolonged engraftment of transduced

progenitors and continued expression of the transduced gene can occur in human unconditioned recipients[183]. However, efficient gene transfer into long lived HSCs is needed for long-term therapy of chronic granulomatous disease.

Table 1.3: Gene therapy trials for ADA deficiency*.

Investigator	Cells	Protocol	*In vitro* gene transfer	*In vivo* results
Blaese et al.	PB	sup IL-2		• Increased immune repertoire • Number of T cells normalised after 2 years
Bordignon et al.	PB BM	IL-2 sup co-cult, or sup	2-40% T-cells 30-40% CFU	• Multilineage repopulation with marked cells • Increased immune repertoire for 2 years
Hoogerbrugge et al.	$CD34^+$ BM	co-cult IL-3	5-12% CFU	• Transduced cells detected 3-6 months • No expression detected
Kohn et al.	CB $CD34^+$	sup IL-3, IL-6, SCF	12-21% CFU	• Multilineage repopulation with transduced cells • 1-10% T cells marked and expressing ADA >2.5yrs
Onodera et al.	PB	sup IL-2, anti-CD3 antibody	3-7% T-cells	• Improvement in immune function • 10-20% of blood cells carrying provirus >1yr

Legend: *See text Section 1.1.6 for details; PB, peripheral blood mononuclear cells; BM, bone marrow; CB, cord blood; sup, supernatant transduction; co-cult, co-culture with retroviral producer cells.

Gaucher disease

Gaucher disease is a lysosomal storage disorder resulting from a deficiency in glucocerebrosidase. In two glucocerebrosidase gene transfer studies, mobilised blood or marrow $CD34^+$ cells from 6 Gaucher patients were transduced with a glucocerebrosidase containing retroviral vector. Following infusion to non-myeloablated autologous recipients, transduced cells were detected at low levels in blood or marrow leukocytes of all patients[184,185]. One study reported glucocerebrosidase enzyme expression at ~50% of normal for 12 months in one patient[185] while no therapeutic benefit or increased enzyme was detected in the other 5 patients[184,185].

Collectively, the results from the gene transfer studies for genetic disease described above illustrate several points which will impact the success of gene transfer for other single gene disorders: i) the transfer of genes that provide a growth and survival advantage can provide long-term maintenance of transduced cells regardless of the original source of HSCs; ii) a selective advantage of transduced cells can not compensate for poor gene transfer efficiency; iii) the presence of cells carrying provirus for greater than one year has demonstrated the safety and feasibility of gene therapy; and iv) gene delivery and expression levels will need to be increased to provide therapeutic benefit in most inherited diseases.

1.1.7 Strategies to Improve HSC Gene Therapy

As clinical gene transfer studies have clearly demonstrated the limitations of current approaches to HSC gene transfer, many laboratories are investigating ways to improve stem cell transduction *in vitro*. A number of problems associated with poor HSC gene transfer efficiency and methods to overcome them are listed in Table 1.4 and discussed here.

Table 1.4: Strategies for optimisation of gene transfer into HSCs.

Strategy	Method
Inducing cells to cycle	• *ex vivo* cytokine stimulation
	• collection of cells during recovery phase after myeloablation or mobilisation
	• culture on stromal layers
Increasing cell-virus contact	• centrifugation of cells and virus during transduction
	• viral supernatant flow through systems
	• coat dishes with fibronectin
	• higher titres and multiple exposures
Increasing viral receptor on target cells	• increase levels of amphotropic receptor by phosphate depletion
	• transfer amphotropic receptor into cell by adeno or adeno-associated virus
	• target cells that have high levels of receptors
	• use receptor which is highly expressed on target cells
Retroviral backbones which efficiently transduce non-cycling cells	• lentivirus and foamy virus vectors
Positive selection of transduced cells	• add positive selectable marker to vector

Inducing HSCs to cycle

Integration of MMLV and related retroviruses occurs in cycling cells[81,82,84]. However, most cells exhibiting the HSC phenotype are quiescent or slowly cycling[69] and thus, need to be induced into cell cycle to allow MMLV based retroviruses to successfully integrate. Attempts to induce HSCs to cycle generally use various combinations of growth factors. Combinations such as IL-3, IL-6 and SCF and have resulted in higher levels of gene transfer into CFU but less than 2% of hematopoietic cells are marked by 1 year post-transplant[161,173]. It is possible that the combination of cytokines used in these studies stimulates hematopoietic progenitors but not HSCs to cycle resulting in low gene transfer to HSCs. Alternatively the low levels of gene marked cells observed *in vivo* may indicate that although HSCs are retrovirally transduced under these conditions they do not maintain their primitive phenotype.

Two recent primate gene transfer studies have demonstrated efficient gene transfer into HSCs. In the first study, marrow was harvested following *in vivo* mobilisation and priming with SCF and G-CSF. The highest levels of HSC gene transfer were observed when CD34-enriched primate marrow cells were cultured with IL-6, SCF, Flt-3L, and TPO prior to and during 48 hr transduction on fibronectin[169]. Following infusion of the transduced cells, there was maintenance of 20% provirally marked cells in recipients for up to a year. Exclusion of TPO in the cytokine cocktail with otherwise similar gene transfer protocol resulted in approximately one log lower levels of provirally marked cells in recipients, demonstrating the importance of this cytokine in maintaining and/or stimulating HSCs *in vitro*[169].

In the second study, $CD34^+$ rhesus marrow cells were cultured on marrow stroma with the addition of IL-3, IL-6, SCF and Flt-3L and retroviral supernatant for 72 hours. Following infusion to autologous recipients, ~10% of circulating leukocytes carried provirus for at least 6 months. However, increasing the culture period by 14 days or excluding Flt-3L dramatically decreased the ability of marked cells to contribute to both short and long-term engraftment[186].

The results of these studies demonstrate efficient gene transfer into primate HSCs in gene transfer protocols including Flt-3L with either stroma or fibronectin and TPO for 72 hours.

The high level of engraftment in these studies may result from increased maintenance and/or proliferation of HSCs *in vitro*, which improved transduction/integration of recombinant MMLV and MPSV based retroviral vectors.

Cell-virus contact

Increased virus-to-cell contact could result in higher levels of gene transfer to HSCs. Some investigators have increased the local concentration of retroviruses by centrifuging virus particles onto target cells[187-189] or by using flow systems in which virus particles and target cells are brought into close proximity to increase initial non-specific adhesion[190-193]. These physical methods of increasing virus to cell contact have demonstrated increased transduction efficiency into cell lines and primary hematopoietic cells and progenitors[187-189].

Another approach to increasing virus to cell contact is through coating tissue culture dishes with fibronectin. This takes advantage of the physiologic properties of both hematopoietic progenitors and retroviral particles to bind fibronectin. Hematopoietic progenitors bind to the carboxy terminal fragment in the CS-1 and Hep II regions of fibronectin via their endogenous integrin receptors VLA-4 and/or VLA-5[194]. Culturing hematopoietic progenitors and HSCs on fibronectin may also increase the maintenance of HSCs during *ex vivo* culture and transduction[195].

Performing transduction in dishes coated with the carboxy terminal fibronectin fragment has resulted in efficient gene transfer to murine, primate and human hematopoietic progenitors[195-197]. For example, gene transfer into ~100% of human hematopoietic progenitors was achieved when $CD34^+$ mobilised peripheral blood cells were pre-stimulated with IL-6 and SCF for 24 hours and transduced for 16 hours on fibronectin pre-loaded with retroviral particles[198]. Cells transduced in the absence of fibronectin had gene transfer to less than 5% hematopoietic colonies[198].

Efficient gene transfer into murine[196] and baboon[197] HSCs cultured on fibronectin coated dishes has been achieved. For example, 17/20 mice transplanted with syngeneic marrow transduced on a recombinant fibronectin fragment with IL-6 and SCF expressed proviral sequences at one year post-infusion. None of 12 mice receiving marrow transduced using a

similar protocol without the addition of fibronectin fragments were positive for proviral expression[196]. The primate study described in the above section by Kiem and colleagues with efficient gene transfer to baboon HSCs also used fibronectin coated dishes in its gene transfer protocol[169]. The inclusion of fibronectin in gene transfer protocols is a safe, clinically applicable method of increasing gene transfer into HSCs.

Pseudotyped recombinant retroviruses

Retroviruses are classified according to their host range, which is largely determined by binding of the retroviral envelope protein to a cellular receptor as discussed above in section 1.1.3[199]. Commonly used retroviral packaging lines produce retroviruses with the amphotropic envelope protein[87,96,97]. Amphotropic viruses utilise an inorganic phosphate transporter as a cell receptor, referred to as the amphotropic receptor (Ram or Pit-2). Ram is expressed at low levels on marrow $CD34^+$ cells. This may be a factor limiting efficient retroviral transduction of HSCs with amphotropic retroviral vectors[200-202]. The expression of Ram may be increased on hematopoietic progenitors by culturing cells in phosphate depleted media[188], or transfer of the Ram gene into hematopoietic cells by adenovirus or adeno-associated virus vectors[203,204]. Following increased Ram expression, retroviruses are internalised at a much higher frequency resulting in higher levels of gene transfer.

Another approach is to use a cellular receptor for viral entry that is expressed at higher levels on HSCs. The gibbon ape leukemia virus receptor (GALVR) is found at higher levels on candidate HSCs than Ram[200,201]. Using GALV pseudotyped retroviral particles may increase viral internalisation in hematopoietic progenitors and HSCs[98]. Several studies have compared the HSC gene transfer efficiencies of retroviral vectors carrying the amphotropic and GALV envelope proteins. Significantly higher levels of gene transfer into CFU-GM[205,206] and HSCs[169,207] has been detected with GALV pseudotyped viruses. For example, in a competitive repopulation experiment, marrow from a baboon was split and half was transduced with amphotropic and the other with GALV pseudotyped retroviruses. Following autologous transplantation of the two transduced aliquots, consistently higher levels of gene marking were detected with the vector packaged with the GALV envelope[207]. In one animal for example, 1-5% of blood leukocytes carried the provirus packaged with the GALV

envelope, while only 0.5-0.8% carried the provirus packaged with the amphotropic envelope at 2-6 weeks after transplant[207].

Retroviral particles pseudotyped with the vesicular stomatitis envelope glycoprotein G (VSV-G) can also transduce hematopoietic cells efficiently. VSV-G does not require a specific receptor, but recognises phospholipid components of the cell membrane. VSV-G pseudotyped viral particles are internalised by membrane fusion[208]. VSV-G pseudotyped particles are very stable and can be concentrated up to 10^9 CFU/ml[209]. Efficient transfer of $CD34^+$ cells with VSV-G pseudotyped MMLV and lentiviral vectors has been described[210,211]. Together with improvements in isolation and increased cycling of HSCs, improved viral uptake may greatly improve current approaches to HSC gene transfer.

Gene delivery by lentiviral vectors

As discussed above MMLV-based retroviral vectors integrate in cells undergoing mitosis. This diminishes their efficacy for gene transfer to target cells that are non-dividing, such as the majority of HSCs[174,212]. This has led to the development of replication incompetent retroviral vector systems based on foamy viruses and lentiviruses, which can integrate into quiescent cells[212-214]. Foamy viruses have a large genome, do not cause any known disease, and can be used for *in vivo* gene transfer because foamy viruses are not inactivated by human serum[213]. Preliminary studies show that they mediate transfer into murine, baboon and human *in vitro* assayable hematopoietic progenitors at higher levels than MMLV[215].

Lentiviruses can efficiently transduce non-cycling cells. Gene transfer vectors based on HIV have been developed which contain less than 25% of the HIV-1 genome[216]. Lentiviral vectors stably integrate into non-cycling cells and maintain long-term expression of transgenes *in vitro*[212,217-220]. A tetracycline-inducible VSV-G pseudotyped lentivirus packaging cell line which can generate viral titres of $>10^6$ has recently been described[221]. Preliminary studies demonstrate that the recombinant lentiviruses produced in this system can efficiently transduce non-dividing cells *in vitro* and *in vivo*[221]. There is considerable optimism that lentiviral and/or foamy viral vectors will overcome the shortcomings of MMLV-based retroviruses and deliver genes to a wide spectrum of cell types, including quiescent HSCs.

Positive selection of transduced cells

HSC gene transfer studies commonly infuse recipients with all cells exposed to retroviral supernatant. This results in competition between the transduced and untransduced HSCs for engraftment. The proportion of retrovirally marked cells contributing to hematopoiesis in mice has been increased through either *in vitro* selection and the exclusive transplantation of transduced cells or *in vivo* selection for cells expressing proviral genes.

In vitro non-toxic selection can be achieved through inclusion of a cell surface molecule gene on the retroviral vector. This facilitates the isolation and selective transplantation of transduced HSCs which are expressing the transgene. The transgene expressing cells engraft without competition from untransduced HSCs. Cell surface molecules which have been evaluated for positive pre-selection include murine heat stable antigen[222], human CD24 antigen[223], truncated low affinity nerve growth factor receptor[224], modified CD4[225], or green fluorescent protein[226]. This approach has been effective at increasing the proportion of provirally marked cells in murine studies. In one study, for example, murine marrow was transduced with a vector carrying the human cell surface antigen CD24. Hematopoietic cells expressing the proviral cell surface marker were enriched by fluorescence activated cell sorting and selectively transplanted into irradiated syngeneic recipients[223,227]. Mice transplanted with the positively selected transduced cells had proviral CD24 expression in up to 80% of circulating hematopoietic cells at six months. Recipients of unselected cells had <20% cells marked at six months[223,227]. This study indicates that positive selection strategies can dramatically increase the proportion of cells expressing a transgene *in vivo*.

In vivo selection can be performed when a drug resistance gene such as MDR, MRP or DHFR is included in the retroviral vector and recipients are treated with the appropriate drug. In a recent study by Sorrentino and colleagues, mice were transplanted with DHFR transduced syngeneic marrow. Following several rounds of treatment with methotrexate, ~90-100% of leukocytes carried the provirus. Control mice transplanted with similar numbers of DHFR transduced marrow but not treated with methotrexate had 10-20% of leukocytes provirus positive. *In vivo* selection provides a means to increase the number of genetically modified cells after transplant[228].

1.1.8 Conclusions

Hematopoietic stem cell gene therapy is being developed as a treatment for some genetic disorders. The results of large animal and human clinical trials discussed in this section have demonstrated that long-term HSC gene therapy is safe and feasible. Recent primate HSC gene transfer studies have demonstrated promising results with the maintenance of clinically relevant levels of genetically modified hematopoietic cells for months at levels predicted to be therapeutic for many disorders. A greater understanding of the basic biology of retroviruses and hematopoiesis will further enhance the development of more advanced and efficient HSC gene delivery systems.

1.2 Biochemical, Genetic and Clinical Features of MPS I

1.2.1 The Mucopolysaccharidoses

The first clinical descriptions of mucopolysaccharide disorders were by Hunter and Hurler. Two brothers with hepatosplenomegaly, inguinal hernias, stiff hands, deafness, dwarfism, noisy respiration and severe skeletal abnormalities were seen by Hunter at Winnipeg General Hospital in 1915 and reported in 1917[229]. Hurler described two cases similar to Hunter's that included gibbus formation, corneal clouding and mental impairment[230]. The report by Hurler was well distributed and the disease widely recognised as Hurler syndrome. These disorders were later described as "gargoylism" in 1936[231,232] and subsequently as Hunter-Hurler disease in 1940[233]. It was later recognised by Njå that some patients had an X-linked form of the disease and others an autosomal recessive inheritance[234]. By 1954, more than 200 cases of Hunter and Hurler syndromes had been reported and the clinical and pathologic features of the two syndromes were delineated[233,235]. Hurler syndrome was characterised by hepatosplenomegaly, corneal clouding, skeletal abnormalities, mental retardation and autosomal recessive inheritance[236-238]. Hunter syndrome was characterised by hepatosplenomegaly, skeletal abnormalities and mental retardation, without corneal clouding and was inherited in an X-linked manner[229,234,239]. Biochemical analysis of livers from Hurler syndrome patients indicated that the disease resulted from excessive amounts of dermatan and heparan sulfate in these patients, and was designated a mucopolysaccharide disorder[240,241]. Electron microscopic analysis demonstrated that Hurler syndrome was a lysosomal storage disease[242].

A variation of Hurler syndrome was described in children and adults in 1962 by Scheie and colleagues[243]. Diffuse corneal clouding was the main presenting feature[243]. Biochemical and histopathologic analysis of these patients demonstrated excessive mucopolysaccharides in urine, and lysosomal inclusions in corneal stroma and skin, similar to those found in Hurler syndrome. However, these patients had no or little intellectual dysfunction and few other symptoms of Hurler syndrome, suggesting that this was a much milder form of disease[243].

By 1965 six distinct mucopolysaccharide disorders were recognised by combined clinical, genetic and biochemical study (reviewed by McKusick in[235]). They were categorised as

mucopolysaccharidosis (MPS) type I through VI: Hurler, Hunter, Sanfilippo, Morquio, Scheie and Maroteaux-Lamy, respectively[235]. Further support for this classification was provided by Neufeld and colleagues following the demonstration that each syndrome had a distinct "corrective factor" except for Hurler and Scheie syndromes, which had the same corrective factor[244]. For example, *in vitro* experiments demonstrated that cells from a patient with Hurler syndrome could correct the metabolic defect in cells from Hunter, but not cells from other Hurler or Scheie patients *in vitro*[244,245].

Classification of the mucopolysaccharidoses

The mucopolysaccharidoses (MPS) are now recognised as a family of lysosomal storage diseases resulting from a deficiency in one of the lysosomal enzymes necessary for the degradation of glycosaminoglycans. A deficiency in any one of these enzymes causes a lysosomal accumulation of glycosaminoglycans such as dermatan, heparan, keratan and/or chondroitin sulfates. High amounts of partially degraded glycosaminoglycans are also excreted in urine. Table 1.5 (modified from[246]) summarises the current clinical, biochemical and genetic classification of six MPS disorders. For example, MPS I and MPS II result from deficiencies in iduronidase and iduronate-2-sulfatase respectively. Both enzymes are involved in the catabolism of dermatan and heparan sulfates. Thus a deficiency in either of these enzymes results in lysosomal accumulation of undegraded dermatan and heparan sulfate. All MPS disorders are inherited in an autosomal recessive manner except Hunter syndrome, which is X-linked. There is a wide variation in the severity of the phenotype in most of the MPS disorders, for example, there are three clinical subtypes in MPS I: MPS IH-Hurler Syndrome, MPS IH/S-Hurler-Scheie syndrome and MPS IS Scheie syndrome, corresponding to severe, intermediate and mild clinical phenotypes respectively (Reviewed by McKusick[235] and Neufeld[246]).

The remainder of this section will focus on the normal biosynthesis, biochemistry and enzymology of iduronidase before discussing the clinico-pathology and genetics of the disorder resulting from its deficiency, MPS I. The chapter will conclude with a discussion of current and proposed treatments for MPS I.

Table 1.5: Classification of Mucopolysaccharidoses (modified from Neufeld et al[246]).

Disorder	Syndrome	Clinical Manifestations	Genetics	Enzyme Deficiency	GAG
MPSI H	Hurler	-corneal clouding -dysostosis multiplex -intellectual dysfunction -childhood mortality	AR	α-l-iduronidase	HS HS
MPSI S	Scheie	-corneal clouding -stiff joints -normal intelligence -normal lifespan	AR	α-l-iduronidase	HS DS
MPSI H/S	Hurler-Scheie	-intermediate phenotype	AR	α-l-iduronidase	HS, DS
MPS II-severe	Hunter-severe	-dysostosis multiplex, -organomegaly -no corneal clouding -intellectual dysfunction -death before 15 yrs	X	iduronate-2-sulfatase	HS DS
MPS II-mild	Hunter-mild	-normal intelligence -short stature -survival 20's to 60's	X	iduronate-2-sulfatase	HS DS
MPS IIIA	SanfilippoA	-mental retardation -hyperactivity -mild somatic disease	AR	heparan N-sulfatase	HS
MPS IIIB	SanfilippoB	-similar to MPS IIIA	AR	α-N-acetylglucosaminidase	HS
MPS IIIC	SanfilippoC	-similar to MPS IIIA	AR	acetyl CoA:α-glucosaminide acetyltransferase	HS
MPS IIID	SanfilippoD	-similar to MPS IIIA	AR	N-acetylglucosamine 6-sulfatase	HS
MPS IVA	MorquioA	-skeletal abnormalities -corneal clouding -odontoid hypoplasia -milder forms exist	AR	galactose-6-sulfatase	KS C6S
MPS IVB	MorquioB	-similar to MPS IVA	AR	β-galactosidase	KS
MPS VI	Maroteaux-Lamy	-dysostosis multiplex -hepatosplenomegaly -normal intelligence -survival to teens -milder forms exist	AR	N-acetylgalactosamine 4-sulfatase	DS
MPS VII	Sly	-dysostosis multiplex -hepatosplenomegaly -wide spectrum of severity	AR	β-glucuronidase	DS HS C46S

Abbreviations: AR, autosomal recessive inheritance; X, X-linked inheritance; HS, heparan sulfate; DS, dermatan sulfate; KS, keratan sulfate; C6S, chondroitin 6-sulfate; C46S, chondroitin 4- and 6-sulfate.

1.2.2 Lysosomal Enzymes

Proteins destined for the lysosome have a number of specific carbohydrate modifications which target them to lysosomes. This section will review the biosynthesis, maturation and transport of iduronidase to the lysosome.

Biosynthesis and protein maturation

Newly synthesised iduronidase polypeptides carry a signal peptide of 26-27 amino acids[247,248] which facilitates their transport into the lumen of the endoplasmic reticulum[249,250]. The signal peptide is cleaved after entry of the nascent protein into the endoplasmic reticulum[249,250]. The predicted size of iduronidase protein after removal of the signal peptide is 70kDa, however, the mature protein secreted from human diploid fibroblasts in culture is ~75 kDa[251,252]. The greater size of the mature protein is due to a series of carbohydrate additions and modifications during the maturation process resulting in multiple exposed mannose-6-phosphate residues[253,254].

Analysis of the peptide sequence of iduronidase identifies 6 potential N-linked glycosylation sites. N-linked glycosylation of asparagine residues is the first carbohydrate modification to the nascent iduronidase protein. The first step of N-linked glycosylation is the *en bloc* addition of a large oligosaccharide consisting of three glucose, nine mannose and two N-acetylglucosamine residues from a lipid bound dolichol pyrophosphate intermediate on the luminal side of the endoplasmic reticulum membrane[255]. Properly folded proteins are transported from the endoplasmic reticulum to the *cis* Golgi network. The oligosaccharides are modified in the *cis* Golgi compartment by the addition of phospho-N-acetylglucosamine residues to the mannose residues[256,257] by UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase[258-260]. The N-acetylglucosamine residue is removed by N-acetylglucosamine 1-phosphodiester-N-acetylglucosaminidase in the *trans* Golgi[261,262] which exposes the phosphomannosyl ligand for recognition by the mannose-6-phosphate receptor (MPR). The MPR-enzyme complex targets iduronidase and other lysosomal hydrolases to lysosomes.

The native protein secreted by human diploid fibroblasts carries six N-linked phosphomannosyl residues[251]. The mature 75kDa iduronidase protein is processed within a few hours to an intermediate of 72kDa. A final 66kDa species is detected 4-5 days later[251]. There appears to be no difference in catalytic activity of the various species, however the 75kDa species is rapidly taken via the MPR, while the 66kDa protein does not use the MPR for internalisation[251]. The intracellular half-life of iduronidase is ~9 days in normal diploid fibroblasts[251].

Enzyme targeting to lysosomes

Iduronidase and other lysosomal enzymes carrying mannose-6-phosphate residues are transported to the lysosomes via interactions with MPRs. Two MPRs involved in the sorting of lysosomal enzymes have been identified. The high molecular weight MPR is 300kDa, is cation independent, functions as a monomer and is responsible for both the intracellular and extracellular transport of lysosomal enzymes[263]. Cation independent MPR is also the receptor for IGF II[264].

The low molecular weight MPR is present as a dimer of two 46kDa subunits and is involved in the intracellular transport of lysosomal enzymes. It is cation dependent in some species and has been designated as the cation-dependent MPR[265]. Lysosomal enzymes not carrying mannose-6-phosphate residues are not targeted to the lysosomes and are secreted[266,267]. Lysosomal enzymes carrying mannose-6-phosphate residues can be targeted to lysosomes through either intracellular and extracellular routes.

Following the addition of functional phosphomannosyl residues in the Golgi complex, soluble lysosomal pro-enzymes traverse to the Golgi network where they bind either the cation independent MPR or cation dependent-MPR, and are transported via clathrin coated buds into newly formed endosomes[268-270]. The endosomal vesicles, termed pre-lysosomal compartments[269], are subsequently acidified by a Na+K+-ATPase[271]. The affinity of MPRs for phosphorylated lysosomal enzymes is decreased at a lower pH, causing the release of lysosomal enzymes into this acidified pre-lysosomal compartment. The MPR is subsequently recycled back to the Golgi compartment[272,273]. Targeting newly synthesised proteins to the lysosome is not fully efficient and a portion of the enzyme is secreted.

There are several mechanisms by which extracellular lysosomal enzymes can be taken up by deficient cells. One mechanism is through binding of the mannose-6-phosphate residues on proteins to cation independent MPR in the plasma membrane and entering the cell through endocytosis[254,274]. Detailed study of the internalisation of the lysosomal enzyme β-galactosidase (β-gal) demonstrated that following binding to the MPR, the β-gal: MPR complex was internalised through coated endocytic pits[275]. The enzyme was subsequently and sequentially detected within uncoated receptosomes near the Golgi, the Golgi tubules, coated endosomes, and lastly, in mature lysosomes[275].

Lysosomal enzymes can also be transferred from lymphocytes and macrophages to other cells directly[276,277]. Immuno-gold electron microscopy of direct lymphocyte-to-fibroblast enzyme transfer has facilitated visualisation of this process. Activated lymphocytes secrete lysosomal enzymes into gaps between the cells at the sites of cell-to-cell contact and are taken up in non-coated micropinocytic vesicles[278]. The enzymes are transferred to endosomes and can be subsequently found in lysosomes[278,279]. Interestingly, the MPR is not involved in the direct cell-to-cell transfer of lysosomal enzymes[279].

1.2.3 Glycosaminoglycans

Proteoglycans, a major component of the extracellular matrix, consist of unbranched polysaccharide chains, or glycosaminoglycans, covalently linked to a protein core (Reviewed by Alberts et al[280]). The glycosaminoglycans are added to the core proteins in the Golgi apparatus. The first step in this process is the addition of a link tetrasaccharide to serine residues of the core protein. This link tetrasaccharide serves as a primer for growth of the glycosaminoglycan side chains by glycosyl transferases. Proteoglycans carry from 1 to more than one hundred glycosaminoglycan side chains. Aggrecan, a major proteoglycan in cartilage, carries ~130 chondroitin sulfate and keratan sulfate side chains[281]. Another proteoglycan, serglycin is found in secretory vesicles of leukocytes and carries up to 15 chondroitin sulfate and dermatan sulfate side chains[282]. There is a slow, continual turnover in extracellular matrix molecules, with the degradation of proteins and glycosaminoglycans to base components balanced with the formation of new proteoglycan molecules.

Degradation of heparan and dermatan sulfates

The biochemical defect in the MPS disorders is the accumulation of glycosaminoglycans within lysosomes. Glycosaminoglycans are normally degraded in a consecutive step-wise fashion by a series of exo-glycosidases and exo-sulfatases in the lysosome. The primary biochemical defect in MPS I, deficiency of iduronidase, results in the accumulation of dermatan and heparan sulfates. The basic structure of dermatan and heparan sulfates and the normal role of iduronidase in their catabolic pathways will be reviewed in this section.

Dermatan and heparan sulfates have uronic acid residues alternating with either N-acetylgalactosamine or α-linked glucosamine molecules respectively. The uronic acid residues are either L-iduronic acid, which may be sulfated, or glucuronic acid. In heparan sulfate, the α-linked glucosamine may be sulfated or acetylated. Glycosaminoglycans are degraded in a stepwise manner from the non-reducing end as seen in Figures 1.3 and 1.4 for dermatan and heparan sulfate respectively.

The first enzyme in both pathways, iduronate-2-sulfatase cleaves the sulfate residue from L-iduronic acid[283]. Iduronidase subsequently removes the L-iduronic acid molecule[252]. After this point the catabolic pathways diverge. In dermatan sulfate degradation, N-acetylgalactosamine-4-sulfatase (arylsulfatase) and β-hexosaminidase A,B remove the sulfate group and then the N-acetylgalactosamine respectively[284,285]. The last step in this pathway is the removal of glucuronic acid by β-glucuronidase[286].

In heparan sulfate catabolism, following the removal of L-iduronic acid, heparan-N-sulfatase removes the N-linked sulfate on glucosamine residues[287], and acetyl CoA:α-glucosaminide N-acteyltransferase adds an acetyl group to the amino group on the glucosamine[288]. The α-linked N-acetylglucosamine is next removed by α-N-acetyl glucosaminidase[289]. Glucuronate-2 sulfatase removes the O-linked sulfate from glucuronic acid residues[290] and β-glucuronidase[286] cleaves the remaining de-sulfated glucuronic residue. A proportion of the N-acetyl glucosamine carries an O-linked sulfate, which is removed by N-acetyl glucosamine 6-sulfatase[291].

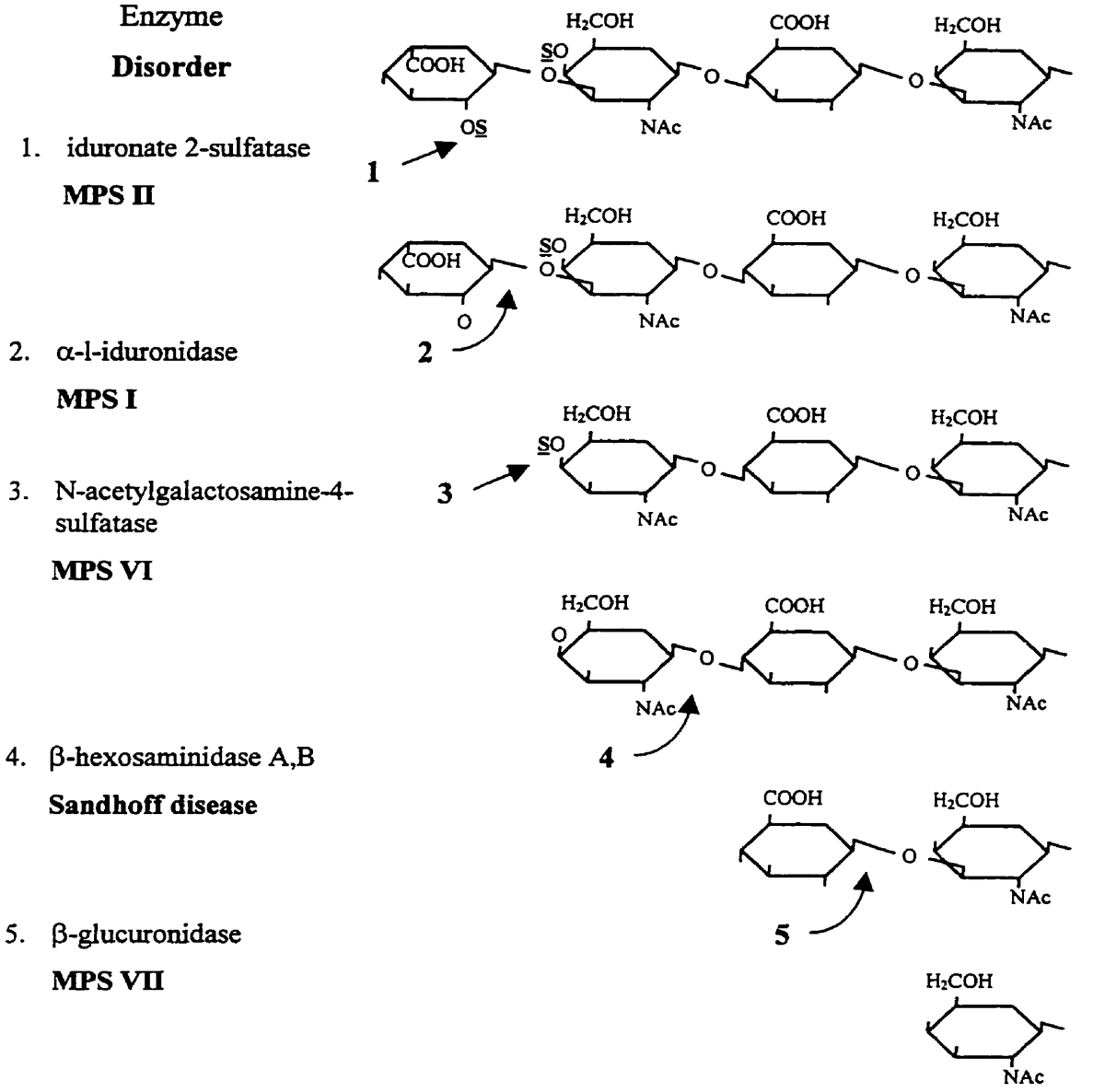

Figure 1.3: Stepwise degradation of dermatan sulfate. The drawing includes all structures known to occur within heparan sulfate but does not imply that they occur stoichiometrically The enzyme which catabolises each step and the MPS disorder resulting from its deficiency are marked. Modified from Neufeld et al[246].

Enzyme

Disorder

1. iduronate 2-sulfatase
   MPS II
2. α-l-iduronidase
   MPS I
3. heparan N-sulfatase
   MPS IIIA
4. acetyl-CoA + acetyl transferase
   MPS IIIC
5. α-N-acetyl glucosaminidase
   MPS IIIB
6. glucuronate sulfatase
   no deficiency known
7. β-glucuronidase
   MPS VII
8. N-acetyl glucosamine 6-sulfatase
   MPS IIID

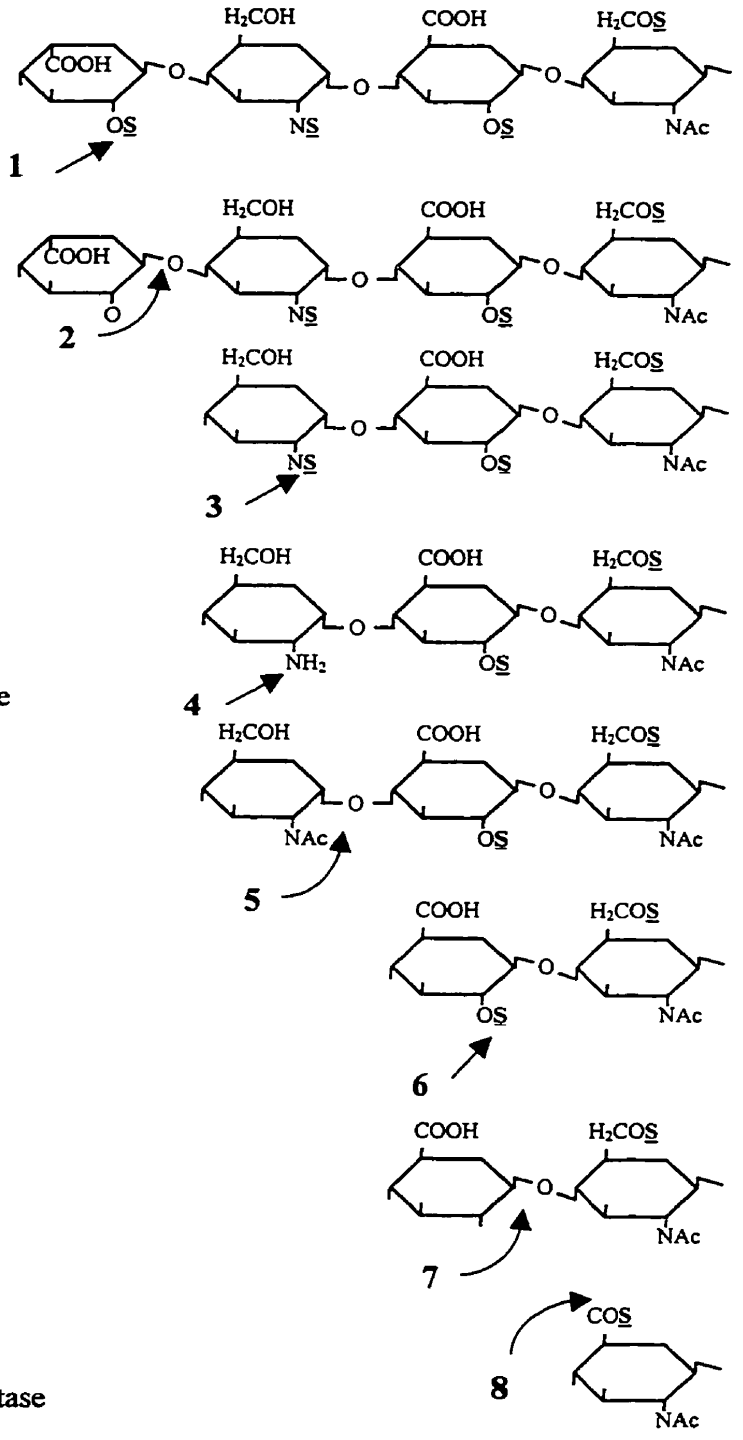

Figure 1.4: Stepwise degradation of heparan sulfate. The drawing includes all structures known to occur within heparan sulfate but does not imply that they occur stoichiometrically The enzyme which catabolises each step and the MPS disorder resulting from its deficiency are marked. Modified from Neufeld et al[246].

The biochemical result of iduronidase deficiency is the accumulation of heparan and dermatan sulfates in the lysosomes of many cell and tissue types. Partially degraded heparan and dermatan sulfate residues are also elevated in the urine of patients with iduronidase deficiency.

1.2.4 Clinical Presentation of MPS I

There is great heterogeneity in the severity of clinical symptoms in MPS I. Hurler syndrome, the most severe form of MPS I, results in intellectual impairment and childhood mortality. The mildest clinical course is seen in Scheie syndrome, in which patients can have normal lifespans with normal intellectual capacity. An intermediate course of disease and lifespan with minor if any intellectual dysfunction is typical of Hurler-Scheie syndrome.

Severe MPS I: Hurler syndrome

Patients with Hurler syndrome appear normal at birth. Onset of clinical symptoms is typically within the first 3-6 months; however, the symptoms are of a generalised nature at this stage and are rarely diagnosed as an MPS disorder at this time unless there is a family history of the disease. The age of diagnosis is usually between 6 and 24 months[292]. The main presenting features are recurrent herniae and upper respiratory tract infections, skeletal abnormalities and hearing deficits. Once diagnosis is made, other symptoms characteristic of Hurler syndrome such as developmental delay, hepatosplenomegaly, facial and skeletal abnormalities are noticed[292]. Hurler syndrome progresses to include corneal clouding, chronic hearing loss, communicating hydrocephalus, shortened stature, dysostosis multiplex and cardiovascular disease in most patients. Mortality is typically due to cardiac and respiratory complications of disease during the first decade of life.

Intermediate and mild MPS I: Hurler-Scheie and Scheie syndromes

Patients with Hurler-Scheie syndrome are generally diagnosed in the first decade of life with corneal clouding, skeletal abnormalities, valvular heart disease and hearing impairment. There is progressive somatic involvement over time and intellectual dysfunction is uncommon. Mortality is generally due to cardiac and respiratory disease during early adulthood.

Scheie syndrome was first diagnosed as an ophthalmologic disease with corneal clouding, glaucoma and retinal degeneration as the main ophthalmologic features in teenagers[243]. Other symptoms may include joint stiffness, deafness, and aortic valvular disease. Intelligence and lifespan are in the normal range.

1.2.5 Pathology of MPS I

The pathological features in MPS I result from the accumulation of glycosaminoglycans in the lysosomes of most cell types. A wide variety of gross anatomical and microscopic changes are seen. Pathological abnormalities have been found in cartilage, periosteum, blood vessels, meninges and cornea. These tissues contain cells with extensive cytoplasmic vacuolation that resemble fibroblasts and histiocytes. Other cell and tissue types such as neurons, Kupffer cells, liver parenchyma, reticular cells of spleen and lymph nodes and epithelial cells are also swollen with lysosomal accumulation of mucopolysaccharides. The main pathological features of MPS I will be reviewed here.

Neurologic pathology

In Hurler syndrome, neurons of the central and peripheral nervous systems have greatly enlarged lysosomes. Accumulations of glycosaminoglycans and sphingolipids are detected in vacuoles and zebra bodies within neurons respectively[293,294]. Histiocytes and fibroblasts with numerous granules and vacuoles are also found in neural tissues and contribute to meningeal thickening[295]. Other cells in the nervous system with increased lysosomal accumulation of glycosaminoglycan include astrocytes and perivascular mononuclear cells[294]. Intellectual dysfunction in Hurler syndrome is likely due to a combination of hydrocephalus and the lysosomal accumulation in the cells of the nervous system. Visual impairment is common in all syndromes associated with MPS I. It is most likely caused by lysosomal storage within cells of the corneal stroma, epithelium and Bowmans membrane[243,296].

Skeletal abnormalities

Severe skeletal abnormalities include stunted growth, lameness and a group of radiologic features known collectively as dysostosis multiplex[297,298]. Cranial changes include an enlarged skull, premature closure of cranial sutures, shallow orbits, abnormal spacing of teeth

and dentigerous cysts. Other bony changes typical of dysostosis multiplex are anterior hypoplasia of the lumbar vertebrae with kyphosis, poorly formed pelvis and clavicles, abnormal formation of long bones, short trapezoidal shaped phalanges and oar-shaped ribs. The diverse skeletal malformations are the result of enlargement and excessive vacuolation in chondrocytes, osteocytes and periosteal cells[231,293,299]. Respiratory disease is common in MPS I with mortality frequently due to bronchopneumonia[237]. Susceptibility to respiratory infections may result from abnormalities in tracheobronchial cartilage from excessive lysosomal accumulations[235].

Cardiovascular pathology

There are many cardiovascular abnormalities in MPS I. Valvular regurgitation caused by nodular thickening of the aortic and mitral valves due to the presence of the highly vacuolated fibroblasts and macrophages is common[300]. Lysosomal accumulations in smooth muscle cells of the arteries and occlusion of the aorta and coronary arteries by intimal deposits of mucopolysaccharide and vacuolated cells are also common[238,300-302]. Lysosomal accumulations of glycosaminoglycans in smooth muscle cells of the epicardium and endocardium cause a generalised thickening of the mural endocardium and stiffening of myocardial walls[293,300,303].

Spleen, lymph nodes and liver pathology

Severe lysosomal accumulation is found in Kupffer cells, liver parenchyma, reticular cells of spleen and lymph nodes causing hepatosplenomegaly[304-309]. The highly vacuolated histiocytes and fibroblasts are found scattered in these tissues. Granulocytes and lymphocytes generally appear normal, although cytoplasmic granulations may be detected[310].

1.2.6 The Iduronidase Gene (IDUA)

The human iduronidase gene (IDUA) was mapped to chromosome 4p16.3 (Figure 1.5)[311,312]. It has 14 exons that span 13kb of genomic DNA (Figure 1.6)[248]. IDUA is arranged in two non-contiguous segments separated by a 9kb intron 2 and is transcribed toward the centromere[311,313].

There are 78% and 77% homologies between the human and murine cDNA and protein respectively. The full length transcripts are 2.3kb, and 3.1kb with open reading frames of 653 and 634 amino acids for human and murine respectively[248,314]. The murine cDNA encodes a 1193bp 3'untranslated region as opposed to the 100bp region in the human cDNA. Exon 2 of both the human and murine IDUA overlap with the 3' untranslated region of the SAT-1 gene, which is transcribed in the opposite orientation[314,315]. Low levels of alternatively spliced transcripts have been detected in human cells, however their biological significance is unknown[248]. Analysis of human IDUA sequences suggests that iduronidase expression is initiated from a housekeeping type promoter with "GC" box type consensus sequence with multiple potential "CA" transcription start sites.

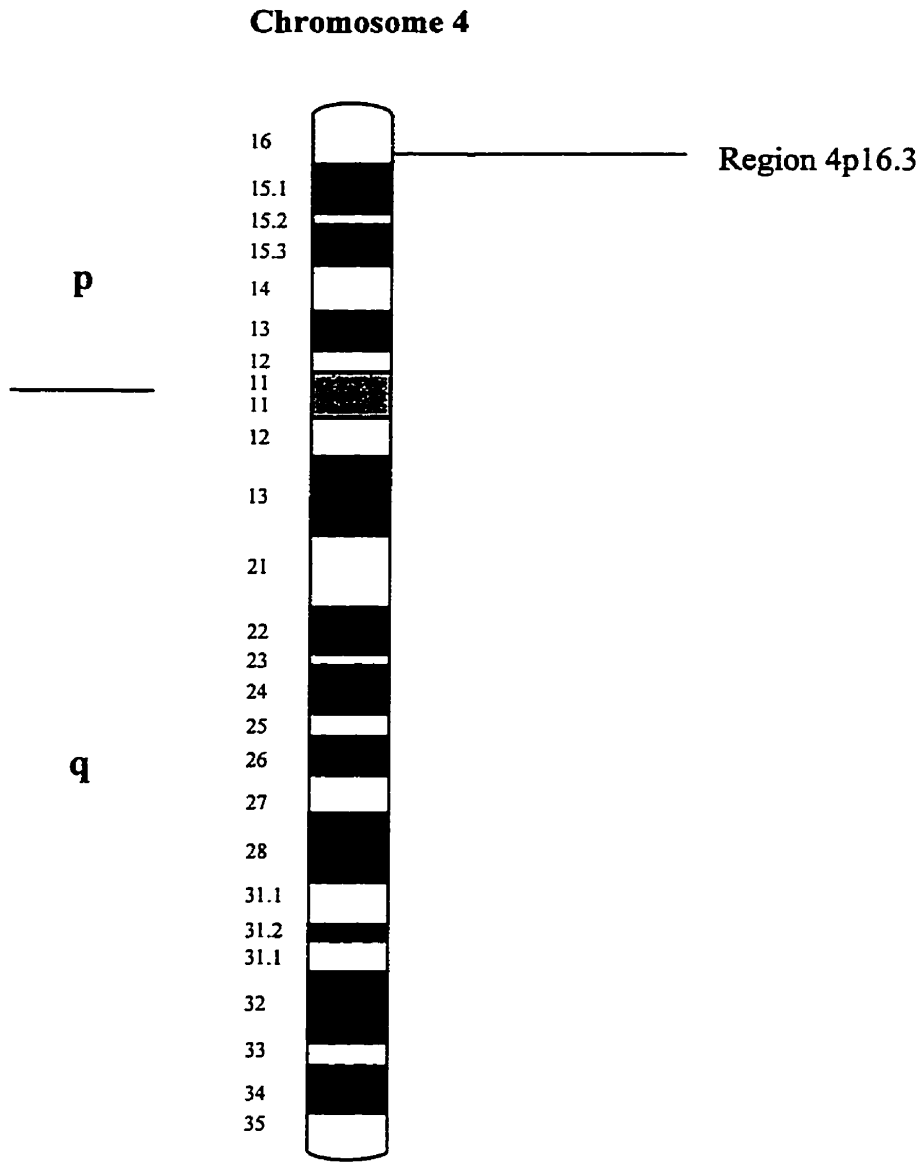

Figure 1.5: Ideogram of human chromosome 4, at standard ISCN resolution of 400 bands. The region 4p16.3 contains the iduronidase gene (IDUA).

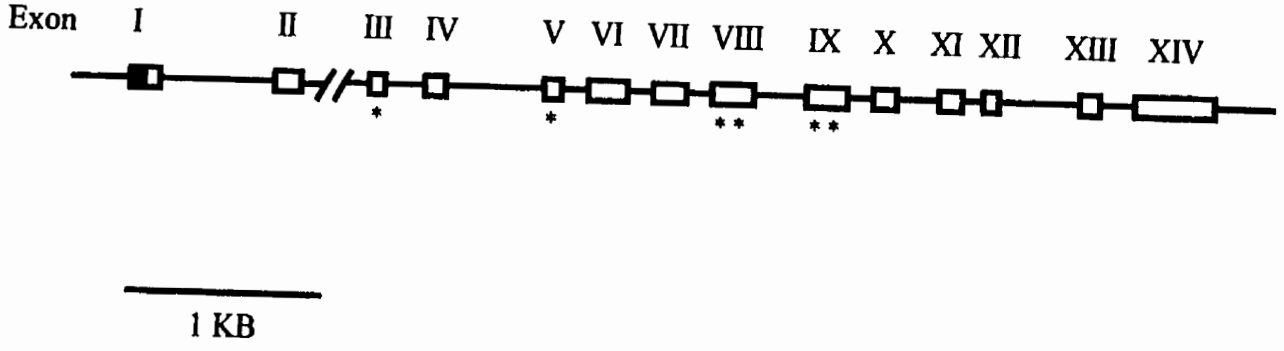

Figure 1.6 Schematic representation of the human and canine α-L-iduronidase genes. Legend: *potential N-linked glycosylation sites; ■ signal peptide sequence; □ exon; — intronic sequences; // intron 2 is 9kb in human IDUA and 13kb in canine IDUA.

Genetic mutations in MPS I

To date 48 IDUA mutations causing MPS I have been identified (reviewed in Scott et al[315]). Mutation analysis has identified nonsense, missense, splice-site, insertion and deletion mutations. There are ethnic and/or geographic differences in allelic distribution. The W402X allele, for example, accounts for ~50% of MPS I alleles in patients of European origin, but has not been found in patients of Japanese origin[315,316]. Another example is the T366P allele, which accounts for about half of the MPS I alleles in Arab populations and has not been detected in patients of Japanese or European origin[317].

The molecular heterogeneity of MPS I has made it difficult to provide simple genotype/phenotype correlations, but some trends have been identified. As expected, the alleles resulting in the most severe phenotypes cause a complete lack of enzyme function. For example, the Trp402 → Ter mutation truncates the protein, and causes a severe Hurler phenotype in patients homozygous for this mutation[315,316]. Patients with milder phenotypes tend to have mutations that permit a trace of enzymatic function such as the missense mutations (R89W) Arg89→ Trp or (R89W) Arg89→ Gln[318,319]. Mutations which produce small amounts of normal transcripts are also associated with milder Scheie phenotypes. For example, the 678-7g->a mutation creates a new out-of-frame acceptor splice-site in intron 5 without changing the original splice site. This mutation results in the production of ~1% of normal levels of transcript and enzyme activity and is associated with a mild Scheie phenotype[319,320]. The wide clinical heterogeneity in MPS I is likely due in part to the large number of common MPS I causing alleles and the many possible combinations of these alleles[315].

Biochemical diagnosis of MPS I

The presenting clinical features in MPS I are very similar to those of the other mucopolysaccharidoses and differential diagnosis ultimately includes biochemical evaluation. The most definitive method for determining the specific enzyme deficiency is by testing the activity of each individual enzyme. For evaluation of iduronidase activity, fibroblasts, leukocytes and serum can be assayed with a fluorogenic (4-methylumbelliferyl substrate) substrate.

1.2.7 Treatment of MPS I

Studies by Neufeld and colleagues in the 1960's demonstrated that Hurler fibroblasts could uptake a "corrective factor", later identified as iduronidase, from culture media conditioned on normal cells and resolve lysosomal accumulation of glycosaminoglycan[245,321]. This finding demonstrated two key biological features instrumental in designing therapy for MPS I: 1) normal cells secrete iduronidase and 2) deficient cells can uptake enzyme and utilise it to decrease storage. The first therapeutic strategies evaluated for Hurler syndrome were plasma transfusion[322-324] and fibroblast transplantation[325,326]. However, neither therapy had any long-term benefit likely due to the short duration and small quantities of iduronidase present in the infusion.

Allogeneic bone marrow transplantation has been developed as a treatment for inherited metabolic disorders including lysosomal storage diseases. In allogeneic marrow transplantation, the recipients hematopoietic system is ablated and repopulated with cells of donor origin. Donor HSCs engrafted in the marrow provide a continual source of normal mature leukocytes that produce normal enzyme. Bone marrow transplants for genetic disease were first successfully performed in 1968 for two lethal genetic immunodeficiencies, Wiskott-Aldrich syndrome and severe combined immunodeficiency[327,328]. The first allogeneic bone marrow transplant for Hurler syndrome resulted in normal levels of iduronidase in the circulating leukocytes and serum, with evidence for a dramatic decrease in the levels of lysosomal storage in many tissues within one year after transplantation[329]. Since the success of marrow transplant for this first patient, allogeneic bone marrow transplant has been performed on over one hundred Hurler syndrome patients. The remainder of this chapter will review the results of bone marrow transplantation for Hurler syndrome.

Several studies have analysed the clinical outcomes of MPS I patients treated with allogeneic marrow transplantation[330-334]. The outcome of these patients has varied considerably. Poorer overall survival has been detected with unrelated and haploidentical donors compared to genotypically HLA identical sibling donors[331,332,334]. Engraftment with donor cells homozygous for normal IDUA alleles and/or endogenously higher levels of enzyme has

generally resulted in quicker resolution of symptoms and a greater chance of retaining cognitive ability[332,334].

In general, patients with sustained engraftment of donor cells had significantly improved disease by 1 year post-transplant. Increased leukocyte iduronidase activity and decreased urinary glycosaminoglycan secretion to the normal ranges has been observed by several months to a year after transplant[335]. The biochemical correction was typically accompanied by resolution of hepatosplenomegaly, reduction in communicating hydrocephalus and cardiomyopathy, and partial or complete clearing of corneas[330-332,334,336]. In contrast to the improvement in lysosomal storage in soft tissues, skeletal abnormalities were not reversed and some patients had continued disease progression following bone marrow transplantation[330-335,337].

There is a wide variation in the effects of bone marrow transplantation on neurological and intellectual status. However it appears that the age and intellectual status of the patient at the time of transplant played significant roles[331,332,334,335]. It is difficult to determine whether the perceived improvements or maintenance of intellectual capacity are within the normal variation in phenotype, or represent true improvements because of the clinical heterogeneity of Hurler syndrome. Several studies have monitored the mental development index or intelligence quotient (IQ) of patients before and after bone marrow transplantation[331-335]. Normal or near normal scores were observed in approximately half of the patients receiving bone marrow transplant prior to 2 years of age or the onset of intellectual dysfunction. There was a lowering of IQ scores in one quarter of patients transplanted after 2 years of age[331,332,335]. These studies suggest that bone marrow transplant does not undo existing damage to the central nervous system, and the younger the patient at the time of transplant the better the observed neurological outcome[331,332,334,335].

The overall survival of transplant patients is related to he histocompatability of the donor-recipient pair. Studies by the Storage Disease Collaborative Study Group demonstrated that the overall survival in Hurler syndrome patients transplanted with related HLA identical, related haploidentical, and unrelated HLA identical donors was 70%, 40% and 50%, respectively[331,338]. These levels are comparable to those previously reported for other

lysosomal storage diseases with approximately 75%, 40%, and 20% for related HLA identical, related haploidentical, and unrelated HLA identical donors respectively[333].

The success of bone marrow transplant may be attributed to a number of unique features of this tissue. The differentiated progeny of HSCs includes lymphocytes, Kupffer cells[339], alveolar and other resident tissue macrophages[340], microglial cells[341], osteoclasts[342,343] and dermal Langerhans cells[344]. Donor derived cells can secrete and possibly directly transfer normal lysosomal enzymes to the deficient tissues in which they reside or through which they traffic[279].

1.2.8 Canine α-L-Iduronidase deficiency

Dr. R. Shull in the Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, identified iduronidase deficiency in a Plott hound and established a breeding colony for this disease. This identification has provided an *in vivo* model for the study of the biochemistry, pathology and therapeutic strategies for human MPS I.

Clinical examination of juvenile iduronidase deficient dogs revealed stunted growth, progressive lameness and corneal opacity similar to patients with Hurler and Hurler-Scheie syndromes in humans[345]. The disease progressed by 6-12 months to include effusions within the joints, severe secondary joint disease with extensive periarticular bone proliferation, and lytic areas within the femoral chondyles and along the thoracic vertebrae. Cardiac symptoms included heart enlargement and valvular thickening. Manipulation of the head and neck caused pain[345]. Hepatosplenomegaly is not a factor in canine MPS I. Thus, clinical similarities between human and canine iduronidase deficiency include: stunted growth, severe bone disease, degenerative joint disease, corneal clouding and heart disease[345,346].

Biochemical analysis of MPS I dogs revealed excessive urinary excretion of dermatan and heparan sulfates, and a complete deficiency of iduronidase activity[346]. Histopathologies of the human and canine diseases are also similar. Fibroblast and macrophage cells with massive lysosomal accumulation are found in most tissues, as well as lysosomal accumulation within neurons, Kupffer cells, parenchymal cells, chondrocytes, osteocytes, and periosteum in canine MPS I. Canine MPS I is clinically, biochemically and pathologically similar to human

MPS I. The somatic disease in dogs is most similar to patients Hurler-Scheie syndrome and the neurologic lysosomal accumulation more similar to Hurler syndrome[345,346].

Canine α-L-iduronidase gene

The canine IDUA gene (cIDUA) is arranged similar to its human counterpart, IDUA. cIDUA has 14 exons that span 19kb of genomic DNA in with two non-contiguous segments separated by a 13kb intron 2[347] (Figure 1.6). There is 82% homology between both the human and canine cDNAs and proteins[247]. The normal cIDUA full length transcript is 2.2kb with an open reading frame of 655 amino acids[247] which is comparable to 2.3kb and 653 amino acids for IDUA[248]. Low levels of alternatively spliced transcripts have been detected, however their biological significance is unknown[247,248]. Iduronidase expression is initiated from a housekeeping type promoter with "GC" box type consensus sequence with one potential "CA" transcription start site[247,248].

Genetic mutation in canine MPS I

The canine iduronidase deficient colony arose from a single Plott hound founder and the genetic mutation has been characterised in detail[347]. There is a single base substitution of G → A which changes the conserved donor splice site in intron 1 from GT to AT and disrupts intron I splicing. Thus the mutant transcript retains the 450bp intron 1 which introduces a stop codon (TGA) at the intron1-exon1 boundary. The predicted peptide for the mutant transcript is 51 amino acids in length. There is a trace amount of transcript spliced at a cryptic splice site 165bp downstream from the normal site, however no immunoprecipitable protein or iduronidase enzymatic activity has been detected[347].

Bone marrow transplantation for canine MPS I

In canine bone marrow transplantation studies, three MPS I dogs received transplant at 5 months of age and were evaluated at least one year post-transplant[348,349]. All dogs engrafted with normal marrow and had greatly improved clinical outcomes. There was also a biochemical correction. Urinary glycosaminoglycan excretion decreased to normal levels and iduronidase activity was detected in many tissues including the central nervous system[348,349]. The iduronidase activity resulted in reduced lysosomal storage in cardiovascular, renal, hepatic and brain tissues[348-350]. There was decreased clinical degenerative arthropathy,

however many of the radiographic lesions were still present[351].

Enzyme replacement therapy

The engineering of a Chinese hamster ovary cell line to secrete large quantities of recombinant iduronidase has facilitated large scale production and purification of iduronidase[352,353]. This has also facilitated the evaluation of the therapeutic benefit of recombinant iduronidase infusion in canine MPS I[354,355]. The clinical outcome of one dog receiving human iduronidase infusions (0.1mg/kg/week) for one year was monitored. The dog started treatment at 5 ½ months of age. Over the year of treatment the dog gained more weight, was more active and had less joint stiffness than control untreated MPS I littermates. Decreased lysosomal storage was detected histologically in liver, kidney, spleen, lymph nodes, synovium and lung[355]. However histologic improvement was not detected in brain, cartilage or heart valve[355]. Treatment with short or long-term recombinant human or canine iduronidase induced complement activating anti-iduronidase antibody formation in MPS I dogs after the second weekly infusion. Antibodies to human iduronidase were associated with complement-mediated anaphylactoid reactions when infusions were given too quickly. The promising results of the canine enzyme infusion study have led to a trial of recombinant infusion in patients with MPS I (Dr. E. Kakkis, personal communication). It is unfortunate that the beneficial effects of bone marrow transplant for canine MPS I in the brain, heart valves and cartilage were not achieved with recombinant enzyme infusion, however it emphasises the importance of *in situ* iduronidase production by the progeny of HSCs.

1.2.9 Conclusion

MPS I is a lysosomal storage disease resulting from a deficiency in iduronidase. A naturally occurring canine model has been identified and developed which has clinical, biochemical and pathological similarities to Hurler and Hurler-Scheie syndromes in humans. The canine model has been instrumental in the development of bone marrow transplantation and recombinant enzyme infusion therapies for patients with MPS I. The experiments undertaken in this thesis have evaluated the therapeutic potential of HSC gene transfer in canine MPS I.

1.3 Rationale

Gene therapy has been proposed as a treatment for single gene inherited disorders such as enzyme deficiencies. Although recent advances have increased gene transfer efficiencies to a variety of cell types, there is limited evidence that gene therapy can actually be of therapeutic benefit in human disease or in large animal models[178,183,356].

An appropriate model system to evaluate the potential clinical benefit of gene therapy would be an enzyme deficiency in which the normal cDNA is cloned and there is a wide range of enzyme levels compatible with a normal or mild phenotype. One large animal model of human disease that meets these criteria is the lysosomal storage disease, canine iduronidase deficiency. Iduronidase deficient dogs are clinically similar to human patients with moderate to severe mucopolysaccharidosis type I (MPS I)[345,346]. The canine iduronidase cDNA and gene have been cloned and characterised[247]. It is predicted that as little as 0.1-1% of the normal iduronidase activity will result in mild disease[315,319,357].

Canine iduronidase deficiency has been treated by allogeneic bone marrow transplantation and enzyme replacement therapy. The rationale for these treatment approaches was based on the observation by Neufeld and colleagues that normal cells secrete iduronidase and deficient cells can uptake enzyme and utilise it to decrease glycosaminoglycan storage[245,321]. Similar to patients with MPS I, allogeneic marrow transplantation in iduronidase deficient dogs resulted in improved clinical and biochemical outcomes. Iduronidase enzyme activity was detected and decreased lysosomal storage was observed in most tissues[348]. The successful outcomes of MPS I patients and dogs treated with allogeneic bone marrow transplantation demonstrated that adoptive transfer of HSCs expressing the normal iduronidase can significantly ameliorate or halt disease progression. In theory, modification of autologous cells to express the desired gene product eliminates the need for allogeneic recipients. The research undertaken in this thesis was designed to further our understanding of the potential of HSCs to serve as vehicles for therapeutic gene delivery using a canine model of iduronidase deficiency.

1.3.1 Hypotheses

On the basis of previous studies from this laboratory demonstrating gene transfer into canine HSCs[74] and the understanding of the biology and treatment of canine iduronidase deficiency[345,346,348] the following hypotheses were put forward:

1. Infusion of transduced HSCs to normal autologous unconditioned recipients would result in long-term engraftment of provirally marked cells at levels predicted to be therapeutic for enzyme deficiencies.

2. Adoptive transfer of iduronidase deficient HSCs, genetically modified to carry and express the normal canine iduronidase cDNA, to MPS I dogs, would result in expression of iduronidase enzyme and amelioration of MPS I disease.

3. Attenuation or prevention of anti-iduronidase immune responses in MPS I dogs would result in higher levels of iduronidase enzyme, and thus, greater therapeutic benefit in MPS I dogs.

1.3.2 Objectives

Three objectives and corresponding experiments were designed to test these hypotheses:

1. Evaluate the potential of retrovirally transduced HSCs to contribute to long-term hematopoiesis following adoptive transfer to unconditioned autologous recipients.

Experiment 1: Marrow was harvested from three normal dogs, retrovirally transduced during LTMC, and infused over 4-5 weeks into unablated autologous recipients. Recipients received post-transplant cyclosporine A treatment to abrogate any anti-graft or anti-transgene immune responses. Recipients were monitored post-infusion for the contribution of provirally marked cells to hematopoiesis and anti-graft immune responses.

2. Assess the potential of adoptive transfer of autologous HSCs, transduced with the normal canine iduronidase cDNA, to ameliorate disease symptoms in MPS I dogs.

Experiment 2: Autologous marrow cells, genetically modified to carry and express the normal canine iduronidase cDNA, were adoptively transferred to six MPS I dogs. The dogs received post-transplant cyclosporine A therapy to abrogate anti-iduronidase immune responses. The dogs were evaluated for engraftment of transduced progenitors, proviral iduronidase expression, amelioration of disease and anti-iduronidase and anti-graft immune responses.

3. Determine the ability of pre-immune fetal transplant to prevent the development of anti-iduronidase immune responses in MPS I dogs, and thereby increase the therapeutic impact of adoptive transfer of the iduronidase cDNA to transduced HSCs.

Experiment 3: Mid-gestation fetal MPS I and normal control pups were injected with allogeneic transduced marrow cells. After parturition, all recipients were monitored for engraftment of provirally marked cells. MPS I recipients were also evaluated for proviral iduronidase expression, amelioration of disease and the induction of immune responses against iduronidase and other graft antigens.

The goal of this thesis was to promote the development of clinical gene therapy protocols by acquiring new knowledge about conditions under which HSCs may be transduced *in vitro*, contribute to hematopoiesis following adoptive transfer and provide therapeutic benefit in a canine model of iduronidase deficiency.

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CHAPTER 2

Materials and Methods

2.1 Veterinary Procedures

2.1.1 Animal Husbandry and Pathology

The MPS I dogs used in this study were bred at either the University of Guelph or the University of Tennessee, and were not treated with recombinant iduronidase prior to this study. Dams were bred by two to four natural breedings. All normal and MPS I dogs were maintained at the University of Guelph for the duration of study. Veterinary care at the University of Guelph was provided by Drs. Kruth and Abrams-Ogg. Protocols were performed in accordance with the guidelines set by the Canadian Research Council on Animal Care and the Animals for Research Act (Ontario 1980). A list of dogs used in these studies and their source is shown in Table 2.1. Dr. Robert Foster of the University of Guelph performed pathologic analysis of MPS I dogs at euthanasia.

2.1.2 Marrow Harvests

Large-scale marrow harvests were performed on 2-12 month old dogs from either the iliac crest or proximal humeri and femora under general anesthesia. The dogs were pre-medicated with intramuscular acepromazine, meperidine and atropine. Anesthesia was induced with intravenous propofol and maintained with isoflourane. Marrow was aspirated into multiple 10mL syringes with 0.1mL preservative-free heparin (200u/mL) through 16-gauge marrow aspiration needles (J-116T Jor-Vet Bone Marrow Needle Jorgensen Laboratories, Inc.). Marrow, equivalent in volume of up to 10% total blood volume, was collected at each large-scale harvest (range: 100-300mL) into a final concentration of 2u/mL preservative-free heparin. Following LTMC cell infusion, 10-25mL aliquots of heparinised peripheral blood and marrow, and serum were collected at 1-3 month intervals for analysis. Dogs were anesthetised for their follow-up marrow samples with intramuscular acepromazine, butorphanol and intravenous propofol.

Table 2.1: Summary of dogs used in gene transfer studies.

Experiment	Experimental reference number	Name	Source
1. Adoptive transfer of transduced HSCs to normal recipients (Chapter 3)	N1	Carmelia	Random source
	N2	Happy	Random source
	N3	Tang	Random source
2. Adoptive transfer of iduronidase transduced HSCs to MPS I recipients (Chapter 4)	M1	Cameo	U of Ten*
	M2	Mork	U of Ten
	M3	Quincy	U of Ten
	M4	Quicha	U of Ten
	M5	Allyssa	U of G^
	M6	Belmont	U of G
3. *In utero* adoptive transfer of transduced HSCs to normal and MPS I pups (Chapter 5)	N4	Fidget	U of G
	N5	Fonz	U of G
	N6	Fudge	U of G
	C1 (Control)	Frolic	U of G
	N7	Coal	U of G
	N8	Diamond	U of G
	N9	Dino	U of G
	N10	Drayton	U of G
	N11	Duffy	U of G
	N12	Dutch	U of G
	M7	Comet	U of G
	M8	Cosmos	U of G
	M9	Denise	U of G

Legend: *College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee; ^Central Animal Facility, University of Guelph, Guelph, Ontario.

2.1.3 Infusion of Autologous LTMC Cells

Cryopreserved or fresh LTMC cells were prepared for infusion by three washes in phosphate buffered saline pH 7.2 (PBS, GIBCO). Cells were resuspended at ~$2x10^7$/mL in Hanks buffered saline (GIBCO) and placed on ice until infusion approximately 2 hours later. Cells were infused into the cephalic vein at a rate of ~1-2mL per minute. Where dogs received multiple large-scale infusions, time points post-infusion for PCR analysis were calculated from the first infusion.

2.1.4 *In Utero* Adoptive Transfer and Parturition

On days 32-38 of gestation pregnant dogs were given 0.05mg/kg butorphanol tartrate intramuscularly for sedation. Anesthesia was induced with 4-6mg/kg intravenous propofol and maintained by isoflourane. The following were evaluated for predicting the day of gestation at the time of fetal injection: vaginal cytology, macroscopic appearance of vaginal wall, serum progesterone levels and abdominal ultrasound. However, the reported day of gestation in this study was retrospectively determined by assuming the date of parturition to be 63 days after mating. The pregnant dogs underwent midline laporotomy and the uteri were exteriorised. The pups were imaged through the uterine wall with an Ultramark 8 Ultrasound System (GE Medical Systems). The transducer and cord were placed in a sterile sheath and cord cover. Sterile lubricant used for the interface. The yolk sac was identified and electronic markers on the image monitor used to guide injection of LTMC cells through a 26 gauge, 3.5-inch spinal needle. The stylet was removed, aspiration attempted to reduce the volume in the yolk sac, and the LTMC cells were injected in a volume of 0.3-0.5mL of PBS. Following closure of the abdominal wall pregnant bitches were examined by ultrasound 7 days after the *in utero* procedure and again several days prior to parturition.

Gestation was normal in all *in utero* transplanted pups. Parturition was normal for the pups in experiment 1. In the second experiment birth of the first three pups was uncomplicated, but the next four pups were born at two-hour intervals and repetitive injections of oxytocin were required to stimulate contractions. The eighth pup was delivered by Cesarean section using standard anesthetic and surgical techniques[1]. All six pups of experiment 3 were delivered by elective Cesarean section, as the mother had a history of uterine inertia. Standard post-operative neonatal care was performed[1].

2.2 Primary Hematopoietic Cell Culture

2.2.1 Primary Hematopoietic Cell Processing

Marrow from large-scale harvests was diluted in an equal volume of PBS, layered over a 65% Percoll (Pharmacia) density gradient (1.075g/cm^3) and centrifuged at 400*g* (centrifugation of cells was always performed in a Beckman TJ-6 centrifuge) for 30 minutes at room temperature. Light density marrow cells (<1.075g/cm^3) were recovered and washed in PBS[2,3]. Cells were counted and established in LTMC or CFU assays as described below.

At one to three month intervals post-infusion, 10-25mL heparinised marrow and peripheral blood, and serum were collected for analysis. Serum samples were centrifuged for 10 minutes at 800*g* and the serum phase aliquoted and placed at minus 80°C until use. Mononuclear cells were obtained from heparinised blood and marrow following ficoll (Pharmacia) density separation. Mononuclear cells were washed three times in PBS and subsequently used in assays for gene transfer and proviral gene expression as described below. When possible, aliquots of cells were cryopreserved in 90% fetal bovine serum (FBS, Sigma), 10% dimethylsulfoxide, and stored at minus 150°C until use. Cryopreserved aliquots of cells were thawed rapidly in a 37°C water bath and immediately diluted to 10mL in PBS or media. Viable cells were determined by trypan blue exclusion. All cell numbers refer to viable cells.

2.2.2 Long Term Marrow Cultures and Transductions

Marrow was set up in long-term marrow cultures (LTMCs) and transduced as described previously[2]. Briefly, 1x10^8 cells were seeded in 150cm^2 tissue culture flasks (Corning) in LTMC media consisting of 10% FBS and 15% horse serum (HoS, Sigma) in McCoy's 5A media (GIBCO) base. LTMC media was supplemented with the following reagents from GIBCO: 2mM L-glutamine, 1X penicillin/streptomycin/fungizone, 0.4X MEM-non-essential amino acids, 0.4X MEM essential amino acids, 1X MEM vitamin solution, 0.9mM sodium bicarbonate, 1mM sodium pyruvate, 10mM HEPES and 5x10^{-7}M hydrocortisone[4]. Control flasks were initiated and maintained with LTMC media alone and transduced cultures were initiated in a 50:50 mix of fresh LTMC media and retroviral supernatant. LTMCs were maintained by removal of half of the non-adherent cells and media on days 7 and 14, and replenishment with fresh LTMC media. Twenty-four hours after feeding, on days 8 and 15,

half of the non-adherent cells and media were again removed and replaced with retroviral supernatant.

Adherent LTMC cells were harvested on day 21 by enzymatic digestion with 0.25% trypsin-1mM EDTA (GIBCO). The cells were washed at least twice with large volumes of PBS. Aliquots of cells were set-aside for molecular assays, histology, and marker rescue assay for replication competent retrovirus (RCR; see below in Section 2.3.2). The remaining cells were either cryopreserved until use or infused directly.

2.2.3 Secretion of Provirally Derived Iduronidase

The ability of provirally derived iduronidase to be secreted by transduced LTMC cells and taken up by MPS I cells was evaluated in a co-culture experiment shown schematically in Figure 2.1. Transduced day 21 LTMC cells were trypsinised, washed and seeded at $1x10^5$ LTMC cells in 2.0mL fresh LTMC media per well in a 6-well plate (Costar). Untransduced day 21 LTMC cells were also trypsinised, washed and seeded at $1x10^5$ cells in fresh LTMC media on each collagen coated 0.22μm filter inserts (Costar). The inserts were suspended over transduced and untransduced control MPS I LTMC cells. The co-cultures were maintained at 33°C. The untransduced LTMC cells in the suspended filter were trypsinised after 5 days of co-culture and iduronidase activity assessed by the 4MUαID fluorimetric assay in each cell fraction as described in Section 2.5.1.

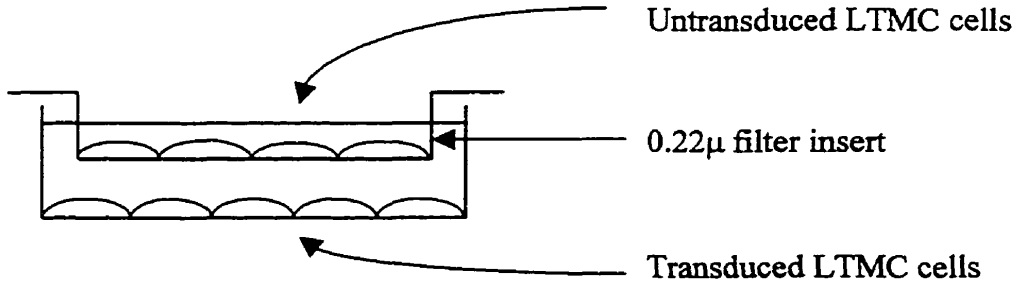


Figure 2.1: Schematic diagram of LTMC co-culture experiment. Untransduced LTMC cells were plated on 0.22μ collagen coated filter inserts and suspended over LTMC cells transduced with iduronidase and/or neo^r containing retroviral vectors, or untransduced controls. The 0.22μ filter allows the diffusion of media, nutrients and iduronidase enzyme without mixing cell populations.

2.2.4 Hematopoietic Progenitor Assays

Marrow mononuclear or LTMC cells were plated in quadruplicate at a density of $1x10^5$ cells/mL in "complete" methylcellulose with recombinant cytokines (MethoCult GF M3434 or H4435, Stem Cell Technologies). Methylcellulose cultures were supplemented with 10% canine leukocyte conditioned media. The leukocyte conditioned media was prepared by culturing ficoll separated blood mononuclear cells, pooled from at least three unrelated dogs, at $1x10^6$/mL in Iscove's Modified Dulbecco's Media (I-MDM, GIBCO) with 15% FBS and 20mg/mL phytohemagglutinin (PHA, GIBCO) at 37°C. After 7 days of culture, the cells were removed by centrifugation and the media filtered (0.22μ, Costar), aliquoted and stored at minus 20°C until use. CFU-GM and BFU-E were assessed using standard criteria on days 10-12 of culture[5].

G418 resistance assays

In some experiments, the proportion of LTMC derived progenitors expressing proviral neo^r was determined by the proportion of colonies able to grow in toxic concentrations of G418, a neomycin analog. In G418 resistance assays LTMC cells were plated in methylcellulose progenitor assays as described above. Samples were plated in quadruplicate with and without the addition of 2.0mg/mL G418 (Sigma). The percentage of colonies resistant to G418 was determined by the formula:

$$\frac{\text{average \# CFU in 2.0mg/mL G418}}{\text{average \# CFU without G418}} \times 100\%$$

2.3 Retroviral Producer Cell Lines

2.3.1 Retroviral Producer Cells

Three Moloney murine leukemia viral based retroviral vectors were used in this study (Figure 2.2). In M48ID, the normal canine iduronidase cDNA[6] was expressed from the murine phosphoglycerate kinase-1 promoter[7]. The M48ID vector was produced by ΨCRIP[8] packaging cell line and provided by Drs A. Salvetti and O. Danos (Paris, France). The LCIDSN vector, based on LXSN[9], has the normal canine iduronidase cDNA expressed from the viral LTR and bacterial neo^r from the SV40 promoter and was provided by Dr. D. Kohn (Los Angeles, California). In LN, neo^r was expressed from the promoter LTR[9] and was provided by Dr. A.D. Miller (Seattle, Washington). Both LN and LCIDSN were packaged by PA317[10].

Retroviral producer cells were maintained in D-MEM with 4.5g/L glucose (GIBCO), supplemented with 2mM L-glutamine, 1X penicillin-streptomycin and 10% FBS at 37°C, in 5% CO_2 in a humidified environment. Retroviral supernatant was prepared by conditioning LTMC media on confluent producer cells for 18-24 hours at 33°C. Viral supernatants were filtered (0.45μm, Costar) and either used directly or stored at minus 70°C until use. All producer cell lines were re-established from selected cryopreserved stocks every 4-6 weeks. LN and LCIDSN producer cell lines were selected for neo^r expression for 7-10 days in 0.4mg/mL G418 prior to cryopreserving stocks of producer cells. Packaging function was selected in Ψ-Crip and PA317 based producer cell lines with HXM (hypoxanthine, xanthine and mycophenolic acid) and HAT (Hypoxanthine, aminopterin and thymidine) media supplements, respectively.

Retroviral titres for the neo^r containing retroviruses, LN and LCIDSN, were determined by exposure of semi-confluent NIH3T3 cells to serial dilutions (10^{-2} to 10^{-5}) of viral supernatant in D-MEM 10% FBS with 8μm/mL polybrene followed by selection in 0.5mg/mL G418. Resistant colonies were enumerated 7-10 days later following washing with PBS and fixation and staining for 15 minutes with XC stain (50% v/v methanol, 0.3% w/v methylene blue and 0.1% w/v basic fuscin). The titre was determined from the number of colonies arising per mL of viral supernatant. The titres of M48ID retroviral supernatants were estimated following Southern blot analysis of DNA extracted from the transduced NIH3T3 cells and comparison to the signal from a producer cell line carrying ~1 copy M48ID vector per cell.

LCIDSN

Packaging cell line : PA317

Titer: 1-5x10^5

M48ID

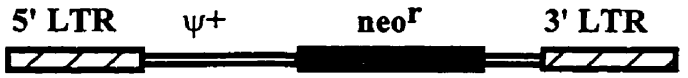
Packaging cell line : ψ Crip

Titer: 1-5x10^5

LN

Packaging cell line : PA317

Titer: 1-5x10^5



500bp

Figure 2.2: Illustration of retroviral vectors. Abbreviations: LTR, long terminal repeats; ψ+, packaging signal; SV, SV40 promoter; neo^r, neomycin phosphotransferase; PGK, murine phosphoglycerate kinase-1 promoter.

2.3.2 Replication Competent Retrovirus Testing

To assess for the generation of replication-competent retrovirus, supernatants were subjected to testing in a marker rescue assay[4,11]. Samples were assayed in duplicate. Filtered supernatant from producer cell lines, LTMCs or serum from experimental or control dogs were incubated with semi-confluent *Mus dunni* fibroblast cells for 10 days to permit the amplification of any infectious particles present. One mL of supernatant from the amplified cultures was filtered and incubated for 48 hours with semi-confluent cultures of NEORSQ cells that have the LN vector stably integrated. One mL of cell free supernatant from the NEORSQ cultures from each sample was transferred to semi-confluent cultures of *Mus dunni* cells. Twenty-four hours after incubation, 0.5mg/mL G418 was added to each culture and incubated for 7-10 days. The cultures were fixed and stained with XC stain as described above, with the presence of colonies indicating the presence of RCR in the test sample. Serial dilutions of wildtype Friend murine leukemia virus supernatant (kindly provided by Dr. Y Ben-David, Sunnybrook Health Science Centre) served as a positive control.

2.4 Molecular Biology and Genetic Assays

2.4.1 α-L-iduronidase Genotyping

Genotypes were determined through a PCR based assay developed by Neufeld and colleagues,[12] which is shown schematically in Fig 2.3. In brief, an 87bp fragment of genomic DNA from exon 1 to intron 1 which spans the mutation is amplified through PCR with sense primer (CA-1): 5'-CGCTGCGGCCCCTGCGGCCCTTCT-3', and an antisense primer (CA-2): 5'-GGGCCGGGCCTGTCGCTCAGT-3'. The PCR cycling conditions were: denaturation at 94°C for 3 min, followed by 40 cycles of amplification: denaturation at 94°C for 1 min and annealing and extension at 72°C for 1 min. PCR reactions were performed in PCR buffer 'C' (Bios), with 0.25mM dNTPs, 1 unit taq and 100ng each primer in a 100μl reaction. PCR amplification products were analysed in 5% agarose gel electrophoresis (metaphor agarose, FMC). PCR products from normal alleles digest to provide two fragments, 32bp and 55bp. MPS I alleles do not digest, as the mutation eliminates the HpHI site.

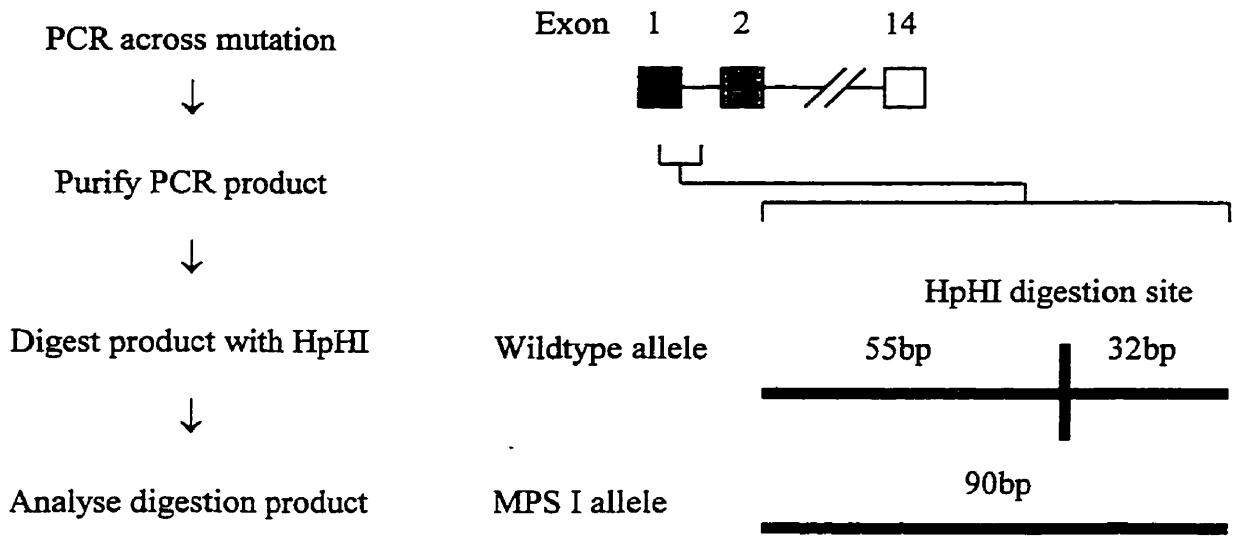


Figure 2.3: Schematic diagram of PCR based genotype analysis for iduronidase alleles. Mutant (MPS I) alleles have lost a HpHI restriction enzyme digestion site present in the wildtype gene.

2.4.2 *In Vitro* Assays of Gene Transfer

Gene transfer was assessed in LTMC cells, blood, marrow and hematopoietic colonies by PCR amplification of proviral specific iduronidase and neo^r sequences. DNA was purified from bulk cell populations using standard phenol-chloroform extraction. One negative control sample was included in each set of extractions. Individual colonies and background methylcellulose controls were plucked into 40μL of lysis buffer (0.5% NP-40, 0.5% tween-20 and 0.9mg/mL proteinase-K). The colonies were incubated at 56°C for 1-2 hours and boiled for 10 minutes to inactivate the proteinase-K. Five microlitres of each sample was used per PCR amplification.

Proviral iduronidase sequences were detected by PCR amplification of a 422bp amplicon from exon 3 to exon 6 of the iduronidase cDNA sequence with a sense primer (cID3S): 5'-CAAGGCCTGAGCTACAACTTC-3', and an antisense primer (cID6AS): 5'-GTGCCGTTGTGACAATGCTCC-3'. The neo^r PCR amplifies a 471bp fragment with a sense primer N1: 5'- GAACAAGATGGATTGCACGCAG-3', and an anti-sense primer

YJR2: 5'-GTCCAGATCATCCTGATCGACAAG-3'[13]. The quality of genomic DNA was assessed by PCR amplification of the canine dystrophin gene with a sense primer: 5'-ACAGTCCTCTACTTCTTCCCACCA-3' and an antisense primer: 5'-AATTCACAGAGCTTGCCATGC-3'. All PCR amplifications were performed with an initial 3 minute denaturation at 94°C, followed by 42 cycles of amplification: denaturation at 94°C for 20 seconds, annealing at 61°C for 25 seconds and extension at 72°C for 30 seconds. PCR reactions were performed in PCR Buffer 10 (Stratagene), with 0.25mM dNTPs, 0.25 units taq and 10pmol each primer in a 25μL reaction. PCR amplification products were analysed following agarose gel electrophoresis.

2.4.3 Southern Blot Analysis

PCR product blots

Gels were transferred onto nylon membrane (Hybond N^+, Amersham) in 0.4M NaOH solution by over night capillary transfer. Membranes were pre-hybridised in Rapid-hybe buffer (Amersham) for 2-4 hours at 50°C. Blots were hybridised with radioactively labelled probe for either 2 hours (PCR samples) or overnight (genomic samples) at 50°C. The hybridised blots were washed for 10 minutes in 2X sodium citrate solution (SSC) 0.1%SDS solution, and 0.5X SSC, 0.1%SDS. Membranes were exposed to BioMax X-ray film (Kodak) at minus 80°C for 1hour-1 week before developing.

The sequences of the neo^r and iduronidase probes used were: neo^r 5'-CGACCTGTCCGGTGCCCTGAATGAACTGG-3' and iduronidase 5'-CCAGGCTGACCGCTATGACC-3'. Oligonucleotide probes were labelled with γ-^{32}P-ATP by T4 polynucleotide kinase (GIBCO) according to manufacturer's specifications.

Genomic Southern blots

For genomic Southern blots, 10μg genomic DNA was digested with KpnI (which cuts in both the 5' and 3' LTRs) overnight at 37°C. The samples were run on a 1% agarose gel and transferred as described above. The cIDUA probe for genomic southern blots was a 1.3kb Sac I fragment from the 3' region of the cIDUA cDNA. This probe detects 4.8kb and 4.6kb fragments from integrated LCIDSN and M48ID proviruses, respectively. For neo^r genomic Southern blots the 235bp EcoRI-PstI and 352bp PstI-SphI neo^r fragments were used as

probes. These probes detect 4.8kb and 2.7kb fragments from integrated LCIDSN and LN proviruses, respectively. The probes were labelled with α^{32}P-dCTP using the Rediprime DNA Labelling System according to manufacturer's instructions (Amersham).

2.4.4 Cytogenetic Analysis

Cytogenetic analysis was performed on leukocytes from animals receiving opposite sex-grafts. Peripheral blood (0.5mL) or samples were stimulated with 2% PHA in 10mL IMDM 15% FBS for 72 hours at 37°C. The cells were pelleted by centrifugation at 400g for 10 minutes and resuspended in 0.5mL I-MDM. PHA stimulated blood cells or fresh marrow cells were incubated in 10mL potassium chloride hypotonic solution (0.075M KCl) with colcemid (Karyomax, GIBCO) for 10minutes at 37°C. Cells were pelleted by centrifugation at 400*g* and were subsequently fixed with three changes of 3:1 methanol: acetic acid. Chromosome analysis was performed as described[14].

2.4.5 *In Situ* PCR Analysis

In situ PCR was performed as described[15]. Cell samples were fixed in methanol:acetic acid as described above for cytogenetic samples in Section 2.4.4. Slides were prepared by dropping 20-50µl of cell suspension with a transfer pipette onto clean slides and subsequently air dried at room temperature. PCR amplification of neo^r was performed with a forward primer: 5'-GTGGAGAGGCTATTCGGCCTATGA-3' and reverse primer: 5'-GTCCAGATCATCCTGATCGACAAG-3'. The reactions were performed in 50µl with: 1X PCR buffer (Perkin Elmer), 4.5mM MgCl2, 0.4µM each dNTP, 8ng each primer, 0.1% BSA, and 10units of taq polymerase. The reaction mixture was added to pre-warmed slides and sealed with AmpliCover discs and clips on the Perkin-Elmer GeneAmp 100 PCR system assembly tool. Fifteen cycles of PCR amplification were performed in a GeneAmp 1000 *In Situ* PCR Thermal Cycler (Perkin-Elmer) with one minute incubations at 94°C, 61°C, and 72°C for denaturation, primer annealing and extension respectively.

Amplified neo^r sequences were detected by hybridisation with 2 fluorescently labelled neo^r probes. The probes were the 235bp EcoRI-PstI and 352bp PstI-SphI fragments described for genomic Southern blotting. The probes were labelled with fluorescein-dUTP with the Primit fluorescent random labelling kit (Stratagene) according to manufacturer's instructions. The

slides were hybridised overnight at 37°C. The slides were washed in 2XSSC at 62°C for 10 minutes, followed by a 5 minute wash in PBS at room temperature. The cells were counterstained with 0.6μg/mL propidium iodide and coverslipped. Cells were visualised using a triple band pass filter on a Zeiss Axioscope fluorescent microscope. The nuclei of negative cells stained red and positive cells appeared yellow-green. At least three hundred individual cells were counted for each sample from at least four different regions of the slide. Only non-overlapping cells that had good morphology and uniform staining were scored[15].

In situ *PCR validation*

The reliability of the neo^r *in situ* PCR protocol used in these studies was evaluated by Dr. Charles Catzavelos and colleagues through blind scoring of mixes of between 0-100% provirus positive and negative Jurkatt cells[15]. However, in the current study several assays, indicated that the experimental samples contained 10% or less neo^r positive cells. Using controls in the actual range of samples can increase the validity of linear regression method comparisons[16]. The data from this control-validation experiment for the whole range from 0-100% and the area of interest between 0-10% are shown in Figure 2.4. The correlation coefficient on the controls between 0-100% was r=0.996 and coefficient of variability r^2=0.98, indicating a strong linear relationship between the actual and observed percent positives over the whole range. However, the correlation coefficient for control samples between 0-10% is lower at r=0.74 and coefficient of variability r^2=0.55, indicating that although there is still a linear correlation between the actual and observed percent positives in this sample range, there is a much greater variability than for 0-100%. Table 2.1 compares the relative frequency of scored controls that underestimated, overestimated or were the same as the actual mix. For example, 8/11 of the 5% positive mix samples were overestimated by *in situ* PCR. The tendency for samples between of 0.001-5% was to overestimate the actual percent positive while at >5% positives the tendency was to underestimate the actual percentage of positive cells (Table 2.1). Thus, it is likely that the percentage of blood and marrow cells scored positive by *in situ* PCR from the experimental dogs overestimates the actual percentage of positive cells.

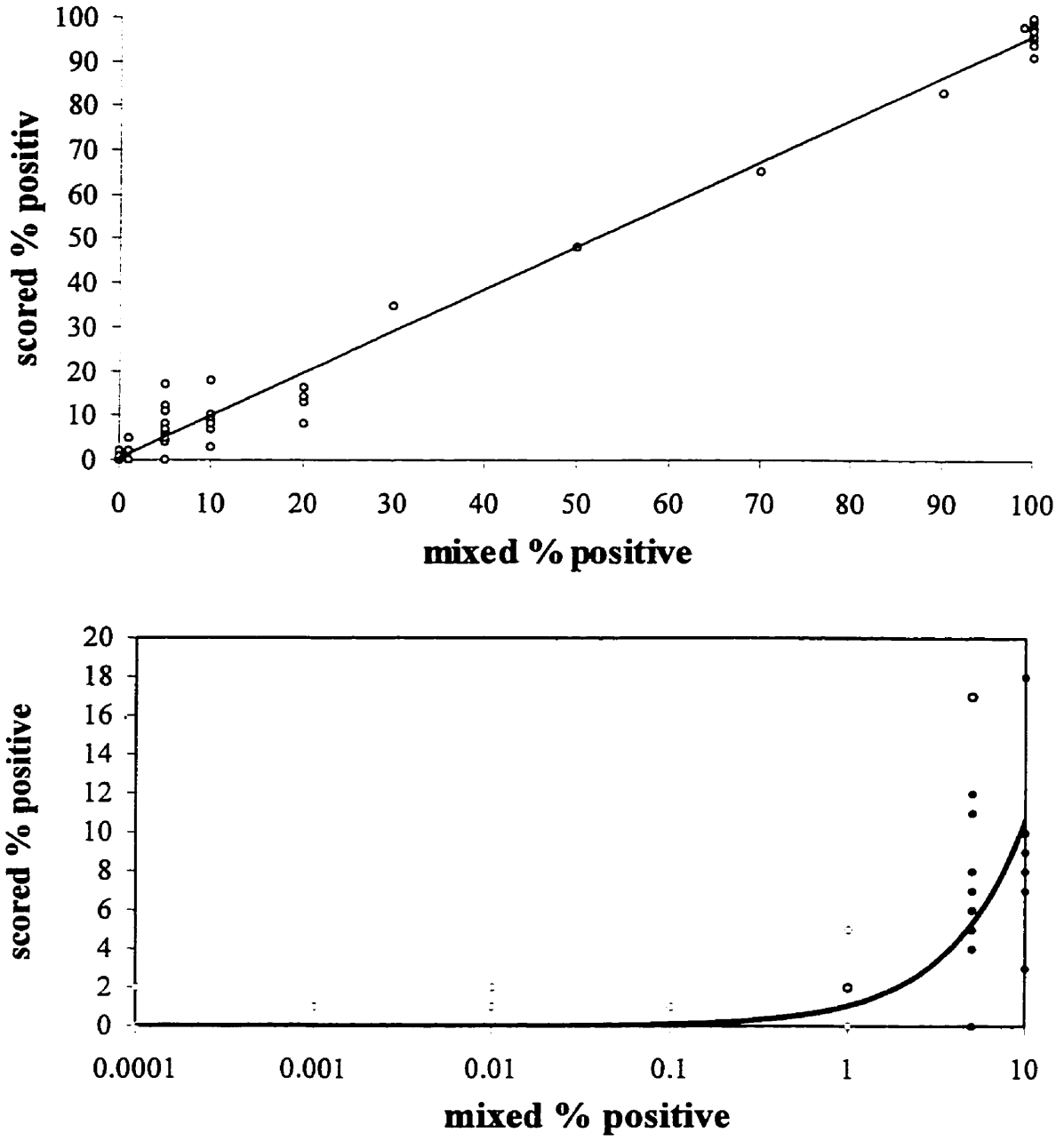


Figure 2.4: Comparison of actual and scored percentages of positive cells by *in situ* PCR in mixing experiments. The scored percentages were calculated following *in situ* PCR analysis and scoring of ≥300 cells. Graphical representation of actual positive verses scored positive percentages from Catzavelos et al[15] in the following ranges: a) linear scale with 0-100% mixes, b) semi-log scale with 0-10% mixes. Solid symbol (•) represents three data points at this value.

Table 2.2: Comparison of the frequency of scored *in situ* PCR samples which vary from the actual percentage of positive cells in the sample.

	Number of control samples scored by *in situ* PCR^			
Actual Mix	Underestimate	Exact	Overestimate	Total*
0	0	11	1	12
0.0001-1%	3	0	9	12
5%	2	1	8	11
10%	5	1	1	7

Legend: ^, 100-1200 cells analysed from each sample; *, total samples scored.

2.5 Assays for Proviral Iduronidase Expression

2.5.1 Iduronidase Activity Assay

Iduronidase activity was assessed in duplicate by the 4-methylumbelliferyl α-iduronide (4MUαID) fluorometric assay[17,18]. In brief, samples were suspended in PAD buffer (10mM phosphate buffer pH5.8, containing 0.1mM dithiothreitol and 0.01% triton-X 100) and lysed by either 4 cycles of freeze-thawing (for assessment of individual CFU) or sonication (blood, marrow and LTMC samples). The reaction was performed in 0.2M formate pH3.5, with 25μM 4MUαID (Sigma) at 37°C for 1-24 hours. Serial dilutions of 4-methylumbelliferone (4MU) were used to calibrate the fluorometer. Negative controls included pre-treatment samples, tissue blanks and reagent controls. Positive controls consisted of matched tissues from normal dogs. Liberated 4MU was detected fluorometrically with 365nm excitation and 440 emission filters (assay sensitivity ~1% of normal levels) in either a Turner Diagnostics cuvette reader for tissue samples and Fluoroskan II 96 well reader for CFU. 1 unit (u) of iduronidase activity corresponded to the release of 1nmol of 4MUαID substrate per mg protein per hour at 37°C. Tissue samples were assayed in duplicate with one tissue control per sample. For analysis of iduronidase activity in CFU-GM, individual colonies were plucked in 3-5μl methylcellulose and resuspended in 25μl PAD buffer. The whole colony was used for the assay. Background methylcellulose and reagent controls were included as negative controls.

2.5.2 Reverse Transcriptase PCR (RT-PCR)

RT-PCR was performed to detect proviral iduronidase transcripts. RNA was prepared from tissue samples (Trizol reagent, GIBCO), treated with ribonuclease-free deoxyribonuclease (Promega) to eliminate genomic DNA and heated to 75°C for 5 minutes to inactivate the deoxyribonuclease. Iduronidase cDNA was synthesised by reverse transcriptase (Superscript II, GIBCO) using an antisense primer derived from exon 7: 5'-GGTCCGCCTCGTCGTTGTAA-3'. The mutation in the canine iduronidase gene (cIDUA) in MPS I dogs eliminates the splice site in intron 1. Therefore endogenous transcripts from the mutant gene retain intron 1 and are 450bp longer than transcripts from the normal canine iduronidase cDNA present in the provirus. PCR amplification of the cDNA with a forward

primer from exon 1 (primer CA-1 from[12]): 5'-CGCTGCGGCCCCTGCGGCCCTTCT-3' and the reverse primer cID6AS (described above in Section 2.4.2) were used for amplification. This reaction unambiguously distinguishes transcripts derived from the provirus (618bp) and those from the endogenous mutant gene which retains intron 1(1068bp). The PCR reaction and cycling conditions were the same as described above in section 2.4.2. The cDNA quality was assessed by PCR amplification of exons 3-6 of iduronidase (described in Section 2.4.2) which amplifies the same size fragment from both the normal and the MPS I genomic transcripts. For each sample, a control was manipulated identically but without the addition of reverse transcriptase (RT-) to confirm that PCR amplicons arose from cDNA and not contaminating genomic DNA.

2.6 Immunoassays

2.6.1 Enzyme Linked Immunosorbent Assay (ELISA)

The presence of humoral immune responses against iduronidase, HoS, FBS and bovine serum albumin (BSA) were evaluated by ELISA on serum samples from dogs prior to and at 1-3 month intervals post-infusion. Pre-infusion serum was collected from each adult recipient dog and used as an individual baseline control. However, pre-infusion serum samples were not available from fetal recipients, therefore the average reading from serum samples from three untreated MPS I dogs was used as a baseline reading for assay of sera from MPS I fetal recipients.

ELISA for iduronidase specific antibodies was performed as described[17] with modifications. Wells were coated with 0.2μg of recombinant iduronidase (kindly provided by Dr. Emil Kakkis, Los Angeles, California), in 100μl PBS pH5.8 (aPBS) overnight at 4°C. Plates were blocked with 0.1% tween-20 in PBS pH5.8 for 2 hrs. Blocking and all subsequent steps were performed at room temperature. Serum samples were serially diluted in 2.5% FBS in aPBS. One hundred μl samples of diluted sera were assayed in triplicate or quadruplicate wells for 1 hour. After primary antibody binding the samples were washed six times with aPBS. The secondary antibody, goat anti-canine IgG conjugated to alkaline phosphatase (Chemicon), was diluted at 1:1000 in PBS pH5.8 0.1% tween-20. The wells were incubated with 100μl of a secondary antibody (anti-canine IgG) for 1 hour. The plates were washed four times in aPBS and twice in a 10mM diethanolamine (Sigma), 1mmol $MgCl_2$ (Sigma) buffer. Each well received 100μl substrate solution (1mg/mL p-nitrophenyl phosphate (Sigma) in diethanolamine buffer). Following a one-hour incubation the plates were read in a Molecular Devices Vmax Microplate reader with a 405nm filter. The OD readings from triplicate or quadruplicate samples were averaged. The titre was reported as the greatest average dilution of serum with an OD of greater than twice that of the pre-immune or control serum. Negative controls included wells with aPBS substituted individually for iduronidase antigen, blocking reagent, primary antibody, secondary antibody, or substrate solution. Serum from a dog treated with recombinant iduronidase known to carry high levels of anti-iduronidase IgG was used as a positive control (kindly provided by Dr. Emil Kakkis, Los Angeles, California).

For the detection of specific antibodies directed against HoS, FBS or BSA, wells were coated with 100µl antigen solution containing 2.5% of the test serum in PBS. The subsequent steps were performed as described above for the detection of iduronidase specific antibodies, except serum samples were diluted in PBS without FBS and washes were performed in PBS.

2.6.2 Cell Proliferation Assay

Cellular immune responses were assessed by the proliferation of peripheral blood mononuclear cells in response to transduced autologous stroma. Marrow stroma was grown similarly to LTMCs except that 4-24 hours after culture initiation the non-adherent fraction was removed and replaced with fresh LTMC media. The 'stromal cultures' were maintained in LTMC media by twice-weekly half volume media changes. At confluence (10-14 days) the stroma was trypsinised and replated at a 1:3 dilution. Twenty-four hours later the stroma was transduced by replacing the media with retroviral supernatant twice daily for two days. Control untransduced stroma was manipulated similarly to transduced stroma, however, fresh LTMC media was used instead of retroviral supernatant. Stroma was maintained by 1:3 dilutions. At passage 3-4, the stromal cells were plated in 100µl of IMDM, 15% FBS in 96 well plates. Responder cells were fresh or cryopreserved autologous ficoll gradient-separated peripheral blood mononuclear cells. Responder cells were added in triplicate at each dilution to each transduced and control stroma sample. The positive control wells received 2% PHA, while blank wells, peripheral blood cells alone and stroma alone served as negative controls. Cells were incubated at 37°C for 6 days with [^{3}H]-thymidine added for the last 18 hours. Thymidine incorporation was analysed on a Wallac 1205 Betaplate counter and reported as the mean counts per minute (cpm$\pm$SD) for triplicate measurements.

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CHAPTER 3:

Adoptive Transfer of Genetically Modified Hematopoietic Stem Cells to Autologous Unconditioned Canine Recipients

3.1 Summary

Hematopoietic stem cell (HSC) gene therapy for many single gene inherited disorders requires efficient transfer of therapeutic genes to HSCs and contribution of these genetically modified HSCs to long-term hematopoiesis following adoptive transfer. The start of this research project coincided with the publication of a study from this laboratory reporting a significantly improved level of gene transfer into HSC. Bienzle et al[1] reported that adoptive transfer of canine marrow cells genetically modified during long-term marrow culture (LTMC) resulted in dogs with 5 to 10% long-lived hematopoietic progenitors derived from genetically modified HSCs. To date, no laboratory has published data to corroborate these findings in a large animal or human system. The experiments undertaken in this chapter were designed to repeat and extend the previous experiment, as the methodological approach developed in this work was proposed for evaluation of HSC gene therapy for canine iduronidase deficiency, the subject of this thesis.

Marrow was harvested from three normal dogs, established in LTMCs, and exposed to neo^r containing retroviral supernatants during three weeks of culture. The latter vector also carried the canine iduronidase cDNA. Efficient gene transfer to committed progenitors during LTMC was demonstrated by the resistance of up to 50% of LTMC derived progenitors to 2.0mg/mL G418, with >80% of colonies growing in 2.0mg/mL G418 PCR positive for proviral neo^r sequences. A total of ~10^8 LTMC cells/kg were infused into each autologous, cyclosporine A treated, non-myeloablated recipient over 4-5 weekly infusions. Similar to previous recipients of a single infusion of cells[1], 5-10% of hematopoietic colonies from all dogs carried proviral sequences at 1-2 years. This confirms that primitive canine hematopoietic progenitors may be transduced during LTMC and contribute to hematopoiesis in unconditioned recipients for greater than 2 years. However, division of the LTMC graft into 4-5 weekly infusions and treatment with cyclosporine A did not result in higher engraftment than previous studies with a single infusion. Humoral immune responses against LTMC media components were detected in recipients following infusion, but did not prevent engraftment of provirally marked hematopoietic progenitors. The levels of provirally marked progenitor engraftment achieved in this study is predicted to be in the therapeutic range for some single gene disorders such as enzyme deficiencies[2-4].

3.2 Introduction

Genetically modified hematopoietic stem cells (HSCs) have been proposed as vehicles for gene delivery for a variety of genetic disorders. Effective long-term HSC gene therapy for patients with single gene inherited disorders requires efficient transfer of genes to HSCs, engraftment and contribution of the genetically modified cells to hematopoiesis, and long term transgene expression. Early HSC gene transfer studies were aimed at transferring genes into HSCs of large animals using transduction protocols similar to those used successfully in murine studies[5-8]. Marrow cells were incubated for a few hours or days with retroviral producer cells or cell free supernatants and then infused into myeloablated recipients. Results from these large animal studies were disappointing, as proviral sequences were undetectable within days to weeks after transplantation[9-11]. One problem identified in these studies was the low virus-to-stem cell ratio when whole marrow populations were used. Increasing the virus-to-target cell ratio through CD34+ selection of hematopoietic cells prior to transduction resulted in increased gene transfer efficiency[12-14]. Another problem identified in early gene transfer studies was the low numbers of HSCs cycling in steady-state marrow[9,10]. The proportion of hematopoietic progenitors and stem cells undergoing mitosis was increased through cyto-reduction or hematopoietic growth factor mobilisation *in vivo*[15] and/or inclusion of hematopoietic growth factors during *ex vivo* transduction[13,16,17].

While the above approaches demonstrated reasonably high levels of gene transfer into *in vitro* assayable hematopoietic progenitors, gene transfer into HSCs assessed by *in vivo* reconstitution experiments produced disappointing results. Studies in large animal and non-human primate models failed to demonstrate clinically relevant levels of HSC gene transfer[12,13,17-20]. In the majority of these studies, less than 2% of hematopoietic cells and progenitors in the marrow and blood contained proviral sequences by one year post-transplant.

A number of studies demonstrated increased efficiency of gene transfer into HSCs using stromal based gene transfer systems[1,14,21-23]. In one gene transfer approach developed at this Institution, a three-week, three-exposure long term marrow culture (LTMC) protocol resulted in gene transfer into primitive canine hematopoietic progenitor cells[1,21]. Infusion of 10^7-10^8

neo^r transduced LTMC cells per kg bodyweight to autologous unconditioned canine recipients resulted in the maintenance of 5-10% marrow derived hematopoietic progenitors resistant to G418 at one to two years post-infusion[1]. The approach was unique in the following ways: marrow mononuclear cells were cultured in long-term marrow cultures (LTMC) where a marrow derived stromal layer developed which promoted the survival and proliferation of progenitors and stem cells in the absence of added growth factors; during LTMC, hematopoietic progenitors and stem cells were exposed to retroviral supernatant at the time they were most likely cycling, 24 hours after weekly demi-depopulation and media replenishment; and, transduced LTMC cells were infused into unconditioned recipients. To date, no laboratory has published data to corroborate these findings in any large animal or human system.

Experiments in the field of gene therapy have been fraught with problems. Often, these have been related to difficulties documenting low levels of engraftment using colony formation in the presence/absence of drug selection and PCR analysis; the former as a tool for identifying progenitors and the latter as a sensitive assay for assessing the presence of proviral genomes. Both techniques are subject to intra-laboratory variations depending on a myriad of factors ranging from methylcellulose quality to PCR buffer. Perhaps not surprisingly, data from reports documenting incremental increases in gene transfer have been met with scepticism and are held in abeyance until confirmed. The experiments described in this chapter were designed to confirm and extend the encouraging results of the study by Bienzle et al[1] prior to undertaking experiments to evaluate HSC gene therapy for canine deficiency. They were also designed to improve the engraftment potential of provirally marked hematopoietic progenitors and stem cells through two modifications: 1) fractionating the graft into multiple weekly infusions, and 2) treating recipients with cyclosporine A. The results of this study confirm that primitive hematopoietic progenitor cells can be genetically modified during LTMC, engraft in unconditioned autologous recipients and contribute to hematopoiesis for greater than 2 years at levels thought to be clinically relevant. Fractionating the graft into multiple weekly doses and treatment of recipients with cyclosporine A in the current study resulted in similar levels of engraftment as previous studies with a single bolus injection of cells. The results presented here indicate that gene transfer to HSCs during LTMC may be of therapeutic value in some deficiency disorders.

3.3 Experimental Design

Marrow was harvested from three normal dogs and maintained in LTMC for three weeks. During LTMC, each set of cultures were exposed to retroviral supernatant on three occasions, at culture initiation and once a week for two weeks, twenty-four hours after weekly demi-depopulation and media replenishment. Two recombinant retroviruses were used in this study, LN and LCIDSN, both of which carry the neo^r gene (Figure 2.1). The latter vector also carried the normal canine iduronidase cDNA. Each LTMC was exposed to both retroviral vectors. The last set of cultures from each dog were infused directly while earlier cultures were cryopreserved until use. Dogs received weekly infusions for 4–5 weeks. The dogs were unconditioned but received post-transplant cyclosporine A.

Following adoptive transfer of transduced autologous LTMC cells dogs were monitored for the contribution of provirally marked hematopoietic progenitors to hematopoiesis through the presence of provirally marked leukocytes and hematopoietic progenitors. Immune responses against components of the graft such as LTMC medium components and canine iduronidase were also evaluated.

3.4 Results

3.4.1 Marrow Harvests and LTMCs

Four to five marrow harvests were performed per dog. A total of $3.3x10^8$, $3.9x10^8$ and $3.7x10^8$ marrow mononuclear cells per kg and $1.4x10^5$, $1.1x10^5$ and $1.4x10^5$ CFU-GM per kg were harvested from dogs N1, N2 and N3, respectively. The average cell and progenitor yields per kg at marrow harvest were similar for each dog and are summarised in Figure 3.1. The average cell yields per kg were $6.6x10^7$ (SD: $\pm 3.0x10^7$), $9.8x10^7$ (SD: $\pm 8.2x10^7$) and $3.7x10^8$ (SD: $\pm 5.6x10^4$) while average progenitor yields per kg were $2.8x10^4$ (SD: $\pm 2.5x10^4$), $2.8x10^4$ (SD: $\pm 2.2x10^4$) and $3.5x10^4$ (SD: $3.4x10^4$) for N1, N2 and N3, respectively.

The marrow mononuclear cells at each harvest were established in LTMC. The LTMCs developed normally with stromal layers nearing confluence and the emergence of hematopoietic foci during the second week. A typical photomicrograph of a day 21 LTMC from N1's second marrow harvest is shown in Figure 3.2. At the time of LTMC culture

harvest on day 21 an average of $1.3x10^7$ (SD: $\pm 1.3x10^7$) cells per kg and $2.0x10^3$ (SD: $\pm 2.3x10^3$) CFU-GM per kg were recovered from N1, $1.5x10^7$ (SD: $\pm 9.7x10^6$) and $8.8x10^2$ (SD: $\pm 2.5x10^2$) from N2, and $3.3x10^7$ (SD: $2.6x10^6$) and $1.8x10^3$ (SD: $\pm 1.3x10^2$) from N3's LTMCs (Figure 3.1). These numbers correspond to 19.7% and 7.1% recovery of the input cells and progenitors of the LTMCs from N1. The respective average cell and progenitor recoveries were 15.3% and 3.1% for N2, and 8.9% and 5.1% for N3.

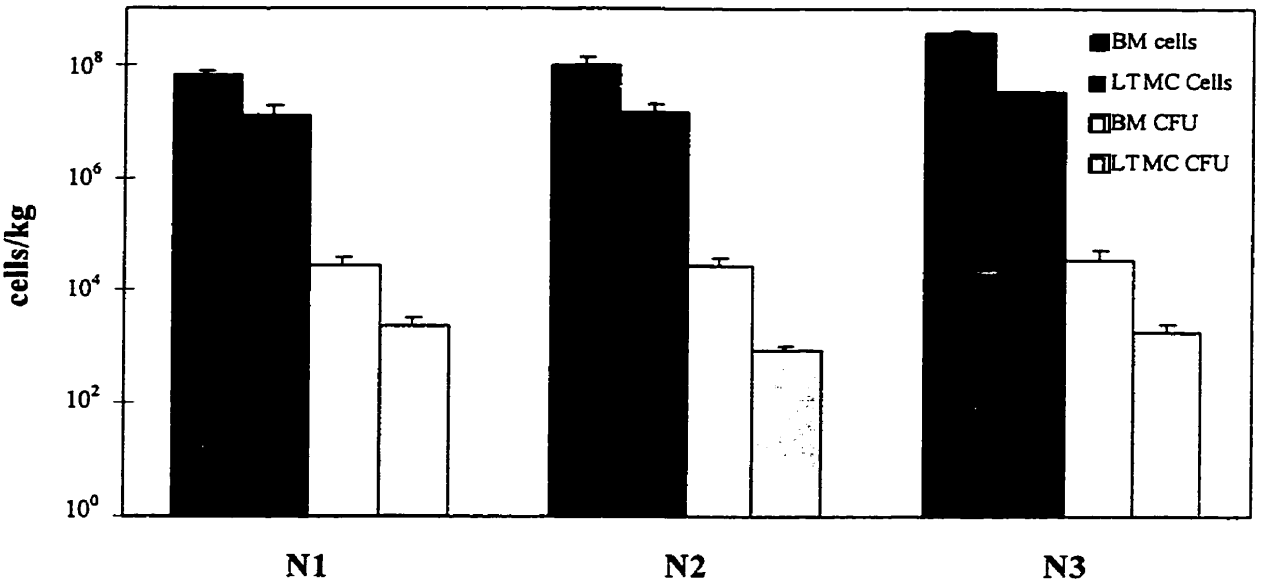

Figure 3.1: Average cell recoveries from marrow harvest and following 21 days of LTMC for each normal dogs. Standard deviation is shown. Note log scale. Legend: BM cells, mononuclear cells in marrow harvest; LTMC cells, cells at day 21 of LTMC; BM CFU, Number of CFU-GM present in marrow harvest; LTMC CFU, number of CFU-GM present at day 21 of LTMC.

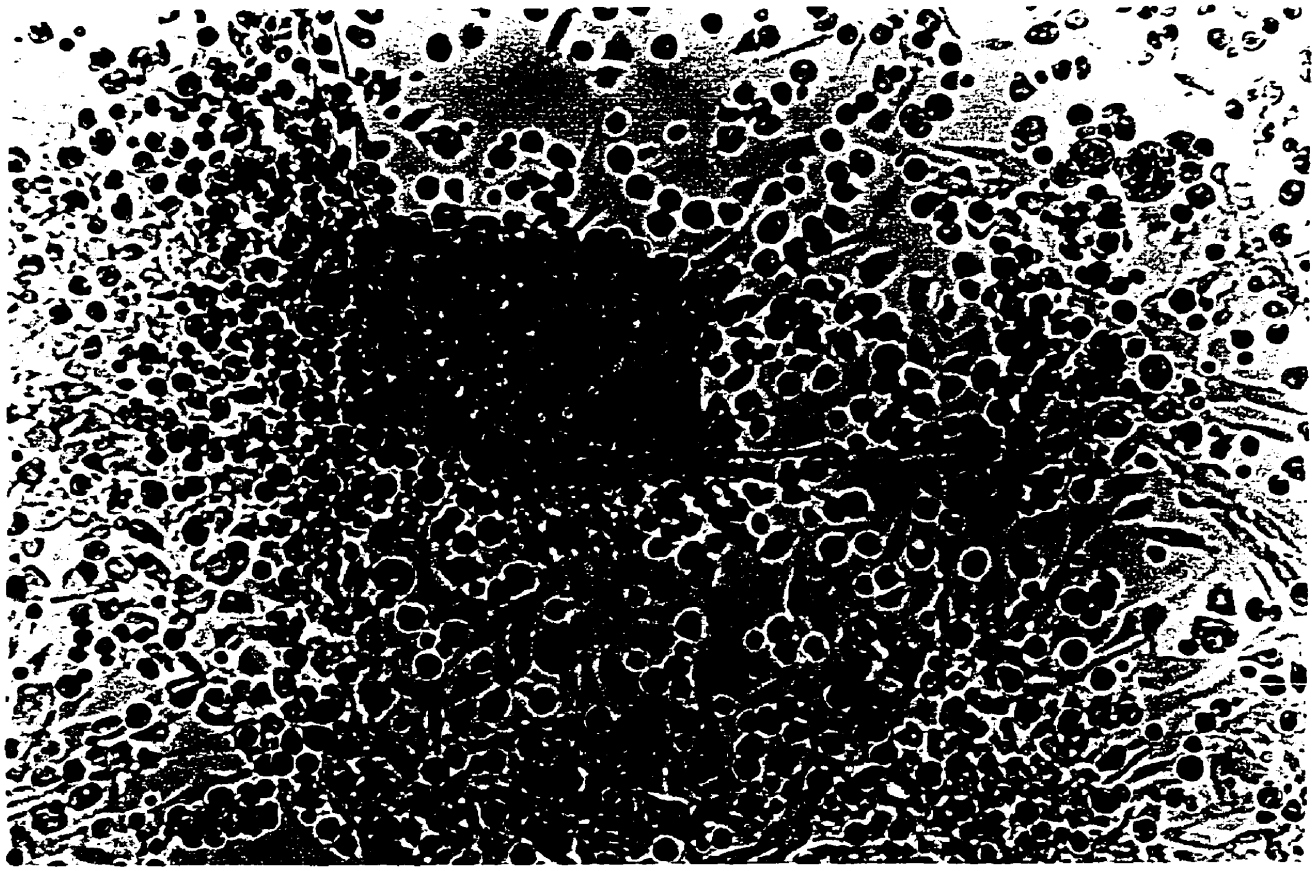

Figure 3.2: Photomicrograph of a day 21 long term marrow culture from N1.

3.4.2 *In Vitro* Assays of Gene Transfer

Marrow cells were exposed to retroviral vector containing supernatants on three occasions during LTMC. The proportion of LTMC derived progenitors carrying and expressing the provirus was determined by percentage of colonies growing in 2.0mg/mL G418. The lowest concentration of G418 in which normal, untransduced, control hematopoietic colonies were unable to grow was identified. In three control marrow samples tested there were few colonies surviving at 1.0mg/mL and no breakthrough colony growth at 2.0 mg/mL G418 (Figure 3.3). In another experiment transduced LTMC derived colonies growing in methylcellulose containing 2.0mg/mL G418 underwent PCR analysis to confirm that the colonies carried neo^r sequences. In two samples, 16/17 (94.1%) and 8/10 (80.0%) CFU-GM growing in 2.0 mg/mL G418 carried the neo^r gene. The PCR data from the former experiment is shown in Figure 3.4. These results indicated that ≥80% of hematopoietic colonies growing at a concentration of 2.0mg/mL G418 carried the provirus.

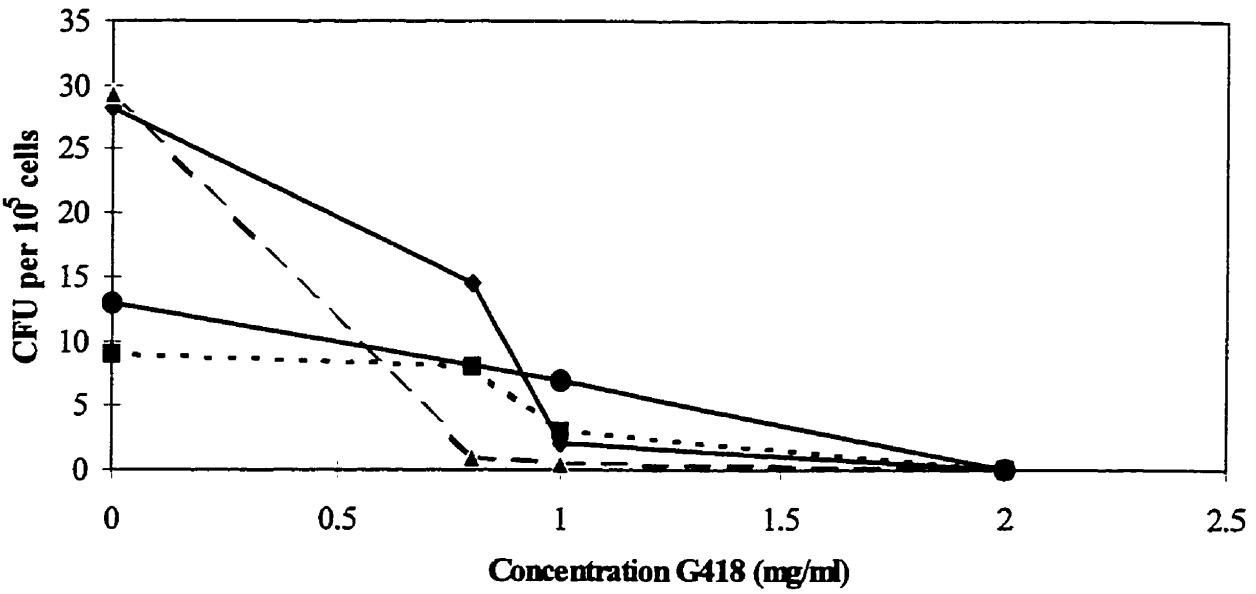

Figure 3.3: Resistance of normal untransduced canine progenitors to G418.

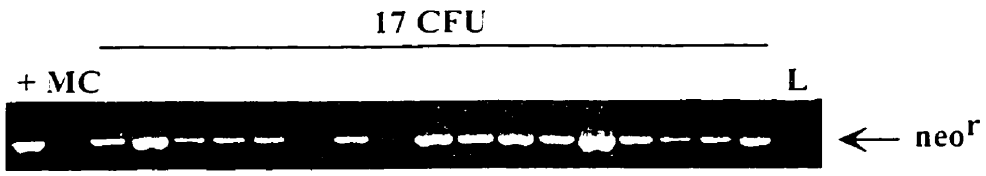

Figure 3.4: Neo^r PCR amplification of G418 resistant CFU from transduced LTMCs. This sample demonstrates 16/17 G418 resistant CFU positive for neo^r sequences. CFU were cultured in 2.0mg/ml G418. Abbreviations: L, DNA ladder; MC, methylcellulose control; +, 200pg positive control DNA. This assay was performed as described in Section 2.4.2.

The gene transfer efficiency was determined for each set of LTMCs from each dog. The average gene transfer efficiencies for each dog are shown in Table 3.1. On average, 32.4% (range: 15.1-50.9%), 39.6% (range: 27.8-48.1%) and 25.6% (range: 12-56.3%) of LTMC derived hematopoietic progenitors were resistant to 2.0 mg/mL G418 for N1, N2 and N3 respectively. The gene transfer efficiencies determined in this study were similar to those from previous canine gene transfer studies with the same LTMC gene transfer protocol[1,21]. The demonstration of gene transfer into hematopoietic progenitors indicated that infectious retroviral particles were present in the viral supernatant, but was not predictive of levels of gene transfer into HSCs.

Table 3.1: Summary of gene transfer efficiency in LTMC derived hematopoietic colonies measured by resistance to 2.0mg/mL G418.

	No. colonies resistant to G418/total number of colonies* (%)					
LTMC	1	2	3	4	5	Average†
N1	28/55 (50.9)	NE	24/120 (20.0)	13/86 (15.1)	13/30 (43.3)	32.4±8.7%
N2	NE	25/52 (48.1)	15/54 (27.8)	2/7 (28.6)	ND	39.6±6.0%
N3	3/15 (20.0)	12/79 (15.1)	3/25 (12.0)	15/61 (25.6)	18/32 (56.3)	25.6±8.0%
Untransduced control	0/21	0/33	0/20	0/20		0

Legend: *number of colonies from a total of $4x10^5$ cells plated; NE, not evaluable as colonies didn't grow; ND, a fifth marrow harvest was not performed:, †average ±standard deviation.

3.4.3 Autografts

On average, each unconditioned recipient was infused with a total of $1.0x10^9$ autologous LTMC cells (range $9.0x10^8$-$1.2x10^9$). In two dogs, N1 and N2, the cells were infused over four weekly infusions while N3 received five weekly infusions. The total cell dose per kg bodyweight was $7.0x10^7$, $5.3x10^7$ and $4.3x10^7$ for N1, N2 and N3 respectively (Figure 3.5).

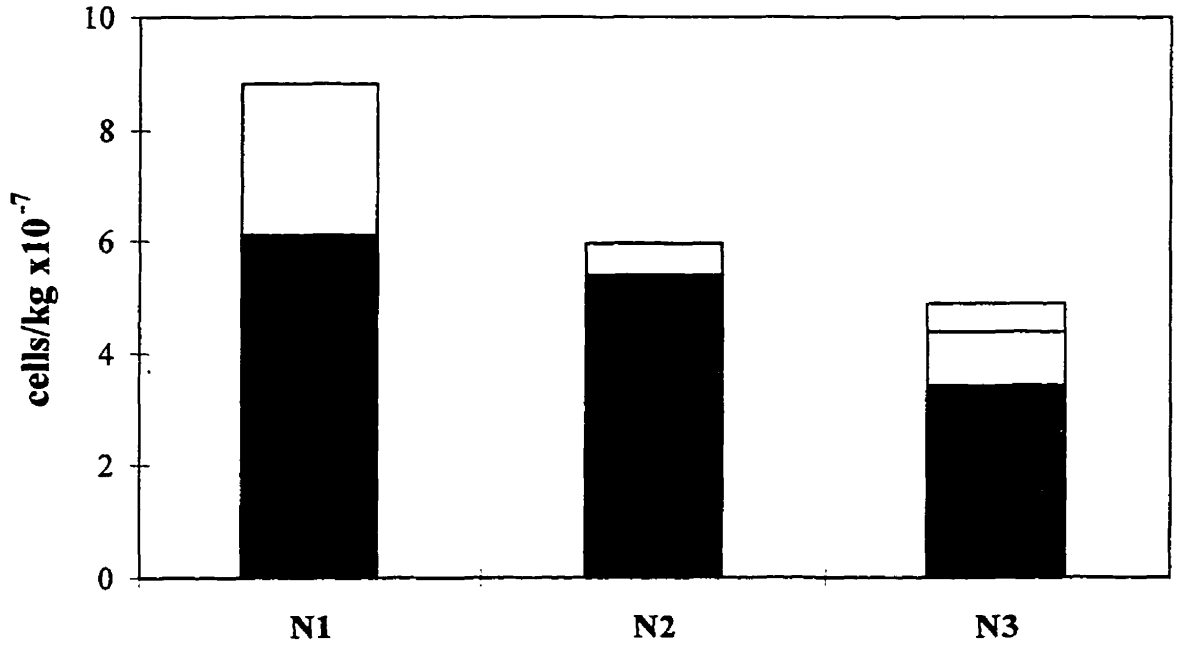

Figure 3.5: Summary of LTMC cells infused into normal dogs. Infusions were separated by one week. Each infusion is indicated by different a shading pattern.

3.4.4 Gene Transfer Into *In Vivo* Repopulating Cells

The engraftment of provirally marked primitive hematopoietic progenitors in unconditioned recipients was evaluated by assaying for the continued production of provirally marked progeny in recipients for 27-29 months post-infusion.

Proviral marking of hematopoietic colonies

The engraftment of provirally marked hematopoietic progenitors was evaluated by PCR analysis for neo^r sequences on individual hematopoietic colonies between 7-29 months post-infusion. The levels of proviral marking in colonies was similar among the 3 dogs with 3.5-10% of the colonies marked at up to 29 months post-infusion. In N3, for example, 6.6% (15/225) of colonies were neo^r positive during the 7-12 month period and the level remained fairly constant with 5.0% (7/140), 7.1% (4/56) and 5.6% (1/17) at 13-18, 19-24, and 25-29 months after infusion, respectively(Table 3.2). A sample PCR analysis from N1 at one year post-infusion is shown in Figure 3.6. Each assay included reagent and plucking methylcellulose controls with a total of 222 reagent, 96 methylcellulose and 104 random blinded untransduced control colonies. None were positive for proviral sequences.

Table 3.2: Detection of proviral sequences in marrow derived hematopoietic colonies by PCR analysis in months post-infusion.

		No. (%) of hematopoietic colonies positive for proviral sequences				
Dog	Infusions	Pre-infusion	1-12*	13-18	19-24	25-29
N1	4	0/21	10/121 (8.2)	18/298 (6.0)	4/45 (8.9)	3/77 (3.9)
N2	4	0/33	8/189 (4.2)	10/115 (8.7)	5/50 (10)	2/57 (3.5)
N3	5	0/24	15/225 (6.6)	7/140 (5.0)	4/56 (7.1)	1/17 (5.6)
Controls[†]	1		11%	3%	7%	

Legend: *Months post-infusion.[†] Data from controls (N=11) were previously published[1].

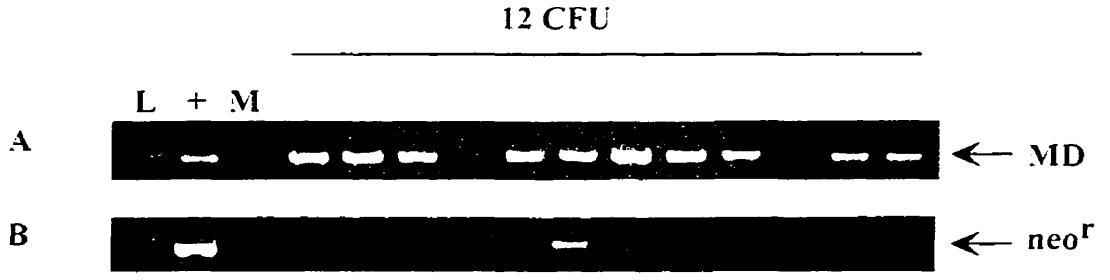

Figure 3.6: Neo^r PCR amplification of marrow derived CFU from N1 at 12 months post-infusion. This sample demonstrates 10/12 CFU positive for endogenous dystrophin (MD) sequences indicating amplifiable DNA in 10 CFU. 1 of 10 MD positive colonies was positive for neo^r sequences: L, DNA ladder; MC, methylcellulose control; +, 200pg positive control DNA. This assay was performed as descibed in Section 2.4.2.

During the neo^r PCR analysis of these colony samples, the same colony plucking, handling and PCR techniques were evaluated for the possibility of contamination of samples in an independent study by Drs. A. Keith Stewart and Ian Dubé at this Centre and Drs. Cindy Dunbar and John Tisdale at the N.I.H.[24]. No false positives were detected and positives correlated with expected results. The results of this validation study and the multiple negative controls included in the PCR analysis of these samples suggests that contamination during colony plucking, handling and PCR analysis was unlikely with the protocols used in this study.

PCR and Southern blot analysis

PCR amplification and genomic neo^r Southern blot analyses evaluated the proportion of neo^r positive cells in hematopoietic tissues post-infusion. Blood and marrow leukocyte samples from the first 12 months post-infusion were consistently positive for proviral sequences. During the second year after infusion samples from all dogs were intermittently positive for proviral sequences, indicating that at the later time points the proportion of positive cells approached the limit of detection for this PCR assay (~0.01%)(Table 3.3). Genomic Southern blot analyses on samples from the first six months post-infusion were all negative for proviral sequences (data not shown).

Table 3.3: Detection of proviral sequences by PCR amplification of neo^r in blood (PB) and marrow (BM) leukocytes post-infusion.

	Pre-infusion		1-12 Months		13-18 Months		19-24 Months		25-29 Months	
	BM	PB	BM	PB	BM	PB	BM	PB	BM	PB
N1	-	-	+	+	+	+	+	+	+/-	-
N2	-	-	+	+	+	+	+/-	+/-	-	-
N3	-	-	+	+	+	+	+/-	+	+/-	+/-

Legend: +, positive for neo^r sequences; +/-, at least one sample in this time period was positive and at least one negative; - negative for proviral sequences.

Proviral in situ PCR analysis

In situ neo^r PCR analysis on blood and marrow leukocytes detected neo^r positive cells at up to 29 months post-infusion in all three dogs. For example, analysis of leukocyte samples from N2 demonstrated 7% of marrow leukocytes marked at 6 months and the level dropped to 2-5% between 12-18 months and to <2% by 20-27 months (Figure 3.7). The controls in each experiment included: 100% positive Jurkatt cells, mix of 10% positive Jurkatt cells and 90% untransduced Jurkatt cells, and 100% negative Jurkatt cells. The controls included in each experiment were not scored blindly.

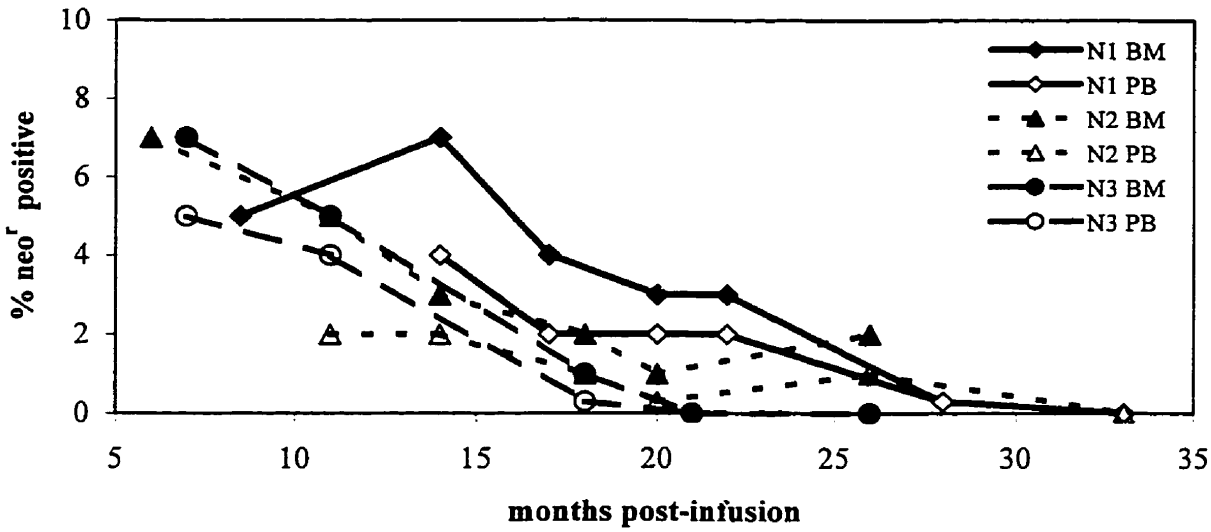

Figure 3.7: Detection of neo^r sequences in blood and marrow leukocytes from normal dogs by *in situ* PCR. A minimum of 300 cells were analysed for each time point. For each experiment, positive controls were 10% Jurkat neo^r positive: 90% Jurkat negative, and 100% Jurkat neo^r positive. Negative controls were 0% Jurkat negative cells.

Summary of engraftment data

Engraftment of provirally marked hematopoietic progenitors was demonstrated by the continued presence of provirally marked cells by several assays for greater than 2 years post-infusion. Between 3.5-10% of hematopoietic colonies carried proviral sequences for the evaluation period of 27-29 months. Direct quantitation of the proportion of blood and marrow leukocytes carrying proviral sequences was more difficult. *In situ* PCR data suggested that the level of proviral marking in leukocytes was ~5% during the first year and dropped off by the end of the second year to less than $\leq$1%. Genomic Southern blots were negative for proviral neo^r with sensitivity of 5%, indicating that there was less than 5% positive cells. In support of the genomic Southern data, semi-quantitative PCR indicated that ~1% of leukocytes carried provirus during the first year post-infusion and the level decreased to ~0.1-0.01% during the second year.

3.4.5 Immune Responses Against Graft Antigens

Enzyme linked immunosorbent assay (ELISA) was used to evaluate immune responses against putative graft antigens. Specific IgG directed against iduronidase, HoS, FBS and bovine serum albumin (BSA) were evaluated. Humoral immune responses against iduronidase protein were not detected in any dog. In contrast, specific antibodies to LTMC media components were detected in all dogs at similar levels, with representative data from N1 presented in Figure 3.8. The antibody titres against all LTMC media components peaked at the end of the series of infusions. The highest antibody titres in all dogs were against FBS with the peak levels at 1:8000 at one month post-first infusion in N1 and N2, and 1:16000 in N3. The anti-BSA titres were lower and peaked at 1:2000 in all dogs. The anti-HoS titres were intermediate with 1:4000 after all infusions, and dropping to 1:1000-2000 at later times.

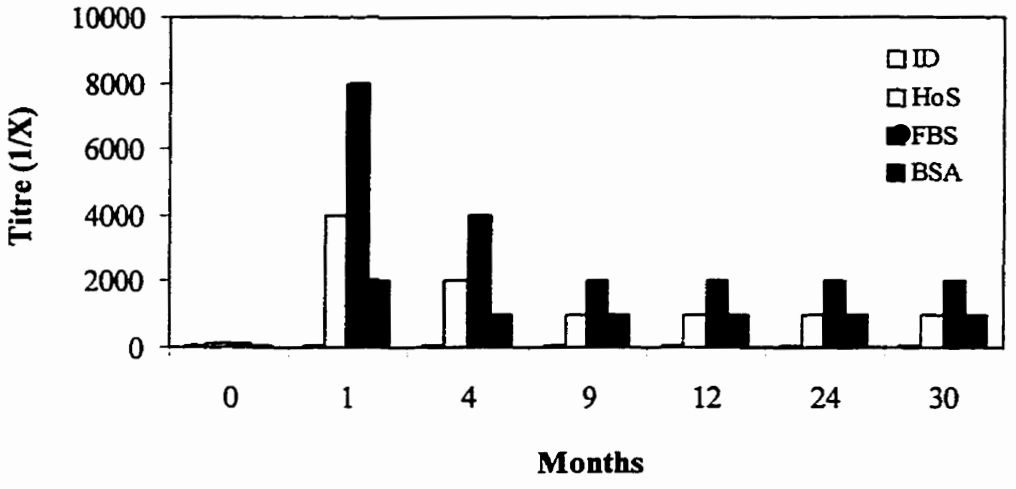

Figure 3.8: Detection of serum IgG specific for iduronidase (ID), horse serum (HoS), fetal bovine serum (FBS) and bovine serum albumin (BSA) in N1 by ELISA. Titres are shown as the dilution of serum which gave a corrected OD reading of >2X the dog's pre-infusion serum. Month 0 corresponds to immediately prior to the first infusion, and 1 month is at the time of the fourth infusion.

3.5 Discussion

The development of reliable approaches for transferring genes into HSCs is a requirement for the development of HSC gene therapy for most single gene disorders. A number of studies have demonstrated increased efficiency of gene transfer into HSCs when transduced in the presence of marrow stroma[1,14,21-23,25]. In a stromal based gene transfer approach developed at this Institution, marrow cells were efficiently transduced during LTMC[1,21]. In a previous study, Bienzle et al demonstrated that up to 5% of the marrow derived hematopoietic progenitors carried provirus at 18-24 months post-infusion, following a single infusion of 10^7-10^8 LTMC cells per kg into unconditioned canine recipients[1]. The current sets of experiments were designed to confirm the Bienzle study, which until very recently were not independently corroborated[26]. In the current studies the autograft infusion schedule was modified from a single infusion to multiple weekly infusions to cyclosporine A treated recipients.

The average cell and progenitor yields at marrow and LTMC harvest from each dog were within the range observed for other large scale harvests from normal dogs in other studies at this Institution[1,21,27] and elsewhere[28]. For example, in this study the average cell and progenitor recoveries at the end of LTMC for each dog were 9.1-19.7% and 3.1-7.0%, respectively. These yields are comparable to recoveries in the study by Bienzle and colleagues of canine LTMC gene transfer in which cell and progenitor recoveries at the end of LTMC were 12% and 7%, respectively[21].

During LTMC, the marrow cells were exposed to recombinant retroviruses carrying the bacterial neo^r gene. Several titration experiments were performed to determine which concentration of G418 was toxic to untransduced progenitors, yet enabled the growth of colonies expressing the neo^r gene. These experiments identified 2.0mg/mL as the optimal concentration of G418 in methylcellulose to evaluate the gene transfer efficiency to hematopoietic progenitors. On average, 31% of LTMC derived hematopoietic progenitors were resistant to 2.0mg/mL G418. This level of gene transfer is similar to two previous canine gene transfer studies at this Institution where on average 39% or 44% of the CFU-GM were resistant to G418[1,21]. The level of gene transfer into *in vitro* assayable progenitors using

the LTMC gene transfer protocol is favourable to canine gene transfer protocols at other centres in which ~10% of progenitors are routinely transduced[29,30]

All dogs engrafted with genetically modified hematopoietic progenitor cells capable of contributing to hematopoiesis for at least 2 years post-infusion. For example, between 5-10% of individual marrow derived hematopoietic colonies carried proviral sequences at ~2 years post-infusion. Provirally marked blood and marrow cells were detected by PCR analysis at up to 29 months post-infusion. These levels of engraftment are similar to the dogs receiving a single injection of LTMC cells in the previous study by Bienzle[1]. For example, all three dogs in the current study had 5-10% of hematopoietic colonies marked at time points up to and greater than 2 years post-infusion. These results were similar to those of Bienzle et al where 5-10% hematopoietic progenitors were resistant to G418 for 21-24 months in three of eight dogs[1]. Comparison of the engraftment in these 2 studies indicates that there is not increased engraftment with the cyclosporine-multiple infusion protocol. However comparison of larger numbers of animals would be necessary to distinguish a significant difference between the levels of engraftment seen in these two studies. The similar engraftment using these 2 infusion schedules is in contrast to murine studies where fractionating a dose of $2x10^8$ cells into five infusions of $4x10^7$ marrow cells resulted in significantly higher levels of engraftment than a single injection of $2x10^8$ cells in unconditioned recipients[31-33]. A major difference between these two studies is that a proportionately higher number of cells were infused into the mice, a total of ~10^{10}cells/kg compared to ~10^7 cells/kg in the dogs. Other differences between the studies include fresh uncultured/untransduced marrow and daily infusion in the murine study and LTMC cultured/transduced cells with weekly infusion in the canine study.

The recipients in this study did not receive marrow conditioning prior to infusion, as earlier studies at this Institution demonstrated that unconditioned recipients had similar or higher levels of engraftment than those receiving lethal total body irradiation[21]. The results described in this chapter support the earlier studies by demonstrating that unconditioned dogs engrafted with provirally marked cells for at least 2 years post-infusion. The engraftment of gene marked canine hematopoietic progenitors in unconditioned recipients in this study and the study by Bienzle et al[1] are in direct contrast to other canine studies. For example,

Schuening and colleagues have also evaluated the effects of increasing doses of myelosuppression on the engraftment of gene marked cells in autologous canine recipients[30]. In that study, mobilised peripheral blood cells were stimulated with recombinant cytokines and transduced by co-culture with retrovirus producer cells for 48 hours followed by supernatant transduction for 4 to 7 days. Evaluation of the percentage of granulocyte samples provirus positive at >4 weeks post-transplant indicated the highest level of engraftment with dogs conditioned with sub-lethal total body irradiation. Gene marked cells were not detected in samples from the unconditioned recipients[30]. A number of differences in the experimental designs may account for the different results in unconditioned recipients such as the starting population of hematopoietic cells, transduction protocol and assays and length of follow up. The inclusion of growth factors in Schuening et al's study may have inhibited the engraftment into unconditioned recipients. Murine unconditioned transplant studies have shown that incubation with recombinant cytokines prior to infusion results in significantly lower levels of engraftment than infusion of cells not treated with cytokines prior to infusion[34,35].

Hematopoietic cell engraftment has been observed in the absence of marrow ablative conditioning in humans including: recipients of liver transplants can also become hematopoietic chimaeras due to the presence of HSCs in the graft[36], women carrying, or who have carried a male fetus may have circulating male hematopoietic cells for up to 27 years[37], and patients with SCID undergoing allogeneic bone marrow transplantation engraft without conditioning[38]. Several human hematopoietic cell gene transfer studies have also demonstrated that retrovirally transduced autologous marrow can engraft in unconditioned patients with adenosine deaminase deficiency[39-42], and chronic granulomatous disease[43]. Thus, the demonstration of successful unconditioned hematopoietic cell transplantation in the current study supports an expanding body of data and observations indicating that ablative marrow conditioning is not required for hematopoietic stem cell engraftment.

Higher levels of proviral marking were observed in progenitors (~5-10%) than circulating hematopoietic cells (0.01-1%) in this study. This proportional difference has also been identified in the human clinical gene transfer studies at this Institution[24] and elsewhere[41,42,44,45]. In a study by Brenner and colleagues for example, 1-10% of progenitors

and ~0.01% circulating leukocytes were provirally marked for up to 4 years in pediatric recipients of transduced HSCs[44,45]. A similar phenomenon was also described in pediatric ADA patients infused with retrovirally transduced cord blood[41,42]. The biological basis for this difference is unknown. One hypothesis is that differentiated cells carrying or expressing proviral genes may have shortened survival due to immune responses against the transgene or viral peptides. However, a study by Dunbar and colleagues suggests that immune responses against the transgene is an unlikely cause of this discrepancy[46]. Cohorts of mice were transplanted with syngeneic marrow transduced either with a neo^r containing vector or an identical vector in which the translation start site was mutated. Similar levels of provirally marked differentiated cells were observed in recipients of both vectors suggesting that neither expression of proviral neo^r nor immune responses against it are responsible for differences in levels of marking[46]. Alternatively, it has been suggested that the observed differences in engraftment and maintenance of progenitors and differentiated cells may result from altered or blocked differentiation pathways in progenitors carrying proviral sequences.

Another hypothesis suggests that the difference is a feature of normal biological characteristics of these cell populations and not as a result of genetic marking. The results of an unconditioned murine transplant study by Wu and Keating supports this hypothesis[47]. In this study, female recipients had an average of 7.5% donor-derived (male) hematopoietic progenitors and less than 0.01% donor-derived circulating cells[47]. However, further understanding of the normal hematopoietic differentiation pathway and evaluation of effects of retroviral transduction and proviral gene expression on this process is needed to determine the cause of this phenomenon.

One concern in unconditioned transplants is the induction of immune responses against the transgene[48] or other graft antigens[49-50] in recipients. For example, following detection of immune responses against a neo^r-thymidine kinase transgene[48] or fetal bovine serum media components[49] subsequent infusions of gene modified cells failed to engraft. In the experiments in this chapter, recipient dogs were treated with post-infusion immunosuppressive therapy to abrogate immune responses against autologous transduced autograft cells. Humoral immune responses against LTMC media components and iduronidase were evaluated in the dogs in this study. Antibodies against iduronidase were not

observed, however, high titres of specific IgG against FBS and HoS, and BSA were detected. Humoral immune responses against neo^r protein were not evaluated since neo^r ELISA assays are known to be unreliable, however cellular immune responses against neo^r were not detected in the canine gene transfer study presented in Chapter 4 suggesting that neo^r protein has low antigenicity[50]. The presence of anti-FBS and anti-HoS antibodies did not prevent the maintenance of provirally marked cells for greater than two years.

The results of this study indicate that long-lived hematopoietic progenitors are transduced with recombinant retroviruses during LTMC. These transduced primitive progenitors can engraft and contribute to hematopoiesis in autologous unconditioned recipients for at least two years post-infusion. Fractionation of the LTMC graft into 4-5 weekly infusions and cyclosporine A treatment of recipients resulted in engraftment of 5-10% provirally marked progenitors for at least 2 years. The engraftment in these animals is comparable to infusion of similar numbers of cells in a single infusion without cyclosporine A treatment in previous studies. The high level of long term engraftment of provirally marked progenitors in normal dogs is within the range thought to be therapeutic for a number of single gene inherited disorders such as the lysosomal storage diseases[2]. The experiments presented in chapter 4 evaluated the therapeutic potential of HSC gene transfer to alleviate disease in a canine model of iduronidase deficiency.

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CHAPTER 4

Genetically Corrected Autologous Stem Cells Engraft, But Host Immune Responses and Low Transgene Expression Limit Their Utility for Canine α-L-Iduronidase Deficiency.

4.1 Summary

HSC gene transfer is being developed as a treatment for genetic disease. In experiments described in the previous chapter, canine hematopoietic progenitors and stem cells were genetically modified during long-term marrow culture (LTMC) and contributed to hematopoiesis for greater than 2 years post-infusion. In the current set of experiments, the therapeutic potential of HSC gene transfer for a canine model of a single gene disorder, α-L-iduronidase (iduronidase) deficiency was evaluated.

Large volume marrow collections were performed on five iduronidase deficient dogs. Marrow mononuclear cells were established in LTMCs and exposed on three occasions during three weeks of culture to retroviral vectors bearing the normal canine iduronidase cDNA. Transduced LTMC cells from deficient dogs expressed enzymatically active iduronidase at 10-200 times the levels seen in normal dogs. An average of 32% of LTMC-derived clonogenic hematopoietic cells were provirus positive by PCR and about half of these expressed iduronidase. Approximately 10^7 autologous gene-modified LTMC cells/kg were infused into non-myeloablated recipients. Proviral DNA was detected in up to 10% of individual marrow-derived hematopoietic colonies and blood and marrow leukocytes for up to 3 years. Despite good evidence for engraftment of provirally marked cells, neither iduronidase enzyme nor proviral transcripts were detected in any dog. Immune responses against iduronidase protein, iduronidase transduced cells, and culture media serum components were evaluated. Humoral responses to iduronidase protein, fetal bovine and horse sera, and bovine serum albumin were identified by ELISA in all dogs. Cellular immune responses to autologous iduronidase but not neo^r transduced cells were demonstrated by lymphocyte proliferation assays. To abrogate potential immune phenomena, four affected dogs received post-transplant cyclosporine A. While immune responses were dampened in these dogs, iduronidase activity remained undetectable. In none of the dogs engrafted with genetically corrected cells was there evidence for clinical improvement. These data suggest that while the iduronidase cDNA may be transferred to long lived hematopoietic progenitors the potential of this approach appears limited by the levels of provirally derived enzyme that are expressed *in vivo* and by the host's response to cultured and transduced hematopoietic cells expressing foreign proteins.

4.2 Introduction

Gene therapies delivered by hematopoietic stem cells (HSCs) offer several theoretical advantages over other methods of drug delivery. These include *in vivo* production of a wide variety of therapeutic agents derived from DNA permanently integrated into the genomes of cells with life-long capacities for extensive proliferation and multilineage differentiation[1]. Recent progress towards this goal has been tenuous, with apparent advances generally followed by the identification of largely unforeseen obstacles. To date there is limited evidence that genetically modified HSCs can actually be of therapeutic benefit in human disease or in large animal models[2-4].

Over the last decade, several approaches have been taken to develop HSCs as vehicles for gene delivery. These include the use of HSC-enriched cell populations[5] and methods for selecting gene-modified HSCs[6]. One recurring concern is that HSCs, in contrast to their committed progeny, appear to express low levels of the receptor required for successful gene transfer mediated by the retroviral vectors used in most applications[7-9]. Another problem limiting practical applications of HSC gene transfer is that the vast majority of HSCs are likely to be quiescent[10] and consequently resistant to stable gene transfer. Furthermore, the likelihood that any given gene-modified HSC would be among the small minority that undergo expansion *in vivo* under steady-state conditions seems remote[11,12].

Other researchers have focused on maintaining HSCs *in vitro* and inducing them to exit quiescence and enter the cell cycle. Data from a number of studies have demonstrated that exposure of hematopoietic cells to retroviral vectors in the presence of marrow-derived stromal cells resulted in efficient gene transfer to committed progenitors[13-17]. In a canine model system, autologous marrow, transduced by multiple exposures to retroviral vectors in long-term marrow cultures (LTMC) engrafted in the absence of myeloablative conditioning and gave rise to reporter gene-marked committed progeny that were maintained at ~5% levels for up to two years[15]. Similar results were more recently obtained from the canine gene transfer studies described in chapter 3[18] and a human clinical trial of LTMC stem cell gene marking in multiple myeloma[19]. These studies suggested that while the goal of achieving large populations of genetically modified hematopoietic cells *in vivo* remains elusive, the

levels of gene transfer achievable using current technology may alleviate disease symptoms in some deficiency disorders.

It has been suggested that a good model system to evaluate the potential clinical benefit of low numbers of genetically corrected hematopoietic cells would be an enzyme deficiency with the following characteristics: the disorder is a single gene defect for which the normal cDNA is cloned; there is a wide range of enzyme levels compatible with a normal or mild phenotype; bone marrow transplantation and/or recombinant enzyme infusion is of clinical benefit. Canine iduronidase deficiency meets these criteria. In this autosomal recessive disorder there is a complete deficiency of iduronidase due to a single base substitution in the iduronidase gene which disrupts intron I splicing and gives rise to a stop codon[20]. Dogs homozygous for the iduronidase mutation do not have any iduronidase enzyme as determined by either iduronidase activity or immunoprecipitation[21]. Iduronidase deficiency results in lysosomal accumulation of heparan and dermatan sulfate glycosaminoglycans in many tissues including the central nervous system[22,23]. Iduronidase deficient dogs are clinically similar to human patients with the Hurler-Scheie phenotype of mucopolysaccharidosis type I (MPS I) with progressive cardiac abnormalities, corneal clouding, stunted growth and degenerative joint disease, all of which progress to severe states within 2-3 years[22,23].

The canine iduronidase cDNA has been cloned and characterised[24]. An oligonucleotide developed from the protein sequence was used to probe a canine testis cDNA library and three partial cDNAs were aligned to create a composite full length iduronidase cDNA[24]. The cloning of the canine iduronidase cDNA has facilitated the development of retroviral vectors carrying and expressing normal iduronidase.

There is a wide range of iduronidase enzyme levels compatible with a normal or mild disease. For example, phenotypically normal heterozygotes with a mutant allele and a non-pathologic allele have been identified with ~1% of normal iduronidase activity[25]; iduronidase deficient patients with as little as 0.1% of the normal iduronidase activity have Scheie syndrome with significantly milder disease symptoms than patients with no detectable iduronidase activity[26,27].

Canine iduronidase deficiency has been treated by allogeneic bone marrow transplantation and enzyme replacement therapy. The rationale for developing these treatments was based on the demonstration by Neufeld and colleagues that normal cells secrete iduronidase and deficient cells can uptake enzyme and utilise it to decrease storage[28,29]. In canine bone marrow transplantation studies, three MPS I dogs receiving allogeneic marrow transplantation engrafted with normal marrow and had greatly improved clinical and biochemical outcomes. For example, cardiovascular, renal and hepatic tissues had significantly decreased lysosomal glycosaminoglycan accumulation[30,31]. Dogs receiving weekly infusions of recombinant iduronidase for up to one year demonstrated improved clinical outcomes despite the induction of complement activating antibodies. Decreased lysosomal storage was detected histologically in liver, kidney, spleen, lymph nodes, synovium and lung[32,33]. However, histologic improvement was not detected in brain, cartilage or heart valve[32].

In previous experiments described in chapter 3, hematopoietic progenitors genetically modified during LTMC contributed to hematopoiesis for at least 2 years post-infusion in canine recipients. The experiments undertaken in this chapter were designed to evaluate whether adoptive transfer of hematopoietic progenitors and stem cells, genetically modified to carry the normal canine iduronidase cDNA, to MPS I dogs could result in *in vivo* delivery of enzyme and prevention of severe clinical symptoms.

4.3 Results

4.3.1 Mating and Genotype Analysis

This set of experiments utilised five iduronidase deficient dogs. Three MPS I dogs, M2, M3 and M4 were bred and genotyped at the University of Tennessee by Dr. Robert Shull and were transported to the Ontario Veterinary College at 2-6 months of age prior to the initiation of studies. During this time an MPS I breeding colony was also established at the Ontario Veterinary College by Drs. Kruth and Abrams-Ogg with one male and two female iduronidase heterozygotes donated by Dr. Shull.

For this experiment both female heterozygotes were mated to the male heterozygote and each produced a litter of four pups. Genotype analysis of the litter 'A' detected one homozygous

iduronidase mutant genotype (-/-), one heterozygous (+/-) and two wildtype (+/+) normal pups. Leukocyte expression of iduronidase was also examined. As expected, M5, the dog with two mutant iduronidase genes demonstrated 0 units (nmol substrate/mg protein/hr) iduronidase activity, while samples from the other dogs were in the normal range of 1.1-1.9units (Table 4.1). In the second or 'B' litter, genotype analysis detected one homozygous iduronidase mutant genotype, one wildtype and two heterozygous pups. Assay of iduronidase activity in leukocytes from 'B' litter dogs demonstrated 0 units of activity in the sample from the homozygous iduronidase mutant dog, M6, and normal iduronidase activities of 0.2-1.1units in the three other dogs (Table 4.1; Figure 4.1).

Table 4.1: Iduronidase genotype analysis on pups resulting from the mating of iduronidase heterozygotes.

Litter	Dog	Genotype	Iduronidase activity
A	M5	-/-	0u
	A1	+/+	1.7u
	A2	+/+	1.9u
	A3	+/-	1.1u
B	M6	-/-	0u
	B1	+/-	0.2u
	B2	+/-	1.0u
	B2	+/+	1.1u

Legend: +, wildtype allele; -, mutated MPS I allele; u, units, nmol/mg/hr.

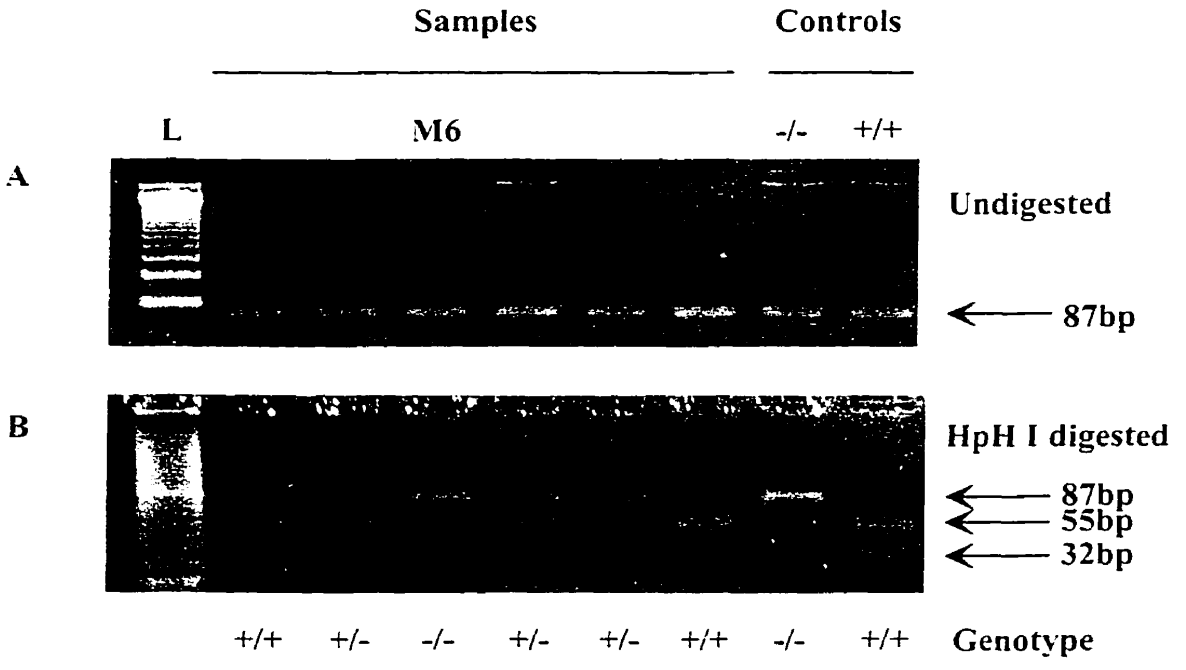

Figure 4.1: Iduronidase genotype PCR analysis of M6 and littermates. A) PCR amplification of an 87bp genomic iduronidase sequence with CA1-CA2 primers. B) Analysis of PCR products following digestion with HpHI. PCR amplicons from the MPS I iduronidase allele do not digest with HpHI and those from wildtype iduronidase allele digest with HpHI into 55bp and 32bp fragments. Controls included samples from wildtype (+/+) and MPS I (-/-) dogs. Abbreviations: L, DNA ladder. Genotypes shown at bottom: wildtype, +/+; +/-, heterozygote; -/- MPS I heterozygote. This assay was performed as described in Section 2.4.1.

4.3.2 Marrow Harvests and LTMCs

Large volume marrow harvests were performed on five MPS I affected juvenile dogs (~2-8 months of age). The first dog, M2, had marrow harvested on three occasions. For all other dogs multiple harvests were performed only when yields of marrow mononuclear cells were less than 10^8 cells per kg. The time intervals between collections ranged from 1 to 3 months and in all cases subsequent harvests were done after blood cells counts had normalised. Three dogs, M3, M5 and M6, underwent one marrow harvest, while M4 and M2 underwent two and three marrow aspirations respectively.

On average, $1.0x10^8$ (SD: $\pm 5.1x10^7$) mononuclear cells per kg and $4.9x10^4$ (SD: $\pm 4.4x10^4$) CFU-GM per kg were obtained from each MPS I marrow sample after density gradient separation and washing. Marrow mononuclear cells were established in LTMCs and maintained for three weeks as described in Section 2.2.2. The cell and progenitor recoveries at the end of the three week LTMC and transduction period were $1.5x10^7$ cells (SD: $\pm 1.9x10^7$) per kg and $6.8x10^3$ CFU-GM (SD: $\pm 6.8x10^3$) per kg corresponding to 15% and 14% recovery of cells and CFU-GM respectively (Figure 4.2).

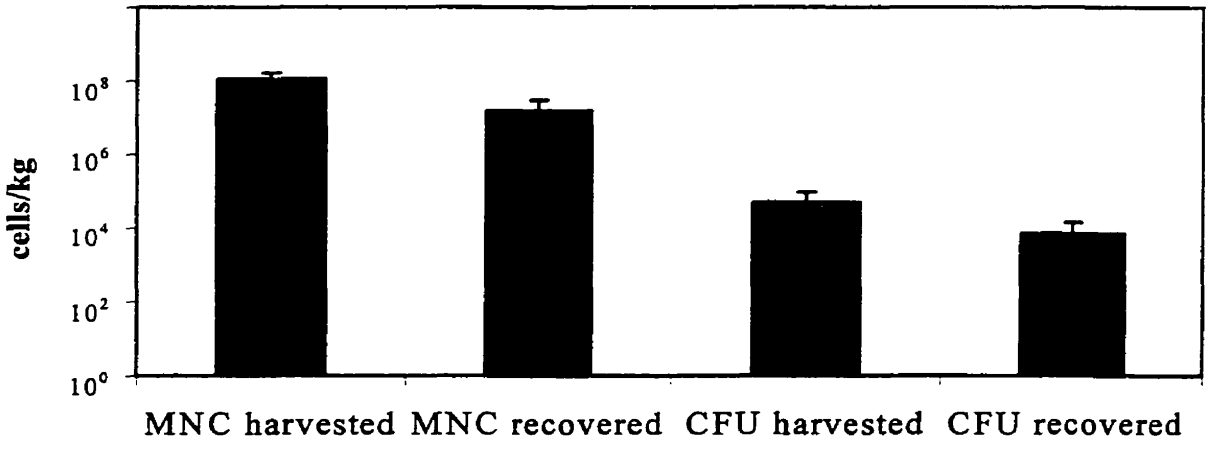

Figure 4.2: Average mononuclear cell (MNC) and hematopoietic progenitor (CFU) yields from marrow harvests (harvested) and recovered at the end of 21 days of LTMC (recovered) from 8 marrow harvests from MPS I dogs. Means with standard deviations are shown.

4.3.3 Exposure to Retroviral Containing Supernatant During LTMC

LTMCs were transduced by three exposures to retroviral vectors bearing the canine iduronidase cDNA as described in Section 2.2.2. During three weeks of culture, LTMCs from M3, M4 and M5 were transduced with M48ID. For the remaining two dogs, LTMCs from the first two marrow harvests from M2 were transduced with M48ID, and the third with LCIDSN, while for M6, approximately one-half of the LTMCs were transduced with each vector. Aliquots of retroviral and LTMC supernatants were routinely tested for the presence of RCR. All samples were negative.

4.3.4 Gene Transfer and Proviral Expression in LTMC Cells

LTMCs were assayed for levels of retroviral gene transfer and proviral gene expression with the data summarised in Table 4.2. Successful transduction of LTMC cells was confirmed by the detection of proviral DNA by PCR in cells from all adherent layers (Figure 4.3). Transduced LTMCs were assayed for proviral iduronidase expression. LTMC cells from transduced cultures had iduronidase activities of 10-198 units, corresponding to ~10-100 times normal levels. No iduronidase activity was detected in untransduced MPS I control LTMC cells. All transduced LTMCs had proviral iduronidase transcripts by RT-PCR (Table 4.2).

To evaluate the levels of gene transfer and expression in the hematopoietic progenitor sub-population of LTMCs, individual hematopoietic colonies were assayed. In test experiments, 75-100% of CFU-GM from normal marrow samples were positive for iduronidase activity, while no activity was detected in any of 60 CFU-GM from untransduced MPS I marrow samples, or methylcellulose controls. A sample was scored as positive when its fluorescence was 2-fold higher than methylcellulose and reagent controls. Iduronidase activity was not consistently detected in normal BFU-E (Table 4.3). Only CFU-GM were assayed for iduronidase activity in these experiments. The average percentage of LTMC-derived CFU-GM positive for provirus was 32.8% (range: 20-62%) (Table 4.2). Iduronidase activity was also assayed in LTMC derived CFU-GM and on average 15% (range: 8-20%) of CFU-GM from transduced LTMCs had proviral iduronidase activity. The range of iduronidase activity in colonies from normal marrow samples was 71-511 raw fluorescence units. Iduronidase

activities from transduced MPS I LTMC derived colonies were similar 76-412 raw fluorescent units. Examination of the proviral marking and expression data from individual colonies indicates that approximately half of number of progenitors carrying proviral sequences express iduronidase activity.

Table 4.2: Evaluation of gene transfer and proviral gene expression into canine MPS I LTMC adherent layers and individual CFU-GM derived from transduced LTMCs.

	Adherent layer Cells			LTMC derived CFU-GM	
	PCR	iduronidase	RT-PCR	PCR (%)	iduronidase (%)
M2	+ve	>10u	+ve	13/21 (62)	5/30 (17)
M3	+ve	101u	+ve	4/15 (27)	2/25 (8)
M4	+ve	84u	+ve	3/15 (20)	5/32 (16)
M5	+ve	198u	+ve	5/21 (24)	2/10 (20)
M6	+ve	100u	+ve	13/42 (31)	ND
Untransduced control	-ve	0u	-ve	0/20	0/12

Legend: +ve, positive; -ve, negative; iduronidase, iduronidase activity; u, nmol/mg/hr, normal activity=~1u; PCR, PCR amplification of proviral neo^r or iduronidase sequences; ND, sample not done; Untransduced control, untransduced canine LTMC control.

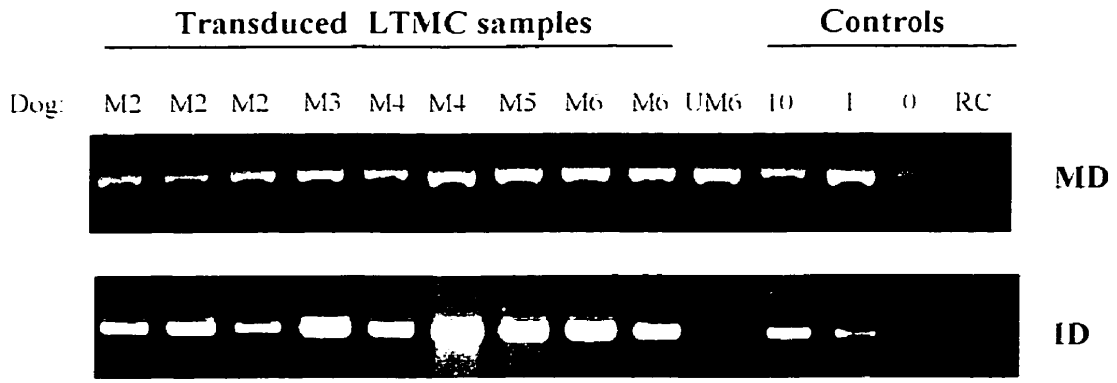

Figure 4.3: PCR amplification of endogenous dystrophin (MD) and proviral iduronidase (ID) sequences from day 21 LTMC cells. Dog M2 had 3 LTMC samples, and dogs M4 and M6 had 2 LTMC samples. Positive controls were: 10, 1 and 0% positive control M48ID DNA mixed with untransduced canine DNA. Legend: untransduced LTMC DNA from M6 (UM6); reagent control (RC). This reaction was performed as described in Section 2.4.2.

Table 4.3: Detection of iduronidase activity in individual hematopoietic colonies from normal and MPS I marrow samples*.

	Number of CFU positive for proviral sequences / total CFU analysed (%)					
	Negative controls		Normal dog samples		MPS I dog samples	
Expt	Plucking	Tissue	CFU-GM	BFU-E	CFU-GM	BFU-E
1	0/9	0/10	3/4 (75)	1/6 (17)	0/16	0/14
2	0/13	0/32	9/12 (75)		0/16	
3	0/12	0/17	3/3 (100)	3/11 (27)	0/12	0/16
4	0/4		4/4 (100)	1/6 (17)	0/16	0/14

Legend: Plucking controls, aliquots of background methylcellulose, Tissue, tissue samples incubated without substrate; *Colonies were scored positive when the raw fluorescence reading was greater than 2-fold higher than methylcellulose and reagent controls.

The ability of provirally derived iduronidase to undergo normal post-translational processing, cellular trafficking, secretion by transduced cells, and uptake by deficient cells was evaluated. In this experiment untransduced MPS I LTMCs were cultured on collagen coated 0.22μ filter inserts suspended over iduronidase transduced MPS I LTMCs (as described in Chapter 2.2.3 and Figure 2.1). Physically separating the cell populations by a 0.2μ filter insert allowed diffusion of nutrients, media and proteins without mixing of the cell populations. The mean iduronidase activity in M48ID and LCIDSN transduced MPS I LTMCs from 3 experiments were 79.5u (SD: ±24.1) and 30.0u (SD: ±7.0) respectively. In untransduced and LNc11 transduced controls activities were 0.17u (SD: ±0.15) and 0.28u (SD: ±0.08). Untransduced MPS I LTMC cells expressed 30.9u (SD: ±5.8) of iduronidase activity after 5 days of co-culture with M48ID transduced MPS I LTMC cells (Figure 4.4). This demonstrates that transduced cells can secrete significant amounts of iduronidase, which can be taken up by deficient cells. Control samples in which untransduced MPS I LTMC cells were co-cultured with LN transduced MPS I transduced cells had 0.2u (SD: ±0.12) of enzyme activity (Figure 4.4). Untransduced LTMCs from normal dogs had an average activity of 2.9u (SD: ±1.2).

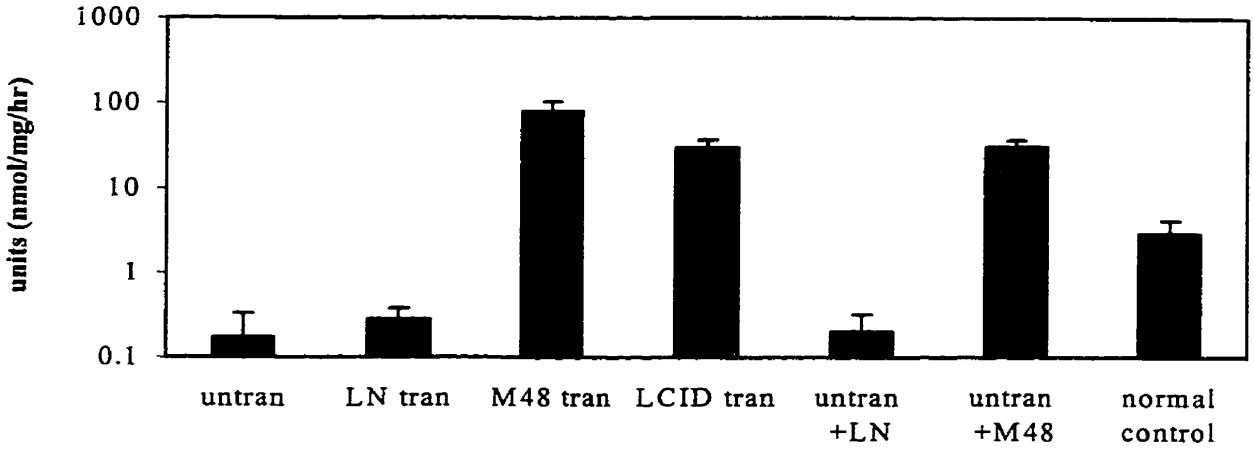

Figure 4.4: Transfer of iduronidase enzyme from iduronidase transduced LTMC cells to untransduced MPS I LTMC cells through a 0.2μ filter. All LTMCs were MPS I except for the normal control. Abbreviations for transduced cultures: untransduced LTMCs (untran), transduced LTMCs (tran); LNc11 transduced LTMCs (LN tran); M48ID transduced LTMCs (M48 tran); and LCIDSN transduced LTMCs (LCID tran). For co-culture experiments, enzyme activity in untransduced LTMCs which had been co-cultured with: M48ID transduced (untran +M48 tran); LCIDSN transduced (untran +LCID); and LNc11 transduced (untran + LN) LTMCs. Means from three experiments and standard deviations are shown.

4.3.5 Autografts

An average of 1.67×10^7 LTMC cells per kg (range: 9.6×10^6 to 3.1×10^7 cells per kg) were infused into each unconditioned autologous recipients (Figure 4.5). Three dogs, M3, M5 and M6 each received one and M4 received two infusions of iduronidase transduced LTMC cells. After M2 received three infusions of transduced cells, treatment with cyclosporine A and prednisone was initiated and two more infusions of 5×10^6 LTMC cells were given. All other dogs received immediate post-transplant cyclosporine A and M6 received, in addition methotrexate[34].

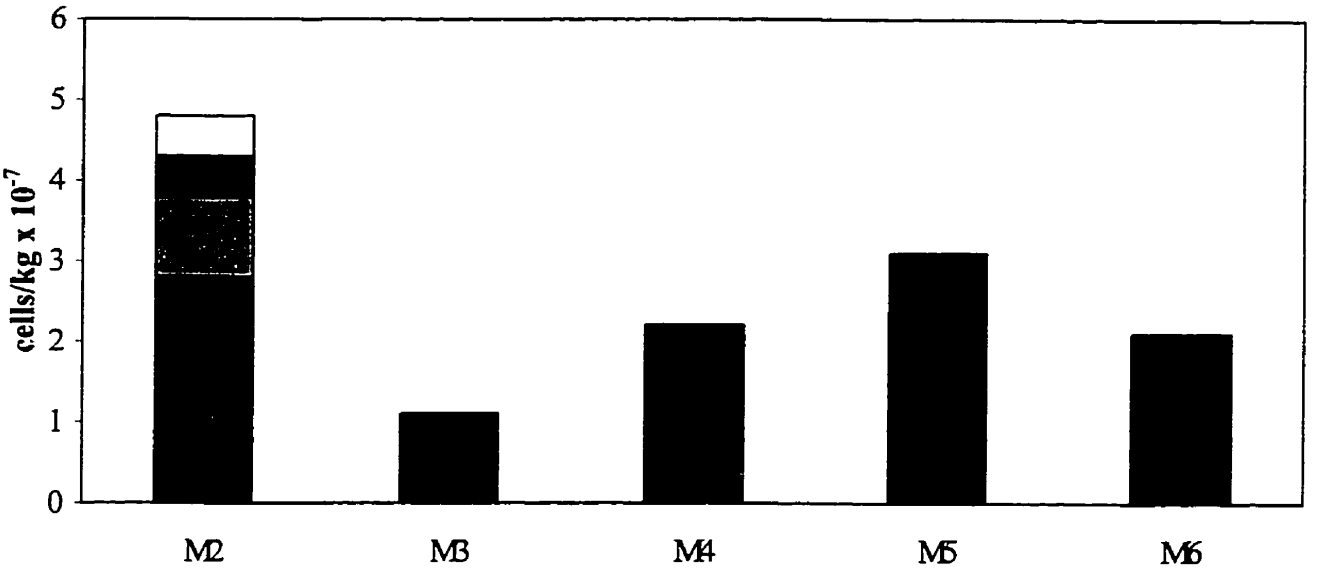

Figure 4.5: Summary of LTMC cells infused per kg into MPS I dogs. Multiple infusions in M2 and M4 were separated by 3-4 weeks. Cell doses given in multiple infusions are indicated by different shading patterns.

4.3.6 Gene Transfer into *In Vivo* Repopulating Cells

To assess the engraftment of provirally marked progenitors PCR analyses for proviral sequences were performed on blood and marrow samples from dogs at up to 36 months post-infusion.

The proportion of differentiated hematopoietic cells carrying proviral sequences was determined by neo^r *in situ* PCR on marrow and blood leukocytes from the two dogs receiving the neo^r containing LCIDSN, M2 and M6. Provirus positive cells were detected in leukocytes from the blood and marrow at time-points extending to 2 years (Table 4.4). There was a general trend to higher levels in the one to three month follow-up samples (2-9%) than in those samples obtained between one and two years post-infusion (0.3-7%). For example, neo^r marked cells were highest at 1-3 months post-infusion in M6 at 9% and 6% for blood and marrow leukocytes and steadily dropped to 6% and 5% at 4-6 months post-infusion and ~3-1% at 7-13 months.

For each sample, at least 300 cells were analysed. Controls were: pre-infusion samples, negative untreated dog samples, untransduced Jurkat cells, Jurkat mix of 10% neo^r positive and 90% untransduced Jurkat cells, and 100% positive Jurkatt cells. A discussion of the validity of *in situ* PCR is presented Section 2.4.5. In general, in samples with less than 10% cells positive, there is great variability in the scored positive cells and the actual mixed percentage of positive cells with the scored value tending to overestimate the proportion of positive cells. Thus, it is likely that the *in situ* PCR results overestimate the actual proportion of provirally marked cells. Genomic Southern blot analysis of proviral neo^r or iduronidase sequences on blood and marrow samples from M2 were negative with an assay sensitivity of ~10% (data not shown).

Engraftment of provirally marked cells was confirmed by semi-quantitative neo^r PCR analysis on blood and marrow mononuclear leukocytes from M2 (Figure 4.6A). The proportion of blood and marrow leukocytes carrying the proviral genome was ~1% at time points up to 26 months post-infusion. The levels of neo^r positive mononuclear cells generally dropped to ~0.1% at 28 months post-infusion, and were undetectable at 36 months.

Table 4.4: The percentage of cells positive for proviral neo^r sequences by neo^r *in situ* PCR in peripheral blood (P) and bone marrow (M) leukocytes from M2 and M6 post-infusion.

	Percentage cells positive for neo^r by *in situ* PCR											
Months*	Pre-infusion		1-3		4-6		7-12		13-18		19-24	
	P	M	P	M	P	M	P	M	P	M	P	M
M2	0	0	6	2	3	3	4	8	4	5	3	1
M6	0	0	9	6	6	5	3	1	4	1	NA	NA

Legend: *, time points are calculated from last infusion; NA, sample not available; Controls in each experiment were mixes of 100%, 10% and 0% neo^r positive and negative Jurkat cells.

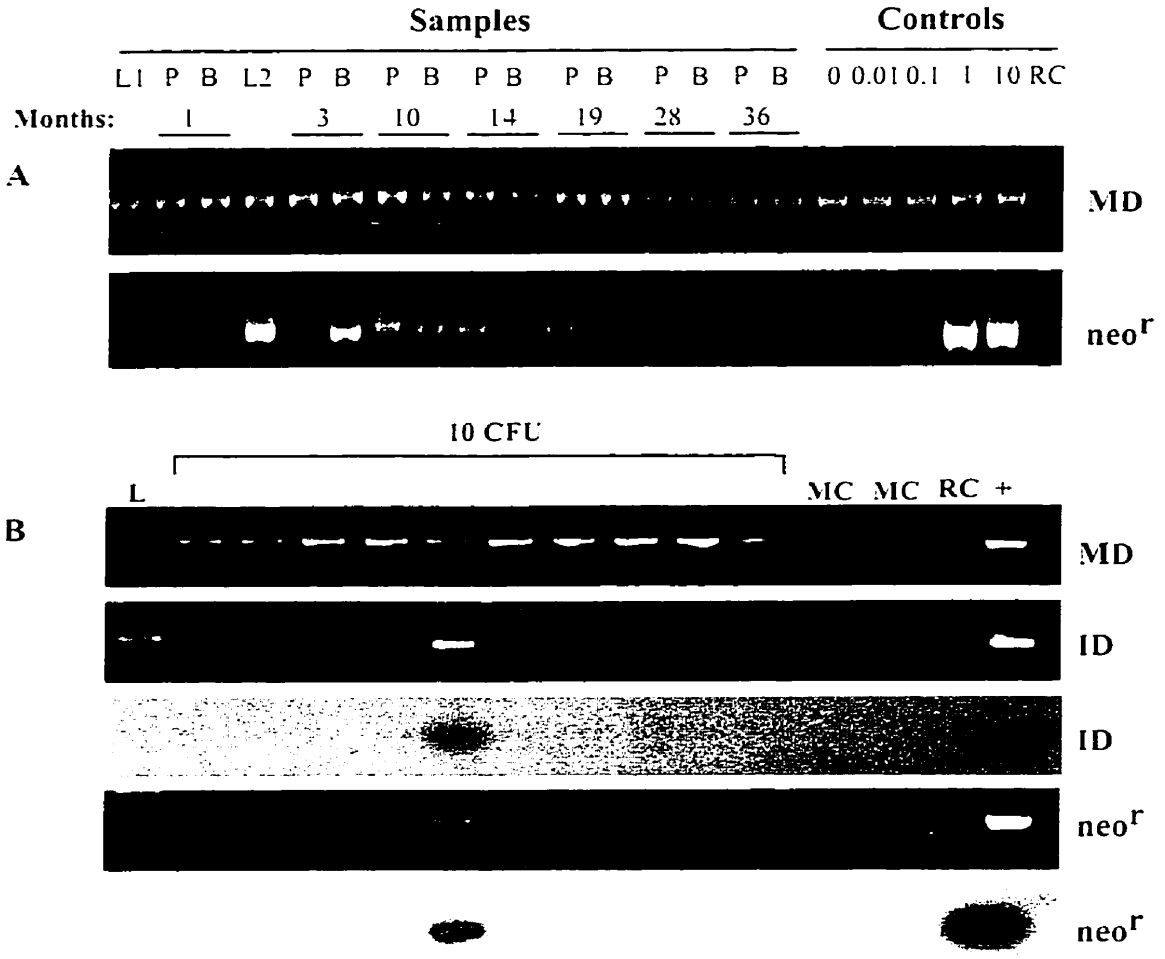

Figure 4.6: PCR amplification and Southern blot analysis of dystrophin (MD) and proviral iduronidase (ID) and neo^r sequences from M2. A) Dystrophin PCR analysis for each sample is shown in top panel. Semi-quantitative neo^r PCR on LTMCs transduced with M48ID (L1) and LCIDSN (L2), post-infusion peripheral blood (P) and bone marrow (B) cells from M2. Positive controls were 0.01, 0.1, 1, and 10% positive DNA mixed with untransduced canine DNA. B) PCR amplification and Southern blot analysis of CFU from M2 at 1 year. Ten CFU were positive for MD and 1 CFU positive for both ID and neo^r sequences. Abbreviations: L, DNA ladder; MC, methylcellulose control; RC, reagent control; +, 200pg positive control DNA; mos, months post-infusion. PCR was performed as descibed in Section 2.4.2 and Southern blotting as described in Section 2.4.3.

The proportion of hematopoietic progenitors carrying the provirus was evaluated by PCR amplification of proviral iduronidase and/or neo^r sequences in marrow derived hematopoietic colonies (CFU-GM and BFU-E) at time-points after adoptive transfer. A representative CFU PCR analysis with amplification of the dystrophin gene as an internal genomic DNA control, proviral neo^r and iduronidase PCRs from M2 at 12 months post-infusion is shown in Figure 4.6B. Blood and marrow leukocytes bearing proviral DNA was detected in all dogs for at least one year post-infusion. For example, M5 maintained between 2-3% provirally marked CFU throughout the 12-month period post-infusion. Other dogs generally had a peak of 5-10% positive CFU in the first six months post-infusion with a gradual decline to 2-5% at time points greater than one year (Table 4.5, Figure 4.7). M2 was followed the longest and maintained levels of up to 6% provirus positive CFU between 12 and 24 months, and 2-5% at up to 24-36 months post-infusion. The level of progenitors carrying provirus in the MPS I dogs in this study were similar to those observed in normal dogs used in previous marker gene studies at this Institution[15]. For negative controls, background methylcellulose from each plate and colonies from control untreated dogs were harvested, plucked, and analysed by PCR. A total of 139 colonies from untransduced control dogs, and 177 background methylcellulose plucking controls from treated dogs underwent neo^r and/or iduronidase PCR analysis and all were negative.

Table 4.5: Detection of proviral specific sequences in marrow derived hematopoietic colonies from MPS I dogs at time points post-infusion[†].

	Number of CFU positive for proviral sequences /total CFU analysed (%)				
Time (Months)	Untransduced control	1-4	5-8.	9-12	13-24
M2	0/21	3/40 (7.5)	2/47 (4.3)	1/37 (4.3)	3/51 (5.9)
M3	0/14	1/78 (1.4)	5/63 (7.9)	1/40 (2.5)	2/72 (2.7)
M4	0/18	3/50 (6.0)	1/27 (3.7)	1/40 (2.5)	2/52 (3.8)
M5	0/26	2/80 (2.5)	2/100 (2.0)	1/48 (2.1)	NA
M6	0/36	9/191 (4.7)	2/24 (8.3)	7/128 (5.5)	3/120 (2.5)

Legend: NA, sample not available as dog was euthanised before this time point; *time points are calculated from the first infusion; [†]Where colonies were available for multiple time points during each range of times the total number of colonies were added together.

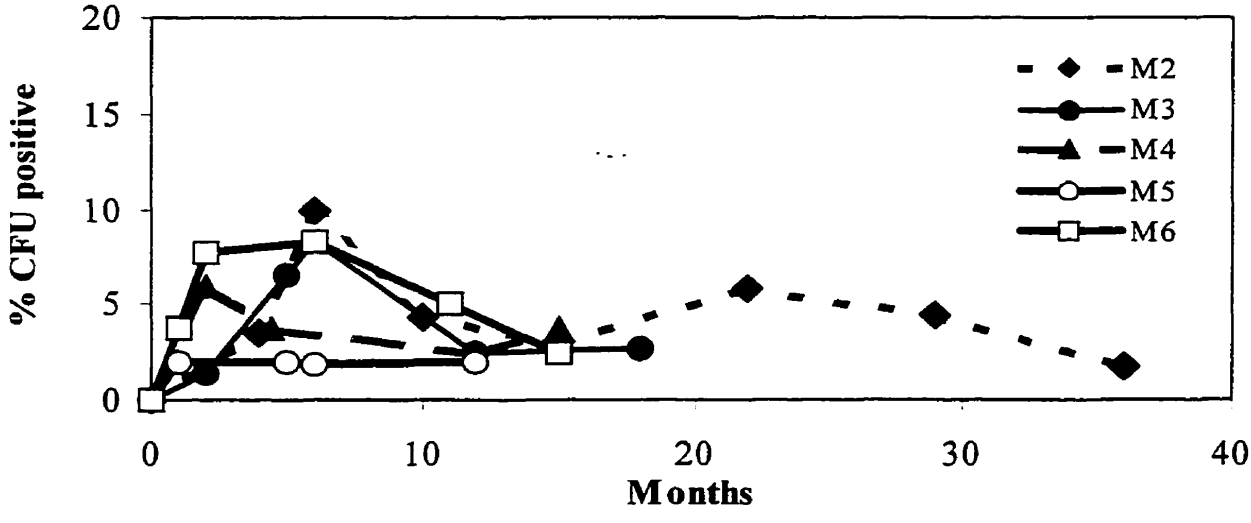

Figure 4.7: Percentage provirus positive progenitors in five MPSI (M2-M6) dogs followed for 1-3 years post-infusion. At each time point up to 100 colonies (CFU-GM and/or BFU) and 10 methylcellulose controls were plucked from methylcellulose plates and subject to iduronidase or neo[r] PCR.

4.3.7 Proviral Iduronidase Expression *In Vivo*

Peripheral blood and marrow leukocytes from all five dogs were assayed for expression by iduronidase enzyme activity and RT-PCR on multiple occasions post-infusion. Iduronidase enzyme activity was not detected in blood or marrow mononuclear cells from any dog (sensitivity ~1%). Proviral specific RT-PCR on blood and marrow mononuclear cells collected during the first 3 months post-infusion from all dogs were consistently negative, with data from M2 shown in Figure 4.8.

Proviral iduronidase activity was also assessed in hematopoietic progenitors. The iduronidase activity assay was performed on individual CFU-GM from M2 and M5 at various time points post-infusion with expression data form these dogs summarised in Table 4.6. A total of 399 CFU-GM from 1-24 months were assayed from M2. One CFU-GM out of 155 was positive for iduronidase activity at one month, while 244 CFU-GM from time points up to 24 months were negative. In colonies from M5, iduronidase activity was not detected in any of 193 CFU-GM from 1-4 months post-infusion (Table 4.6). Controls from these experiments included CFU-GM from normal dogs as positive controls, and reagent and tissue blanks as negative controls.

Table 4.6: Iduronidase activity in peripheral blood (PB) and bone marrow (BM) leukocytes and individual CFU-GM from M2 and M5 post-infusion.

Dog	Time (Months)	PB	BM	CFU-GM
M2	1	-ve	-ve	1/155
	8	-ve	-ve	0/87
	12	-ve	-ve	0/85
	24	-ve	-ve	0/72
M5	1	-ve	-ve	0/90
	4	-ve	-ve	0/103
MPS control*		-ve	-ve	0/100
Normal control†		+ve	+ve	22/23

Legend: -ve, sample negative; *, CFU-GM from untransduced marrow from M2; †, CFU-GM from a normal littermate control.

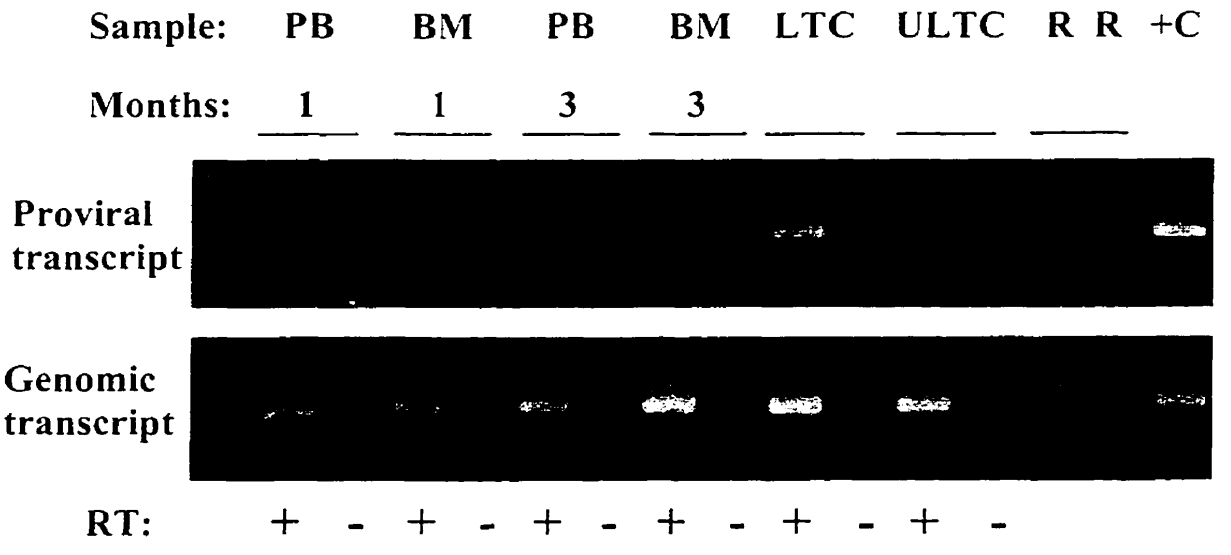

Figure 4.8: Proviral iduronidase expression evaluated in leukocytes from M2 post-infusion by reverse transcriptase PCR (RT-PCR). A) PCR amplification of a 618bp proviral specific iduronidase transcript (proviral transcript) from blood (PB) and marrow (BM) leukocytes at 1-3 months post-infusion. B) PCR amplification of the 422bp transcript arising from either the normal or mutant genomic iduronidase cDNA (genomic transcript) was performed to confirm the presence of amplifiable cDNA in these tissues. A control for each sample was not treated with reverse transcriptase (RT-) to ensure that there was not genomic DNA contamination of samples. Abbreviations: LTC, transduced LTMC from M2; ULTC, untransduced LTMC control from M2; RC, reagent control; +C, positive control DNA.

4.3.8 Immune Response Assays

Immune responses against provirally marked cells, proviral gene products and sera components were evaluated. Specific IgG antibodies against iduronidase enzyme were detected by ELISA in sera from dog M2 at all time points post-infusion. The titre peaked after the third infusion (~2 months post-infusion) at 1:3200, and dropped to 1:800-1600 after initiation of treatment with cyclosporine A at ~3 months post-infusion (Table 4.7). Humoral immune responses against fetal bovine serum (FBS), horse serum (HS) and bovine serum albumin (BSA) were assayed after the third infusion. IgG titres of 1:3200-6400 were observed (Figure 4.9). The anti-fetal bovine and anti-horse sera antibodies observed were maintained in M2's serum for at least 1 year after the infusion of transduced cells at titres of 1:1600-3200.

Table 4.7: Serum anti-iduronidase IgG titre detected by ELISA in serum samples from MPS I dogs post-infusion*.

	Time-post infusion (Months)						
	0[†]	1	2	3	4-6	7-9	10-12
M2	0	200[‡]	3200[‡]	3200[§‡‡]	1600	800-1600	800
M3	0[§]	100	100	100	100	100	100
M4	0[§]	100[‡]	100	100	200	100	100
M5	0[§]	100	400	ND	100	100	ND
M6	0[§]	<100	<100	<100	<100	<100	<100

Legend: * The titre is recorded as the dilution of serum giving a corrected OD reading of >2X each dog's pre-treatment serum OD; [†] Pre-infusion serum was taken from each dog directly before the first infusion and used as an individual background OD for each dog; [‡] Marks the timing of later infusions where dogs received more than 1; [§]Cyclosporine A treatment initiated; ND-sample not done.

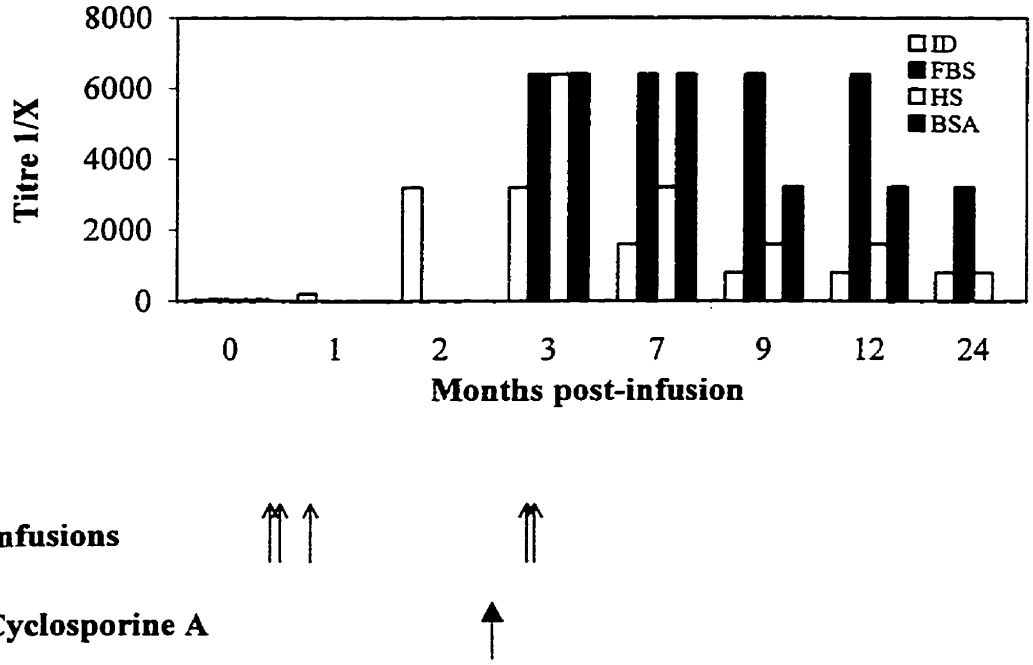

Figure 4.9: Detection of serum IgG specific for iduronidase (ID), horse serum (HS), fetal bovine serum (FBS) and bovine serum albumin (BSA) in dog M2 by ELISA. Titres are shown as the dilution of serum which gave a corrected OD reading of >2X the dog's pre-infusion serum. Analysis of anti-FBS, HS and BSA antibodies were not evaluated prior to 3 months. The approximate timing of infusions and initiation of treatment with cyclosporine A are shown with arrows.

Cellular immune responses to autologous transduced cells in M2 were evaluated by peripheral blood mononuclear cell proliferation assays. Two experiments were performed with blood mononuclear samples from dog M2, two and six weeks after the third infusion of LTMC cells. In these experiments peripheral blood mononuclear cells were overlaid on transduced and control autologous stroma and the cell proliferation under each condition was measured by incorporation of [^{3}H]-thymidine and reported as mean counts per minute (cpm) $\pm$ standard deviation. In the first experiment, fresh peripheral blood mononuclear cells from two weeks after the third infusion were exposed to LCIDSN transduced and untransduced

control stroma. There was a ~3-fold higher [^{3}H]-thymidine incorporation in blood mononuclear cells exposed to autologous LCIDSN transduced stroma than untransduced stroma (7020±719 cpm LCIDSN transduced stroma versus 1850±383.1 cpm untransduced stroma, $P<0.005$) (Figure 4.10A). In the second experiment, three separate stimulatory autologous stromal cells were used: untransduced, transduced with LNc11 (a neo^r only containing vector), and M48ID (the vector containing only iduronidase). Cryopreserved blood mononuclear cells from six weeks after the third infusion of LTMC cells were used as responders. There was ~2-fold higher proliferation when blood mononuclear cells were stimulated with M48ID transduced autologous stroma versus control untransduced stroma (5367±1792 cpm M48ID transduced stroma *v* 3053±812 cpm untransduced stroma, $P<0.05$) or versus LNc11 transduced stroma (5367±1792 cpm M48ID transduced stroma *v* 2635±487 cpm LNc11 transduced stroma, $P<0.05$) (Figure 4.10B). There was no significant difference between stimulation with LNc11 transduced stroma or untransduced stroma ($P=0.167$). The results from these two experiments are consistent with a cellular immune response to iduronidase but not neo^r transduced cells. The higher rate of cell proliferation in the second experiment may be due to a down regulation of the immune response with time.

The demonstration of immune responses against iduronidase, the serum components of the LTMC media and autologous cells expressing iduronidase in dog M2 following multiple infusions led to treatment of the remaining dogs with fewer infusions of transduced cells and post-transplant immunosuppressive therapy. Four dogs received standard immunosuppressive chemotherapy during the immediate post-transplant period. As expected, the titres of iduronidase specific IgG were lower than those for M2, at 1:100 in M3, M5 and M6 receiving one infusion, and a peak at 1:400 in M4 following a second infusion (Table 4.7).

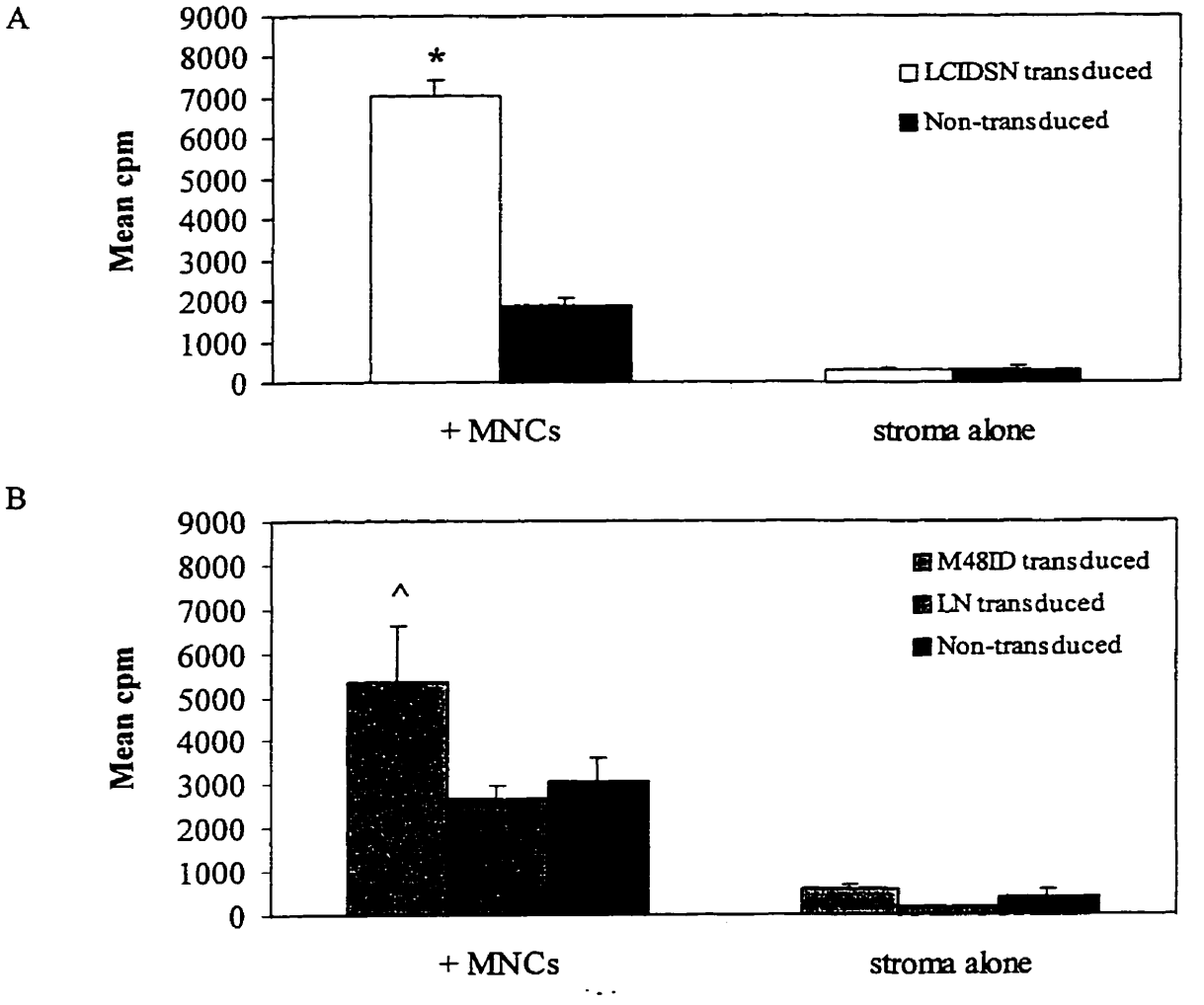

Figure 4.10: Peripheral blood mononuclear cell proliferative response to autologous LCIDSN transduced stroma in dog M2. Blood mononuclear cells from dog M2, which received infusions of both LCIDSN and M48ID transduced LTMC cells, were co-cultured *in vitro* with $1.5x10^3$ autologous LCIDSN transduced (□), M48ID transduced (▩), LNc11 transduced (▨) and non-transduced (■) stroma. MNC proliferation was measured by the incorporation of [^{3}H]-thymidine and reported as the mean counts per minute (cpm) of triplicate wells with standard deviations. The times post-third infusion were A) 2 weeks and B) 6 weeks. *P<0.005 and ^P<0.05.

4.3.9 Pathology

Three MPS I dogs, M4, M5 and M6 were euthanised at 12-24 months post-adoptive transfer due to worsening clinical status, while dog M2 died naturally from disease related causes at 42 months post-infusion. M3 is alive at 34 months post-infusion, with advanced but stable disease.

Pathologic examination of MPS I dogs was performed by Dr. Robert Foster (Guelph, Ontario). The pathology results were consistent with progression of MPS I disease in all dogs. For example, vacuolated cells were prominent in the connective tissues from skin, gastrointestinal tract, liver, pancreas, heart and arteries, respiratory system, reproductive system, urinary and hemolymphatic systems. Representative histologic photomicrographs of liver, aortic smooth muscle and spleen at euthanasia were taken by Dr. Colin McKerlie (Toronto, Ontario) and are shown in Figure 4.11.

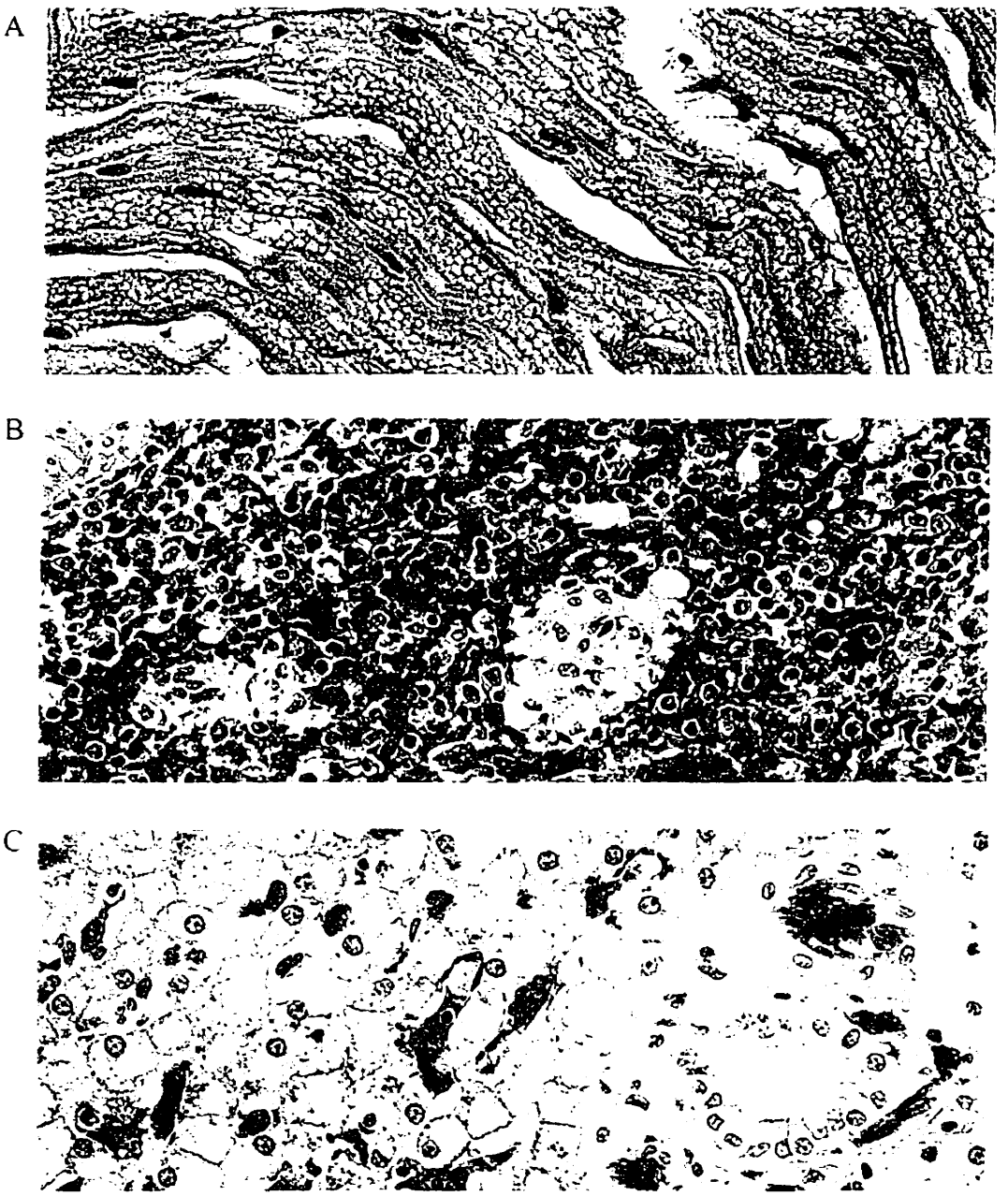

Figure 4.11: Histopathologic photomicrographs of tissues from dog M6 at the time of euthanasia (20 months of age, 16 months post-infusion).
A) Stromal cells of the aorta showed extensive vacuolation.
B) Vacuolated histiocytes around the penicillary arterioles of the spleen.
C) Vacuolation of periportal hepatocytes. (Hematoxylin and eosin, 200X)

4.4 Discussion

Canine iduronidase deficiency was first described in 1982 as a new mutation in three Plott hound dogs from Tennessee[22]. This canine model for human MPS I has been used to evaluate the efficacy of bone marrow transplant and recombinant iduronidase infusion for MPS I. The promising results of allogeneic bone marrow transplantation[30] and evidence for clinical improvement in recombinant enzyme infusion studies, despite the presence of an immune response,[32,33] suggested that iduronidase deficient dogs might be ideal for developing and evaluating therapies delivered by genetically corrected HSCs. The demonstration of significant disease amelioration in affected iduronidase deficient dogs receiving genetically corrected HSCs would represent a much-needed proof of principle in the arena of HSC gene therapy.

A canine MPS I colony has been established at the University of Guelph through breeding of iduronidase heterozygotes. In this experiment, two litters resulting from the mating of two iduronidase heterozygotes produced four pups each, with a total of two iduronidase deficient, three heterozygotes and three wild type pups. Three MPS I dogs bred at the University of Tennessee and two bred at the University of Guelph colony were used in this study.

Marrow was harvested from 5 iduronidase deficient juvenile dogs and transduced during three weeks of LTMC. The cell and progenitor yields at marrow aspiration and the end of LTMC were comparable to yields discussed in the normal marrow samples discussed in Chapter 3[18] and previously published[13,15,35]. The LTMC cells were exposed to retroviral vectors bearing two different canine iduronidase cDNA expression cassettes. One vector, LCIDSN, had iduronidase expressed from the viral LTR and neo^r from an internal SV40 promoter/enhancer (Figure 2.1). In the M48ID vector, iduronidase was expressed from the murine PGK promoter. Both were shown to successfully transduce primary cell types such as stromal fibroblasts and hematopoietic cells, and cell lines such as NIH 3T3 fibroblasts[36].

There was good evidence that hematopoietic cells and progenitors were transduced as bulk populations of LTMC cells and up to 60% of individual LTMC derived CFU-GM carried proviral sequences. Proviral iduronidase activity was detected in up to 20% of individual

LTMC derived hematopoietic colonies and bulk LTMC cells expressed greater than 10 times the normal iduronidase activity at the time of infusion. The high levels of proviral iduronidase activity detected in this study have also been demonstrated in other canine and human iduronidase gene transfer studies. For example, a 10-50 fold increase in iduronidase activity was detected following retroviral mediated transfer of iduronidase to human MPS I marrow samples[37,38]. High levels of proviral iduronidase activity have also been demonstrated following retroviral gene transfer of the iduronidase cDNA into canine MPS I marrow cells [36] and myoblasts[39].

Neufeld and colleagues demonstrated in the 1960's that normal cells secreted iduronidase enzyme, which could in turn be taken up by deficient cells in tissue culture. Successful HSC gene therapy for canine MPS I requires the production and secretion of normal iduronidase enzyme from cells carrying proviral iduronidase and for the enzyme to be taken up by deficient cells in other tissues. This experiment evaluated whether provirally produced iduronidase enzyme was secreted by transduced LTMC cells and taken up by iduronidase deficient MPS I cells. The results of this co-culture experiment demonstrated that iduronidase deficient cells physically separated from iduronidase transduced cells could uptake high levels of provirally derived iduronidase. Two other studies have demonstrated that retrovirally transduced human MPS I marrow cells can secrete functional iduronidase which is in turn taken up by deficient fibroblasts[38], neuronal or glial cells[40] resulting in reduced glycosaminoglycan storage.

The efficiencies of iduronidase gene transfer demonstrated in this experiment were similar to the levels of neo^r gene transfer to normal canine marrow observed in other studies using the same gene transfer protocol (Chapter 3[18] and [13,15,41]). Adoptive transfer of the neo^r transduced LTMC cells to normal autologous recipients resulted in the maintenance of up to 10% provirally marked hematopoietic progenitors *in vivo* for two years post-infusion (Chapter 3 and[15,18]). Similar results were observed in the experiments described here. Monitoring of engraftment for up to 3 years in MPS I recipients demonstrated 2-10% of hematopoietic progenitors carried proviral iduronidase and/or neo^r sequences. *In situ* neo^r PCR analysis on marrow and blood leukocytes also demonstrated 2-10% of cells carrying proviral sequences. However, semi-quantitative neo^r PCR analysis on blood and marrow mononuclear cells

indicated ≤1% of cells carried proviral sequences. The lower proportion of provirally marked blood and marrow mononuclear cells by semi-quantitative PCR cells than in blood and marrow leukocytes by *in situ* PCR may reflect differences in either the two cell populations or the sensitivity of the assays. This is discussed in detail in Section 2.4.5.

Despite the engraftment of significant levels of provirally marked cells, there was no iduronidase activity detected in blood or marrow leukocytes. At the time of infusion, LTMC cells expressed up to 100 times the normal level of iduronidase activity and an average of 15% of individual hematopoietic colonies had proviral iduronidase enzyme activity. However, neither iduronidase transcripts nor activity were detected in blood or marrow leukocytes from any dog. The iduronidase activity assay was optimised for the detection of activity from individual CFU-GM which were 100 cells or greater, however iduronidase activity could not reliably be detected in BFU-E colony with hundreds to thousands of cells. Thus, evaluation of iduronidase activity in individual hematopoietic colonies was restricted to CFU-GM. A total of 592 CFU-GM from M2 and M5 were evaluated for iduronidase activity and one CFU-GM from M2 was found to be expressing iduronidase. PCR analysis for proviral sequences at the same time points indicated that 2-10% of the individual CFU-GM suggesting that proviral gene expression had been strongly down-regulated or silenced *in vivo*.

The progression of MPS I disease evident at autopsy supports the conclusion that there was little, if any, iduronidase produced *in vivo*. In canine MPS I muscle gene transfer studies, Shull and colleagues reported high levels of proviral iduronidase activity in cultured MPS I myoblasts transduced with a vector similar to the LCIDSN[39]. Upon autografting the transduced cells into affected dogs, enzyme was detected in peripheral blood leukocytes for 1-3 weeks after transplant but was undetectable after this time[39]. Following infusion of LTMC cells transduced with the arylsulfatase B cDNA in cats with MPS VI, provirally marked cells were detected for greater than 2 years[42]. Low levels of enzyme activity were detected for several months post-infusion. However, unlike the canine studies described in this chapter, the MFG vector, optimised for proviral gene expression was used.[42]

Silencing of MMLV based promoters, has been described in other *in vivo* studies[43-45]. For example, Cory and colleagues demonstrated significantly less neo^r expression from single gene MMLV based vectors in individual CFU-S than MPSV based vectors. Transgene expression was not detected in CFU-S from double gene MMLV based vectors despite high levels of expression *in vitro*[43]. Suppression of transgene expression in CFU-S has also been demonstrated by Kohn and colleagues with only 10-20% of provirally marked secondary spleen colonies expressed the transgene in the MMLV based LN vector[44].

Humoral immune responses against iduronidase protein were identified. As expected, the titre of anti-iduronidase IgG was highest in the dog receiving multiple infusions of cells. All subsequently infused dogs were treated with post-transplant cyclosporine and fewer infusions in an attempt to prevent anti-graft immune responses. It is unclear whether the anti-iduronidase immune responses contributed to the lack of clinical benefit observed in these dogs. In a clinical MPS I HSC gene therapy trial in Manchester by Fairbairn and colleagues, proviral iduronidase enzyme was detected in blood derived leukocytes during the first week after infusion[46]. A sudden decrease in iduronidase activity during the second week coincided with the detection of anti-iduronidase antibodies. A similar phenomenon is seen in hemophilia A, where patients who develop complement activating antibodies against clotting factor FVIII require larger infusions of factor to abrogate bleeding episodes[47]. In contrast, MPS I dogs treated with recombinant iduronidase enzyme had clinical benefit despite the development of very high levels of anti-iduronidase antibodies after multiple infusions of enzyme[32,33]. It is possible that the large quantities of enzyme infused in these dogs overcomes the inhibitory effects of anti-iduronidase antibodies, while the antibodies observed in the gene transfer study inhibit the continual release of low amounts of enzyme in the gene transfer study. Further evaluation of the effects of anti-iduronidase antibodies on enzyme levels in MPS I recipients of iduronidase gene therapy and enzyme infusion is necessary to determine how anti-iduronidase antibodies affect levels of available iduronidase *in vivo*.

Cellular immune responses against autologous transduced cells expressing iduronidase but not neo^r were also observed in this study. Cellular immune responses against autologous cells expressing a retroviral transgene (thymidine kinase-neo^r fusion) have been identified following adoptive transfer of transduced cells to HIV+ patients[48]. In these patients all

transgene expressing cells were eliminated[48]. It is possible in this MPS I dog study that the cellular immune responses eliminated iduronidase expressing cells, and only cells with silenced iduronidase expression survived. Further characterisation of the cellular anti-iduronidase immune responses would be needed to identify the role of immune responses in the lack of clinical benefit observed in these MPS I dogs.

Humoral immune responses have been detected against a variety of non-therapeutic antigens presented by genetically modified cells such as serum components of culture media as described by Muul et al[49] and discussed in Chapter 3. Humoral immune responses against the LTMC media components, HoS, FBS and BSA were detected in all MPS I dogs infused in this study. As expected, antibody titres were highest in dogs receiving multiple infusions and conversely lowest in dogs receiving one infusion. Similar to the results in normal dogs presented in Chapter 3, the development of anti-culture media antibodies did not prevent the engraftment and maintenance of provirally marked cells.

The recipients in this study were non-ablated. It has been suggested that anti-iduronidase and anti-media protein immune responses may be prevented by myeloablating recipients. However, it is unlikely that the anti-iduronidase immune response is due to the lack of marrow conditioning. In an independent study at the Fred Hutchinson Research Centre MPS I dogs were myeloablated before receiving M48ID transduced marrow cells. Humoral anti-iduronidase immune responses and a lack of clinical benefit similar were also detected in this study[36]. In the ongoing clinical trial in Manchester, the patients developing humoral anti-iduronidase immune responses were conditioned with busulfan prior to adoptive transfer of autologous iduronidase transduced marrow[46]. These results suggest that myeloablation may not have prevented anti-iduronidase immune responses. This study in dogs with iduronidase deficiency demonstrated significant humoral and cellular immune responses against the normal canine iduronidase protein, autologous cells expressing iduronidase and LTMC culture media serum components. As expected, the strongest immune responses in this study were detected after multiple infusions of cells in the absence of immunosuppressive therapy. This study was one of the first demonstrations of humoral and cellular immune responses against cells genetically modified to express a normal cellular protein in an animal deficient for this protein[50].

The presence of cellular immune responses and lack of detectable iduronidase expression *in vivo* may indicate that only cells which have silenced proviral expression survived. Alternatively, two independent mechanisms may account for the lack of sustained enzyme production, silencing of proviral gene expression or immune responses against iduronidase produced *in vivo* and/or cells expressing proviral antigens. In the next set of experiments described in Chapter 5, the therapeutic potential of HSC gene therapy was evaluated in the absence of immune responses, following adoptive transfer to pre-immune iduronidase deficient fetal pups.

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CHAPTER 5

Adoptive Transfer of Genetically Corrected Hematopoietic Progenitors to Fetal MPS I Pups Results in Engraftment but Not Amelioration of Disease

5.1 Summary

Genetically modified HSCs have been proposed as vehicles for gene therapy of single gene disorders. In previous studies, iduronidase deficient dogs infused with autologous marrow cells genetically modified to express iduronidase had long-term engraftment with provirally marked cells, but there was no evidence for proviral iduronidase expression or clinical improvement. The presence of humoral and cellular immune responses against iduronidase in these dogs may have prevented a clear evaluation of the therapeutic potential of HSC gene therapy in these experiments (Chapter 4). In the study undertaken here, adoptive transfer of iduronidase transduced MPS I marrow cells into pre-immune fetal pups has enabled evaluation of the therapeutic potential of *in utero* adoptive transfer of genetically modified HSCs, in the absence of a confounding immune response.

In three separate experiments, 17 mid-gestation fetal pups were injected with 0.5-1.5x10^7 normal or MPS I allogeneic long term marrow culture (LTMC) cells which had been transduced with neo^r or iduronidase containing retroviral vectors. Nine normal and three MPS I pups survived the neonatal period and demonstrated engraftment of provirally marked progenitors at levels of up to 12% for up to 12 months. Neither iduronidase enzyme or proviral specific transcripts were detected in blood or marrow leukocytes of any MPS I dog. Humoral immune responses to iduronidase were not detected in neonates, even after "boosting" with autologous iduronidase transduced LTMC cells. All MPS I dogs died at 8-10 months of age from complications of MPS I disease with no evidence of amelioration of MPS I disease progression.

These results suggest that iduronidase transduced primitive hematopoietic progenitors can engraft in fetal recipients, contribute to hematopoiesis and induce immunologic non-responsiveness to iduronidase in MPS I dogs. However, the therapeutic potential of HSC gene transfer in this model of iduronidase deficiency appears to be limited by poor maintenance of proviral iduronidase gene expression and relatively low levels of genetically corrected cells.

5.2 Introduction

Allogeneic HSC transplantation has been a successful treatment for a variety of enzyme deficiencies such as the mucopolysaccharidoses[1-4]. Two obstacles which have reduced the success of this treatment in some patients are graft rejection and graft versus host disease despite the use of myeolablation and immunosuppression, and non-reversal of pre-graft damage in some tissues. The pre-immune fetus has been proposed as an ideal recipient for hematopoietic stem cell transplantation. Two features of fetal biology enhance the success of the fetal transplant system: tolerance can be induced to allogeneic cells because the recipient is immunologically immature[5], and the fetal hematopoietic environment facilitates the expansion of endogenous and possibly transplanted HSCs to populate the neonatal hematopoietic system[6,7]. Two benefits of early treatment include treatment prior to the onset of symptoms and the induction of tolerance to the graft without chemical immunosuppression.

An experimental *in utero* sheep transplantation model has demonstrated that human HSCs can engraft in fetuses following intra-peritoneal injection and expand and contribute to neonatal and adult hematopoiesis[6-8]. However, the level of donor cell engraftment in the fetal sheep has been quite low, in general less than 1%[9-11]. The results from human *in utero* HSC transplantation are also disappointing as of 21 *in utero* transplants reported in the literature only four recipients have successfully engrafted[12-15]. Although the clinical *in utero* transplants were for a number of genetic diseases such as hemoglobinopathies, immune deficiencies, and Hurler syndrome, all four successful *in utero* HSC transplants were in fetuses with severe immune deficiencies. Immune mediated graft rejection by immunocompetent fetuses may have been a factor in the failed fetal transplants[12-15]. The disappointing results of *in utero* HSC transplant for disorders other than immune deficiencies indicate that *in utero* transplant protocols need to be both optimised and evaluated for therapeutic efficacy in large animal models of human disease.

Engraftment of high levels of human hematopoietic cells following adoptive transfer to the yolk sacs of pre-immune fetal pups has recently been demonstrated[16]. For example, at 10-12 weeks after birth 6-38% of blood and marrow cells were human, demonstrating high levels of

engraftment using this yolk sac injection protocol. The level of human cells in the canine recipients declined to less than 1% at 19 weeks, possibly because the canine hematopoietic environment was not optimised for maintaining human hematopoiesis[16]. It was hypothesised that injection of allogeneic cells into the yolk sacs of fetal canine pups would result in high levels of engraftment of donor marrow and allogeneic cells would likely continue to contribute to hematopoiesis in juvenile and adult dogs.

A canine model of mucopolysaccharidosis I (MPS I) which results from a deficiency in α-L-iduronidase (iduronidase) has been identified and characterised. MPS I dogs have similar clinical features to patients with moderate to severe clinical phenotypes MPS I (Hurler-Scheie and Hurler Syndromes)[17,18]. Symptoms of canine MPS I include progressive cardiac abnormalities, lysosomal storage in the central nervous system, corneal clouding, skeletal abnormalities, degenerative joint disease and progression of disease to such severe states that euthanasia is generally indicated within 1-3 years. In these dogs the germline mutation in the iduronidase gene (cIDUA) leads to a premature stop codon in intron 1, thus resulting in a complete deficiency of iduronidase protein[19].

The canine iduronidase deficiency model is appropriate for the evaluation of the clinical benefit of genetically corrected hematopoietic cells for the following reasons: the normal and mutant canine cDNA have been cloned and well characterised; there is a wide range of enzyme levels compatible with a normal or mild phenotype; allogeneic bone marrow transplantation is of known clinical benefit in affected dogs[20], and; iduronidase deficient cells can uptake iduronidase from the circulation resulting in markedly reduced lysosomal storage[21,22].

In previous experiments, the therapeutic potential of HSC gene therapy for canine iduronidase deficiency was evaluated. Five juvenile iduronidase deficient dogs were injected with autologous marrow cells genetically modified during LTMC to carry and express the normal canine iduronidase cDNA. All dogs had long-term engraftment with genetically modified cells however, neither iduronidase enzyme expression or disease amelioration were observed. Furthermore, immune responses against iduronidase enzyme and iduronidase expressing cells were detected following the infusion of transduced LTMC cells. It was

hypothesised that *in utero* transplantation of the iduronidase expressing grafts would induce tolerance to iduronidase as well as provide insight into the potential of *in utero* HSC gene therapy. In the experiments described in this chapter, the potential of adoptive transfer of genetically modified MPS I marrow cells to iduronidase deficient fetal pups to prevent anti-iduronidase immune responses and contribute to disease amelioration was evaluated.

5.3 Experimental Design

The yolk sacs of mid-gestation fetal pups were injected with transduced LTMC cells. Following birth fetal recipients were monitored for the contribution of marked cells to hematopoiesis. Prior to undertaking *in vivo* experiments with MPS I dogs the first experiment utilised LTMCs from normal donors and normal fetal recipients and evaluated the ability of allogeneic cells to engraft and contribute to neonatal hematopoiesis. In the second and third experiments, fetal pups resulting from the mating of two iduronidase heterozygotes were injected with transduced LTMCs which had been established with marrow from adult MPS I dogs.

5.4 Results

5.4.1 Marrow Harvests, LTMCs and Transductions

In the first experiment ~60 mL marrow was harvested from one female and one male donor, yielding $1.8x10^8$ and $3.8x10^8$ marrow mononuclear cells and $5.9x10^4$ and $8.1x10^4$ CFU-GM respectively. The marrow mononuclear cells were established in LTMC. The male LTMCs were transduced with LNc11 and the female LTMCs were transduced with LCIDSN. The recoveries at the time of culture harvest on day 21 were $2.4x10^7$ and $4.2x10^7$ cells corresponding to 13% and 11% for the female and male samples respectively. No growth was observed in the progenitor assays (Table 5.1).

In the second and third experiments, the marrow donors were female and male MPS I dogs respectively. Approximately 175-200 mL marrow was harvested from each dog with cell and progenitor yields of $1.2x10^9$ and $7.5x10^8$ mononuclear cells and $1.8x10^5$ and $2.0x10^5$ CFU-GM for the donors of experiment 2 and 3 respectively. Half of the LTMCs from each dog were transduced with the LCIDSN vector and the remainder with the M48ID vector (Table 5.1). After 21 days of LTMC cell yields were $1.6x10^8$ and $1.1x10^8$ cells and the progenitor yields were $1.3x10^4$ and $1.2x10^4$ for experiments 2 and 3, respectively. This corresponds to 13% and 15% cell and 7% and 6% CFU-GM recoveries at day 21 for the second and third experiments, respectively.

Table 5.1: Summary of donor allograft marrow harvests, LTMCs and transductions.

	Donor		Vector	Marrow Harvest		Day 21 LTMC cells	
Expt/ LTMC	Sex	Genotype		MNC	CFU	MNC	CFU
1	M	WT	LN	$3.8x10^8$	$8.1x10^4$	$4.2x10^7$	NG
	F	WT	LCIDSN	$1.8x10^8$	$5.9x10^4$	$2.4x10^7$	NG
2*	F	MPS I	½LCIDSN ½M48ID	$1.2x10^9$	$1.8x10^5$	$1.6x10^8$	$1.3x10^4$
3*	M	MPS I	½LCIDSN ½M48ID	$7.5x10^8$	$2.0x10^5$	$1.1x10^8$	$1.2x10^4$

Legend: NG, no growth in methylcellulose was observed; * half of the LTMC flasks from experiments 2 and 3 were transduced with M48ID and the other half with LCIDSN.

5.4.2 Gene Transfer in LTMC Allografts

Analysis of aliquots of LTMCs cells at the end of culture/transduction demonstrated that all three LTMC samples were PCR positive for proviral sequences (Table 5.2). The LTMC cells from experiments 2 and 3, established with marrow from MPS I, expressed ~100 units (1 unit=1 nmol substrate/mg protein/hour) of iduronidase activity. This corresponds to a 10-100 fold increase over iduronidase activity from LTMCs established from normal canine marrow, which typically have ~1-5 units of iduronidase activity. Untransduced controls for both LTMCs had no detectable activity. The proportion of LTMC derived CFU-GM positive for proviral sequences for experiments 2 and 3 were 31% (13/42) and 20% (3/15) respectively. In the LTMC from the third experiment, 8% of individual CFU-GM expressed iduronidase activity. The levels of gene transfer and iduronidase expression in LTMC cells and progenitors observed in this study are similar to other canine gene transfer studies using the same approach[23,24].

Table 5.2: Gene transfer and proviral iduronidase expression in transduced LTMC cells and LTMC derived CFU-GM.

	Total LTMC cells		LTMC-derived CFU-GM	
EXPT/LTMC	PCR	ID activity*	PCR positive (%)	ID activity (%)
1	+	ND	NG^	NG
2	+	100 units	13/42 (31)	NE
3	+	100 units	3/15 (20)	2/25 (8.0)
Untransduced normal control	-	1.8 units	0/24	9/12 (75)

Legend: LTMC+, sample positive for proviral sequences by PCR analysis; *, units=nmol substrate/mg protein/hr; NG, no growth of colonies in methylcellulose; NE, expression not evaluated.

5.4.3 Adoptive Transfer of LTMC Cells into Fetal Canine Recipients

Transduced LTMC cells were injected into the yolk sacs of fetal pups of 3 pregnant females. In the first experiment three pups from a normal mating were injected with $1.5x10^7$ LTMC cells each on day 32 of gestation (Table 5.3). The graft consisted of equal numbers of LTMC cells from each of the two normal donors. The fetal recipient pups had normal gestation and parturition (N4, N5 and N6).

In experiments two and three, fetal pups arising from the mating of two iduronidase heterozygotes were injected (Table 5.3). The LTMC grafts for experiments 2 and 3 consisted of equal numbers of LTMC cells transduced with the M48ID and LCIDSN vectors. In experiment 2, eight fetal pups were each injected on day 38 of gestation with $5x10^6$ LTMC cells derived from a female dog with iduronidase deficiency. The pups subsequently underwent normal gestation, however the dam experienced difficulty whelping and only three pups survived the neonatal period. Genotype analysis identified 3 MPS I, 3 heterozygotes and 2 wildtype pups (Figure 5.1). Of surviving pups, one was heterozygous (N7) and two were

MPS I (M7 and M8). In the third experiment, each of six pups from the mating of iduronidase heterozygotes were injected on day 35 of gestation with $5x10^6$ male iduronidase deficient LTMC cells. All six pups, {one MPS I (M9), four heterozygous and one wild type pup (N8-N12)} survived the neonatal period (Table 5.3).

Table 5.3: Adoptive transfer of allogeneic LTMC cells to mid-gestation normal and iduronidase deficient fetal recipients.

Expt	Genotype of parents	Fetal day of gestation*	Fetuses injected	cells per fetus	Pup genotype	Surviving pups
1	WT/WT	32	3	$1.5x10^7$	3 WT	N4-N6
2	Het/Het	38	8	$5x10^6$	3 MPS I	M7, M8
					3 Het	N7
					2 WT	
3	Het/Het	35	6	$5x10^6$	1 MPS I	M9
					4 Het	N8-12
					1 WT	

Legend: WT, wild type; Het, iduronidase heterozygotes; MPS I, ID deficient; *timing of *in utero* injection.

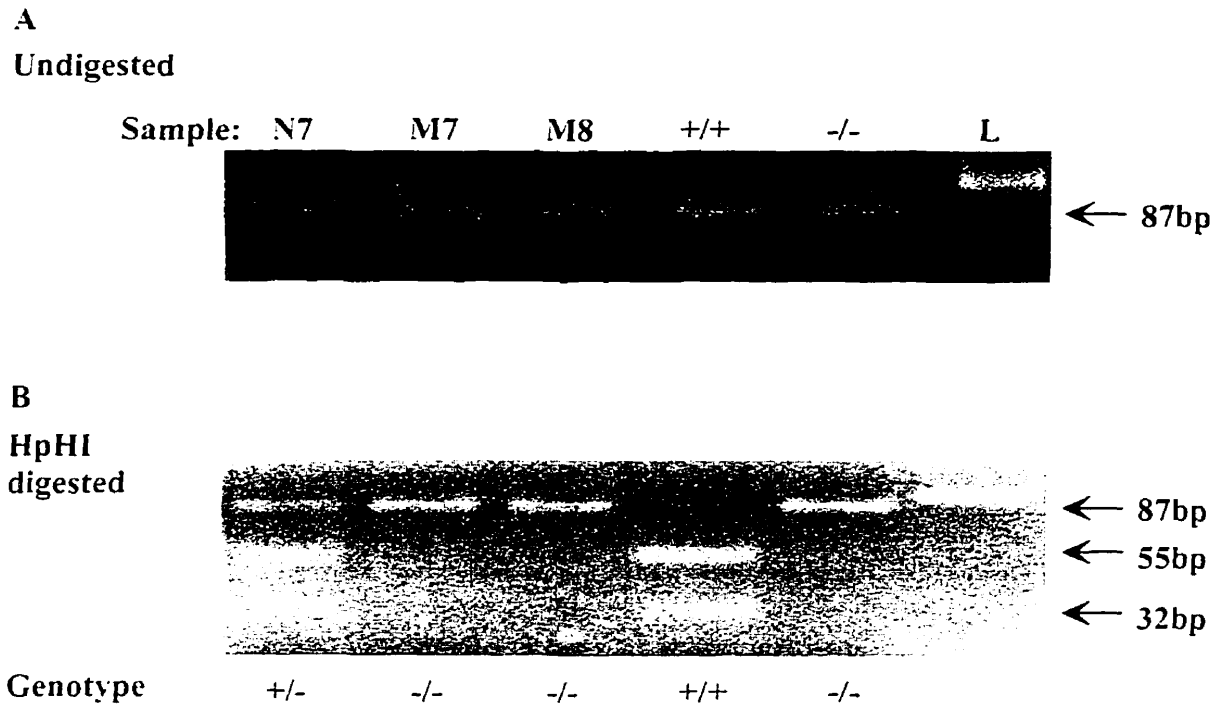

Figure 5.1: Iduronidase genotype PCR analysis on Experiment 2 pups. A) PCR amplification of an 87bp genomic iduronidase fragment with CA1-CA2 primers. B) Analysis of PCR products following digestion with HpHI. PCR amplicons from the MPS I iduronidase allele do not digest with HpHI and PCR amplicons from the wildtype iduronidase allele digest with HpHI to 55bp and 32bp fragments. Samples from heterozygote N7 and MPS I homozygotes M7 and M8 are shown. Controls included samples from wild-type (+/+) and MPS I (-/-) dogs. Genotypes are shown at the bottom: wildtype, +/+; heterozygote (+/-); MPS I (-/-). Abbreviations: L, DNA ladder. This assay was performed as described in Section 2.4.1.

5.4.4 Engraftment of LTMC Cells in Normal Fetal Recipients

The experiment determined whether transduced allogeneic LTMC cells could engraft in fetal canine recipients and contribute to hematopoiesis after birth. Individual hematopoietic colonies from nine normal pups injected *in utero* with allogeneic LTMC cells were monitored for the engraftment of provirally marked cells for up to 52 weeks post-partum. Short term engraftment was evaluated in 3 dogs, N4-6. Peripheral blood and marrow leukocytes samples and individual hematopoietic colonies were subjected to provirus specific neo^r PCR analysis during the first 15 weeks after parturition. Blood and marrow leukocytes were positive for proviral sequences at all times tested, with a sample PCR analysis at 5 weeks shown in Figure 5.2.

Three weeks after birth, which corresponds to ~7 weeks after injection, 7.1% (5/70), 7.4% (4/54) and 3.2% (2/62) of the marrow-derived hematopoietic colonies were PCR positive for proviral neo^r sequences for pups N4, N5 and N6 respectively. Over the next 12 weeks the proportion of colonies carrying proviral sequences remained stable at 1.7-6.0% for all three pups (Table 5.4). PCR analyses of 50-100 colonies were performed on each follow up sample at each time point. One littermate pup (C1) which was not injected with canine LTMC cells *in utero* was used as a negative control for PCR analysis, and neither blood, marrow nor hematopoietic colonies from the control pup were ever positive for proviral sequences.

Long term engraftment of provirally marked progenitors was assessed by provirus specific colony PCR on 3-6 time points during the first year after birth on the 6 remaining normal dogs (N7-N12). All pups engrafted with transduced cells as evidenced by proviral PCR analysis on a total of 615 hematopoietic colonies (Table 5.5). On average 3.4% (range 0-12%) of hematopoietic colonies carried proviral sequences from each dog at each time point. In dogs N7, N8 and N10 hematopoietic colonies were consistently PCR positive over the year of analysis with higher percentages observed during the first six months (2.5%-12%) than in months 6-12 (1.3%-4.5%). Provirus positive colonies from dogs N6, N8 and N9 were only intermittently detected in the range of 0-6.1% (Table 5.5).

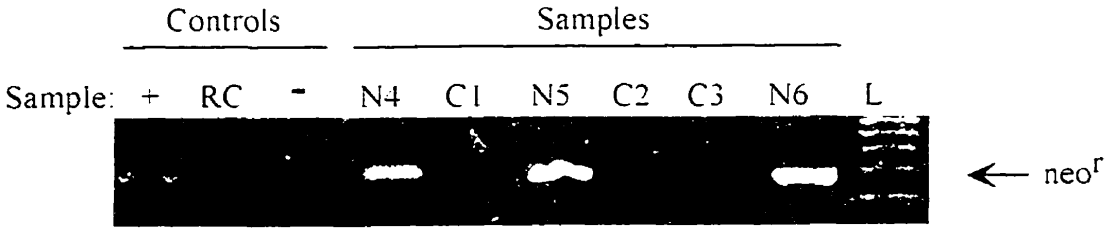

Figure 5.2: Detection of neo^r sequences by PCR amplification of genomic DNA from marrow mononuclear cells from Experiment 1 pups at 5 weeks of age. Three of six dogs were infused at mid-gestation with neo^r transduced allogeneic LTMC cells (dogs N4, N5 and N6) and the other three were controls (C1, C2 and C3). PCR controls: 20pg positive DNA (+), canine genomic DNA negative for neo^r sequences (-) and reagent control (RC). Abbreviations: L, DNA ladder. This assay was performed as described in Section 2.4.2.

Table 5.4: Detection of proviral neo^r sequences in hematopoietic colonies of normal neonatal pups by PCR analysis (experiment 1).

	No. (%) hematopoietic colonies PCR positive for proviral sequences		
Pup	3 weeks	10 weeks	15 weeks
N4	5/70 (7.1)	2/100 (2.0)	2/50 (4.0)
N5	4/54 (7.4)	3/100 (3.0)	3/50 (6.0)
N6	2/62 (3.2)	4/83 (4.8)	1/60 (1.7)
C1	0/36	0/34	

Table 5.5: Long-term engraftment of provirus positive hematopoietic colonies in pups injected with transduced LTMC cells *in utero*.

		No. (%) colonies PCR positive for proviral sequences[†]		
	Dog	1-3 Months	4-6 Months	7-12 Months
Normal pups	N7	3/25 (12)	5/48 (10.4)	3/67 (4.5)
	N8	1/28 (3.5)	1/40 (2.5)	1/80 (1.3)
	N9	1/27 (3.7)	0/54	1/56 (1.8)
	N10	1/29 (3.5)	1/23 (4.3)	
	N11	0/21	4/65 (6.1)	
	N12	0/28	(0/14)	2/64 (3.1)
MPS I pups	M7	4/60 (6.6)	4/34 (11.8)	3/45 (6.6)
	M8	1/29 (3.5)	6/56 (10.7)	3/136 (2.2)
	M9	3/54 (5.6)	1/42 (2.4)	1/36 (2.8)

Legend: *Months post-partum; Blanks, no data available; [†]Where data was available at multiple time points the number of colonies were added together.

In situ neo^r PCR analysis was performed on N7. Provirus positive cells were detected in the blood and marrow at all time-points up to 29 weeks (Figure 5.3). The levels of provirally marked leukocytes peaked at six weeks after birth at 8-12%. The percentage positive cells were maintained at ~5% for the remainder of study. Despite the detection of high levels of neo^r marked leukocytes by *in situ* PCR, neo^r Southern blots were negative at similar time points (sensitivity ~5%, data not shown). This suggests that the proportion of provirally marked cells is lower than indicated by the *in situ* PCR results, indicating an overestimate of provirally marked cells by *in situ* PCR (discussed in Section 2.4.5.).

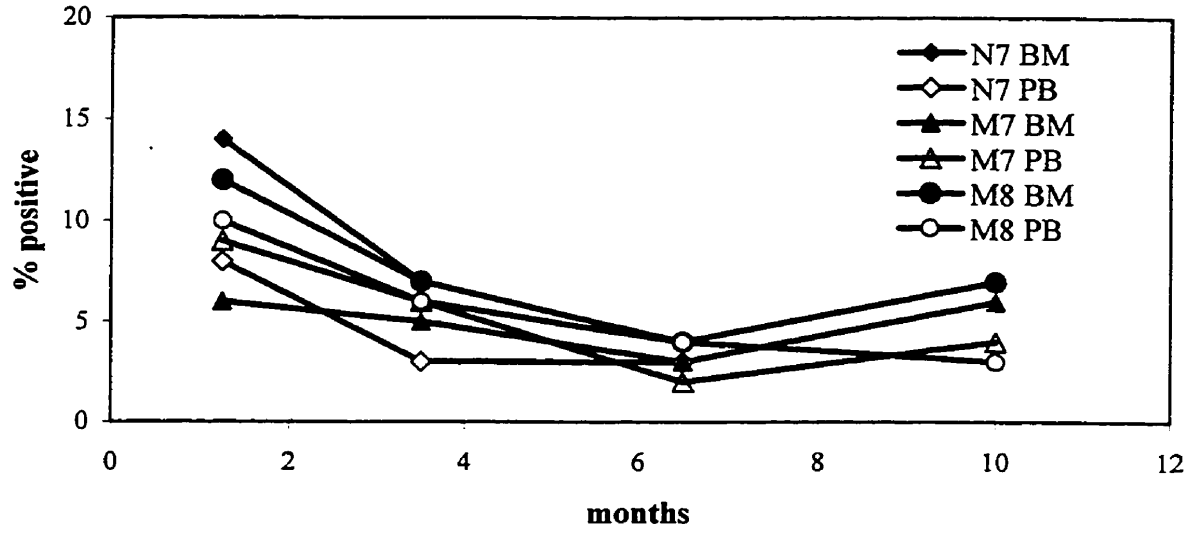

Figure 5.3: Percentage neo^r positive cells by *in situ* PCR on blood and marrow leukocytes in experiment 2 pups. Percentage positive cells based on the analysis of a minimum of 300 cells. Control samples in each experiment were combinations of 100%, 10%, and 0% neo^r positive Jurkatt cells.

Cytogenetic analysis was performed on the two evaluable opposite sex recipients. Analysis of metaphases from marrow and PHA stimulated blood cells from the male recipient N7 demonstrated the presence of female cells at 10-20 weeks (Table 5.6). Between 8-14% of metaphases from blood or marrow leukocytes from dog N7 were female and thus of donor origin (analysis of 12-38 metaphases at each time). A comparison of the proportion of hematopoietic progenitors and blood and marrow leukocytes provirally marked, and of donor cells by cytogenetic analysis in dog N7 are shown in Figure 5.4. Dog N7 was euthanised at 29 weeks of age due to juvenile onset renal failure.

Donor female metaphases were not detected in marrow from N7 at 32 weeks of age, however there were only nine analysable metaphases. In the female recipient N8, cells with donor (male) karyotypes were not detected in PHA stimulated blood or marrow samples at any time point up to 37 weeks, with analysis of 15-50 metaphases at each time point (Table 5.6).

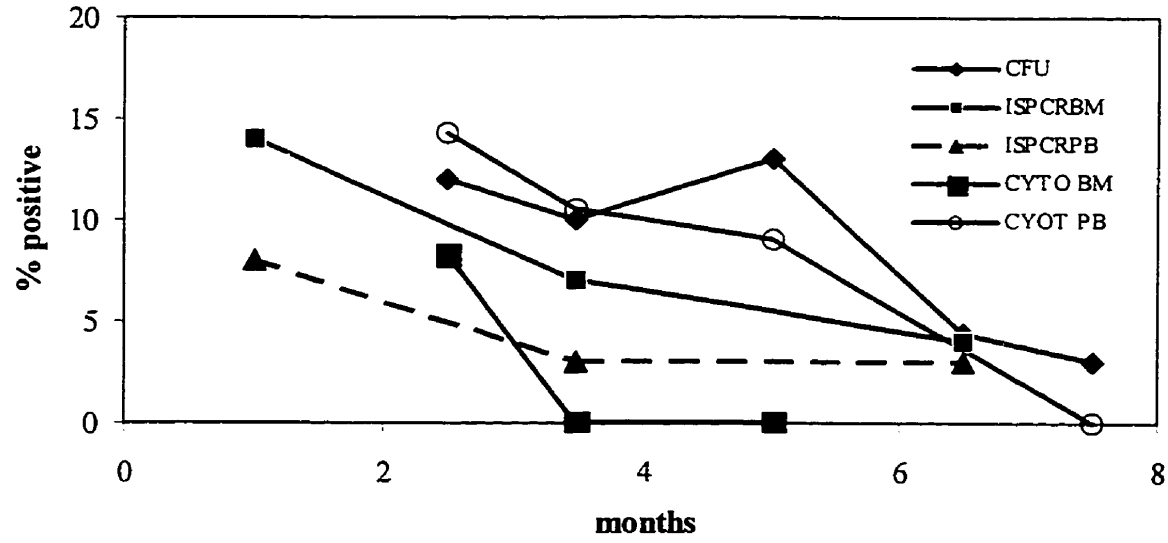

Figure 5.4: Summary of engraftment in dog N7. Neo^r *in situ* PCR analysis on blood and marrow leukocytes, cytogenetics on direct marrow and PHA stimulated blood and hematopoietic progenitors by PCR amplification of neo^r.

Table 5.6: Detection of opposite sex donor karyotypes from blood and marrow leukocytes in normal and MPS I fetal recipient pups[†].

	No. (%) opposite sex donor karyotypes in PHA stimulated blood (B) and marrow (M) by cytogenetic analysis									
	10 Weeks		12-14 Weeks		17-20 Weeks		23 Weeks		>28 Weeks	
Dog	B	M	B	M	B	M	B	M	B	M
N7	4/28 (14)	1/12 (8)	4/38 (10)	NM	3/35 (9)	NM			0/9	NM
N8	0/15		0/22	NM	0/22	0/17	NM	0/50		
M9	0/64		0/18	0/13	NM	0/27	0/17	0/40	0/65	0/15

Legend: [†] cytogenetic analysis was performed on all opposite sex donor-recipient pairs; *Weeks post-partum; NM, fixed sample did not contain analysable metaphases; Blanks, no data available.

5.4.5 Engraftment of LTMC Cells in Iduronidase Deficient Pups

Engraftment was assessed in three MPS I pups (M7, M8 and M9) by PCR analysis for proviral sequences from blood, marrow and hematopoietic colonies. The 3 dogs were sampled over 7-11 months with a total of 492 colonies assessed for the presence of provirus by PCR. The proportion of colonies positive for proviral sequences peaked at 5.6%-11.8% during the first 6 months. At time points greater than six months 2.2%-6.6% of colonies were provirus positive until the time of euthanasia (Table 5.5). A representative colony PCR and Southern blot analysis of dystrophin, proviral iduronidase and neo^r on 10 CFU-GM from dog M1 at 29 weeks of age are shown in Figure 5.5. All 10 CFU were positive for genomic DNA by dystrophin PCR. One colony was carrying the LCIDSN provirus as it was positive for both iduronidase and neo^r sequences.

To determine the proportion of differentiated hematopoietic cells carrying proviral sequences neo^r *in situ* PCR analysis was performed on marrow and blood leukocytes from two dogs M7 and M8. Provirus positive cells were detected in the blood and marrow from both dogs at all time-points up to 43 weeks (Figure 5.3). The levels of provirally marked leukocytes peaked at six weeks after birth for all three dogs at between 5-10%. The percentage positive cells dropped and were maintained at between 2-7% for the remainder of study to 40 weeks after birth. For example, in dog M8, the proportion of marrow and blood leukocytes carrying the neo^r containing provirus were 10-12% at six weeks of age. The levels of provirally marked leukocytes dropped to 5-8% at 10 weeks and were maintained at this level for up to 43 weeks, the length of study. For each sample, at least 300 cells were analysed. Negative controls were untreated dog samples and untransduced Jurkat cells. Untransduced Jurkat cells mixed with of 10% neo^r positive Jurkat cells and 100% positive Jurkat cells served as positive controls. For these samples, the 10% mix positive control was scored on average as 8.4% (range 7-11%), and the 100% neo^r positive controls were scored on average as 97.4% (range 96-100%). The negative controls were all scored as negative in this experiment. Donor engraftment was evaluable in one MPS I pup (M9) by cytogenetics. Donor male chromosomes were not detected in any metaphases from M7 at up to 37 weeks (analysis of 13-65 karyotypes at each time point).

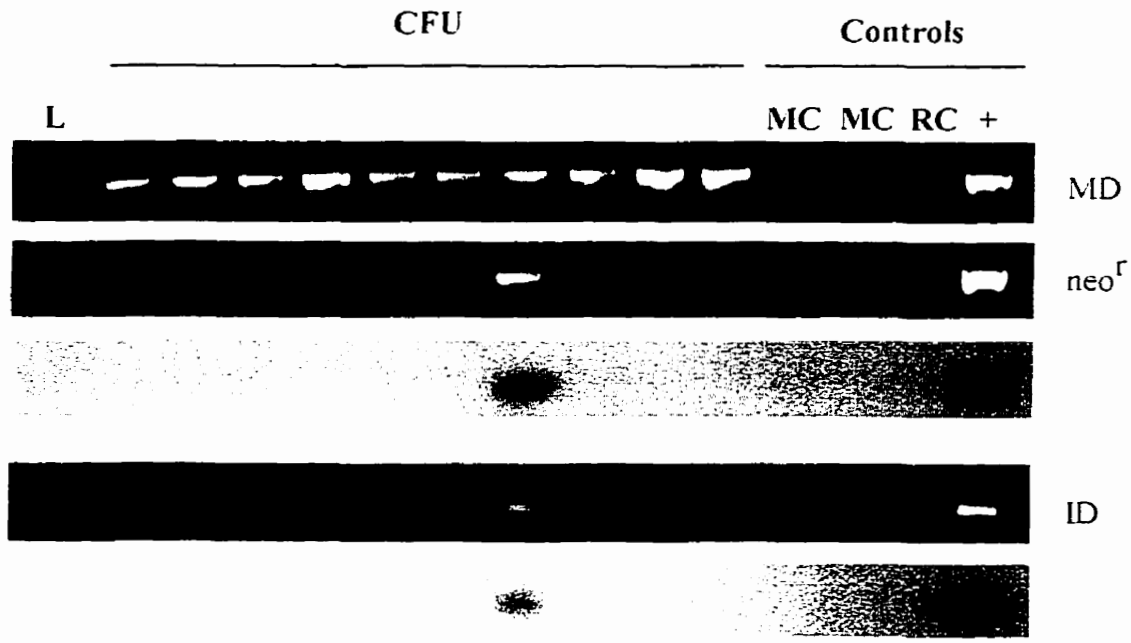

Figure 5.5: PCR amplification and Southern blot analysis on 10 marrow derived CFU-GM from dog M8 at 20 weeks post-infusion. Genomic DNA control for canine dystrophin (MD), proviral iduronidase (ID), and neo^r PCR analysis. This sample demonstrates 10 CFU positive for genomic DNA (MD), and 1 CFU positive for both iduonidase and neo^r sequences. Abbreviations: L, DNA ladder; MC, methylcellulose control; RC, reagent control; +, 200pg positive control DNA. The PCR assay was performed as described in Section 2.4.2 and Southern blotting as described in Section 2.4.3.

5.4.6 Proviral Iduronidase Expression

Iduronidase enzyme activity was not detected in blood or marrow leukocytes from any of the three fetal MPS I recipient pups at any time. The sensitivity of this assay was ~1% of normal. RT-PCR analysis was performed on blood and marrow leukocytes obtained during the first 35 weeks after birth. The internal control PCR that amplifies the same fragment from both the genomic and proviral iduronidase demonstrated the presence of amplifiable cDNA in all samples. However, proviral specific RT-PCR analyses were negative in all samples. A representative RT-PCR analysis on M7 and M8 is shown in Figure 5.6. Reverse transcriptase minus (RT-) controls from all samples were negative, demonstrating that there was no contamination of cDNA samples with genomic DNA.

Comparison of proviral marking and expression in CFU-GM

Individual hematopoietic colonies from M7 and M8 were evaluated for iduronidase activity. The proportion of colonies expressing iduronidase activity was consistently less than the proportion of colonies that were carrying proviral sequences. In general the proportion of colonies expressing iduronidase activity are low, with one or no colonies positive out of a minimum of 36 and maximum of 89 colonies (Table 5.7). For example in colonies from M7, the proportion of CFU-GM positive for iduronidase activity were 1/88, 0/36 and 1/36 at 10, 20 and 30 weeks of age. The levels of proviral marking at similar time points in dog M7 were 1/18, 4/34 and 3/45 for 10, 20 and 30 weeks of age. The level of iduronidase activity was similar in colonies derived from normal (range: 52-667 raw fluorescence units) or transduced MPS I (range: 65-421 raw fluorescence units) marrow samples.

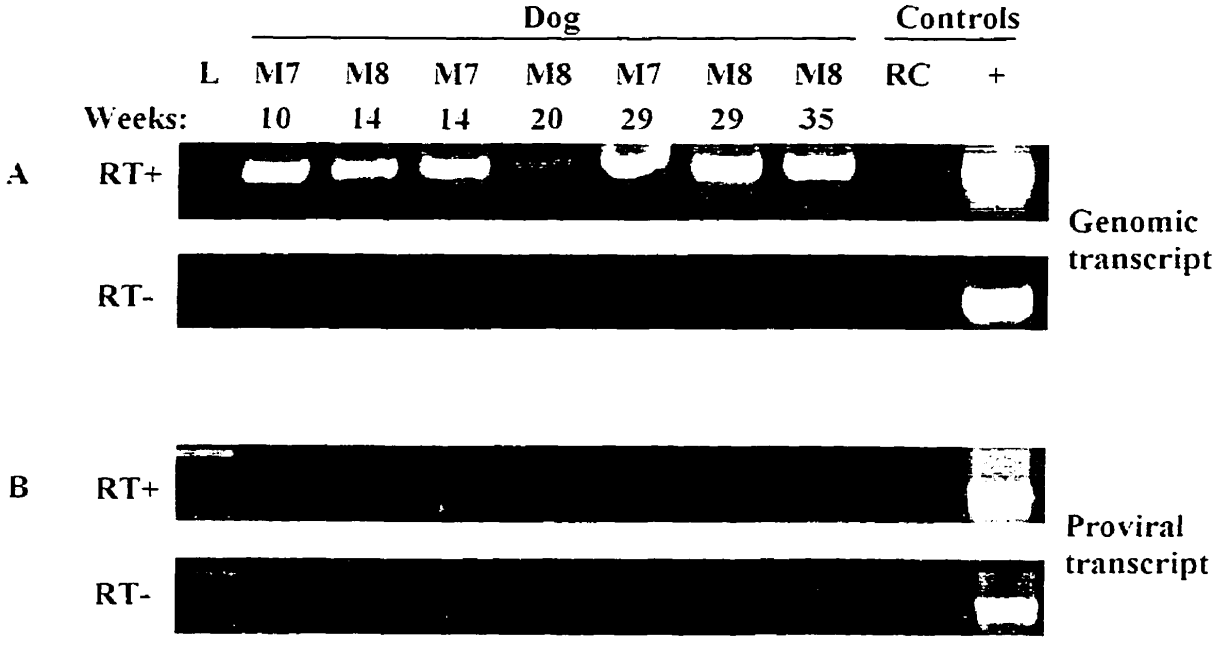

Figure 5.6: Expression of proviral iduronidase transcripts detected by RT-PCR analysis on marrow leukocytes from dogs M7 and M8. A) Assessment of the quality of cDNA demonstrated by exon 3-6 PCR which amplifies a 422bp fragment from both the genomic and proviral iduronidase transcript (genomic transcript). B) Proviral iduronidase expression detected by exon 1-6 PCR which amplifies a 618bp from the proviral specific transcript. Sample specific negative controls which were not treated with reverse transcriptase (RT-) were performed for each sample in each PCR. Abbreviations; L, DNA ladder; RC, reagent control; +, positive control for PCR amplification. This assay was performed as described in Section 2.5.2.

Table 5.7: Number (%) hematopoietic colonies positive for proviral sequences (PCR) and iduronidase activity (ID) at 10, 20 and 30 weeks post-partum.

	Untransduced MPS I control		10 weeks*		20 weeks		30 weeks	
Dog	PCR†	ID^	PCR†	ID^	PCR†	ID^	PCR†	ID^
M7	0/24	0/16	1/18 (5.5)	2/88 (2.3)	4/34 (11.8)	1/36 (2.7)	3/45 (6.6)	1/36 (2.8)
M8	0/32	0/12	1/29 (3.4)	1/74 (1.3)	5/56 (10.7)	1/81 (1.2)	2/54 (3.7)	0/89

Legend: *weeks post-partum; †PCR, neo^r and/or proviral iduronidase PCR analysis; ^ID, assay for ID activity.

5.4.7 Adoptive Transfer of Transduced LTMC Cells to MPS I Recipients

In an effort to both increase the number of proviral containing cells and evaluate immune responses following antigen challenge two *in utero* MPS I recipients, M8 and M9, were infused with autologous iduronidase transduced and expressing LTMC cells at 5 and 7 months respectively. The characteristics of the LTMC-transduction are detailed in Table 5.8. Marrow was harvested from M8 and M9 at 4 and 5 months of age respectively. Analysis of the marrow samples demonstrated no detectable iduronidase activity or proviral iduronidase transcripts at the time of harvest. The marrow mononuclear cells were established in LTMCs and transduced with the M48ID vector. At the end of culture on day 21 the LTMCs expressed greater than 100 units of iduronidase activity. However, upon infusion of the iduronidase transduced LTMC cells, no iduronidase was detected.

Table 5.8: Proviral iduronidase expression in marrow autografts from *in utero* recipient MPS I dogs at marrow harvest, after transduction with M48ID in LTMC and 1 month post-infusion.

		Marrow harvest				Transduced LTMC autograft†				1 Mo. post-infusion		
Dog	age*	Cells harvested	PCR	ID activity	RT-PCR	PCR	ID activity	RT-PCR	Cells infused	PCR	RT-PCR	ID activity
M2	4	$6.0x10^8$	pos	0u	neg	pos	~100u	pos	$1.5x10^8$	pos	neg	neg
M3	6	$7.0x10^8$	pos	0u	neg	pos	~100u	pos	$2x10^8$	pos	neg	neg

Legend: *in months; ID, iduronidase activity; u, nmol substrate/mg protein/hr; pos, positive; neg, negative; †Marrow autograft following 21 days of LTMC and transduction with M48ID.

5.4.8 Evaluation of Immune Responses

To determine whether *in utero* transplanted dogs mounted immune responses to iduronidase protein or LTMC media components, the presence of specific IgG against iduronidase and LTMC media components were evaluated by ELISA. Monthly serum samples taken from neonatal pups had only background levels of IgG directed against iduronidase, fetal bovine serum (FBS) and horse sera (HoS) in all three MPS I pups (Table 5.9).

The ability of fetal injection of transduced LTMC cells to induce long term immunological non-responsiveness toward iduronidase and fetal bovine and horse sera following antigen challenge was also evaluated. Immune responses against iduronidase protein and LTMC media components were evaluated monthly, and prior to and at 1 month post-infusion. A control for this experiment was an age matched MPS I dog, not previously treated with iduronidase enzyme of transduced cells, infused with similar numbers of autologous M48ID transduced cells. Following infusion of the transduced LTMC cells into *in utero* recipients of iduronidase transduced LTMC cells, specific anti-iduronidase IgG were not detected in either dog (Table 5.9). In the control MPS I dog, anti-iduronidase IgG titres were 1:400 after a single infusion of M48ID transduced autologous LTMC cells.

Humoral immune responses against LTMC media components FBS and HoS were at background levels until the time of infusion of transduced LTMC cells. However, serum samples from one month post-infusion demonstrated the development of anti-FBS and anti-HoS antibodies at titres of 1:1600-3200. Normal and MPS I uninfused littermates did not develop antibodies against FBS or HoS. In the control MPS I dog the anti-FBS and anti-HoS titres were 1:1600. This data indicates that contrary to results for cells expressing iduronidase, *in utero* infusion of LTMC media antigens does not induce long-term immunological non-responsiveness.

Table 5.9: Summary of IgG titres detected by ELISA in serum samples from MPS I dogs before and after infusion of a transduced LTMC autograft.

Dog	Boost*	ID	FBS	HS
M7		0	0	0
M8	Pre	0	0	0
M8	Post	0	1600	1600
M9	Pre	0	0	0
M9	Post	0	1600	3200
MPS I control‡	Pre	0	0	0
MPS I control‡	Post	400	1600	1600

Legend: *refers to sample prior to infusion of transduced autograft (Pre) or following autograft (post); †Titres are expressed as the dilution of serum giving an OD reading of >2X control dog serum; ‡MPS I control dog is a previously untreated MPS I dog receiving an M48ID transduced LTMC graft.

5.4.8 Pathology

All three MPS I dogs were euthanised at 8-10 months due to worsening clinical status. Pathologic examination was consistent with normal progression of MPS I disease. Vacuolated cells were prominent in the connective tissues from skin, gastrointestinal tract, liver, pancreas, heart and arteries, respiratory system, reproductive system, urinary and hemolymphatic systems. The heterozygote from experiment 2 was euthanised at 32 weeks due to juvenile onset renal failure.

5.5 Discussion

Lysosomal enzyme deficiencies are candidates for human hematopoietic cell gene therapies[25,26]. A canine model for the lysosomal storage disease, iduronidase deficiency, has been developed[17,18]. This canine model of MPS I has been used to evaluate several treatments for human MPS I such as bone marrow transplant[20], recombinant iduronidase enzyme infusion[21,22] and myoblast gene transfer[27]. Gene transfer to canine and human primitive hematopoietic progenitors during LTMC has been described with ~1-5% of hematopoietic progenitors carrying the provirus at up to 2 years post-infusion[24,28,29]. It has been predicted that as little as 1% of normal iduronidase expression will have therapeutic impact in iduronidase deficiency[25,30,31]. Thus it was hypothesised that transfer of the iduronidase gene to MPS I marrow cells during LTMC would ameliorate disease symptoms in canine MPS I.

In previous studies, iduronidase transduced LTMC cells were infused into autologous juvenile and adult iduronidase deficient recipients. These dogs had long term engraftment with low levels of marked cells but proviral iduronidase expression was not detected[28]. Immune responses against iduronidase protein, autologous cells expressing iduronidase and serum components of the LTMC media were detected. Clinical improvement was not seen in any dog. It was unclear from the latter experiments whether the lack of proviral iduronidase expression was due to either the presence of an humoral immune response eliminating provirally produced iduronidase enzyme, an insufficient level of provirally marked cells, silenced proviral iduronidase expression, or a cellular immune response eliminating cells expressing iduronidase[28].

Long-term engraftment of hematopoietic cells following adoptive transfer of HSCs into the peritoneal cavity of fetal sheep has been described[9,32]. Such studies documented that injection into mid-gestation pre-immune fetal animals can result in tolerance to a variety of cells and cellular antigens[32]. A pre-immune fetal dog transplant system was recently developed at this Institution in which injection of hematopoietic cells into the yolk sacs of mid-gestation pre-immune fetal dogs resulted in engraftment of xenogeneic cells[16]. In this experiment it was hypothesised that injection of LTMC cells, derived from allogeneic MPS I affected dogs and transduced with the normal canine iduronidase cDNA, in MPS I deficient fetal pups would

result in long term engraftment of genetically corrected cells and induction of tolerance to iduronidase enzyme and cells expressing it. Thus, the therapeutic potential of hematopoietic stem cell gene therapy for canine MPS I in the absence of immune responses could be evaluated.

Gene transfer into hematopoietic progenitors during LTMC was demonstrated by the presence of proviral sequences in the adherent layer LTMC cells and LTMC derived progenitors. The level of gene transfer into LTMC cells was comparable to others using the LTMC gene transfer protocol (Chapters 3 and 4;[22-24,28]). For example, 20-31% of hematopoietic progenitors derived from transduced LTMCs carried the provirus, which is comparable to the averages of 40%[23] and 44%[24] demonstrated in other canine LTMC gene transfer studies. The transduced LTMC cells expressed up to 100 fold greater than normal levels of iduronidase activity at the time of transplant[28]. High levels of expression from retroviral vectors *in vitro* has been demonstrated in other human and canine iduronidase gene transfer studies[27,33-35].

The LTMC cells were adoptively transferred to the yolk sacs of individual fetal pups using ultrasound guidance. The *in utero* adoptive transfer protocol was developed at this Institution for the study of human hematopoiesis in human-canine hematopoietic chimaeras. This approach takes advantage of the migration of hematopoietic progenitors from the yolk sac to the fetal liver after mid-gestation[36]. Thus, it was predicted that injection of hematopoietic cells to the yolk sac would lead to a similar migration of hematopoietic progenitors to the fetal liver. The *in utero* sheep transplant model uses intraperitoneal injection of test cells, however there is high mortality in the fetal sheep. In one study, for example, 2/7 fetal recipients died *in utero* following injection[37]. In the yolk sac gene transfer protocol used here there is lower fetal mortality. For example, all thirteen fetal pups receiving human LTMC cells in the xenogeneic study completed gestation[16], and in the current study all seventeen pups receiving allogeneic LTMC cells completed gestation. In the allogeneic studies described here pups died neonatally, consistent with difficulties during whelping and unrelated to the transplant procedure. The results of this experiment confirm the safety of the yolk sac injection demonstrated previously[16] as there was long-term engraftment with no loss of fetal pups during the transplant procedure.

Allogeneic canine LTMC cells engrafted following yolk sac injection in mid-gestation fetal recipients and contributed to hematopoiesis for 1 year. The results of this experiment were comparable to those achieved following the injection of human LTMC cells into fetal canine recipients[16]. Following injection, provirally marked cells engrafted and contributed to neonatal hematopoiesis in all normal and iduronidase deficient pups. The contribution of provirus-containing cells to hematopoiesis was up to 12% of hematopoietic colonies for up to 1 year. However, lower levels of differentiated cells were detected with ~1% of blood or marrow leukocytes carrying proviral sequences. Donor cell engraftment was confirmed by cytogenetic analysis in one of three evaluable dogs.

Pre-immune fetal recipients are susceptible to graft versus host disease following injection of unprocessed marrow or cord blood[6,39]. T cell depletion or selection for CD34 positive hematopoietic cell populations reduces risk of graft versus host disease, but results in lower levels of engraftment[9,37,40]. In the three allogeneic fetal transplant experiments described here, all recipients engrafted with provirally marked LTMC cells with no evidence for graft versus host disease. This finding is consistent with the injection of human LTMC cells to fetal pups in which there was no evidence for graft versus host disease[16]. This is likely due to the low maintenance of B- and T- lymphocytes in LTMC[41].

Factors that could affect the level of engraftment include the cell dose and gestational day at administration. In this experiment, each recipient received 5-15x10^6 LTMC cells. The pups receiving the higher number of cells had similar levels of engraftment to pups receiving lower numbers of cells in the other two litters. This may be because there are a limited number of "receptive sites" in the mid-gestation fetal hematopoietic environment for hematopoietic progenitors and stem cells to engraft and that these sites are saturated in this range. In the studies described here, sustained engraftment in canine recipients occurred following injection at days 32-38 in a 63 day gestation. The level of engraftment does not appear to be affected by the specific day of injection between 32-38, as the litter with the lowest engraftment was injected at an intermediate day 35. This indicates that injection of allogeneic hematopoietic cells on or before day 38 of gestation in the yolk sacs of fetal canine pups can result in graft maintenance.

The MPS I dogs transplanted *in utero* did not mount immune responses against iduronidase protein or horse and fetal bovine sera *in utero*. These results are similar to other studies in which fetal dogs injected with soluble antigens such as bovine serum albumin at 40 or 48 days of gestation[42], or keyhole limpet hemocyanin at 41-55 days of gestation[43] did not mount humoral immune responses against the respective antigens.

Two MPS I dogs in this study were infused with iduronidase transduced cells after birth. These dogs did not mount immune responses against iduronidase, suggesting that the dogs maintained long term non-responsiveness against iduronidase. However, the dogs did mount immune responses against fetal bovine and horse sera. These contrasting results may be due to the large amount of iduronidase enzyme protein expressed in the graft and the continued expression of the iduronidase *in vivo* for several weeks after infusion, while the bovine and horse derived antigens were at low levels on the graft and were not produced *in vivo*. Alternatively, the differences may be due to the antigenicity of the antigens, as stronger immune responses are induced against the serum antigens than iduronidase in juvenile MPS I recipients of iduronidase transduced LTMC cells[28].

Despite the engraftment of cells genetically modified to carry the normal canine iduronidase cDNA and the absence of anti-iduronidase immune responses there was no evidence for disease amelioration in the *in utero* transplanted iduronidase deficient pups. Evaluation of proviral iduronidase expression demonstrated that neither iduronidase activity nor proviral iduronidase transcripts were detected in blood or marrow mononuclear cells from MPS I recipients. Iduronidase activity was detected in 0-2.8% of hematopoietic colonies. However, the absence of clinical benefit of *in utero* HSC transplantation in any iduronidase deficient dog demonstrates that the low level of iduronidase activity detected in progenitors was not enough to ameliorate disease symptoms.

Down regulated transgene expression from MMLV based retroviral vectors has been demonstrated in other studies[44-46]. Several regions of the MMLV LTR used in these experiments are associated with proviral gene suppression. For example, the LTR enhancer repeats[44,47,48], and two regions upstream of the enhancer repeats, a negative control region and a binding site for embryonal carcinoma cell factor 1 binding site[47,49] are associated with repression of proviral gene expression in MMLV based vectors. The MMLV primer binding

region has a repressor binding site that inhibits LTR directed transcription[50,51]. Methylation of the proviral MMLV genome is closely associated with transcriptional inactivation of proviral gene expression. Inclusion of a hypomethylation signal in the proviral LTR increases proviral gene expression[52]. Several new retroviral vectors have been generated which utilise an MPSV LTR and have modified many of these negative acting regions and sequences[47,52,53] and result in extended high level expression *in vivo*[46,54].

This study demonstrated that hematopoietic progenitors transduced in LTMCs can engraft *in utero* and contribute to hematopoiesis for at least one year after birth. Furthermore, adoptive transfer of iduronidase transduced LTMC cells *in utero* appears to abrogate the development of the anti-iduronidase immune response seen in dogs transplanted at 2-12 months of age. The absence of iduronidase enzyme activity in MPS I fetal recipients, despite engraftment of progenitors carrying the provirus, suggests that higher levels of iduronidase expression in higher proportions of cells would be necessary to achieve clinical benefit in iduronidase deficient dogs.

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Summary and Future Directions

Gene therapy has been proposed as a treatment for single gene inherited disorders such as enzyme deficiencies. Although recent advances have increased gene transfer efficiency to a variety of cell types, there is limited evidence that gene therapy can actually be of therapeutic benefit in human disease or in large animal models. The research undertaken in this thesis was designed to further our understanding of the potential of HSCs to serve as vehicles for therapeutic gene delivery using a canine model of iduronidase deficiency. The experiments evaluated the potential of retrovirally transduced HSCs to contribute to long-term hematopoiesis following adoptive transfer to canine recipients. The latter two experiments also assessed the therapeutic potential of HSCs, genetically modified to carry the normal canine iduronidase cDNA, to engraft and ameliorate disease in juvenile and fetal canine MPS I recipients.

Following adoptive transfer of genetically modified HSCs to juvenile or adult recipients, up to 10% of CFU and 1% of leukocytes carried proviral sequences for up to 3 years. However neither iduronidase enzyme activity nor therapeutic benefit was detected in any MPS I dog. Humoral and cellular immune responses against iduronidase, and autologous cells expressing it, were detected in juvenile MPS I recipients despite cyclosporine A treatment. It was hypothesised that the anti-iduronidase immune responses eliminated the iduronidase protein and/or cells expressing it thereby abrogating the therapeutic benefit of HSC gene therapy in these dogs.

Pre-immune fetal transplant has resulted in the induction of tolerance to donor cells in other studies. Thus, adoptive transfer of iduronidase transduced MPS I marrow cells was performed in mid-gestation fetal recipients to evaluate therapeutic impact of this treatment in the absence of immune responses. Following engraftment with iduronidase transduced HSCs recipients remained immunologically non-responsive to iduronidase protein. However, iduronidase activity was not detected and all MPS I dogs had continued disease progression. The absence of iduronidase enzyme activity in MPS I fetal recipients, despite engraftment of progenitors carrying the provirus, indicates that low or absent transgene expression contributed to the lack of therapeutic benefit in these MPS I dogs. The level of engraftment

and long-term retention of genetically modified progenitors is within the range thought to be therapeutic for iduronidase deficiency. However, higher levels of iduronidase expression in a greater number of cells will be necessary to achieve clinical benefit in iduronidase deficient dogs.

The results of the experiments undertaken in this thesis highlight the importance of efficient gene transfer to HSCs, sustained transgene expression and the potential for immune responses against therapeutic transgenes to mitigate therapeutic benefit in deficiency disorders. Adoptive transfer to fetal MPS I recipients resulted in the induction of immunologic non-responsiveness against proviral derived iduronidase overcoming the later obstacle. However, evaluation of other methods of tolerance induction such as oral tolerance or thymic infusion may provide a more widely applicable method for induction of tolerance to transgenes. Future studies need to focus on achieving higher levels of HSC gene transfer with vectors optimised for long-term transgene production. Recent studies have demonstrated efficient gene transfer to HSCs from non-human primates and their contribution to up to 20% of circulating cells for one year post-transplant. It is predicted that this level of HSC marking and contribution to hematopoiesis would be therapeutic in a number of single gene disorders including MPS I. The studies undertaken in this thesis have also demonstrated the necessity of long-term reliable transgene expression. Several promising new retroviral vectors have demonstrated long-term transgene expression in murine hematopoietic cells, however, these vectors need to be evaluated in large animals and xenogeneic human model systems to determine their potential for long-term transgene expression. A greater understanding of the biology of hematopoietic stem cells and hematopoiesis, gene delivery and transgene expression are needed for the development of reliable and efficacious clinical HSC gene therapy protocols for single gene disorders.