Retroviral Mediated Gene Transfer and Expression of the Multidrug Resistance-Associated Protein 1 for Hematopoietic Chemoprotection: Preclinical Trials in a Canine Model

by

Tarja Alison Juopperi

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of
Laboratory Medicine and Pathobiology
University of Toronto

This thesis is dedicated to my parents, Pentti and Carol Juopperi and my grandparents,

Kenneth and Geraldine Abbott.

Abstract

Retroviral Mediated Gene Transfer and Expression of the Multidrug

Resistance-Associated Protein 1 for Hematopoietic Chemoprotection:

Preclinical Trials in a Canine Model

Master of Science, 2000

Tarja Alison Juopperi

Department of Laboratory Medicine and

Pathobiology, University of Toronto

Multidrug Resistance-Associated Protein 1 (*MRP1*) is a member of the ABC superfamily of transporters that confers multidrug resistance. We hypothesized that the transfer and expression of the *MRP1* gene to hematopoietic stem cells (HSCs) might provide protection from the myelosuppressive effects of chemotherapy. We developed a retroviral producer cell line, MRP1-PG13, and optimized conditions for transfer of the human *MRP1* cDNA into HSCs. We demonstrated that MRP1-PG13 is able to transfer *MRP1* to human cultured cells and that expression gives rise to drug resistance. We also established that *MRP1* gene transfer into canine hematopoietic progenitors (CD34$^+$) was feasible *in vitro* at a level of ~13%. For *in vivo* studies, *MRP1* transduced autologous CD34$^+$ cells were infused into two dogs. Dogs received multiple drug challenges. Despite the lack of hematopoietic chemoprotection, we demonstrated that vector-containing cells engrafted and proliferated, as *MRP1* positive CFUs were detected in blood and marrow after drug challenges.

Acknowledgements

I would like to thank my supervisor Dr. Ian Dubé for giving me the opportunity to expand my horizons and pursue my interest in research. Dr. Dubé has continually encouraged and challenged me to work hard and strive for excellence. He has been a great role model and mentor. I feel very fortunate to have been able to learn from him not only about science, but life.

I would also like to thank Dr. Stephen Kruth for believing in me and supporting my career choices. Dr. Kruth has played an important role in my graduate studies. As a member of my committee he has provided veterinary expertise, insightful comments and constructive criticism. On a personal level, he has listened, given advice, encouraged and helped me to succeed. He has been instrumental in directing my future path.

My committee members: Drs. Margaret Hough, Stephen Kruth, Megan Lim, Marciano Reis, Keith Stewart and Paul Woods have provided support and direction for this project and have challenged me with their stimulating questions and concerns. I am grateful for their expertise, advice and commitment to my project. Special thanks to Drs. Stephen Kruth and Paul Woods for their excellent veterinary care.

I would like to express my thanks to current and previous members of our laboratory: Kin Chan, Liheng Li, Shaherose Nanji and Yongjun Zhao, for taking the time to explain and demonstrate the techniques that were essential for my research. They have spent a great deal of time working with me and have all contributed significantly to this project. I would also like to thank fellow laboratory members Carolyn Lutzko, Lisa Cechetto and Suzana Kablar. They have all made this experience an enjoyable and memorable one. A special thanks to Mrs. Renita Yap for her excellent administrative support. Renita has always offered her help and has frequently provided her assistance.

Fellow students, Mary Anne Viani and Ali Shahnavaz have provided emotional support during the last 18 months and have always been available to talk, listen and give advice. Their friendship has been, and will continue to be very important to me.

Finally, I would like to express my deepest appreciation and thanks to my parents, grandparents and sister. They have all provided emotional and financial support during my studies. They have always encouraged me to pursue my goals and have believed that I could achieve them. I would not have been able to complete this work without them.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

AAV	adeno-associated virus
ABC	ATP-binding cassette
ADA	adenosine deaminase
AML	acute myelogenous leukemia
BM	bone marrow
BSO	buthionine sulfoxide
CBC	complete blood count
CFU	colony forming unit
DHFR	dihydrofolate reductase
FANCC	Fanconi anemia type C
FLT3L	FLT-3 ligand
GALV	gibbon ape leukemia virus
GC	glucocerebrosidase
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
HIV	human immunodeficiency virus
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSC	hematopoietic stem cell
IL-3	interleukin-3
IL-6	interleukin 6
LTMC	long term marrow culture
LTR	long terminal repeat

MDR	multidrug resistance
MGDF	megakaryocyte growth and development factor
MMLV	Moloney murine leukemia virus
MRP	Multidrug Resistance-Associated Protein
MTX	methotrexate
Neo	neomycin phosphotransferase
NGFR	nerve growth factor receptor
PB	peripheral blood
PCR	polymerase chain reaction
P-gp	P-glycoprotein
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
TBI	total body irradiation
TK	thymidine kinase
TPO	thrombopoietin
VCR	vincristine
VEGF	vascular endothelial growth factor
WBC	white blood cell

Chapter 1

Literature Review

1.1 Historical Perspective of the Field of Gene Therapy

Somatic cell gene therapy is currently an extensively studied approach to the treatment of inherited and acquired disorders. The concept of gene therapy is however, not new. Practical support for its potential application to human diseases can be traced back to the 1960's. Data from several laboratories demonstrated that mammalian cell lines containing single gene defects, such as hypoxanthine-guanine phosphoribosyl transferase (HPRT) or thymidine kinase (TK) deficiency could be corrected by the introduction of genomic DNA from wild type cells[1]. In these early applications, foreign genetic material was transferred to deficient cells by creating conditions that favoured DNA uptake and by applying appropriate selection pressures in tissue culture. These early, relatively simple, experiments clearly demonstrated the feasibility of gene transfer into mammalian cells for correcting genetic defects and paved the way for further studies[1].

More powerful methods of gene transfer were developed in the 1970's and 1980's when viruses were recognized as prime candidates for gene delivery vehicles[1]. By then, virologists had determined that some viruses had optimized conditions for inserting their genetic material into that of the host avian or mammalian cell and for some viruses, the viral genome became permanently integrated into the target cell's genome. Several investigators realized the potential for viruses to serve as vehicles for gene transfer and initiated studies designed to modify viruses for therapeutic purposes[2-7]. In these applications, undesirable and pathogenic viral genes were replaced with DNA encoding the genes of interest. Subsequently, viral packaging cell lines were developed that were capable of producing non-pathogenic viral vectors containing the transgene[8,9]. Inherent in the design of these vectors was the requirement that once successful targeting had occurred there was minimal chance of further viral spreading. Such viral vectors were used to successfully correct hereditary enzymatic defects in tissue culture by specifically transferring wildtype genes to cell lines established from affected patients. Other studies involving a variety of genes and mutant cell lines clearly demonstrated that engineered viruses had great potential as vehicles for the genetic modification of mammalian cells[1].

By the end of the 1980's, investigators were able to seriously consider the therapeutic applications of genes, as the progress in mammalian gene transfer techniques (including the development of viral and non viral vectors) and the advances in recombinant DNA technology made all the essential tools available[1]. Candidate diseases and potentially therapeutic genes were identified and gene delivery systems became more widely accessible[10]. Blood cells, particularly hematopoietic stem cells, were also recognized as ideal targets for the delivery of gene therapies[10]. Extensive *in vitro* and pre-clinical studies followed, with many of these studies providing promising results. The information gathered stimulated further investigations and provided the impetus to initiate clinical trials. Currently, there are a large number of clinical trials underway worldwide and gene therapy is being developed for the treatment and prevention of a variety of inherited and acquired disorders[2,5,7,11].

1.2   Current Status of Gene Therapy for Human Disease

The first human gene therapy clinical trial was initiated in 1990[12]. A decade later, there are approximately 300 clinical trials worldwide utilizing gene transfer[2,11,13]. Although gene transfer is potentially a very versatile therapy, the majority of clinical trials are directed towards the treatment of cancer. The remaining trials are focused on monogenic inherited or infectious diseases.

There are two main approaches to the transfer of therapeutic genes: *in vivo* and *ex vivo* gene transfer. Various methods have been employed, however it appears that viral vectors are more effective than non-viral vectors[5,6,14,15]. Many of the human clinical protocols have utilized murine retroviruses and adenoviruses as vectors for gene transfer. The results from phase I clinical trials have been rather disappointing[5,7,11,13]. Though the main objective of determining the feasibility of gene transfer was achieved, few investigations have had any impact on clinical outcome.

Barriers or obstacles to successful gene transfer have been identified. One of the main problems facing gene therapy is the poor efficiency of gene transfer[5,6,14,16]. Inadequate delivery of therapeutic genes and transient transgene expression are critical limitations for many of the commonly used viral vectors. Optimizing viral vectors requires enhancing the accuracy of vector targeting, increasing transduction efficiency and increasing the magnitude and duration of transgene expression. Other issues that need to be addressed are limiting the immunogenicity, reducing the toxicity, and increasing the safety of gene delivery systems[6].

Recently, impressive results have been obtained in clinical trials of critical limb ischemia. Baumgartner et al. injected naked plasmid DNA encoding the angiogenesis promoting factor vascular endothelial growth factor (VEGF) into ten limbs of nine patients[17]. Collateral blood vessel development was reported in seven limbs. Ischemic ulcers healed in four of seven limbs and limb salvage was possible for three patients that were recommended for below-knee amputation. These results are encouraging and provide evidence that gene therapy may one-day play a significant role in clinical medicine once technical obstacles have been resolved.

Many of the current gene therapy trials utilize retroviruses as vectors and hematopoietic stem cells as targets for gene transfer. The following section will review the use of these vectors, the problems associated with hematopoietic stem cell gene therapy and the strategies used to overcome the barriers to successful gene transfer.

1.3   Retroviral Mediated Hematopoietic Stem Cell Gene Therapy

1.3.1   Hematopoietic Stem Cells as Targets for Gene Therapy

Hematopoiesis is the process by which blood cells are maintained at physiological levels. This tightly regulated process is maintained by long-lived, primitive hematopoietic stem cells (HSCs). HSCs are defined as cells that are capable of self-

renewal and have the ability to give rise to progeny that can differentiate into all cells of the lymphoid, erythroid and myeloid lineages[18,19]. Although HSC are found at very low numbers (approximately 1 per 10^6 bone marrow cells), they are relatively easy to access as they can be found in adult and fetal bone marrow, umbilical cord and peripheral blood. HSCs retain their ability to proliferate and differentiate after *ex vivo* manipulation, which has enabled their use in various clinical protocols[18].

The hematopoietic stem cell (HSC) is an optimal target for many gene transfer protocols. The genetic modification of these cells for the prevention and treatment of acquired and congenital diseases is being actively pursued. The insertion of a therapeutic gene into the genomes of pluripotent stem cells could provide long term persistence of genetically modified cells in the hematolymphoid compartment[20,21]. Theoretically, any genetic disease correctable by HSC transplantation could be considered a candidate disease for HSC gene therapy[10,22]. Utilization of HSCs for disease treatment is an exciting idea, however there are certain inherent characteristics of these cells that provide challenging obstacles for their use in gene therapy.

HSCs are rare cells and acquiring sufficient numbers to achieve significant clinical benefits is a major concern when working with this cell target population[23]. Several procedures have been developed to overcome this limitation, including the use of mobilization regimens (chemotherapy and/or hematopoietic growth factors) combined with leukapheresis. Clinical studies have shown that the number of circulating hematopoietic progenitor cells in the peripheral blood may be increased with the use of various mobilization regimens[24]. Hematopoietic cytokines such as granulocyte colony stimulating factor (G-CSF) or granulocyte macrophage colony stimulating factor (GM-CSF) are frequently used to induce large numbers of hematopoietic progenitor cells into circulation[25,26]. Once mobilized, HSC can be isolated using *in vitro* techniques such as fluorescence activated cell sorting. This approach of isolating stem cells is based primarily on the fact that HSCs express the cell surface antigen CD34 and lack expression of cell surface markers associated with lineage commitment[27].

CD34 is a surface glycophosphoprotein expressed primarily on primitive hematopoietic progenitor cells, although it is also found on small-vessel endothelial cells and embryonic fibroblasts[26,28]. A small subset of cells expressing CD34 have been shown to be capable of differentiating into various blood cell lineages, however the function of this protein is not fully understood. Enrichment for CD34$^+$ cells may aid in isolating a subpopulation of cells that have the ability for long-term hematopoietic reconstitution. Recent investigations using a human/sheep competitive engraftment model and a murine competitive repopulation assay, have demonstrated that a CD34$^-$ cell population is capable of multilineage hematopoietic reconstitution[21,29-32]. These studies suggest that a CD34$^-$ cell population might contain the earliest precursors of all hematopoietic lineages[29]. Further studies are required to resolve this controversy.

The majority of HSCs are in a state of quiescence, which poses an additional difficulty in employing these cells as targets for gene therapy[10,33]. Many clinical gene therapy trials utilize retroviruses as vectors for gene transfer. These viruses require the target cell to be actively cycling to achieve proviral integration[10,21]. The use of recombinant hematopoietic cytokines that have been shown to regulate cycling of primitive progenitors such as thrombopoietin and FLT-3 ligand or tissue culture systems that promote cell cycling, are current methods used to overcome this problem[21,30].

The lack of adequate human *in vitro* and *in vivo* stem cell assay systems is another impediment to the use of HSCs in gene therapy protocols. Currently, human HSCs are studied indirectly by examination of their progeny using clonogenic assays or by using long-term culture systems to detect more primitive hematopoietic cells[21,34]. *In vivo* stem cell assays are available in animal systems such as the mouse, however they are not practical in human applications. For example, HSCs can be assayed by their ability to reconstitute the hematopoietic systems of lethally irradiated animals[35]. Variations of these assays using xenogenic recipients such as severe combined immunodeficient (SCID) mice or fetal sheep, have been developed as a means of investigating human hematopoiesis *in vivo*. These reconstitution assays, while limited, are important, as presently there are no *in vivo* stem cell assays for humans[34].

Using HSCs as targets for gene transfer is challenging and many problems have been identified. However, the potential therapeutic use of genetically modified HSCs provides incentive to continue developing approaches to harness the tremendous proliferation potential of these unique cells.

1.3.2 Methods of Gene Delivery

Efficient gene delivery systems are required to deliver foreign genes to target cells. It is essential that such vectors are able to insert the gene of interest into specified cells and that transgene expression occurs for an appropriate length of time. Two general categories of vector systems exist: viral and non-viral vectors[6,14,16]. Viral vectors include adenoviruses, adeno-associated viruses, herpes viruses, murine retroviruses and more recently lentiviruses. Non-viral techniques include chemical and physical methods such as electroporation, calcium phosphate precipitation, DNA-protein complexes and the use of liposomes. Each of these gene transfer techniques has certain advantages and the decision to select one over the other is largely based on the particular application. The delivery system chosen should be relatively easy to use, safe and provide non-toxic and non-immunogenic delivery of DNA to selected cells at specified doses and times[15,36].

Many of the current clinical gene therapy trials employ *ex vivo* methods to deliver therapeutic genes in which cells are removed and genetically modified outside of the patient[15]. *Ex vivo* methods minimize the chances of inadvertent germ-line cell modification and may yield a higher gene transfer efficiency while enabling the analysis and selection of genetically modified cells before they are returned to the patient[14,15]. Safety concerns may also be addressed before re-infusion of genetically altered cells. This approach however, may be limited to specific cell types such as HSCs that are capable of being manipulated *ex vivo*. They are also time consuming and complex. *In vivo* methods of gene delivery may provide a better alternative to *ex vivo* manipulation, as they may allow the introduction of a therapeutic gene to a wider range of target cells[15]. These methods may prove to be simpler, however gene transfer efficiency and expression

may be reduced by a number of complicating factors such as competitive uptake by non target cells.

All gene delivery systems presently available have specific limitations or drawbacks. Further refinement of existing technology is necessary for the development of the ideal vector.

1.3.2.1 Retroviral Vectors

Retroviral vectors have proven to be invaluable tools for the genetic modification of cells. RNA viruses, particularly the murine retroviruses, have been selected as the vector of choice for many protocols[8]. Retroviruses have a diploid RNA genome and virus replication enzymes that are contained within a viral protein core. They replicate through a DNA intermediate and have developed the ability to insert their genetic material into the host cell genome[37]. Gene therapy protocols employing retroviruses utilize this feature for their advantage, allowing stable integration of therapeutic genes into target cells. Most clinical gene therapy protocols use vectors based on the Moloney Murine Leukemia Virus (MMLV)[8]. The genome of this virus is relatively simple, consisting of three genes designated *gag*, *pol*, and *env*[37]. The *gag* gene encodes the viral core proteins and the *pol* gene encodes the viral replication enzymes reverse transcriptase and integrase. The *env* gene encodes the viral envelope, a glycoprotein that covers that surface of the virion. The envelope proteins mediate virus absorption to the target cell and determine the viral tropism or target cell specificity of the virion. Viruses with ecotropic *env* can infect murine cells, whereas amphotrophic *env* facilitates entry into a variety of mammalian cell types including human, murine and canine cells.

In the construction of a retroviral vector, deletion of the viral genes allows for the insertion of 6-8 kb of foreign DNA[14-16]. These alterations disable the virus in such a manner that it is replication incompetent, a feature that is essential for clinical safety.

Vectors must include the packaging signal (Ψ) and elements that are necessary for the integration of the virus contained in the long terminal repeats (LTRs)[9,38,39].

Packaging cell lines have been generated to produce infectious replication incompetent retroviral vectors[9,38,39]. These cells have been designed to form retroviral structural proteins, but lack the packaging signal required to transmit the RNAs encoding these functions. Packaging cell lines are able to produce retroviral vectors containing the transgene that are capable of one-time cell infection. Several modifications have been made to these cell lines to prevent the formation of replication competent retroviruses through rare recombinational events.

Though retroviral vectors are capable of achieving stable and efficient transduction of a gene into target cells, limitations to their use as gene delivery vehicles in HSC gene therapy exist. Retroviral vectors require cells to undergo mitosis in order for proviral integration to occur[8,40]. HSC quiescence can limit the efficiency of gene transfer with retroviral vectors. Another significant limitation is the number of amphotrophic retroviral receptors present on the surface of HSCs. The interaction between the virus envelope surface protein and the host cell membrane receptor protein is fundamental for viral internalization[8,40]. Amphotrophic MMLVs are able to infect human cells, however it appears that HSCs express a relatively low number of these receptors. Inadequate numbers of viral receptors on HSCs may result in low retroviral gene transfer efficiency[41,42].

Retroviral vectors insert randomly in the host cell genome[8]. As a result, there is a risk of cell transformation by insertional mutagenesis, possibly due to the activation of proto-oncogenes or the disruption of a tumour suppressor gene. Disruption of a gene essential for normal cell function may also occur. Considering that the development of cancer is a multistep, multifactorial process, the risk of malignancy developing appears to be low[15].

Investigation into improving retroviral mediated gene transfer is ongoing. Methods to induce cell cycling and the use of pseudotyped retroviral vectors are strategies that have been used to overcome some of the limitations of retroviral vectors[9,42,43]. New techniques are currently being developed and integrated into HSC gene transfer protocols.

1.3.2.2 Other Viral Vectors

Retroviruses have been used extensively in clinical HSC gene therapy protocols, however the limitations experienced with their use has initiated the quest for more efficient viral vectors. Many viruses have been studied and several have been selected as promising candidates for the development of alternative vehicles for gene delivery[14,15]. Among these viruses are the adenoviruses, the adeno-associated virus and lentiviruses.

1.3.2.2.1 Adenoviral Vectors

Adenoviruses belong to the Adenoviridae family and their members share the following basic characteristics: they are nonenveloped viruses that have a DNA genome and an icosahedral symmetry[15,44]. Several features make them useful vectors for gene therapy. They are relatively safe viruses that can be used for *in vivo* or *ex vivo* gene delivery and they can be produced at fairly high titers. Adenoviruses have significant advantages over retroviral vectors in HSC gene therapy. They are able to infect both actively dividing and quiescent cells, making them suitable for targeting HSCs. Recent studies have also demonstrated that adenoviral vectors may be able to achieve a higher level of transgene expression in hematopoietic progenitor and precursor cells than retroviral vectors[45-48]. The major limitation to their use in HSC gene therapy is the transient nature of the transgene expression. Adenoviral vectors are unable to integrate their viral DNA into the host cell genome. Long-term expression of the transgene is

essential for many HSC gene therapy protocols. Adenoviral vectors may be more suited for other applications of gene therapy where this feature is not critical[49].

The occurrence of serious and potentially life-threatening adverse effects is another important consideration in the use of adenoviruses as vectors for gene transfer[50-54]. Though adverse reactions may not occur, mild influenza-like symptoms and severe inflammatory reactions have been associated with the use of adenoviral vectors *in vivo*. Recently, a gene therapy related death has occurred in a clinical trial utilizing adenoviral vectors. More stringent guidelines to their use in clinical protocols and further safety evaluations are necessary to avoid this tragedy[52,54].

1.3.2.2   Adeno-Associated Viruses

Adeno-associated viruses (AAV) are small single-stranded DNA viruses that are members of the parvovirus family. These viruses have not been associated with human disease and wildtype AAV integrates site-specifically to chromosome 19[55]. Vectors for human gene transfer have been designed using AAVs. These vectors have been demonstrated to have low immunogenicity and have displayed long-term transgene expression after *in vivo* administration[56]. There are several disadvantages to using these vectors including the small insert size available (4.5 kb) and the costly price to manufacture them. Also, the recombinant virus appears to integrate more randomly than wildtype AAV. AAV have been shown to be useful vectors for gene transfer to HSCs as they are capable of transducing non-differentiated cells[57]. Studies utilizing AAV vectors have demonstrated successful gene transfer to primitive human and non-human primate hematopoietic progenitors[44,58]. Stable integration and long-term expression was exhibited in some studies, whereas in others, transgene expression was transient. Though the feasibility of HSC gene transfer using AAVs has been established, further work is required to optimize conditions and determine the usefulness of these vectors.

1.3.2.2.3   Lentiviral Vectors

Lentiviruses are members of the Retroviridae family. These retroviruses are currently being explored as vectors for HSC gene transfer[37]. Lentiviral vectors have all of the advantages of the MMLV based vectors, as well as the special ability to transduce nonproliferating cells[59]. Unlike other retroviruses, lentiviruses are able to transport their genetic material through the intact nuclear membrane[37,60]. Many HIV-based vectors have been used to investigate lentiviral gene transfer to HSCs. These vectors have been shown to efficiently transduce human CD34^{+} hematopoietic progenitors and provide long term expression of the transgene[61]. Lentiviral based gene transfer to HSCs is still in its infancy and several concerns such as vector safety, vector production and vector performance still need to be addressed.

1.3.2.3   Physical Methods of Gene Transfer

Physical or non-viral methods of gene transfer are being investigated as tools for HSC gene therapy. Methods such as electroporation or the use of liposomes to transfect HSCs may be simpler and safer to use than traditional viral vectors[14]. These methods are free of some of the limitations and complications of using viruses such as the transfer of viral genes[62]. The transfer of naked DNA to hematopoietic cells has been shown to be feasible using electroporation. Electroporation can be used to transfect a wide variety of cells and though stable integration is possible, the levels of expression are typically low. Studies of liposome-mediated gene transfer to hematopoietic progenitors have demonstrated that transfer of exogenous genes to HSCs occurs, however expression is transient and low levels are common[63,64]. Although physical methods are currently being examined for gene transfer to HSCs, they are not as widely used as viral vectors.

1.3.3 Hematopoietic Stem Cell Gene Transfer: Animal Studies

A majority of the early HSC gene transfer studies consisted of gene marking clinical trials. These investigations were not designed for therapeutic purposes, but rather to advance the state of knowledge about the feasibility and safety of gene transfer and to provide valuable information relevant to stem cell biology and disease pathogenesis[1]. Animal models have been used extensively in gene marking trials and the pertinent data generated from these experiments has contributed immensely to the field.

Murine studies of retroviral mediated gene transfer into hematopoietic stem cells have been remarkably successful and informative. Initial investigations demonstrated that high efficiency retroviral mediated gene transfer and expression was readily achieved in the murine hematopoietic system[65]. In several studies, cytotoxic agents such as 5-fluorouracil and total body irradiation (TBI) were used to induce stem cell cycling. Mobilized marrow was harvested as a source of HSCs and cells were transduced by co-culture with replication incompetent retroviral vectors containing reporter genes such as those for *neomycin phosphotransferase* (*neo*) and *dihydrofolate reductase* (*DHFR*)[65,66]. The genetically modified cells were then injected into lethally irradiated syngeneic recipients. The results of molecular genetic and biochemical assays demonstrated that gene modified marrow could reconstitute the murine hematopoietic system and provide large numbers of blood cells carrying the introduced gene and producing the expected gene product.

These trials were also instrumental in providing insights into retroviral gene transfer and the dynamic and complex process of hematopoiesis. Significant increases in gene transfer efficiency were noted when various methods to induce mobilization of HSCs were used such as the use of cytotoxic agents or TBI[65]. The results supported the concept that cell cycling was imperative for MMLV-based retroviral mediated transduction. Data generated also illustrated that steady state hematopoiesis is most likely oligoclonal and that the majority of hematopoietic stem cells are quiescent with only a small number of clones actively cycling at any given time[40,65-69]. The information

gathered motivated researchers to develop alternate animal models that could potentially serve as pre-clinical models of human gene transfer.

Early gene transfer studies utilizing large animal models were initiated after the success of murine studies and consequently they incorporated many of the techniques and conditions that appeared to result in optimal gene transfer in the murine system. The findings of these studies indicated that there were several limitations that needed to be overcome to improve hematopoietic stem cell gene transfer and expression in a large animal model. Though the problem was greater than anticipated, various strategies to achieve this objective have been investigated. Many studies have been performed using canine and nonhuman primate models[43,70-72].

Early canine gene transfer experiments utilized amphotrophic retroviral vectors containing the marker genes *neo* and mutant *DHFR*. Retroviral mediated gene transfer into canine hematopoietic progenitor cells was first determined to be feasible, as genetic modification of cells was readily achieved *in vitro*[73]. A subsequent study was initiated to develop a canine model of human marrow transplantation. Dogs received lethal doses of radiation and were infused with autologous marrow that had been transduced by co-cultivation with retroviral producer cell lines. Engraftment was noted in all dogs used in the study, however researchers were unable to demonstrate, by Southern blot analysis, the presence of proviral DNA in the hematopoietic cells of surviving dogs. *In vivo* selection to enrich for genetically modified stem cells and their progeny was attempted by administering methotrexate (MTX) to dogs receiving marrow transduced with the DHFR vector. Despite this, CFU-GM colonies analyzed for evidence of the drug resistance phenotype and genotype were generally negative. Drug resistant hematopoietic colonies were noted in one dog that had survived MTX treatment, however they were detected at extremely low levels (0.1% at week three and 0.03% at week five) and for a very short duration (undetectable by week seven)[73,74]. Though investigators were able to successfully engraft dogs with retrovirally transduced autologous marrow, they were unable to demonstrate adequate levels of genetically modified cells in any of the dogs used[74].

Similar results were noted in early primate studies employing retroviral mediated gene transfer. In a study conducted by Kantoff et al., bone marrow was transduced with a vector containing the *neo* gene and the human *adenosine deaminase* gene *(ADA)*[75]. Two different transduction protocols were used for comparison: co-cultivation with the viral producer cell line and exposure to retroviral producer cell supernatants. Variability in gene transfer efficiency and the capacity for hematopoietic stem cell engraftment was observed between transduction protocols. Animals receiving the autologous marrow transduced by co-cultivation were unable to survive the procedure, as successful engraftment did not occur. In contrast, full hematopoietic reconstitution and long term survival was noted for those animals receiving marrow transduced by exposure to retroviral supernatants. The poor hematopoietic recovery observed among the animals in the co-cultivation group was attributed to the loss of bone marrow cells after transduction, resulting in inadequate numbers of hematopoietic progenitor cells being reinfused into the animal. Gene transfer was less efficient using the co-cultivation method, as demonstrated by the lower frequency of G418 resistant CFU-C in this group. Low levels of *neo* and human *ADA* activity were detected in peripheral blood mononuclear cells of one of the animals in the co-cultivation group. Southern blot analysis on peripheral blood and bone marrow cells of two animals in the experimental group transduced via supernatants were repeatedly negative, however human *ADA* activity was detectable in peripheral blood cells at levels of 0.2% and 0.5% of the endogenous primate *ADA* activity. The expression of human *ADA* was transient, as levels of this enzyme were only demonstrated between days 60-129. *Neo* activity was also detected for a short duration; activity was not present beyond day 104. The results of this experiment demonstrated that retrovirally transduced bone marrow cells were able to reconstitute the hematopoietic system of lethally irradiated nonhuman primates and provided evidence for low level gene transfer and expression of the inserted gene[75,76].

Gene transfer experiments utilizing a sheep transplantation model demonstrated an improvement in the duration of the expression of genetically modified cells. However, investigators still did not attain overall results comparable to those achieved in the 1985 murine studies. Kantoff and colleagues, used vectors containing either the *neo*

gene or the human ADA cDNA for transduction of fetal sheep hematopoietic cells[77]. Cells were obtained from lambs *in utero*, transduced *ex vivo* by exposure to retroviral producer cell supernatants and infused into individual fetuses. Lambs were analyzed at various intervals after birth for evidence of the exogenous gene, determined by assessing resistance of bone marrow cells to G418. Six of the ten animals sampled were positive for G418 resistant hematopoietic cells (one week postpartum). Only two of the six lambs were analyzed for a period of eight months and one was followed for 24 months. Both lambs exhibited G418 resistant colonies during the eight-month study period. Significant fluctuations in the appearance of these cells were noted during the 24-month study. Between days 104-153, the numbers of drug resistant cells had decreased and stabilized to ~10-15% of the total number of colonies. Levels observed at later sampling time points had reduced to 4-8%. Further analysis of gene transfer and expression included PCR, Southern blot analysis as well as the determination of *neo* activity. Southern blotting failed to detect proviral integration and *neo* activity was detected in marrow cells of only one animal at six weeks after birth. PCR analysis was used to estimate the presence of vector DNA sequences in whole marrow and it was determined to be between 0.1-10%. Results from this experiment demonstrated that *in utero* gene transfer using a retroviral vector is feasible and long term expression of the genetically modified cells could be obtained, albeit at very low levels.

1.3.4 Strategies to Enhance Gene Transfer into Hematopoietic Stem Cells

1.3.4.1 Inducing Hematopoietic Cells to Cycle

Over the years, numerous strategies have emerged to overcome the obstacles of low gene transfer[11,60,78]. Among these are techniques to induce stem cell cycling[79]. Cells targeted for retroviral mediated gene transfer must be cycling for stable integration of the introduced genetic material to occur[8]. Transduction protocols incorporating prestimulation by incubation of HSCs with combinations of hematopoietic cytokines and the use of long-term bone marrow cultures (LTMC) that simulate the marrow

environment have been integrated into gene transfer protocols to stimulate hematopoietic progenitor cells to enter the cell cycle[43,70-72,80]. Hematopoietic cytokines such as stem cell factor (SCF) and Flt-3 ligand have been demonstrated to act synergistically with other hematopoietic cytokines (thrombopoietin, granulocyte-colony stimulating factor) to promote cell division. Thrombopoietin (TPO) has also been shown to support the survival and proliferation of hematopoietic stem cells[81]. In a study conducted by Murray and colleagues, improved gene transfer into mobilized peripheral blood CD34$^+$ cells was observed using a protocol that incorporated TPO, FLT3L and SCF in the transduction procedure[79]. A retroviral vector was used to transfer the human *nerve growth factor receptor* gene to target cells. A 73-fold increase in *NGFR* expression was observed for CD34$^+$ cells transduced in the presence of TPO, FLT3L and SCF compared to cells transduced with viral supernatant supplemented with IL-3, IL-6 and leukemia inhibitory factor. Investigators concluded that gene transfer into CD34$^+$ cells was significantly enhanced with the addition of TPO, FLT3L and SCF.

The administration of hematopoietic cytokines *in vivo* has also resulted in higher levels of gene transfer into hematopoietic repopulating cells[71]. Hematopoietic growth factors such as G-CSF and GM-CSF have been shown to increase the number of circulating CD34$^+$ cells in the peripheral blood. Mobilized peripheral blood progenitor cells are easily collected and often used as targets for gene transfer. Investigators have observed improved gene transfer into this population of cells and have attributed the increase to a change in the cell cycle status of target cells[43,71].

1.3.4.2 Increased Virus to Cell Contact

Other methods to increase gene transfer into HSCs have focused on improving the virus to cell contact[82]. This can be accomplished by several means including enhancing the virus to cell ratio[78,82-84]. Gene transfer protocols may incorporate high titer vectors or use "spinoculation" in which cells are centrifuged with viral supernatant to concentrate retroviruses on target cells. Transducing cells on fibronectin-coated flasks may also

enhance cell to virus contact[70,71,85]. Fibronectin is an extracellular matrix protein that is involved in the adhesion and migration of hematopoietic cells[86]. Gene transfer into hematopoietic CD34$^+$ cells has been improved by using fibronectin in retroviral-mediated gene transfer[70,71,85]. In a study conducted by Dao and colleagues, human CD34$^+$ cells were transduced with a retroviral vector containing the *neo* gene in the presence of fibronectin, stromal layers or BSA[87]. Higher levels of gene transfer were observed for progenitors transduced on fibronectin (51.4%) compared to cells transduced on stromal layers (13.1%). This enhancement has been attributed to the co-localization of retroviral particles and target cells on fibronectin fragments. The interaction between the viral particle and the cellular receptor that is required for internalization of the virion may be increased with the use of fibronectin[85].

1.3.4.3 Pseudotyped Retroviruses

The expression of viral receptors on target cells correlates with gene transfer efficiency. Amphotrophic viruses used in gene transfer protocols utilize Ram-1, an inorganic phosphate transporter as a cell receptor[8,9,78,83]. As Ram-1 is found at low levels on bone marrow CD34$^+$ cells, researchers have sought other viral receptors that may be more useful for HSC gene transfer. The gibbon ape leukemia virus receptor is expressed at higher levels than the amphotrophic receptor on most HSCs[71]. In an attempt to increase gene transfer to HSCs, investigators have developed pseudotyped retroviral vectors utilizing the GALV envelope[9]. Pseudotyped retroviruses consist of the genome derived from one type of retrovirus encapsidated by the envelope of a second, unrelated virus [88,89]. The gibbon ape leukemia virus (GALV) has been used in conjunction with MMLV to create a vector for HSC gene transfer[9]. It has been demonstrated that this pseudotyped retrovirus has an overall higher gene transfer efficiency into hematopoietic stem cells than amphotropic vectors. Kiem and colleagues have compared gene transfer rates for an amphotrophic retrovirus and a GALV pseudotyped retrovirus using a nonhuman primate model[42]. CD34$^+$ bone marrow cells were divided into equal amounts, transduced with both vectors and transplanted to autologous recipients. Higher levels of

gene marking were observed for cells transduced with the GALV pseudotyped vector (between 1-5% of leukocytes) in two animals. Increased levels were attributed to the higher expression of the GALV receptor than Ram-1 on hematopoietic progenitor cells.

1.3.5 Improvements in Gene Marking: Animal Studies

Several groups using a canine model for gene therapy have employed long-term bone marrow cultures (LTMCs) in an attempt to increase gene transfer[90-95]. Schuening et al. demonstrated (*in vitro*), increased levels of gene transfer into canine hematopoietic progenitors using a LTMC system[94]. Two retroviral vectors were used for these experiments, one containing the *DHFR* gene and the other the *neo* gene. Canine hematopoietic progenitors were co-cultivated with viral producing cells for 24 hours followed by incubation in a LTMC culture. Cultures were fed three times with medium supplemented with viral supernatant. Gene transfer efficiency increased four fold (from ~10% to ~46%) as a result of the inclusion of LTMCs in the gene transfer protocol[94]. The improvement noted was attributed to either the repeated and prolonged exposure to viral supernatant and/or the induction of cell cycling of hematopoietic progenitors present in the culture. These investigators proceeded to demonstrate in a following study[95] that gene transfer into canine long-term repopulating cells was feasible using a protocol that combined the collection of marrow from animals that had been treated with recombinant human G-CSF and a transduction protocol utilizing LTMCs. Transduced autologous marrow was infused into dogs that had been exposed to lethal total body irradiation. Two of the four dogs in this study group survived the experimental procedure and were followed for a period of two years to evaluate the persistence and expression of transduced cells. Both dogs showed persistence of the *neo* gene for two years as assessed by the presence of G418-resistant colonies (between 1-11%) and *neo* specific PCR analysis of lymph node, bone marrow and peripheral blood cells. Samples containing both the *neo* gene and the human *ADA* gene were identified although expression of ADA was not demonstrated. This study provided direct evidence that long-term persistence of genetically modified cells was possible in a large animal model.

Long-term *in vivo* persistence and expression of genetically modified hematopoietic cells carrying the murine *adenosine deaminase* gene (*ADA*) was demonstrated by Bodine and colleagues[96,97], in a non-human primate model. Primates were treated with 5-fluorouracil five days before harvest and CD34$^+$ cells were selected from bone marrow mononuclear cells. CD34$^+$ cells were cultured on a murine stromal cell line that had been engineered to produce human SCF. Cells were transduced every 24 hours with retroviral supernatants in the presence of human SCF and IL-6. After a 96-hour culture period, autologous transduced cells were infused into irradiated recipients. Proviral sequences were detected by PCR analysis of peripheral blood cells and mouse *ADA* activity was detected (~3% the activity of monkey *ADA*) for all three animals sampled up to day 48. Only two animals were available for long term follow up. Both animals showed proviral integration in peripheral blood cells (average~2%) for almost one year. Bone marrow was also PCR positive for both animals up to the 11 month time point. This report documented enhanced gene transfer into non-human primate repopulating stem cells and improved long term expression and persistence of these cells. These advances were facilitated by the inclusion of hematopoietic cytokines in the transduction protocol and co-cultivation of CD34$^+$ cells on cytokine producing stromal cell lines.

More efficient gene transfer into primate CD34$^+$ cells was illustrated in a study by Xu et al.[97,98]. CD34$^+$ cells were transduced with retroviral supernatants in the presence of human hematopoietic cytokines IL-6, IL-3 and SCF. A period of prestimulation in the presence of autologous stroma, separated from the cells by the use of a semipermeable membrane was added to the protocol for two of the four monkey samples. Autologous cells transduced with the human glucocerebrosidase gene (GC) were infused into irradiated recipients and three of the four primates were followed for up to 20 months. For all three animals, proviral sequences were detected by PCR analysis in 2-4% of circulating leukocytes for up to 15 months. Bone marrow cells were found to be 4-16% positive and 12-22% of cells from the popliteal lymph nodes also contained the GC provirus. Expression of the human GC gene in peripheral blood mononuclear cells was

successfully confirmed by RT-PCR. This study demonstrated long-term *in vivo* expression of genetically modified cells in a large animal.

Higher levels of gene transfer into non-human primate CD34$^+$ cells were demonstrated in a study conducted by Dunbar and colleagues[43]. Peripheral blood and bone marrow CD34$^+$ cells obtained from animals mobilized with SCF and G-CSF, were transduced with retroviral supernatants in the presence of human hematopoietic cytokines (IL-6, IL-3, SCF). Genetically modified cells were returned to lethally irradiated non-human primates. Levels of up to 5% gene transfer were detected in the peripheral blood of monkeys by PCR and Southern blot analysis for up to one year. Researchers concluded that CD34$^+$ cells collected from cytokine-primed animals were superior targets for gene transfer.

Major improvements in gene transfer using a non-human primate model were observed when researchers combined a number of strategies into one gene transfer protocol. Kiem and colleagues achieved a high level of gene transfer by using a GALV-pseudo-typed retroviral vector to transfer the *neo* gene to bone marrow derived CD34$^+$ cells[71]. The gene transfer protocol utilized in this study incorporated the transduction of cells on fibronectin-coated flasks in the presence of human hematopoietic cytokines IL-6, FLT3-L, SCF and megakaryocyte growth and development factor (MGDF). These investigators were able to develop conditions that resulted in 20% of peripheral blood and bone marrow cells containing vector sequence by Southern blot analysis for more than 20 weeks in one animal. This study demonstrated that clinically useful levels of genetically modified HSCs were obtainable using a protocol that integrated pseudotyped retroviral vectors, fibronectin and hematopoietic cytokine support to augment gene transfer.

Goerner and colleagues also used a similar protocol to achieve higher levels of gene transfer into HSCs in a canine model[70]. Canine bone marrow CD34$^+$ cells were prestimulated with a combination of hematopoietic cytokines (FLT3-L, cSCF and cG-CSF) for 24 hours and transduced with viral supernatants on fibronectin coated flasks in

cytokine supplemented media. A GALV pseudotyped retroviral vector containing the *neo* gene was used in these studies. Transduced cells were infused into lethally irradiated dogs. PCR and Southern blot analysis were used to detect the presence of vector containing cells in the peripheral blood and bone marrow. Overall improvements in the gene transfer rates into canine hematopoietic cells were observed. Eight months post-infusion of cells, levels of 10% gene transfer were detected in the peripheral blood of one dog. This significant increase was attributed to the use of a gene transfer protocol utilizing GALV pseudotyped retroviral vectors, fibronectin and hematopoietic cytokines.

Extensive investigations of HSC gene transfer have been conducted using animal models and have enabled the development of effective strategies to enhance gene transfer. Encouraging results and advancements in animal models may be applicable to gene transfer to human HSCs.

1.3.6 Gene Transfer to Hematopoietic Stem Cells: Human Trials

Human gene marking trials have been conducted since 1990 and a variety of transduction protocols and approaches to enhance gene transfer to human HSCs have been employed[11,13,99]. Early gene marking studies, using autologous tumour-infiltrating lymphocytes marked with the *neo* gene, demonstrated the feasibility and safety of using gene transfer in humans[100,101]. Gene marking of autologous marrow from patients with various diseases such as acute myeloid leukemia and neuroblastoma have been performed to investigate the contribution of the marrow graft to relapse of the disease[102,103]. These trials were also instrumental in confirming the safety of returning gene marked cells to patients.

Data generated from HSC gene marking experiments in humans have reflected those seen in large animal models. The levels of gene marked cells have typically been low (less than 1%), even with the incorporation of strategies to improve gene transfer.

Though results from gene marking trials have been disappointing, these investigations have provided evidence that gene transfer to human hematopoietic cells is achievable.

Therapeutic HSC gene transfer trials have been initiated for several inherited disorders including Gaucher disease, Fanconi's anemia type C and adenosine deaminase deficiency (ADA)[2,11,88]. Although investigations are still ongoing, very few clinical benefits have been observed. Dunbar et al. conducted a gene therapy clinical trial for patients with Gaucher disease using a retroviral vector containing the human glucocerebrosidase cDNA (GC)[104]. Bone marrow or mobilized peripheral blood were collected from three patients and CD34$^+$ cells isolated. Cells were transduced every 24 hours with viral supernatant in the presence of autologous bone marrow derived stromal cell monolayers. Exogenous hematopoietic cytokines were used to supplement the culture medium for two of the three patients. After 72 hours, cells were re-infused into nonmyeloablated patients. Peripheral blood and bone marrow were sampled periodically and PCR analysis was performed to detect the presence of vector containing cells. Glucocerebrosidase enzyme activity was also monitored for increases. Gene marking was detected for only two of the three patients studied. Vector containing cells were present at one month post-infusion in bone marrow cells of both patients and peripheral blood mononuclear cells of one of the patients. At the two to three month time point, only one patient had gene marked cells present in the peripheral blood and the levels were extremely low (less than 0.02%). Increased levels of glucocerebrosidase enzyme activity were not detected for any patient. This study demonstrated that engraftment of gene marked cells was possible for a short duration in nonmyeloablated patients. No clinical improvements were noted, suggesting that either higher levels of gene transfer or elevated GC expression were required for clinical benefits to be observed.

In an attempt to increase the levels of gene transfer and the frequency of gene marked cells present in patients, Liu and colleagues designed a clinical protocol for the treatment of Fanconi anemia type C (FANCC) that incorporated multiple cell harvests and transduction cycles[105]. Three children and one adult with Fanconi anemia type C received bone marrow and /or peripheral blood CD34$^+$ cells transduced with a retroviral

vector containing the normal FANCC gene. Most patients underwent three or four cycles of mobilization, cell harvest and infusion of gene marked cells. Gene modified cells were only detected transiently and at low levels in the peripheral blood of three patients (ranging from 0.01-3%). Two of these patients also exhibited transient increases in bone marrow cellularity. In the fourth patient, vector-containing cells became evident in the peripheral blood only after she had undergone radiation therapy for a concurrent malignancy. This patient received one infusion of gene-modified $CD34^+$ bone marrow cells. FANCC gene marked cells have been detected at low levels in the peripheral blood of this patient for over one year. The low levels of gene transfer and minor improvements obtained in this study are typical of human clinical gene therapy trials.

Despite the fact that very few patients have benefited from clinical gene transfer protocols, progress has been made in identifying critical limitations and obstacles to effective HSC gene therapy. Great effort has been made to address these problems both in animal models and in human *in vitro* studies. It is anticipated that protocols that have shown promising results in large animal models may be used successfully in humans.

1.4   Canine Model for Hematopoietic Stem Cell Gene Therapy

Information gathered through animal investigations has played a major role in the development of clinical procedures and treatments for humans. Preclinical animal studies are commonly used to develop new therapies before they are evaluated in humans[24]. The dog serves as a useful model for human diseases, since many spontaneous and genetic canine diseases closely resemble those seen in humans[106]. The data obtained from canine experimentation may be predictive and easily extrapolated to human clinical outcome.

The dog has frequently been used as a model system to investigate various aspects of human hematopoiesis including experimental bone marrow transplantation. Major advances in autologous and allogeneic bone marrow transplantation have been

made based on results obtained from experiments conducted in dogs. Studies conducted in the 1960's by several researchers showed that hematopoietic recovery after lethal irradiation was possible with autologous marrow transplants. Mannick et al. reported rapid hematopoietic reconstitution in lethally irradiated dogs receiving autologous marrow infusions shortly after radiation treatment[107]. Numerous attempts by investigators to perform allogeneic transplants in the dog have not been as successful as autologous grafts. In a study by Thomas et al., lethally irradiated dogs receiving allogenic bone marrow infusions were given methotrexate (MTX) in an effort to limit graft rejection and graft-versus host disease (GVHD)[108]. Five dogs treated with MTX showed no evidence of (GVHD) four months after treatment. The results of this preliminary study indicated that immunosuppressive agents might be useful in increasing the survival of allogenic transplant recipients.

The use of peripheral blood as a potential source of cells for engraftment was elucidated in a canine model. In 1964, Calvins and colleagues demonstrated that peripheral blood contains cells that are capable of hematopoietic reconstitution[109]. Peripheral blood samples were obtained from dogs at various intervals and mononuclear cells separated and stored at -80°C in 10% dimethyl-sulfoxide. Dogs were lethally irradiated and infused intravenously with freshly thawed autologous leukocytes. Three of nine dogs survived and had complete hematopoietic recovery. Researchers concluded that the number of cells transplanted was critical for survival and that a large graft was essential. This study illustrated the possible use of peripheral blood as a source of hematopoietic stem cells.

Extensive studies have also been performed in the dog to examine the effect of hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) on hematopoiesis after lethal total body irradiation (TBI). In a study performed by Schuening and colleagues, recombinant human G-SCF was administered to dogs after lethal total body irradiation (TBI)[110]. Four of five animals treated with G-CSF immediately after radiation treatment (400 cGy) had complete hematopoietic recovery. Hematopoiesis was stimulated by the administration of G-CSF and a decrease in the

duration of neutropenia was noted. In a following study by Schuening et al., the effect of recombinant human stem cell factor (SCF) on hematopoietic recovery after TBI was evaluated[111]. Results comparable to those obtained in the G-SCF experiment were noted. 50% of animals receiving SCF immediately post-irradiation (400 cGy) exhibited sustained hematopoietic recovery. These studies demonstrated that hematopoietic growth factors could reverse the myelosuppressive effects of TBI in a canine model.

A broad spectrum of topics related to hematopoiesis have been examined using the canine hematopoietic system. The importance of leukocyte groups in hematopoietic cell transplantation was recognized in a canine model. Many studies of graft-versus-host disease, the establishment of mixed chimerism and induction of tolerance were conducted using dogs[90,112-114]. The use of hematopoietic cytokines to mobilize hematopoietic progenitors into the peripheral blood was also evaluated in a canine model[111,115]. The canine hematopoietic system appears to resemble the human system and therefore may be a valuable tool for further experiments of bone marrow transplantation, graft-versus host-disease and HSC gene therapy.

Recently, a canine CD34 antibody has become available to use as a marker for canine HSCs[116]. Investigators have demonstrated that canine CD34$^+$ cells appear functionally and phenotypically similar to humans. Canine CD34$^+$ cells have been detected in canine bone marrow at levels similar to those seen in human bone marrow, ~1-3%. These cells can provide radioprotection after lethal total body irradiation and can give rise to long-term hematopoiesis[117]. These results are encouraging and provide evidence that the canine hematopoietic system may be a reliable model to investigate HSC gene therapy.

1.5   The Phenomenon of Multiple Drug Resistance in Cancer Treatment

1.5.1   Multidrug Resistance

Chemotherapy is a standard treatment for many malignancies. Unfortunately, drug resistance is a major obstacle in cancer chemotherapy and often prevents complete destruction of neoplastic cells. Drug resistance may be present at the start of antineoplastic therapy (intrinsic) or may be induced or acquired after chemotherapeutic challenge. Various cellular drug resistance mechanisms have been identified including reduced cellular drug uptake, enhanced cellular drug efflux, increased drug metabolism and decreased apoptosis[118,119]. The clinical importance of each of the drug resistance mechanisms has not been fully established as most of the investigations have been performed *in vitro*.

Multidrug resistance (MDR) describes the phenomenon whereby cells display simultaneous resistance to unrelated drugs[119]. Several mechanisms have been described that contribute to the MDR phenotype. These drug resistance strategies can be classified as classical MDR, non P-glycoprotein MDR, atypical MDR and alterations in drug induced apoptosis[118]. The acquisition of the MDR phenotype by malignant neoplasms may result in ineffective chemotherapy. The clarification of the mechanisms of MDR and their importance in clinical drug resistance may enable the development of chemosensitizers or inhibitors of MDR. Modulation of drug resistance may permit more successful treatment of cancer patients[120].

1.5.2   Classical MDR

Classical MDR refers to drug resistance mediated by P-glycoprotein (P-gp) a product of the *MDR1* gene, cloned by Ling et al.[121]. P-gp is a member of the ATP-binding cassette superfamily of transport proteins. It is a 170 kDa transmembrane glycoprotein that is normally expressed at intermediate to high levels in the adrenal

cortex, biliary hepatocytes, proximal renal tubuli, CD34^{+} hematopoietic cells, gastrointestinal epithelium and endothelium of the blood-brain barrier[119]. P-gp is thought to play an important role in protecting normal cells from xenobiotics and toxins. In malignant cells, P-gp can mediate drug resistance by reducing cellular drug accumulation. P-gp functions as an energy dependent efflux pump that actively extrudes drugs from cells. Overexpression of P-gp confers a broad drug resistant phenotype to naturally occurring drugs such as taxanes, anthracyclines and epipodophyllotoxins. Overexpression is thought to result from either amplification or enhanced transcriptional activation of the MDR1 gene[120]. High levels of P-gp have been detected in clinical tumour samples from tissues that normally express the MDR1 gene. The clinical relevance of P-gp mediated drug resistance has been extensively studied and P-gp has been found to be an adverse prognostic factor in some malignancies[122]. As a result of its clinical significance in certain drug resistant tumours, inhibitors or agents that could modulate P-gp activity have been developed[118]. Clinical trials are ongoing to determine if the MDR phenotype could be reversed and the response to chemotherapy improved with the use of these agents[123,124].

1.5.3 Atypical MDR

Atypical MDR is mediated by alterations in the antineoplastic drug target topoisomerase II (topo II)[118]. Topo II is a nuclear enzyme that is involved in DNA replication. Chemotherapeutic agents such as anthracyclines and epipodophyllotoxins are potent topo II inhibitors and exert their cytotoxic effects by inhibiting DNA synthesis via stabilization of the enzyme-DNA complex. Once a stable complex is formed, DNA strand breaks are unable to be religated and eventually cell death occurs. Atypical MDR is the result of altered topo II expression, either due to reduced levels or a mutation that alters the structure of the enzyme. Drug resistance occurs when fewer DNA-enzyme complexes are formed[120]. Although atypical MDR may have clinical relevance, most studies have been performed in vitro and limited data is available describing the

expression of topo II in clinical tumour samples. Further studies are required to determine the clinical importance of atypical MDR[118].

1.5.4 Alterations in Drug Induced Apoptosis

Chemotherapeutic agents have been shown to induce apoptosis by producing DNA damage. Alterations in a cell's sensitivity to apoptosis inducing drugs could result in the MDR phenotype[125]. Overexpression of Bcl-2, an oncogenic protein, has been demonstrated to inhibit programmed cell death[126]. Upregulation of Bcl-2 has been shown *in vitro* to confer MDR in a variety of tumour cell lines[127]. The clinical importance of Bcl-2 overexpression has been examined in a variety of malignancies including leukemias and lymphomas[127]. Increased levels of Bcl-2 have been associated with a poor response to chemotherapy in some human cancers[125]. Other genes that are associated with apoptosis may also play a role in MDR. Identification of theses genes and examination of their role in clinical drug resistance is ongoing.

1.5.5 Non P-glycoprotein MDR

Non-P-glycoprotein MDR is the result of overexpression of the *Multidrug Resistance-Associated Protein 1* (*MRP1*). This gene was first cloned and characterized by Susan Cole and Roger Deeley[128]. Like P-gp, *MRP1* is a member of the ABC family of transport proteins. It is ubiquitously expressed at low levels in normal tissues where it is thought to play an essential physiological role[129]. *MRP1* mediated drug resistance is primarily the result of increased drug efflux or reduced intracellular drug accumulation. Vesicular transport and drug sequestration may also be important in mediating the drug resistance phenotype[130]. Cells overexpressing *MRP1* show increased resistance to naturally occurring drugs. The clinical significance of *MRP1* overexpression has been examined in very few malignancies. Additional studies are required to determine the prognostic value of *MRP1* overexpression and the usefulness of *MRP1* inhibitors.

The following section will review in greater detail, the mechanism of *MRP1* mediated drug resistance and the importance of this protein in clinical drug resistance. The use of the *MRP1* gene as a possible candidate for hematopoietic chemoprotection by gene transfer is also discussed.

1.6 Multidrug Resistance-Associated Protein 1 (MRP1)

1.6.1 Biology of *MRP1*

The *Multidrug Resistance-Associated Protein 1* (*MRP1*) and P-glycoprotein, a product of the *MDR1* gene, are members of the ATP-binding cassette (ABC) superfamily of transport proteins. This family consists of large membrane proteins that contain highly conserved ATP-binding domains and multispanning transmembrane segments[131]. Though they are members of the same superfamily, the genes encoding *MRP1* and P-gp are evolutionarily divergent[130]. These proteins share only 15% amino acid identity and the range of chemotherapeutic agents that they mediate resistance to is not identical[132]. Using a differential hybridization approach, a mRNA species that was overexpressed in a doxorubicin-selected H69AR cells was identified and sequenced[128]. The *MRP1* gene was cloned from a doxorubicin resistant small cell lung carcinoma cell line H69AR that did not overexpress P-gp[128]. This gene resides on the short arm of chromosome 16 at band 13.1 and encodes for a 190 kDa protein[133].

Five variants of *MRP1* have been identified (*MRP 2-6*). Within the mammalian *MRP* family, *MRP3* has the highest homology to *MRP1* (58% amino acid identity), followed by *MRP2* (49%) and *MRP6* (45%)[131]. *MRP1, 4* and 5 have been found to be distributed widely in the body whereas *MRP 2,3,6* are found mainly in the liver, gut and kidney[131]. Cells overexpressing *MRP1* can show increased resistance to naturally occurring drugs such as anthracyclines, *Vinca* alkaloids, epipodophyllotoxins and antifolates[129]. Investigations into the ability of other members of the *MRP* family to confer drug resistance have demonstrated that *MRP2* and *MRP3* can contribute to drug

resistance, whereas *MRP6* does not[134-136]. Additional studies are required to provide further insight into the physiological functions, normal expression, substrate specificity and drug resistance patterns of members of the *MRP* family.

1.6.2 Tissue Distribution, Normal Expression and Physiological Function

Several investigations have been performed to establish the normal distribution and expression of *MRP1* in various tissues and cell types. It has been determined by RT-PCR, Western blot analysis and immunohistochemistry that *MRP1* is ubiquitously expressed in normal tissues[133]. The degree of expression varies depending on tissue type. The highest levels of *MRP1* have been demonstrated in skeletal muscle, lung, testes, heart and kidney. Intermediate levels of *MRP1* are found in the gall bladder, thyroid, adrenal gland and urinary bladder. Low levels have been shown in hematopoietic cells, prostate, brain, liver, small intestine, colon, pancreas, and placenta[137].

Variations in the distribution of the *MRP1* protein have been demonstrated in normal and malignant tissues[129]. In malignant tumour samples or drug selected *MRP1* expressing cell lines, the protein appears to be recruited to the plasma membrane where it may play a role in drug efflux. In normal epithelial cells, it has been reported that *MRP1* is predominately cytoplasmic, which may indicate that *MRP1* is involved in trafficking compounds into intracellular compartments. The function of *MRP1* in normal cells remains to be established, however an essential physiological role is suspected due to the prevalent expression of *MRP1* in most tissues[129,133].

It has been suggested that *MRP1* has several functions in normal tissues including mediating inflammatory reactions and protection from xenobiotics[129,133]. The generation of *MRP1* knock-out mice has enabled researchers to investigate the physiological role of this protein. Studies utilizing *MRP1* knockout mice have confirmed *in vitro* data implicating *MRP1* as a mediator of leukotriene induced inflammation[138,139]. *MRP1* has been demonstrated *in vitro* to transport glutathione conjugates such as cysteinyl

leukotriene LTC_4. LTC_4 is involved in inflammatory reactions by mediating vascular permeability and smooth muscle contractions. *MRP1* knockout mice show a decreased response to leukotriene inducing inflammatory stimuli. Mast cells from these mice exhibit decreased LTC_4 secretion. Results from these studies have confirmed that *MRP1* plays an important physiological role in mediating inflammatory responses *in vivo*.

The ability of *MRP1* to transport a variety of substances such as glutathione S-conjugates and hydrophobic anionic compounds suggests that *MRP1* plays a protective role in normal tissues. *MRP1* can transport GSH-conjugates of the activated form of aflatoxin B_1 suggesting that this protein is important in the prevention of chemical carcinogenesis[137]. *MRP1* not only transports endogenous metabolites and glutathione conjugates, it is also involved in the elimination of natural drug products. Cells in which *MRP1* has been disrupted or knocked out have exhibited increased drug sensitivity to the chemotherapeutic drug etoposide and moderate levels of sensitivity to other chemotherapeutic agents such as doxorubicin and daunorubicin. The increased sensitivity to etoposide has been confirmed *in vivo* in the *MRP1* knockout mice[138,139]. These mice appear to be hypersensitive to etoposide resulting in decreased body weight and increased mortality. Baseline expression of the protein appears to exert a protective role and mediates inflammatory reactions. The exact physiological role of *MRP1* in normal tissues is still uncertain and further studies are required to clarify its function.

1.6.3 Mechanism of *MRP1*-Mediated Drug Resistance

Overexpression of *MRP1* has been demonstrated to confer drug resistance to a wide variety of natural product drugs including epipodophyllotoxins (etoposide, teniposide), anthracyclines (doxorubicin, daunorubicin), *Vinca* alkaloids (vincristine, vinblastine), and actinomycin D^{133}. More recently, *MRP1* has been found to mediate antifolate resistance[134]. Unlike P-gp, *MRP1* is not efficient at transporting agents such as taxol and colchicine. The mechanism of *MRP1* overexpression is unclear[140]. Low levels

of *MRP1* drug resistance are thought to arise due to transcriptional activation, whereas high levels may be due to gene amplification[129,137].

The process by which *MRP1*-mediates drug resistance is also uncertain and requires further elucidation. *MRP1* actively transports a broad range of compounds including glutathione sulfate (GSH) and glucuronide conjugates across cellular membranes via an ATP-mediated process. Drug resistance is primarily the result of reduced drug accumulation by enhanced drug efflux, however drug sequestration and vesicular transport may also be important methods[141,142].

In vitro studies of *MRP1* mediated resistance have demonstrated that this protein is capable of transporting conjugated and unmodified drugs. GSH has been shown to be essential for *MRP1* transport of various substances[143]. The role of GSH in *MRP1* mediated drug resistance is not fully defined. The chemotherapeutic agents vincristine and daunorubicin are transported in a GSH dependent manner, whereas GSH is not required for transport of etoposide, vinblastine or antifolates[144]. GSH is thought to act either as a co-transporter or as an activator, facilitating binding or transport of substances[145]. GSH has also been shown to be required for vesicular transport of vincristine by *MRP1*[145]. The importance of GSH in *MRP1* mediated drug resistance has been examined in studies using agents that deplete cellular GSH. A reduction in GSH results in increased drug sensitivity in *MRP1* overexpressing cells for some chemotherapeutic agents[146]. Results from these studies suggest that GSH and *MRP1* interaction is required for specific drugs to be effluxed.

MRP1 transport of chemotherapeutic agents is thought to be the result of an ATP-dependent process by which drugs are actively extruded from cells by a plasma membrane associated efflux pump. Support for this idea has been generated by studies in which *MRP1* has been found to be primarily located on the cell surface. The cells examined displayed high levels of *MRP1* and high levels of drug resistance[129,137,146]. *MRP1* mediated drug resistance may also be facilitated by vesicular transport or drug sequestration. In cells expressing low levels of *MRP1* and low levels of drug resistance,

MRP1 is found to be predominantly within the cytoplasm. Studies have demonstrated altered intracellular drug distribution of anthracyclines in cells expressing high levels of *MRP1* in the cytoplasm, suggesting a role for *MRP1* in sequestering drugs away from their cellular target[142].

1.6.4 *MRP1* in Malignancies

Since its discovery in the human small cell lung carcinoma cell line H69AR, the role that *MRP1* plays in multidrug resistance has been extensively studied[128]. It has been demonstrated *in vitro* that *MRP1* transfectants display multidrug resistance to naturally occurring drug products[129]. As a result of these studies, *MRP1* expression has been investigated in a variety of drug resistant tumour cell lines and clinical tumour samples. The *MRP1* protein and its mRNA have been detected in several tumours that have been derived from tissues that normally express *MRP1*, such as prostate and lung carcinomas[147]. Expression of the *MRP1* gene has also been found in many hematological malignancies and solid tumours[147]. High levels of *MRP1* expression have been detected in tumour cell lines that are intrinsically drug resistant and respond poorly to chemotherapy including non-small cell lung carcinomas, thyroid carcinomas and neuroblastomas[147-150]. Although many tumour cell lines and clinical samples exhibit moderate to high levels of *MRP1*, the clinical relevance of the expression of this gene is uncertain for many malignancies. Only a few investigations have clearly demonstrated a correlation between expression of the *MRP1* protein and disease outcome.

MRP1 expression has been found to correlate negatively with disease outcome in solid tumors such as non-small cell lung carcinoma, neuroblastoma and primary breast carcinoma[147-153]. Neuroblastoma is a pediatric malignancy that is highly chemoresistant. The expression of *MRP1* is common in both clinical tumour samples and neuroblastoma cell lines. An association has been demonstrated between high levels of *MRP1* expression and poor clinical outcome. Shorter overall survival and disease free survival have been noted. *MRP1* expression has also been shown to correlate with amplification

and overexpression of the N-myc oncogene. Tumours with N-myc amplification are aggressive, have a poor response to chemotherapy and a poor prognosis. They are mainly advanced stage tumours that show high levels of *MRP1* expression[150,153]. *In vitro* assays utilizing non-selected neuroblastoma cell lines have confirmed that *MRP1* expression and the amplification of the N-myc oncogene are associated with drug resistance.

Overexpression of *MRP1* has also been associated with a poor prognosis for non-small cell lung carcinomas[149,154]. Non-small cell lung carcinoma is intrinsically chemoresistant and *MRP1* expression is prevalent. Though *MRP1* is normally expressed in lung tissue, moderate to high levels of *MRP1* have been correlated with a significantly worse clinical outcome of chemotherapy with vindesine and etoposide. Thus, *MRP1* expression could potentially be used to predict prognosis for this malignancy.

Recently, the clinical significance of *MRP1* expression has been investigated in primary breast carcinomas[151,152]. Patients that did not exhibit *MRP1* expression had an excellent prognosis and prolonged survival compared to patients displaying *MRP1* expression. A five fold increased relative risk for death was also determined for patients with *MRP1* expressing tumours. Results from this study have suggested that *MRP1* overexpression is an important prognostic factor in primary breast carcinoma.

In contrast to the above finding, the detection of *MRP1* expression in several other solid tumours such as ovarian and prostate carcinomas does not necessarily have clinical significance or correlate with the prognosis of malignant disease[155,156]. *MRP1* has been demonstrated to be regularly expressed at moderate to high levels in ovarian carcinomas and at low levels in prostate carcinomas but expression does not correlate with response to therapy or progression of these tumours. *MRP1* expression has been found to correlate with the degree of differentiation for esophageal squamous cell carcinoma, gastric and colorectal adenocarcinoma, however no significant difference was noted in patient survival[156,157].

MRP1 expression has also been examined in hematological malignancies. The clinical significance of *MRP1* expression in acute myeloid leukemia (AML) requires further investigation, as there are conflicting results in the literature. Drug resistance is a problem in the treatment of AML. The results of one study suggested an association between *MRP1* expression and clinical outcome[157]. Several patients affected with a subclass of AML (M4Eo) with an inversion in chromosome 16 displayed deletion of the *MRP1* gene and had a more favourable prognosis. An increased duration of disease free and overall survival was also noted and attributed to lack of *MRP1* expression. In support of these findings, another study of AML suggested that *MRP1* expression was increased in samples from patients that had relapsed or in malignancies that were drug refractory[158,159]. In contrast, other studies have reported that there is no relationship between *MPR1* expression and clinical response in AML[160-163]. Results of several investigations have demonstrated that *MRP1* expression has no effect on overall survival rates, disease free survival or complete remission rates. There was no correlation between inversion (16) and *MRP1* expression. Differences were not detected in *MRP1* expression before or after chemotherapy treatment suggesting that *MRP1* is not clinically relevant in drug resistant AML.

Overexpression of *MRP1* has been demonstrated in B-cell malignancies such as chronic lymphocytic and prolymphocytic leukemia[164,165]. High levels of *MRP1* have been detected, however there was no association between overexpression of *MRP1* and chemotherapy treatment. Thus, it appears that *MRP1* has no clinical significance and would not contribute to drug resistance for these hematological malignancies.

1.6.5 Experimental Modulation of *MRP1*

The expression of *MRP1* and possible involvement of this protein in drug resistant malignancies has prompted the search for agents that may reverse or modulate chemoresistance. Compounds that are able to reverse the MDR phenotype maybe very useful and could improve the clinical outcome for some cancer patients[118]. *MRP1* is

ubiquitously expressed at low levels in normal tissues. Studies of *MRP1* knockout mice have demonstrated that a complete block of *MRP1* is compatible with life. *MRP1* knockout mice are viable and fertile, however they do show increased sensitivity to the chemotherapeutic agent etoposide and display decreased response to leukotriene-induced inflammation[138,166]. The results from these studies suggest that the use of *MRP1* inhibitors is feasible, as there are minimal side effects to disrupting the *MRP1* gene.

Chemosensitizers or agents that are able to reverse the MDR phenotype have been used to modulate drug resistance mediated by P-gp[118]. Several of these agents have been utilized to try to reverse *MRP1* mediated drug resistance. Agents such as verapamil and cyclosporine A that have been used to inhibit the activity of P-gp have little to no effect on *MRP1* activity[167]. Since the exact mechanism of *MRP1* drug resistance is not fully defined, numerous inhibitors have been examined.

Glutathione (GSH) has been considered to be essential for the transport function of *MRP1*[141]. Agents that deplete GSH stores or inhibit GSH biosynthesis such as buthionine sulfoximine (BSO) have been used to try to modulate *MRP1* chemoresistance[167]. Cells overexpressing *MRP1* have demonstrated increased sensitivity to various chemotherapeutic agents such as vincristine and daunorubicin, after BSO treatment[130]. Though the exact mechanism is uncertain, BSO could prevent *MRP1* mediated expulsion of these agents resulting in the intracellular accumulation of drug.

Rifampicin is a semi-synthetic agent that is used for the treatment of tuberculosis. This compound and other rifamycins have been used to reverse P-gp activity by direct interaction with P-gp drug binding sites. Rifampicin has been shown to inhibit *MRP1* activity resulting in the intracellular accumulation of the chemotherapeutic agent vincristine[168]. The concentrations of this agent required to achieve this effect were substantially higher than that used clinically in patients. The dosage needed for therapeutic effect would preclude the use of rifampicin due to concerns of toxicity. Genistein is another compound that is capable of mediating reversal of *MRP1* drug resistance, however is not clinically useful[133]. This agent is a potent inhibitor of tyrosine

kinase activity and in *MRP1* overexpressing cells can increase cellular drug accumulation and decrease efflux. Very high concentrations were required to observe these effects and toxicity limits its use. In contrast, probenecid, an inhibitor of organic anion transporters, has been demonstrated to reverse *MRP1* mediated chemoresistance at clinically useful levels[169]. Increased accumulation of daunorubicin and vincristine were noted in *MRP1* overexpressing cells treated with this agent. Probenecid may prove to be a useful chemosensitizer.

An alternate strategy to overcome *MRP1* drug resistance is the use of specific antisense oligonucleotides. Antisense oligonucleotides bind specifically to complementary nucleic acid targets, preventing protein synthesis and therefore expression of the protein. This novel approach has been used to inhibit P-gp activity and has recently been employed to reverse *MRP1* drug resistance[118]. *MRP1* transfectants displayed an increased sensitivity to doxorubicin and decreased levels of *MRP1* mRNA and *MRP1* protein after antisense oligonucleotide treatment[129]. Though these results were encouraging, the increased drug sensitivity was short lived. .

Although the clinical relevance of *MRP1* overexpression in drug resistant cancers is still unclear, experimental modulation of *MRP1* activity is being actively pursued. *MRP1* reversal agents may prove to be essential components to the treatment of various drug resistant malignancies however, there are no highly effective non-toxic inhibitors available to date.

1.6.6 *MRP1* Gene Transfer for Drug Resistance

The ability of *MRP1* to confer drug resistance has previously been demonstrated *in vitro* by *MRP1* cDNA transfection experiments. In a study performed by Cole et al., drug-sensitive Hela cells were observed to have moderate levels of drug resistance to naturally occurring drugs such as doxorubicin and etoposide after *MRP1* transfection[128]. These cells were found to have increased levels of *MRP1* mRNA and the *MRP1* protein.

Other investigators confirmed these results using NIH-3T3 *MRP1* transfectants[142]. The drug resistance phenotype conferred to transfected cells was similar in both studies. These investigations determined that drug resistance to a variety of chemotherapeutic agents could be obtained by transfer of the *MRP1* gene.

Evidence that *MRP1* could confer drug resistance led to the development of retroviral vectors for *MRP1* gene transfer. D'Hondt and colleagues, constructed ecotropic retroviral producer cell lines and demonstrated that their *MRP1* retroviral vector was able to transfer the gene to NIH-3T3 cells[170]. *MRP1* transduced clones exhibited increased expression of *MRP1* by flow cytometry. *MRP1* expressing cells also displayed increased drug resistance to doxorubicin, vincristine and etoposide. Chemoprotection of target cells was obtained in this study by *in vitro* retroviral gene transfer of *MRP1*. These promising results encouraged investigators to pursue *MRP1* as a potential candidate for hematopoietic chemoprotection by gene transfer.

Machiels et al. demonstrated in a murine model that *MRP1* transduced hematopoietic cells could reduce chemotherapy-induced myelosuppression[171]. Murine hematopoietic cells were transduced with a retroviral vector containing the human *MRP1* cDNA and transplanted to lethally irradiated mice. Long-term (nine months) engraftment of gene marked cells was noted by PCR and Southern blot analysis of peripheral blood in *MRP1* transduced mice, without the administration of chemotherapy. *MRP1* expression was evident by flow cytometry in the peripheral blood by five months post-transplant. Hematopoietic protection from doxorubicin-induced myelosuppression was noted in mice exhibiting high levels of *MRP1* in hematopoietic cells. These mice experienced less severe leukopenia than controls. An additional finding in this study was that *in vivo* selection of *MRP1* transduced hematopoietic cells was possible by the administration of doxorubicin. Results from this study illustrated the potential of *MRP1* for hematopoietic chemoprotection and selection.

1.7   Chemotherapy Induced Myelosuppression

1.7.1   Mechanism of Chemotherapy Induced Myelosuppression

Chemotherapeutic agents are commonly used for the treatment of a variety of neoplastic conditions. A frequent and clinically important consequence of chemotherapy is acute hematologic toxicity[172]. The highly proliferative nature of the hematopoietic system renders it susceptible to damage by many anti-cancer agents. This may result in a temporary depression of hematopoietic lineages[172]. Myelosuppression most often manifests as a transient neutropenia, though thrombocytopenia and anemia may also occur. A deficiency in neutrophils and platelets can result in an increased susceptibility to infection and hemorrhage, respectively. These serious therapeutic side effects not only increase the cost of hospitalization, but can also increase patient morbidity and mortality[173].

Hematopoietic cells are damaged directly by chemotherapeutic agents in a similar manner to neoplastic cells, by deregulation of cell division or promotion of cell death. The pharmacologic mechanism of an antineoplastic drug and the site of activity in the cell cycle will dictate the hematologic effects observed[172]. Phase-specific (S or M) agents produce neutropenia and thrombocytopenia rapidly by primarily targeting multilineage restricted progenitors and differentiated cells[173]. Hematologic recovery is usually swift. Agents that are not restricted to a specific phase, but active during the cell cycle, may produce a granulocyte nadir early, but a slower recovery. A delayed nadir and recovery is typically observed with the use of chemotherapeutics that are not cell-cycle specific or function during G_o[33,174].

Acute hematologic toxicity is an expected consequence of many chemotherapy regimens, however long-term bone marrow damage may also result[172]. Residual injury to the bone marrow may occur by direct or indirect mechanisms. HSCs may be damaged directly by chemotherapeutic agents resulting in cell death and a reduction in the numbers of HSCs available in the bone marrow[172,173,175]. Although HSCs are not usually

primary targets, the death of committed cells may stimulate HSCs to cycle, exposing them to the cytotoxic effects of many chemotherapeutic drugs. Indirect mechanisms include damage to the hematopoietic microenvironment. Some chemotherapeutic agents may injure bone marrow stromal cells and as a result many of the factors that regulate normal hematopoiesis and the support provided by stromal cells may be compromised[175]. Chronic bone marrow injury may not be evident until events occur that place additional hematologic stress on the bone marrow reserve.

1.7.2 Current Management of Myelosuppression

Clinical complications of cancer chemotherapy include neutropenia, thombocytopenia and anemia. Various treatments and procedures have been implemented to manage these potentially life-threatening side effects such as the administration of blood products. hematopoietic cytokines and hematopoietic stem cell transplantation[173].

Severe neutropenia may place a patient at risk of infections that may have grave consequences such as septic shock and death. Febrile neutropenia is currently managed by the administration of broad-spectrum antibiotics[173]. Hematopoietic cytokines such as granulocyte colony-stimulating factor (G-CSF) have also been used extensively in the treatment of chemotherapy-induced neutropenia[173,176,177]. G-CSF has been shown to have several effects that can be beneficial to managing neutropenia[173]. G-CSF can promote proliferation and differentiation of neutrophil progenitors as well as stimulation of the release of neutrophils from the bone marrow[110]. The depth and duration of neutropenia may be reduced as a result of these effects and the incidence of infection may be lowered.

Hemorrhage is a serious consequence of chemotherapy-induced thrombocytopenia and current management consists of platelet transfusions[178,179]. Transplantation of autologous hematopoietic stem cells is another method presently used to facilitate platelet and neutrophil engraftment[180,181]. Autologous bone marrow and

mobilized peripheral blood stem cell transplants have been used to accelerate myeloid and platelet recovery. A shorter time to engraftment and a reduction in the duration of hospitalization have been observed with the use of hematopoietic stem cell support.

Although the existing treatments and procedures have been used successfully to minimize the severity of chemotherapy-induced myelosuppression, it is often necessary to reduce the dose or intensity of chemotherapy regimens to avoid acute hematologic toxicity[172].

1.7.3 Dose Intensification and High Dose Chemotherapy

The potential sequelae of chemotherapy-induced myelosuppression can be life-threatening and as a result chemotherapeutic dose or intensity are often altered to circumvent severe myelosuppression[173]. Compensatory dose attenuation may result in sub-optimal anti-tumour effects[182-184]. There are several malignancies that are chemosensitive and may benefit from high dose chemotherapy or dose intensification[185-188]. Hematopoietic growth factors such as G-CSF are used in various ways to facilitate high-dose intensive chemotherapy[189]. G-CSF can be administered after chemotherapy to decrease the episodes of neutropenia and allow for the delivery of chemotherapy doses on time. The use of G-CSF may enable accelerated administration of chemotherapy doses and support the use of high dose chemotherapy by shortening the interval of neutropenia[189]. Hematopoietic growth factors are also used for mobilization of peripheral blood stem cells[115,177,190-192]. Peripheral blood and bone marrow stem cell rescue treatment has been used in combination with high dose or dose-intensive chemotherapy regimens to hasten hematopoietic recovery[189]. Patient morbidity and mortality may be reduced with the use of hematopoietic stem cell support after high dose chemotherapy.

Current management of myelosuppression has facilitated the use of high dose or dose intensive chemotherapy regimens. The feasibility has been demonstrated, however further investigations are required to determine the efficacy of these treatments.

1.7.4 Retroviral Gene Transfer for Hematopoietic Chemoprotection

A major obstacle in cancer chemotherapy is the resistance of tumour cells to antineoplastic agents. Several mechanisms have been characterized and drug resistance genes have been identified[119,120]. Genes that have been considered to be impediments to cancer treatment are now being investigated as a means to prevent chemotherapy-induced myelosuppression. The transfer of drug resistance genes to hematopoietic stem and progenitor cells is a possible strategy to overcome the acute hematologic toxicity observed with the use of cancer chemotherapeutics[122,193].

There are several promising candidate genes for chemoprotection of hematopoietic cells. Chemoresistance genes such as *MDR1*, *DHFR* (dihydrofolate reductase), *glutathione-s-transferase* and *MRP1* (multidrug resistance-associated protein) could be exploited for the purpose of rendering the bone marrow chemoresistant[193]. Genetically modifying hematopoietic cells by introducing drug resistance genes has great potential clinical value[193-196]. If HSCs and their progeny were expressing a chemoresistance gene, serious side effects of chemotherapy treatment such as neutropenia and thrombocytopenia could potentially be avoided. The reduced risk of infection and hemorrhage could result in decreased patient morbidity and mortality[197]. Safer dose intensification may also be facilitated by abrogating life-threatening myelotoxicity[195]. This may enable greater killing of tumour cells, improving the effectiveness of chemotherapy regimens[19,21].

The feasibility of transferring drug resistance genes for hematopoietic chemoprotection has been shown in studies using the *MDR1* gene and *DHFR* variants[193-196,198-200]. Initial experiments using transgenic mice demonstrated the possibility of using *MDR1* for protection from the myelosuppressive effects of chemotherapy[201-204]. Mice transgenic for a human *MDR1* cDNA expressed human P-glycoprotein in bone marrow cells and were resistant to daunomycin-induced leukopenia[201]. Control mice experienced a three fold drop in white blood cell counts whereas cell counts for *MDR1* positive mice remained normal or increased as a result of drug challenge. Many *in vitro* studies of gene

transfer of *MDR1* for chemoprotection have been conducted[122,196,200,205,206]. Retroviral gene transfer of *MDR1* to murine and human hematopoietic cells *in vitro* has been successful and expression of the gene has provided protection from chemotherapeutic agents such as vinblastine and colchicine[122,196,200,205,206]. The *MDR1* gene has also been used as a selectable marker. *In vivo* selection by chemotherapeutic challenge of *MDR1* retrovirally transduced hematopoietic cells has been demonstrated. Mice transplanted with *MDR1* transduced bone marrow cells were treated with taxol[207]. Marrow from recipient mice was then used to transplant other secondary mice. A series of taxol and retransplantation cycles were performed that illustrated the possibility of *in vivo* selection of *MDR1* transduced cells and the corresponding increase in levels of chemoresistance. In light of encouraging pre-clinical trials, human clinical trials of *MDR1* gene transfer to bone marrow cells have been initiated and are ongoing. The main focus of these investigations is to determine the safety and efficacy of the gene transfer procedure.

DHFR variants have also been shown to be useful and effective chemoresistance gene for gene transfer studies[20,198]. Point mutations of the *DHFR* gene have exhibited a reduced affinity for antifolate drugs. *In vitro* and *in vivo* studies have confirmed that retroviral gene transfer of the *DHFR* gene can provide protection from methotrexate induced myelosuppression in a murine model[198]. Corey et al. have successfully demonstrated hematopoietic chemoprotection by retroviral-mediated gene transfer of the *DHRF* cDNA into murine hematopoietic bone marrow cells[208]. When challenged with methotrexate, mice transplanted with *DHFR* transduced hematopoietic cells experienced less mortality (below 20%) and maintained higher hematocrits than control animals. Mortality in control mice reached as high as 60% and significant anemia was observed in this group. Secondary transplantation of methotrexate-resistant bone marrow was performed to confirm transduction of hematopoietic stem cells. Bone marrow was harvested from primary recipients two months after transplantation and injected into secondary recipients. Recipient mice were challenged with methotrexate to assess bone marrow chemoresistance. Higher hematocrits and increased survival were noted in mice receiving transduced marrow as compared to control mice. This study demonstrated

reduced mortality and decreased hematologic toxicity in mice due to retroviral gene transfer of the *DHFR* gene.

Mutant *DHFR* genes have also been shown to provide protection from the newer antifolate drugs such as trimetrexate. In a study conducted by Spencer and colleagues, bone marrow transduced with a retrovirus containing a *DHFR* variant was transplanted into recipient mice[198]. Reconstituted mice were challenged with trimetrexate and blood samples were monitored for hematologic changes. While mice in the control group experienced severe neutropenia and reduced to absent reticulocyte production, hematologic protection was observed in mice receiving *DHFR* transduced marrow. The majority of these mice exhibited preserved erythropoiesis and granulopoiesis. Mice that were not protected against myelosuppression were found to have low to undetectable levels of proviral sequences. Although hematopoietic drug resistance was not observed for all experimental animals, protection from chemotherapy induced neutropenia and reticulocytopenia was demonstrated in this study. The results from murine studies are encouraging and retroviral mediated gene transfer of *DHFR* variants may prove to be a useful strategy for hematopoietic chemoprotection.

The transfer of drug resistance genes for hematopoietic chemoprotection is an exciting idea, however there are drawbacks to this treatment strategy[193,194,200]. A possible consequence of genetically modifying peripheral blood or bone marrow hematopoietic stem cells to be chemoresistant is the accidental modification of tumour cells[101-103]. Studies have shown that autologous marrow or peripheral blood transplants could be contaminated with tumour cells and contribute to relapse when accidentally infused[101-103]. Methods of purging these cells are being intensively studied[209]. The sorting of CD34+ cells by immunomagnetic selection appears to be a promising method of purging tumour cells[210]. The elimination of neoplastic cells from the autograft is critical before the introduction of a chemoresistance gene.

Another important consideration in the transfer of drug resistance genes is that toxicity to other organ systems such as the gut, heart and lungs may be dose limiting[172].

As a result, dose intensification or high dose chemotherapy may not be feasible even with hematopoietic chemoprotection.

Despite the fact that there are possible limitations to the transfer of drug resistance genes, there is great value in reducing the hematological side effects of chemotherapy. Decreased patient morbidity and mortality, as well as safer dose-intensification are paramount. The transfer of drug resistance genes for hematopoietic chemoprotection has been demonstrated in animal models and in *in vitro* studies [171,193-196,200,207,208,211]. Further investigations and improvements in retroviral gene transfer are necessary before this form of treatment becomes a reality.

1.8   Study Rationale

In addition to malignant cells, chemotherapeutic agents commonly damage normal, rapidly dividing cells. The hematopoietic system is a frequent target and hematologic toxicity is a serious side effect of this anticancer modality. Chemotherapy induced myelosuppression is a major dose limiting factor in cancer treatment. Compensatory dose attenuation is common and may result in sub-optimal anti-tumor effects. Transfer of drug resistance genes such as the *Multidrug Resistance–Associated Protein 1* to hematopoietic stem and progenitor cells could render the bone marrow chemoresistant. The short-term myelotoxicity seen with the use of antineoplastic agents could potentially be avoided if hematopoietic progenitors were modified to express a chemoresistance gene. As a result, the risk of infection and hemorrhage would be considerably reduced. Infusion of drug resistant stem cells into a patient should result in a protection level that increases with each cycle of chemotherapy. *In vivo* selection and expansion of resistant hematopoietic stem cells should occur with drug exposure. Unmodified stem cells may also be protected, as they would not be as vulnerable to chemotherapy if they are not forced to cycle. By decreasing the hematologic toxicity, safer dose intensification may be facilitated. This might enable greater killing of tumour cells, improving the effectiveness of chemotherapy regimens.

1.9   Hypothesis

We postulated that the expression of the *Multidrug Resistance-Associated Protein 1* in hematopoietic progenitor and stem cells would provide protection from the myelosuppressive effects of many cancer chemotherapy regimens and permit safer dose intensification.

1.10   Experimental Objectives

The initial objective of this project was to determine if the MRP1-PG13 retroviral vector could transfer the *MRP1* gene to hematopoietic cells and whether expression of the gene would give rise to drug resistance.   The second objective of this project was to determine whether *MRP1* gene transfer to canine hematopoietic progenitor cells could be achieved.   We also wanted to examine, in a canine model, whether genetically modified hematopoietic progenitor cells carrying the *MRP1* gene could provide protection from the myelosuppressive effects of chemotherapy *in vivo*.

Chapter 2

Materials and Methods

2.1 Veterinary Procedures

2.1.1 Animal Husbandry

Seven normal dogs (three mixed breed dogs, four beagles), ranging from eight months to two years of age, were used in these studies. Dogs weighed from 10-32 kg and 10 kg dogs were selected for use in the *in vivo* assays. The dogs were maintained at the Central Animal Facility of the University of Guelph. All experimental protocols met guidelines set by the Canadian Council on Animal Care (Care and Use of Experimental Animals, Volumes 1 and 2), were performed in accordance with the Animals for Research Act (Ontario 1980) and were approved by the institutional Animal Care Committee. Veterinary procedures were performed by Drs. Stephen Kruth (D.V.M.), Paul Woods (D.V.M) and Anthony Abrams-Ogg (D.V.M.).

2.1.2 Hematopoietic Mobilization

Prior to the administration of hematopoietic growth factors, venous blood samples were obtained and complete blood counts performed to establish baseline cell numbers. For hematopoietic mobilization, dogs received recombinant human stem cell factor (rhSCF, Amgen, Thousand Oaks, California) and recombinant human granulocyte colony-stimulating factor (rhG-CSF, Amgen), kindly supplied by Dr. Keith Stewart (Toronto, Ontario). The combination of SCF (25 µg /kg/day) and G-CSF (10 µg/kg/day) was administered subcutaneously for five days. Complete blood counts were performed daily. Total white blood cell and segmented neutrophil counts were monitored to assess hematopoietic mobilization. Bone marrow or peripheral blood harvests were performed on the fifth day of cytokine treatment.

2.1.3   Bone Marrow and Peripheral Blood Harvest

Large-scale bone marrow harvests were performed under general anesthesia in the Small Animal Clinic of the University of Guelph. Dogs were sedated with a combination of acepromazine (0.05 mg/kg) and butorphanol (0.2 mg/kg) intramuscularly. Anesthesia was induced with intravenous propofol (4-6 mg/kg) and was maintained with isoflurane and oxygen. Bone marrow, corresponding to 15% blood volume, was aspirated from the iliac crests and proximal humeri and femora using 16 gauge spinal needles (B-D Spinal Needles, Becton Dickson and Company, Franklin Lanes, New Jersey) for dogs weighing 15 kg or less, or 16 gauge disposable "I" type bone marrow aspiration needles (Jorgensen Laboratories Inc., Loveland, Colorado) for dogs greater than 15 kg.   0.1 ml of preservative free heparin (200 u/ml) was added to the marrow collection to a final volume of 2 u/ml. For follow up studies, small volume harvests (30-40 ml) were performed in the Central Animal Facility of the University of Guelph. Dogs received acepromazine (0.05 mg/kg) and butorphanol (0.2 mg/kg) intramuscularly for sedation and general anesthesia was induced and maintained with intravenous propofol.

Large volume (15% blood volume) blood collections were performed under light sedation with intramuscular acepromazine (0.05 mg/kg) and butorphanol (0.2 mg/kg). Blood was collected from a jugular vein using an 18 gauge butterfly catheter. Preservative free heparin was added to the sample as previously described for bone marrow collection. Small volume samples (~30 ml) were collected without sedation by venipuncture from the cephalic vein using a 20 gauge needle and a 30 ml syringe.

2.1.4   Infusion of Transduced Autologous Canine CD34$^+$ Cells

After retroviral transduction, adherent canine CD34$^+$ cells were harvested from fibronectin coated plates by non-enzymatic digestion with Cell Dissociation Buffer (Gibco, Grand Island, NY). Cells were washed twice with phosphate buffered saline (Gibco), resuspended in Hank's buffered saline (Gibco) and transported on ice to the

University of Guelph. Autologous canine CD34$^+$ cells (ranging from 1 X 10^6-7.5 X 10^6 cells) were infused slowly over a period of 15 minutes into a cephalic vein. Dogs were monitored for adverse reactions to cell infusions.

2.1.5 Chemotherapeutic Challenge

For chemotherapy challenge, dogs received one intravenous injection of 1 or 1.5 mg/m^2 vincristine sulphate (Novopharm, Toronto, Ontario). The drug was administered by bolus infusion via a 22 gauge butterfly catheter in a cephalic vein. Blood samples were collected previous to drug challenge to establish baseline cell counts and daily to detect myelosuppression. General assessments of the health status of the animal were performed daily to monitor for chemotherapeutic side effects.

2.2 Retroviral Producer Cell Line (MRP1-PG13)

2.2.1 Retroviral Vector Construction

The myeloproliferative sarcoma virus (MPSV)[212] U3 region was isolated by digesting pR271 (obtained from Dr. Ostertag, Hamburg University, Hamburg, Germany), which contains the entire long terminal repeat (LTR) of MPSV, with KpnI and NheI. The 448bp KpnI/NheI fragment was inserted into the equivalent site of the pG1Na vector[8] to replace the MMLV U3 of the 3'-LTR. The resulting plasmid was termed pG1Na^{MPSV}. The MRP1 cDNA (originally obtained from Dr. Susan Cole, Queen's University, Kingston, Ontario, Canada) was cloned into the pG1Na^{MPSV} vector to replace the neomycin transferase gene and make plasmid pG7MRP1. The retroviral vector construction was performed by Dr. Fusayuki Omori, a recent post-doctoral research fellow in our laboratory.

2.2.2 Retroviral Production

Retroviral vector packaging cell lines GP+E86[39], PA317[38] and PG13[9] (ATCC, Rockville, Maryland) were grown in Dulbecco's modified Eagle's medium (DMEM: GIBCO) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO) and 1% penicillin/streptomycin (GIBCO) in a 25cm^2 culture flask (CORNING, Corning, NY). GP+E86 cells at 70-80% confluence were transfected with 5 µg of the vector plasmid pG7MRP1 by lipofectin (GIBCO) according to the manufacturer's instruction. Two days later 0.6 µg/ml of etoposide (Novopharm, Ontario, Canada) was added to the culture. Supernatants were harvested, filtered through a 0.45 µm filter (Millipore, Bedford, MA) and used to transduce the amphotropic retroviral vector packaging line PA317, which had been grown to approximately 50% confluence. The transduction was performed three times by changing the viral supernatant every 24 hours for three days with addition of 5 µg/ml of polybrene (Sigma). Two days later 0.6 µg/ml of etoposide was added to the culture for selection of *MRP1*-transduced PA317 cells. The resultant *MRP1* producer line created by Dr. Fusayuki Omori, was maintained in DMEM with 10% FCS at 37°C in 5% CO_2 in air. Supernatant from the PA317 MRP1 producer cell line was similarly used to transduce PG13 cells and the arising etoposide resistant producer line, MRP1-PG13, was used for all future studies. Etoposide selection of the producer cells was intermittently performed thereafter. Aliquots of the producer cells were cryopreserved at -150°C for later use. The MRP1-PG13 producer cell line was made by Mr. Chi-Kin Chan, a research technologist in our laboratory.

2.2.3 Production of Viral Supernatant

Freshly thawed producer cells were grown to ~90% confluence. For the production of viral supernatant to be used in canine assays, the DMEM media was removed and replaced with Iscove's modified Dulbecco's media (IMDM, Gibco) supplemented with 12.5% FCS, 12.5% horse serum (Gibco), 1% penicillin-streptomycin and 1% L-glutamine. For other cell types, DMEM was used. The cultures were

transferred to 32°C and after 24 hours, supernatants were harvested and filtered through a 0.45 μm filter. The supernatants were either used fresh or frozen at –80°C until use.

2.2.4   Retroviral Titering

Two human hematopoietic cell lines were used for retroviral titering; the chronic myelogenous leukemia cell line K562[213] and the acute T-cell leukemia cell line Jurkat[214]. Two million cells (either K562 or Jurkat) were exposed to 4 ml of MRP1-PG13 supernatant in the presence of 5 μg/ml protamine sulphate (Sigma). Two days later, 5 X10^3 cells were cultured in "complete" methylcellulose (StemCell Technologies Inc., Vancouver, BC) supplemented with 20% FCS and incubated at 37°C in 5% CO_2 in air. The methylcellulose used contained recombinant human cytokines SCF, IL-3, IL-6, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF) and erythropoietin (EPO). One week later, colonies were plucked and analyzed by PCR for *MRP1*. The titer was determined by multiplying the total number of cells exposed to 1 ml of viral supernatant by the percentage of provirus positive colonies.

2.2.5   Determination of Gene Transfer Efficiency Using the K562 and Jurkat Cell Lines

One million cells were incubated with 2 ml of MRP1-PG13 supernatant in the presence of 5μg/ml protamine sulphate. Two days later, approximately 5 X 10^3 cells were cultured in   "complete" methylcellulose supplemented with 20% FCS, and incubated at 37°C in 5% CO_2 in air. One week later colonies were plucked and analyzed by *MRP1* PCR. The gene transfer efficiency was calculated as the percentage of colonies that were PCR provirus-positive relative to the total number of colonies present.

2.3   Tissue Culture

2.3.1   Cell Culture

All cells were incubated at 37°C in 5% CO_2 and air. Retroviral producer cells lines PA317 and PG13 were grown in DMEM (Gibco) supplemented with 10% FCS, 1% penicillin-streptomycin and 1% L-glutamine. K562, a human chronic myelogenous leukemia cell line and Jurkat, a human acute T-cell leukemia cell line, were grown in RPMI Medium 1640 (Gibco) supplemented with 10% FCS, 1% penicillin-streptomycin and 1% L-glutamine. Canine bone marrow and peripheral blood mononuclear cells (CD34 positive and negative fractions) were cultured in IMDM supplemented with 12.5% FCS, 12.5% horse serum, 1% penicillin-streptomycin and 1% L-glutamine. Human hematopoietic cytokines SCF, G-CSF, interleukin-6 (IL-6), FLT-3 ligand (FLT-3L), and thrombopoietin (TPO) (all obtained from Medicorp, Montreal, Ontario) were added to the canine culture medium at a concentration of 50 ng/ml. When necessary, cells were cryopreserved at –150°C in FCS and 10% dimethyl sulfoxide (Sigma). Cryopreserved cells were thawed quickly in a 37°C water bath.

2.3.2   Canine Mononuclear Cell Separation

Heparinized canine bone marrow or peripheral blood samples were diluted 1:2 in PBS (Gibco). For each 50 ml centrifuge tube (Becton Dickinson, Franklin Lanes, NJ), 25 ml of diluted sample was layered onto 15 ml of Ficoll-Paque gradient (Amersham-Pharmacia Biotech, Arlington Heights, IL). Samples were centrifuged at 400 X g for 30 minutes at 18°C. The mononuclear cells were removed by aspiration using a sterile transfer pipette. Cells were pooled, washed once with PBS containing 2% horse serum and centrifuged at 1200 rpm for 10 minutes at 4°C. Contaminating red blood cells were lysed by incubating cells with ACK lysis buffer for 10 minutes. Remaining cells were washed twice with PBS and 2% horse serum, centrifuged at 1200 rpm for 10 minutes at 4°C and resuspended in PBS with 2% horse serum for further use.

2.3.3 Canine CD34 Positive Sorting

Fresh or frozen canine mononuclear cells were resuspended in PBS supplemented with 2% horse serum and filtered through a 70 μm nylon cell strainer (Becton Dickinson). Cells were labeled with either a biotinylated or non-biotinylated IgG-1 anti-canine CD34 antibody (1H6) acquired from the Fred Hutchinson Cancer Research Center[116]. Cells labeled with the biotinylated antibody were incubated with 1H6 monoclonal antibody at 40 μg/ 1 X10^8 cells for 20 minutes at 4°C. Cells labeled with non-biotinylated antibody were incubated with 20 μg of antibody /1 X 10^8 cells for 20 minutes at 4°C. After incubation, cells were washed 2X with PBS containing 0.1% BSA and 1mM EDTA (Miltenyi's Buffer, Miltenyi Biotec, Auburn, California) and centrifuged at 1200 rpm for 10 minutes. Mononuclear cells were then incubated with either Rat Anti-Mouse IgG1-Microbeads (Miltenyi Biotec) (200 μl / 1 X 10^8 cells) for nonbiotinylated antibody or Streptavidin coated Microbeads (Miltenyi Biotec) (100 μl / 1 X 10^8 cells) for cells labeled with biotinylated antibody for 20 minutes at 4°C. Cells were washed once with Miltinyi's Buffer and centrifuged for 10 minutes at 1200 rpm. Washed cells were filtered through a 70 μm Nylon Cell Strainer and separated using an immunomagnetic column technique according to the manufacturer's protocol (Miltenyi Biotec). Enriched cells were resuspended in PBS with 2% horse serum for use in various assays.

2.3.4 Transduction of Canine Hematopoietic Cells

Canine CD34 positive and negative cells were prestimulated for 24 hours in IMDM supplemented with 12.5% FCS, 12.5% horse serum, 1% penicillin-streptomycin and 1% L-glutamine in the presence of 50 ng/ml of SCF, IL-6 (dogs 1 and 2), SCF, IL-6, G-CSF and FLT-3L (dogs 3 and 4) or SCF, IL-6, FLT-3L, G-SCF and TPO (dogs 5-7). Following prestimulation, cells were centrifuged at 1200 rpm for 10 minutes, resuspended in viral supernatant and plated on 12.5 cm^2 tissue culture flasks coated with 2 μg/cm^2 of recombinant human fibronectin fragment CH-296 (Takara Biomedicals,

of viral supernatant for twenty minutes. Cytokines used in the prestimuation period were added to the transduction medium at a concentration of 50 ng/ml. Protamine sulphate (5 μg/ml) was added to the transduction medium for dogs 1 and 2. The transduction was performed four times over a period of 48 hours, with full media exchange and cytokine replacement for dogs 1-2 and half-media exchange with replenishment of cytokines for dogs 3-7. After the transduction period, cells were harvested by non-enzymatic digestion using Cell Dissociation buffer, washed twice with PBS and resuspended in Hank's buffered saline. The number of viable cells were determined by trypan blue staining and enumeration with a hemacytometer.

2.3.5   Hematopoietic Progenitor Assays

Canine CD34 positive and negative cells were resuspended in IMDM supplemented with 12.5% FCS, 12.5% horse serum, 1% penicillin-streptomycin and 1% L-glutamine and added to complete methylcellulose at a cell density of 1 x10^5 cells/ml of methylcellulose. IMDM was added to the methylcellulose to achieve a final volume of 100 μl for each ml of methylcellulose. One ml of methylcellulose was cultured in each 35 mm tissue culture grade plate and cells were plated in triplicate. Cells were incubated at 37°C in 5% CO_2 in air.   Between days 10-14, individual hematopoietic colonies were plucked into 40 μl of non-ionic detergent lysis buffer containing proteinase K (GIBCO) (1mg/ml) and incubated at 56°C for 1 hour. Lysates were then boiled for 10 minutes to inactivate proteinase K and frozen at –20°C for further use.

2.3.6   Dose Response Curves for Control K562 Cells and *MRP1* Transduced Clones

To determine the cytotoxic dose of etoposide for control K562 and transduced K562 clones, 5000 cells were suspended in IMDM (Gibco) and plated in one ml of "complete" methylcelluose (Stem Cell Technologies Inc.).   Various concentrations of etoposide were added to the culture plates, ranging from 0 to 5 μg/ml of methylcellulose.

IMDM was added to the methylcellulose to a final volume of 100 ml IMDM/ ml of methylcellulose. Cells were plated in triplicate and incubated at 37°C in 5% CO_2 in air. Colonies were counted at day seven. Drug concentrations where colony formation was not evident were considered to be cytotoxic to K562 cells.

2.3.7 Dose Response Curve for Normal Canine Bone Marrow Mononuclear Cells

To determine the cytotoxic dose of etoposide for untransduced canine bone marrow mononuclear cells, 1 X 10^5 cells were suspended in IMDM (Gibco) and plated in one ml of "complete" methylcelluose (Stem Cell Technologies Inc.). Various concentrations of etoposide were added to the culture plates, ranging from 0 to 0.5 µg/ml of methylcellulose. IMDM was added to the methylcellulose to a final volume of 100 ml IMDM/ ml of methylcellulose. Cells were plated in triplicate and incubated at 37°C in 5% CO_2 in air. Colonies were counted between days 10-14. Drug concentrations where colony formation was not evident were considered to be cytotoxic to canine bone marrow mononuclear cells.

2.4 Molecular Biology Techniques

2.4.1 DNA Extraction

Cells (maximum 10^7) were washed with PBS and centrifuged at 1200 rpm for 10 minutes at 4°C. The resulting cell pellet was resuspended in 330 µl of DNA-A (a solution of 10mM Tris-HCL, 10 Mm EDTA, 10Mm NaCl) and 330 µl of DNA-B (a mixture of DNA-A and 2% SDS), 50 µl of Proteinase K (PK) and 60 µl of RNase A were added. Samples were incubated for one hour at 56°C. A further incubation of 45 minutes at 56°C was performed after the addition of 50 µl of PK. Samples were centrifuged for 10 minutes at 13,000 rpm and a phenol extraction performed. Following two phenol-chloroform extractions, a single chloroform extraction was performed. 5M

two phenol-chloroform extractions, a single chloroform extraction was performed. 5M NaCl and 100% ethanol were added to the samples (resulting ratio was 2.2 ethanol: 1NaCl/DNA solution) and the samples were stored at –20°C overnight until the DNA had precipitated. DNA was recovered by centrifugation at 13000 rpm for 30 minutes. The supernatant was removed, the DNA pellet washed twice with 70% ethanol and samples centrifuged at 13000 rpm for 10 minutes. The DNA pellet was dried and resuspended in sterile water.

2.4.2 RNA Extraction

Cells were lysed with TRIZOL Reagent (Gibco) according to the product instructions and samples were incubated for 15 minutes at ambient temperature. A chloroform extraction was performed and the aqueous phase isolated. RNA was precipitated from the aqueous phase by the addition of isopropyl alcohol (0.5 ml per 1 ml of Trizol Reagent). Samples were incubated for 10 minutes at room temperature and centrifuged at 13000 rpm for 10 minutes. The supernatant was removed and the RNA pellet washed once with 75% ethanol. After centrifugation for five minutes the RNA pellet was dried. The RNA was dissolved in RNase-free water (Qiagen, Valencia, CA) and incubated for 10 minutes at 56°C.

2.4.3 PCR Analysis

Individual colonies in methylcellulose cultures were plucked into 40 μl of non-ionic detergent lysis buffer containing proteinase K (1mg/ml) and incubated at 56°C for 1 hour. Lysates were then boiled for 10 minutes to inactivate proteinase K. To assess the quality of amplifiable DNA for human hematopoietic colonies, PCR amplification of the human β-actin gene was performed using the following primers: 5'CTGCCTGACATGAGGGTTACC-3' and 5'CTAGAAGCATTTGCGGTGGAC-3'. The PCR was run at 94°C for 90 seconds followed by 42 cycles at 94°C for 20 seconds,

61°C for 25 seconds and 72°C for 30 seconds followed by 5 minutes at 72°C. For canine hematopoietic colonies, amplifiable DNA was assessed using PCR amplification of the canine muscular dystrophin gene. PCR was performed on 5μl of lysate with the following primers both derived from exon 1: 5'ACAGTCCTCTACTTCTTC-3' and 5'AATTCACAGAGCTTGCCATGC-3'. The cycling conditions for these PCR reactions were 42 cycles of denaturation at 94°C for 20 seconds, annealing at 61°C for 25 seconds and extension at 72°C for 30 seconds.

To detect the presence of proviral DNA, a 376 bp amplicon from exon 2 to exon 4 of the human *MRP1* cDNA sequence was amplified using the following primers: 5'-TGTCACGTGGAATACCAGCAAC-3' and 5'-TACCAGCCAGAAAGTGAGCATG-3' with 42 cycles of denaturation at 94°C for 20 seconds, annealing at 63°C for 30 seconds and extension at 72°C for 30 seconds. The products of each 20 μl reaction were run and analyzed on a 2% agarose gel.

2.4.4 Southern Blot Analysis

Southern blot analysis was used to confirm proviral integration. Genomic DNA was extracted from control PG13 cells, *MRP1* producer cells, control K562 cells and transduced K562 cells. Ten micrograms of genomic DNA from each was digested with *Hind*III (GIBCO), electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane (Amersham, Arlington Heights, IL) and hybridized with a *MRP1* cDNA probe [^{32}P] labeled by random priming. The *MRP1* probe of 706 bp was derived from the plasmid pG7*MRP1* and corresponds to the *Bam*HI/*Hind*III fragment shown in Figure 2A.

2.5   Assays for Proviral *MRP1* Expression

2.5.1   Western Blot Analysis

To detect the *MRP1* protein, Western blot analysis was performed. 3.0×10^5 cells were washed three times in PBS, lysed with SDS (GIBCO) sample buffer and boiled for 15 minutes. The cell extract was electrophoresed on a 10% SDS-Page gel and transferred to a nitrocellulose membrane (Scheleicher and Schuell, Keene, NH). The membrane was blocked with PBS and 5% skim milk at 4°C overnight. It was then incubated with monoclonal anti-human *MRP1* antibody (Kamiya Biomedical, Seattle, WA) (1:50 dilution), for 2 hours at ambient temperature. The membrane was washed two times with PBS and 2X 0.1% Tween-20 PBS and then incubated with an anti-rat IgG (1:500 dilution) secondary antibody and subjected to the enhanced chemiluminescence assay (ECL) according to the manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ).

2.5.2   Reverse Transcriptase PCR (RT-PCR)

Reverse transcriptase PCR was performed to detect *MRP1* expression in transduced and untransduced canine CD34 negative RNA samples. The cDNA was synthesized using the Superscript II reverse transcriptase (Gibco) and an antisense primer (5'-CATTCACGAACTTGATGAGC-3') derived from exon 9 of the human *MRP1* cDNA. PCR amplification of the cDNA was performed using the following human *MRP1*-specific primers from exon 5 and exon 8: 5'-TCATTCAGCTCGTCTTGTCCTG-3' and 5'-GGCCTTGAAGAAGAAGCTCATG-3' and the following conditions: 42 cycles of denaturation at 94°C for 20 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds. The products of each 20 µl reaction were run and analyzed on a 2% agarose gel and the expected product size was 452 bp.

2.5.3 Flow Cytometry

Flow cytometry was used to assess *MRP1* protein expression. Approximately one million cells were fixed and permeabilized using Intraprep™ Permeabilization Reagent (IMMUNOTECH, Marseille, France) according to the manufacturer's instruction. Cells were incubated for 15 minutes at ambient temperature with monoclonal anti-human *MRP1* antibody (Kamiya Biomedical, Seattle, WA) (1:50 dilution). After washing with PBS, cells were incubated for 15 minutes at ambient temperature with a fluorescein isothyocyanate (FITC)-conjugated goat anti-rat antibody (1:100 – 1:200 dilution) (DAKO, Carpinteria, CA). After three washings with PBS, cells were resuspended in 0.5% paraformaldehyde (Sigma). Fluorescence analysis was performed on a Coulter Epics Flow cytometer (Coulter, Burlington, Canada).

Chapter 3

Results

3.1   Determination of the Gene Transfer Efficiency for the MRP1-PG13 Retroviral
       Vector Using the K562 and Jurkat Cell Lines

3.1.1   Introduction

Efficient and reproducible gene transfer into HSCs is a highly desirable goal for many gene therapy protocols. Retroviruses are one of the viral vectors most commonly used in human gene therapy trials. These vectors are based on the Moloney murine leukemia virus (MMLV) and although they are able to obtain stable integration of their genes into the genomes of target cells, there are important limitations that hamper the usefulness of these vectors[211]. A critical limitation is the low transduction efficiency[42,215].

There are several means of overcoming low gene transfer efficiency, including the use of pseudotyped retroviral vectors. The gibbon ape leukemia virus (GALV) has been used in conjunction with MMLV to create a pseudotyped retrovirus[9]. Higher levels of gene transfer into hematopoietic stem cells has been demonstrated with the use of GALV pseudotyped retroviral vectors[42,83]. Another method of increasing transduction efficiency is the use of polycations such as polybrene and protamine sulfate in transduction protocols. These molecules are known to neutralize the charge on the surface of cells and retroviral particles and diminish the repulsive surface forces, thereby facilitating retroviral-mediated transductions[82,83,216]. We incorporated the use of a GALV pseudotyped retrovirus and polycations in our gene transfer protocol in an attempt to increase gene transfer efficiency into hematopoietic cells. Reliable transfer of the transgene is a necessity for any protocol utilizing gene transfer.

The set of experiments described in this section was designed to determine the gene transfer efficiency of the MRP1-PG13 retroviral vector for two human hematopoietic cell lines (Jurkat and K562). The transfer and expression of the transgene and the ability of MRP1 to confer drug resistance was evaluated.

3.1.2 Experimental Design

It was necessary to demonstrate transfer and expression of *MRP1* *in vitro* to determine the feasibility of using the MRP1-PG13 retroviral vector for chemoprotection by *MRP1* gene transfer. The viral titer and gene transfer efficiency for the MRP1-PG13 retroviral vector were assessed using two human hematopoietic cell lines: the acute T-cell leukemia cell line Jurkat and the chronic myelogenous leukemia cell line K562. Cells were exposed to retroviral supernatants and cultured in methylcellulose for seven days. *MRP1* PCR was used to detect the presence of the provirus in unselected colonies. The gene transfer efficiency was calculated as the percentage of colonies that were PCR positive relative to the total number of colonies analyzed. The viral titer was determined by multiplying the total number of cells exposed to one ml of viral supernatant by the percentage of provirus positive colonies. The K562 cell line was further utilized to evaluate *MRP1* expression and drug resistance of transduced cells. Expression of the *MRP1* protein in transduced K562 clones was evaluated by flow cytometric and Western blot analysis. Drug resistance was assessed by colony formation in presence of cytotoxic doses of the chemotherapeutic agent etoposide.

3.1.3 Results

3.1.3.1 MRP1-PG13 Viral Titer

We developed a gibbon ape leukemia virus (GALV) pseudotyped retroviral vector producer cell line. This PG-13 based cell line produces retroviral vectors bearing the human *MRP1* cDNA. To determine the MRP1-PG13 viral titer, two human hematopoietic cell lines were targeted. The viral titer was estimated to be 1.7×10^5 viral particles/ml using K562 cells as a target. The average viral titer for the Jurkat cell line was 3.0×10^5 viral particles/ml of supernatant (2.8×10^5 for experiment # 1 and 3.2×10^5 for experiment #2). (Table 1A).

Colonies Analyzed	β-Actin Positive	*MRP1* Positive	% Positive (A)	Transduced Cells (B)	Viral Titer (A X B)
K562	60	21	35	5 X 10⁵	1.7 X 10⁵
Jurkat (#1)	117	64	55	5 X 10⁵	2.8 X 10⁵
Jurkat (#2)	144	91	63	5 X 10⁵	3.2 X 10⁵

Table 1A: Gene transfer efficiency (A) and viral titer for the MRP1-PG13 retroviral producer cell line using K562 and Jurkat Cell Lines

3.1.3.2   Gene Transfer Efficiency for the K562 and Jurkat Cell Lines

To assess gene transfer efficiency into hematopoietic cells, 1×10^6 cells (Jurkat or K562) were co-cultured with two ml of viral supernatant and plated for colony formation in methylcellulose. Individual non-drug selected colonies were examined by *MRP1* PCR after methylcellulose culture. Gene transfer efficiency for the K562 cell line was determined to be ~35% based on the presence of proviral DNA (Table 1A). The gene transfer efficiency for the Jurkat cell line was determined to be ~55% in experiment #1 and 63% for experiment #2. The average gene transfer efficiency was ~59%. (Table 1A)

Gene transfer and expression of *MRP1* was determined using the K562 cell line. Transduced K562 cells were plated in methylcellulose containing a cytotoxic dose of etoposide (1.0 μg/ml) for seven days. Quantitation was done by comparing the number of colonies growing in the presence of etoposide to the number of colonies growing without selection. Gene transfer efficiency was determined to be ~6% based on resistance of clonogenic cells to etoposide (Table 1B).

Experiment #	# of Colonies in the Presence of Drug	# of Colonies Without Selection	Gene Transfer Efficiency
1	40	689	5.8%
2	43	694	6.2%
Average	42	692	6.0%

Table 1B: Gene transfer and expression of *MRP1* using the K562 cell line

3.1.3.3 Confirmation of Proviral Integration

The human CML line K562 was also used to assess proviral integration and to demonstrate *MRP1* expression. Experiments were performed using transduced K562 cells clonally selected by plating in methylcellulose with the chemotherapeutic agent etoposide (1.0 µg/ml). Nine K562 clones were plucked from methylcellulose containing etoposide (1 µg/ml) and plated in RPMI medium. After cells had proliferated, DNA was extracted and PCR analysis performed for proviral detection. All nine clones were determined to be *MRP1* positive by PCR analysis. (Figure 1)

To confirm proviral integration, the producer (MRP-PG13) cells and transduced K562 cells were analyzed by Southern blot. K562 genomic DNA samples as well as control cells were digested with *Hind*III and hybridized with a *Bam*HI/*Hind*III MRP cDNA probe (Fig 2A). The probe detected ~3.3kb and ~9kb *Hind*III restriction fragments in PG13 and K562, respectively. The MRP-PG13 producer cell line showed a large smear, consistent with multiple integration sites in a polyclonal population (Fig 2B). Each of seven clones of transduced K562 cells analyzed showed a discrete hybridization pattern. In six clones there was evidence for only one proviral integration but in the remaining clone there were four separate proviral integrations (Fig. 2B).

Figure 1: *MRP1* PCR analysis of producer cell lines (PA317 and PG13) and K562-MRP clones. *MRP1* PCR analysis confirmed the presence of the provirus for producer cell lines and for all nine clones sampled.

MRP1 PCR of Transduced K562 Clones and Control K562
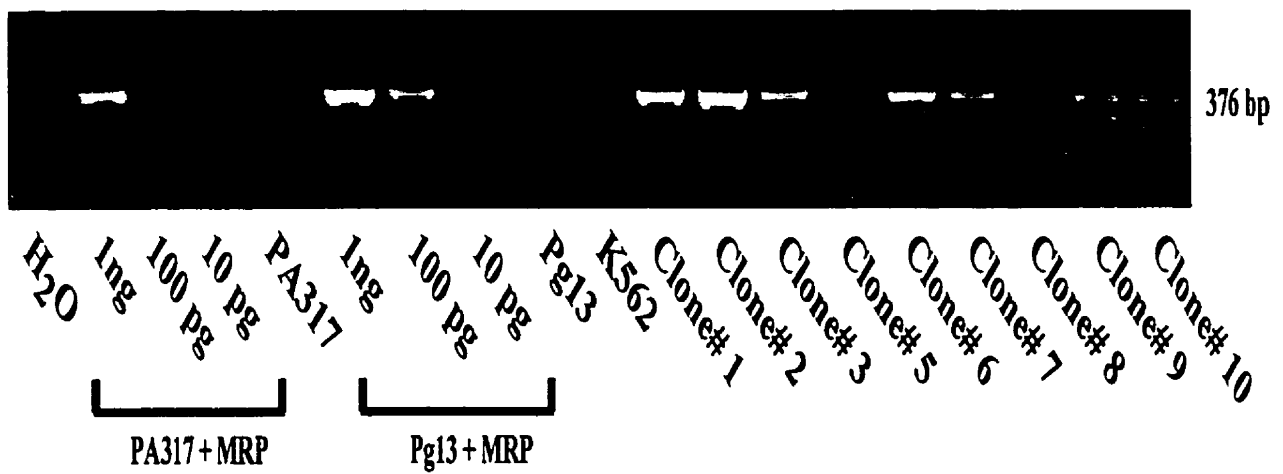
Figure 2A: This sketch depicts the genomic structure of the
             retroviral vector along with the probe used for
             Southern blot analysis

Figure 2B: Southern blot analysis confirmed proviral integration
             for the producer cell line MRP1-PG13 and for *MRP1*
             transduced clones.

Southern Blot Analysis of MRP1 Transduced Clones

Figure 2A
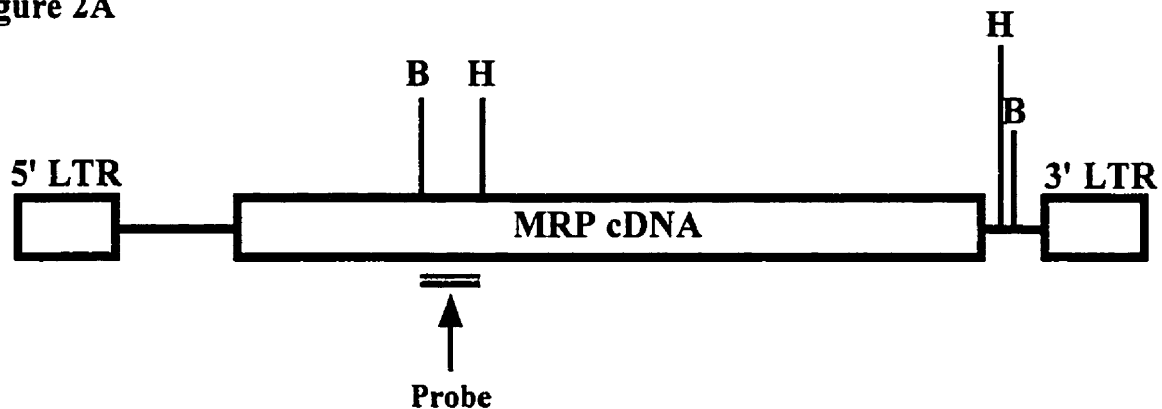
Figure 2B
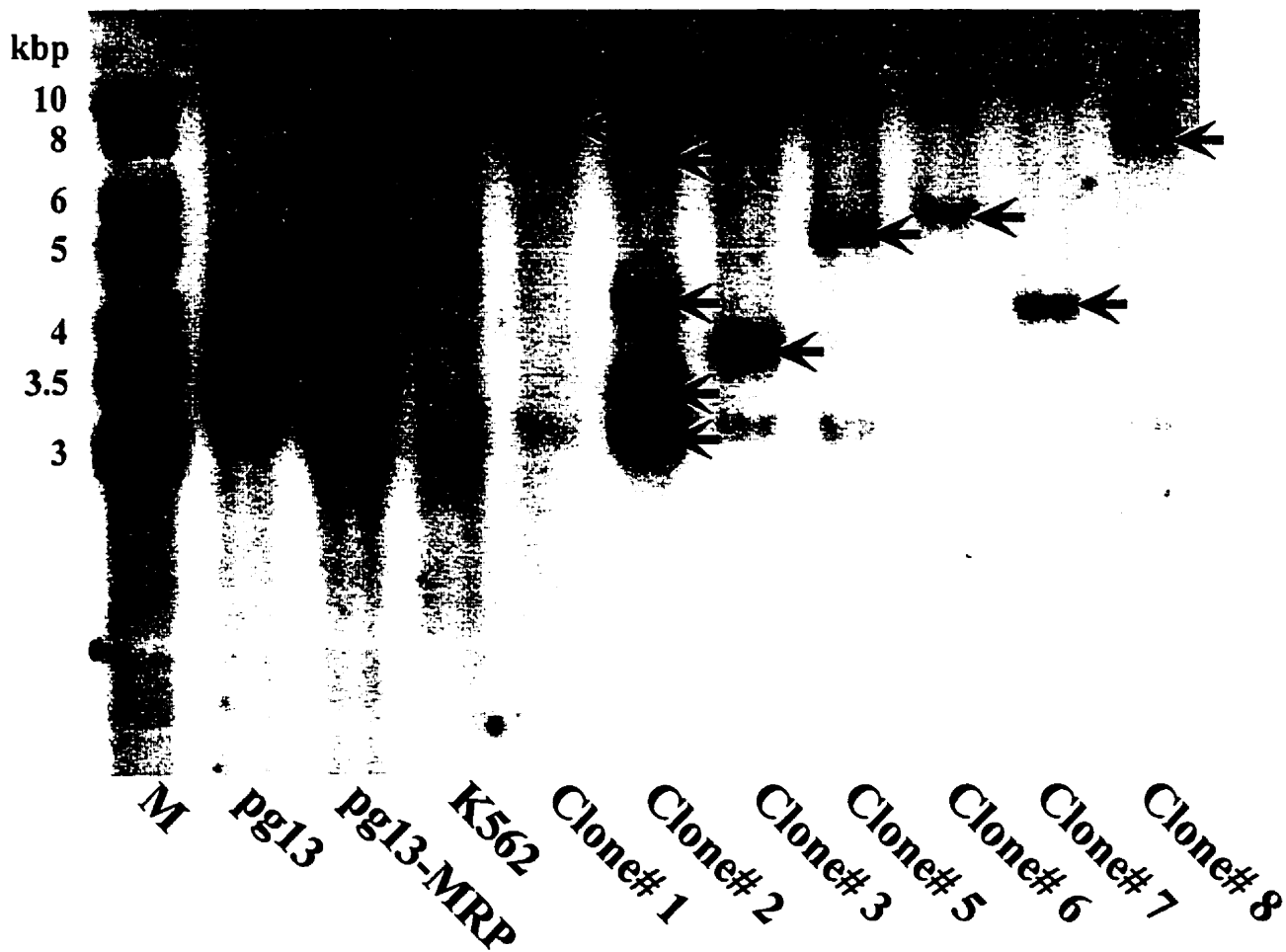
3.1.3.4   Western Blot Analysis and Flow Cytometry

Western blot analysis and flow cytometry were used to assess *MRP1* gene expression in nine transduced K562 clones. Western blot analysis confirmed human *MRP1* protein (190 kDa) expression in all nine clones sampled (Figure 3). The expression of *MRP1* was also analyzed by flow cytometry using an anti-human *MRP1* antibody. K562 cells transduced with MRP1-PG13 supernatants and selected in etoposide containing media showed an increase in fluorescence intensity as compared to control K562 cells. This increase varied from 16 to 54 fold (average ~30 fold) in the nine different clones analyzed. The results of four of the nine clones are represented in Figure 4. Both Western blot and flow cytometry confirmed successful gene expression in transduced cells.

3.1.3.5   Functional Protein Assays

To assess whether *MRP1* expression would result in drug resistance, *MRP1* transduced K562 clones were plated in methylcellulose with various cytotoxic concentrations of etoposide (range 0-5 µg/ml). Dose response curves were performed for untransduced K562 and for eight *MRP1* positive K562 clones (Table 2). The results of five of the nine clones are demonstrated in Figure 5. We determined that untransduced K562 cells did not survive at drug concentrations above 0.4 µg/ml of etoposide. All nine *MRP1* positive K562 clones were able to form colonies in the presence of cytotoxic doses of etoposide. Drug concentrations where colony formation was not evident were considered cytotoxic to untransduced K562 cells. Four clones were able to survive in 5 µg/ml of etoposide, 10 times the minimal toxic concentration for untransduced K562 cells (0.5 µg/ml). *MRP1* expressing clones were thus clearly capable of proliferating in the presence of otherwise toxic concentrations of etoposide.

Figure 3: Western blot showing expression of the *MRP1* protein in nine clones of transduced K562 cells. Untransduced K562 serves as a negative control.

Western Blot Analysis of MRP1 Transduced K562 Clones

MRP
(190 kDa)

Figure 4: Flow cytometric analysis of *MRP1* expression. Cells were stained with an anti-human *MRP1* antibody. Control K562 cells and four of nine *MRP1* transduced K562 cells are shown.

Flow Cytometric Analysis of MRP1 Transduced Clones

■ Unstained

☐ Stained with anti-Human MRP1
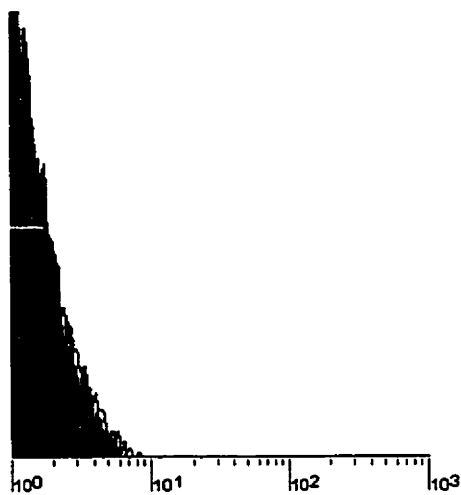
K562 no MRP
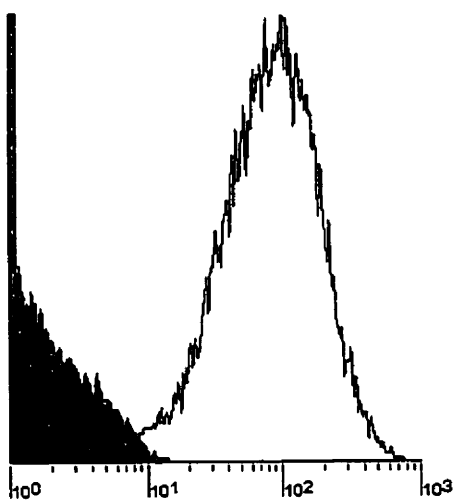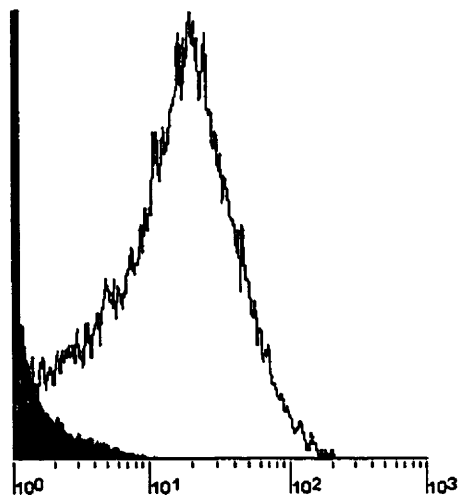
Clone #2

Clone #5
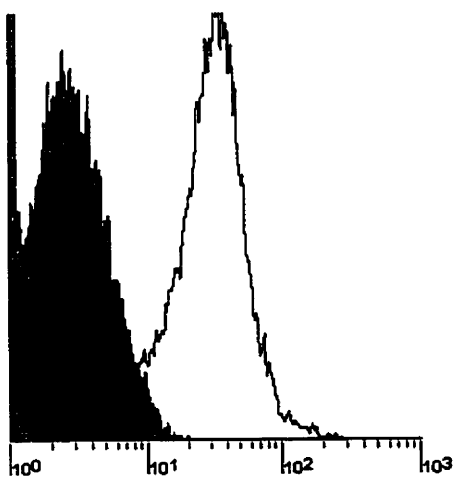
Clone #6
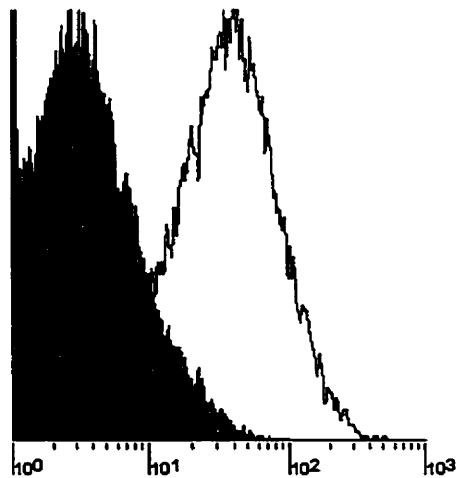
Clone #7

Drug [ ]	Control K562	Clone # 1	Clone # 2	Clone # 3	Clone # 5	Clone # 6	Clone #8	Clone #9	Clone # 10
0	516	260	337	601	802	612	296	552	393
0.5	0	235	268	485	756	524	284	540	368
0.8	0	235	153	169	636	373	268	488	339
1.0	0	117	73	99	444	285	189	447	315
2.5	0	15	36	18	76	100	170	400	288
5.0	0	0	0	0	5	0	163	373	211

Table 2: Dose Response for control K562 and *MRP1* clones using etoposide

Figure 5: A graph illustrating the results of an etoposide dose response curve for untransduced K562 cells and *MRP1* transduced clones. A representative five of the nine clones are shown. Colonies were counted at day seven.

Etoposide Dose Response for K562 and MRP1 Clones

Number of Colonies (y-axis)

Drug Concentration (ug/ml) (x-axis)

Legend:
- Control K562
- MRP10
- MRP9
- MRP8
- MRP6
- MRP3
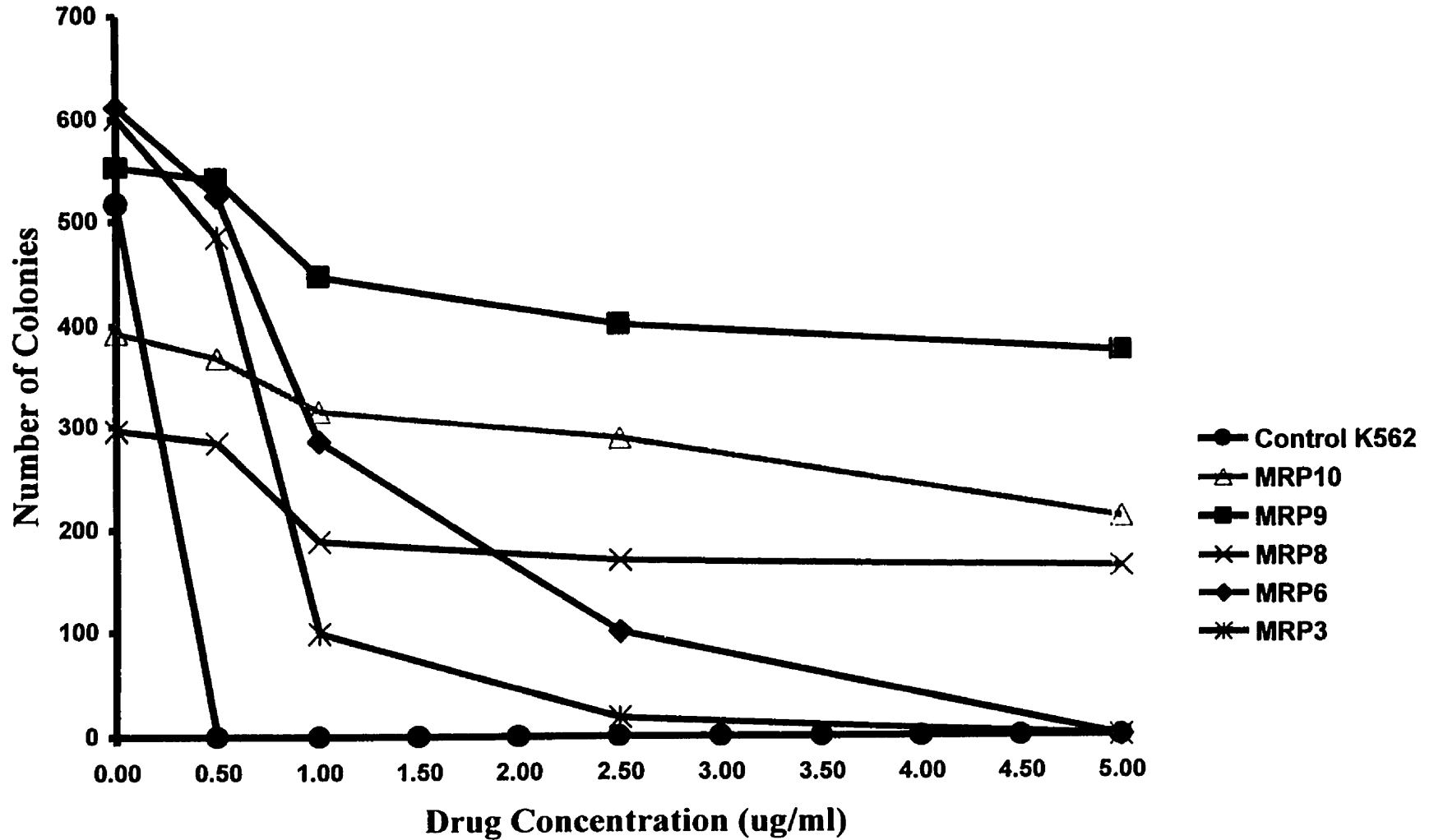
3.2 Determination of the Gene Transfer Efficiency for the MRP1-PG13 Retroviral Vector Using Canine Hematopoietic Progenitor Cells.

3.2.1 Introduction

Clinical studies have demonstrated that the number of circulating hematopoietic progenitor cells in the peripheral blood may be increased with the use of hematopoietic cytokines such as G-CSF or GM-CSF[24]. CD34$^+$ cells sorted from cytokine primed marrow or mobilized peripheral blood are relatively easy to collect and are excellent targets for gene transfer. Several methods to improve gene transfer to CD34$^+$cells have been investigated. Enhanced gene transfer into CD34$^+$cell populations has been obtained by using hematopoietic cytokines *in vitro* and also by incorporating fibronectin in retroviral gene transfer protocols[71]. A gene transfer protocol that combines the use of fibronectin. as well as cytokines *in vivo* and *in vitro* may result in high levels of gene transfer into CD34$^-$ cells.

In previous studies, our laboratory demonstrated that the MRP1-PG13 retroviral vector is capable of transducing normal human bone marrow mononuclear cells and enriched human CD34$^+$ cell populations[217]. *MRP1* expression in transduced human samples resulted in drug resistance. Successful gene transfer and expression of *MRP1* must be demonstrated *in vitro* to determine the potential use of *MRP1* to confer drug resistance *in vivo*. This set of experiments was designed to ascertain whether the MRP1-PG13 retroviral vector could transfer the *MRP1* gene into canine hematopoietic cells and to determine the gene transfer efficiency for both CD34$^+$ and negative fractions.

3.2.2 Experimental Design

Two dogs were treated with recombinant human cytokines G-CSF and SCF for hematopoietic mobilization. The administration of these cytokines has been demonstrated to increase the number of $CD34^+$ cells present in the peripheral blood[115]. A complete blood count was performed prior to initiating mobilization to establish baseline cell counts and then every day until harvesting and one week post-harvest. After five days of growth factor treatment, bone marrow or peripheral blood (15% blood volume) were obtained from the dogs. Mononuclear cells were separated and $CD34^-$ cells sorted using an anti-canine CD34 antibody in conjunction with an immunomagnetic column technique[116]. $CD34^+$ cells were then incubated for 24 hours in the presence of hematopoietic cytokines. Following the prestimulation period, the cells were seeded on flasks precoated with fibronectin and fresh MRP1-PG13 retroviral supernatant was added four times over 48 hours. Cells were harvested on day four. Gene transfer efficiency was determined by the use of clonogenic progenitor assays. Between days 10-14, colonies were counted and individual colonies plucked for *MRP1* PCR analysis. Gene transfer efficiency was calculated as the percentage of colonies that were *MRP1* positive relative to the total number of colonies analyzed. To determine *MRP1* expression, Western blot and flow cytometric analysis were performed on bulk peripheral blood and bone marrow mononuclear cell samples. Further *MRP1* expression analysis was performed using RT-PCR and transduced bone marrow CD34 negative samples from two dogs.

3.2.3 Results

3.2.3.1 CD34 Enrichment of Canine Mononuclear Cells

An unlabelled canine CD34 antibody was used in conjunction with an immunomagnetic column technique to separate $CD34^+$ cells from canine bone marrow (BM) and peripheral blood (PB) samples[116]. Determination of $CD34^+$ purity after sorting was not an option as a second $CD34^+$ antibody recognizing a separate epitope was not

available. The percentage of CD34$^+$ cells was estimated by dividing the total number of CD34$^+$ cells by the starting mononuclear cell number (MNC). Based on previously published reports from the Fred Hutchinson Cancer Research Center, investigators utilizing the canine CD34 antibody and an immunomagnetic separation technique have routinely recovered a CD34$^+$ population with a purity of 98 or 99%[116]. The assumption was made for our studies that a similar purity would be obtained. The recovery of CD34$^+$ cells ranged from 0.4-5.9% (Table 3).

Dog	Sample	Total # of MNC	# of CD34$^-$ cells	% CD34$^-$
Dino	Frozen BM	7.6 X 10^6	4.5 X 10^5	5.9
Leslie	Frozen BM	8.7 X 10^7	3.2 X 10^6	3.7
Sadie	Frozen BM	4.8 X 10^7	4.4 X 10^5	0.9
Jessica	Frozen PB	2.5 X 10^8	4.7 X 10^6	1.9
Julia	Frozen BM	3.9 X 10^8	3.4 X 10^6	0.9
Kodiak	Fresh BM	1.1 X 10^8	3.8 X 10^6	3.5
Oregon	Fresh BM	4.5 X 10^8	5.7 X 10^6	1.3
Annabelle	Fresh BM	5.6 X 10^8	3.6 X 10^6	0.6
Skipper	Fresh BM	1.1 X 10^9	3.7 X 10^6	0.4
Caesar	Fresh BM	3.4 X 10^8	6.8 X 10^6	1.9
Average	Frozen BM	1.6 X 10^8	2.4 X 10^6	2.7
Average	Fresh BM	5.1 X 10^8	4.7 X 10^6	1.5

Table 3: CD34 sorting with unlabelled canine CD34 antibody

A high percentage of CD34$^+$ cells was noted for two of the frozen and one of the fresh samples used (5.9%, 3.7% and 3.5% respectively). The viability of fresh samples was ~85-95% and for frozen samples the viability was variable (range 50-87%).

Clonogenic progenitor assays were also performed for several samples using both CD34$^+$ and negative fractions. No significant difference was noted between the number of hematopoietic colonies observed after 10-14 days in the CD34$^+$ and CD34 negative fractions. A summary of results is presented in Table 4. Details are presented in Appendices A1, A2, A3 and A4.

CD34 Positive CFUs				CD34 Negative CFUs			
Dog	Cell #	CFU-GM	BFU-E	Dog	Cell #	CFU-GM	BFU-E
Leslie	1 X 10^5	29	3	Leslie	1 X 10^5	17	1
Sadie	1 X 10^5	3	1	Sadie	1 X 10^5	10	1
Kodiak	1 X 10^5	18	0	Kodiak	1 X 10^5	4	0
Julia	1 X 10^5	N/A	N/A	Julia	1 X 10^5	37	0
Average		17	1	Average		17	0.5
Leslie	5 X 10^4	14	1	Leslie	5 X 10^4	11	1
Sadie	5 X 10^4	2	0	Sadie	5 X 10^4	5	1
Kodiak	5 X 10^4	5	0	Kodiak	5 X 10^4	2	0
Julia	5 X 10^4	6	0	Julia	5 X 10^4	33	0
Average		7	0	Average		13	0.5

Table 4: Summary of CFU assays for bone marrow CD34$^+$ and negative fractions from 4 canine samples. Two cell plating concentrations were used.

In the course of these experiments, a biotinylated canine CD34 antibody became available to our group. The use of this antibody allowed for the evaluation of CD34

purity after cell sorting. A summary of the results we obtained with the use of this antibody is presented in Table 5. The percentage of $CD34^+$ cells recovered ranged from 0.1-1.7%. The purity after sorting was assessed for two samples (89% and 91%).

Dog	Sample	Total # of MNC	# of $CD34^+$ cells	% $CD34^+$
Jessica	Fresh BM	5.0×10^8	8.4×10^6	1.7
Leslie	Frozen BM	1.0×10^7	7.2×10^4	0.7
Amigo	Frozen BM	3.5×10^7	3.2×10^4	0.1
Kodiak	Fresh BM	4.1×10^7	3.1×10^5	0.8
Boston	Fresh PB	5.4×10^8	3.5×10^6	0.7
Annabelle	Fresh PB	3.5×10^9	1.8×10^7	0.5
Average	BM	1.8×10^8	2.8×10^6	0.8
Average	PB	1.4×10^9	7.3×10^6	0.7

Table 5: CD34 sorting with a canine biotinylated CD34 antibody

3.2.3.2 Transduction of Canine Hematopoietic Cells

Three dogs were used for the canine *in vitro* assays. Dogs #1 and 2 were mobilized with rhG-CSF and rhSCF for five days according to the previously described

protocol[115]. The only modification was the use of human recombinant cytokines instead of canine cytokines. A four-fold increase in the number of neutrophils was observed for dog #1 after five days of cytokine treatment and a nine-fold increase was seen for dog #2. The results are presented in Table 6. Dog #3 was not mobilized.

| Day | Jessica (Dog #1) | | Julia (Dog #2) | |
	WBC X 10^9/L	Neutrophils X 10^9/L	WBC X 10^9/L	Neutrophils X 10^9/L
1	11.9	7.9	11.5	4
5	35.9	30.5	37.7	34.7

Table 6: Cell counts for hematopoietic mobilization of dogs #1 and 2

After five days of cytokine treatment, bone marrow was harvested from dog #1 and peripheral blood was collected from dog #2. A small bone marrow sample was collected from dog #3. Mononuclear cells were separated, CD34$^+$ cells sorted and cells prestimulated for 24 hours with hematopoietic cytokines. CD34$^+$ recovery after sorting ranged from (0.9-3.5%). Additional cytokines (FLT 3 ligand, G-CSF) were added to the prestimulation and transduction protocol, as studies have demonstrated increased gene transfer efficiencies into hematopoietic progenitors with their use. Two different growth factor combinations were used a) SCF and IL-6 and b) SCF, IL-6, G-CSF, FLT3L. Growth factor combinations and cell numbers before and after prestimulation and transduction are shown in Table 7. In two of the three dogs (dogs #2 & 3), prestimulation with growth factors for 24 hours resulted in a reduction in cell number. After the transduction period (72 hours in culture), cell numbers were further decreased. In an attempt to reduce cell loss, protamine sulfate was not used during transduction and half media change was implemented instead of a full media exchange for dog #3. For dog #3,

fibronectin plates were preloaded with viral supernatant four times before the addition of cells.

Dog	Sample	Total # of MNC/ # After Cryopreservation	# of CD34⁻ cells (%)	Growth Factors Used	# of CD34⁺ After 24hr Culture	# of CD34⁻ After 72hr Culture
1 (Julia)	320 ml BM	8.2 X 10⁸ / 3.9 X 10⁸	3.4 X 10⁶ (0.9)	SCF, IL-6	6.8 X 10⁶	7.1 X 10⁵
2(Jessica)	360 ml PB	2.0 X 10⁹ / 2.5 X 10⁸	4.7 X 10⁶ (1.9)	SCF, IL-6	3.0 X 10⁶	5.7 X 10⁵
3 (Kodiak)	30 ml BM	1.1 X 10⁸	3.8 X 10⁶ (3.5)	SCF, IL6, G-CSF, FLT3L	2.0 X 10⁶	1.4 X 10⁶

Table 7: CD34⁺ cells before and after prestimulation and transduction for dogs 1-3.

3.2.3.3   Analysis of Gene Transfer Efficiency for Canine Hematopoietic Cells

To assess gene transfer efficiency into canine hematopoietic cells, cells were co-cultured for 48 hours with viral supernatant on fibronectin coated plates in the presence of hematopoietic cytokines (SCF, IL6, +/- G-CSF, FLT3L). Transduced CD34⁺ and negative cells were cultured in methylcellulose for 10-14 days. Individual non-drug selected colonies were analyzed by *MRP1* PCR after methylcellulose culture. Table 8 summarizes the results of each transduction. The level of gene transfer for dog #1 was determined to ~11% for both CD34⁺ and negative fractions. Gene transfer was not evident for dog #2 as all colonies were negative. However, colonies did not appear as healthy as expected during culture in methylcellulose. Gene transfer for dog #3 was

determined to be ~15% for CD34$^+$ cells and ~24% for CD34$^-$ cells. The levels of gene transfer efficiency may be underestimated, as assessment of the quality and amount of amplifiable DNA in the samples was not performed. PCR amplification of the canine muscular dystrophin gene is typically used in our laboratory to assess amplifiable DNA in canine hematopoietic colonies. This assay was not available as difficulties in optimizing the PCR were encountered. However, successful gene transfer into canine CD34$^+$ and negative cells was demonstrated by *MRP1* PCR analysis for two of the three dogs sampled.

Target	Dog	PCR$^+$/Total Colonies	% Positive
CD34$^-$ cells	1 (Julia)	11/100	11
	2 (Jessica)	0/6	0
	3 (Kodiak)	9/38	24
CD34$^-$ cells	1 (Julia)	4/38	11
	2 (Jessica)	0/36	0
	3 (Kodiak)	3/20	15

Table 8: Gene transfer efficiency (% *MRP1* Provirus Positive) for canine CD34$^+$ and negative cells

3.2.3.4   *MRP1* Expression Studies

Western blot and flow cytometry were performed to detect human *MRP1* protein expression in transduced canine bone marrow samples. From our studies, we determined that the anti-human *MRP1* antibody used in these assays reacts nonspecifically with normal canine mononuclear cells. A single band, similar in size to the human *MRP1* protein was detected in canine untransduced bone marrow samples by Western blot analysis. Moderate levels of *MRP1* expression were also noted using flow cytometry in

normal canine bone marrow samples. Approximately forty to fifty percent of normal canine bone marrow mononuclear cells expressed the *MRP1* protein by flow cytometry. The *MRP1* antibody may have reacted with endogenous canine *MRP1*. As a result of the inability to distinguish canine endogenous *MRP1* from proviral human *MRP1* using these assays, Western blot and flow cytometry were not used to demonstrate human *MRP1* expression in canine samples.

RT-PCR was also used to analyze human *MRP1* expression in transduced canine bone marrow samples. Bone marrow CD34 negative cells from two dogs were transduced and analyzed post-transduction for *MRP1* expression by RT-PCR. In both samples, *MRP1* expression was detected in normal untransduced canine CD34 negative cells and transduced samples. Densitometry was used to determine the increase in *MRP1* expression between untransduced and transduced samples. A 2.4 fold increase in *MRP1* expression was noted for dog #6 and a 1.4 fold increase in *MRP1* expression was noted for dog #7 (Figure 6). An increase in *MRP1* expression was detected for both canine samples after transduction with MRP1-PG13 viral supernatants. The RT-PCR results demonstrate expression of the *MRP1* gene in transduced samples, which may reflect increased *MRP1* expression in transduced cells.

Figure 6: RT-PCR analysis of transduced (Trans) and
Untransduced (Un) canine bone marrow CD34-
cells (dog #6 & 7). *MRP1* expression was
demonstrated for all samples. A 2.4 fold increase
in *MRP1* expression was detected for dog #6 and
a 1.4 fold increase was noted for dog #7. Positive
controls for *MRP1* PCR are also shown (1ng,
100pg, 10pg).

MRP1 Expression in Canine CD34-ve Transduced Samples

DOG #6

| Trans | RT-ve | Un | RT-ve | 1ng | 100pg | 10pg |

DOG #7

| Un | RT-ve | Trans | RT-ve | 1ng | 100pg | 10pg |
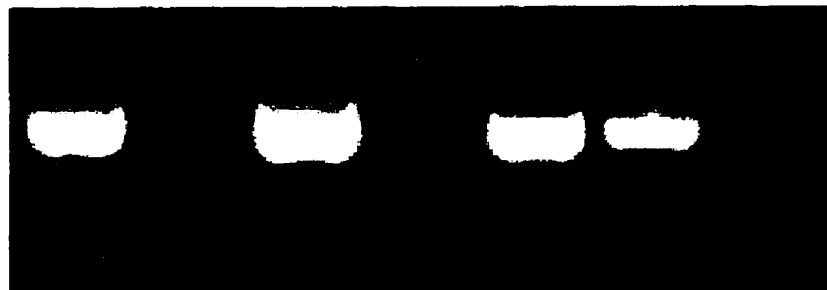
3.2 Assessment of the Ability of Genetically Modified Hematopoietic Stem Cells Carrying the *MRP1* Gene to Resist the Myelosuppressive Effects of Chemotherapy *In vivo.*

3.3.1 Introduction

The transfer of multidrug resistance genes into hematopoietic stem cells has great potential clinical utility including hematopoietic chemoprotection[193]. The infusion of drug resistant hematopoietic progenitors into a patient should result in a reduction in the short-term myelotoxicity that is seen with the use of several chemotherapeutic agents. Long-term bone marrow chemoresistance could also be achieved if stem cells are modified to express a drug resistance gene. Increasing levels of hematopoietic chemoprotection could be obtained with each cycle of chemotherapy, as expansion of resistant hematopoietic stem cells should occur with drug exposure. By preventing or lessening the hematologic toxicity associated with chemotherapy regimens, patient morbidity and mortality may be reduced[193,195].

Preclinical studies using a large animal model such as the dog may provide valuable information that is required before initiating human trials and might contribute to the development of future therapies. *In vivo* assays are critical to establish the optimal method of administering gene therapy for chemoprotection. The set of experiments described in this section was designed to determine if *MRP1* retroviral gene transfer to hematopoietic stem cells could be used for chemoprotection.

Non-conditioned animals were used in our experiments. Many gene transfer protocols incorporate the use of lethal total body irradiation before administration of gene modified hematopoietic cells to condition the animal[42,43,71]. This procedure has several severe side effects including susceptibility to infection and hemorrhage due to myelosuppression and gastrointestinal toxicity. An effective protocol that could avoid the use of lethal irradiation would be less harmful to the patient and would be more clinically applicable.

3.2.3 Experimental Design

For this series of experiments, two dogs were first mobilized with rhG-CSF and rhSCF for five days. Bone marrow was harvested from one dog and peripheral blood harvested from the other. Mononuclear cells were separated and CD34$^+$ cells sorted. CD34$^+$ cells were plated on fibronectin coated dishes and exposed to MRP1-PG13 retroviral supernatants four times during 48 hours in the presence of hematopoietic cytokines (SCF, IL-6, G-CSF, FLT3L +/- TPO). After transduction, CD34$^+$ cells were infused into autologous recipients. To allow for engraftment of the cells, the dogs were challenged 14 days after infusion. Before initiating the drug challenge, complete blood counts (CBCs) were performed to establish baseline cell numbers. Vincristine (VCR) was used to challenge the dogs, as we have shown that this drug can induce myelosuppression in dogs at a single dose of 1 mg/m^2 I.V. The dogs were administered VCR and CBCs were performed daily. Total white blood cell and neutrophil counts were used to monitor for myelosuppression. Bone marrow and peripheral blood were sampled 14 days after the infusion of transduced cells and after the dogs had recovered from the chemotherapy challenge. Clonogenic assays and *MRP1* PCR were performed to detect the presence *MRP1* containing cells. After the initial drug challenge, dogs were challenged between 17-19 days later to assess chemoresistance over the 30 day period of the study.

3.2.3 Results

3.3.3.1 Hematopoietic Mobilization and Recovery of CD34$^+$ Cells

Dogs #4 and 5 were mobilized with rhG-CSF and rhSCF (Table 9 and Figure 7). For dog #4, the total white blood cell count (wbc) was 19.6 X 10^9/L and the segmented neutrophil count was 14.1 X 10^9/L on day five of cytokine therapy. For dog #5, the total white blood cell count and the segmented neutrophil count increased by day three (38.1

X10^9/L and 32.3 X 10^9/L respectively), however cell counts dropped by day five (18.2 X 10^9/L and 14.9 X 10^9/L respectively).

	Oregon (Dog #4)		Boston (Dog #5)	
Day	WBC X 10^9/L	Neutrophils X 10^9/L	WBC X 10^9/L	Neutrophils X 10^9/L
1	9.0	5.3	11.4	6.7
2	9.3	5.7	31.6	26.5
3	18.2	7.9	38.1	32.3
4	21	14.1	42	35.2
5	19.6	14.1	18.2	14.9

Table #9: Peripheral blood cell counts after cytokine induced hematopoietic mobilization of dogs #4 and 5.

On day five of cytokine therapy, primed marrow (BM) was harvested from dog #4 and mobilized peripheral blood (PB) was collected from dog #5. Mononuclear cells were separated and CD34$^+$ cells sorted and transduced as previously described. In addition to the SCF, IL-6, G-CSF and FLT3L, thrombopoietin (TPO) was added to the prestimulation and transduction protocol for dog #5. Results are shown in Table 10. The percentage of CD34$^+$ cells recovered after sorting was 1.3% for dog #4 and 0.7% for dog #5. For dog #4, the number of CD34$^+$ cells had decreased from 5.7 X 10^6 to 1.4 X 10^6 after transduction (72 hours in culture). In contrast, a three fold increase in the number of CD34$^+$ cells was noted for dog #5 after the transduction period. Cells were harvested on day four and 1 X 10^5 CD34$^+$ cells/kg were infused into dog #4 and 6.3 X 10^5 CD34$^+$ cells/kg were infused into dog #5.

Figure 7:   A graph illustrating the daily white blood cell (WBC)
            and segmented neutrophil counts (PMNS) for dog #4
            and 5 during cytokine treatment with rhG-CSF and
            rhSCF. Cytokines were administered for 5 days.

Blood Cell Counts for Hematopoietic Mobilization
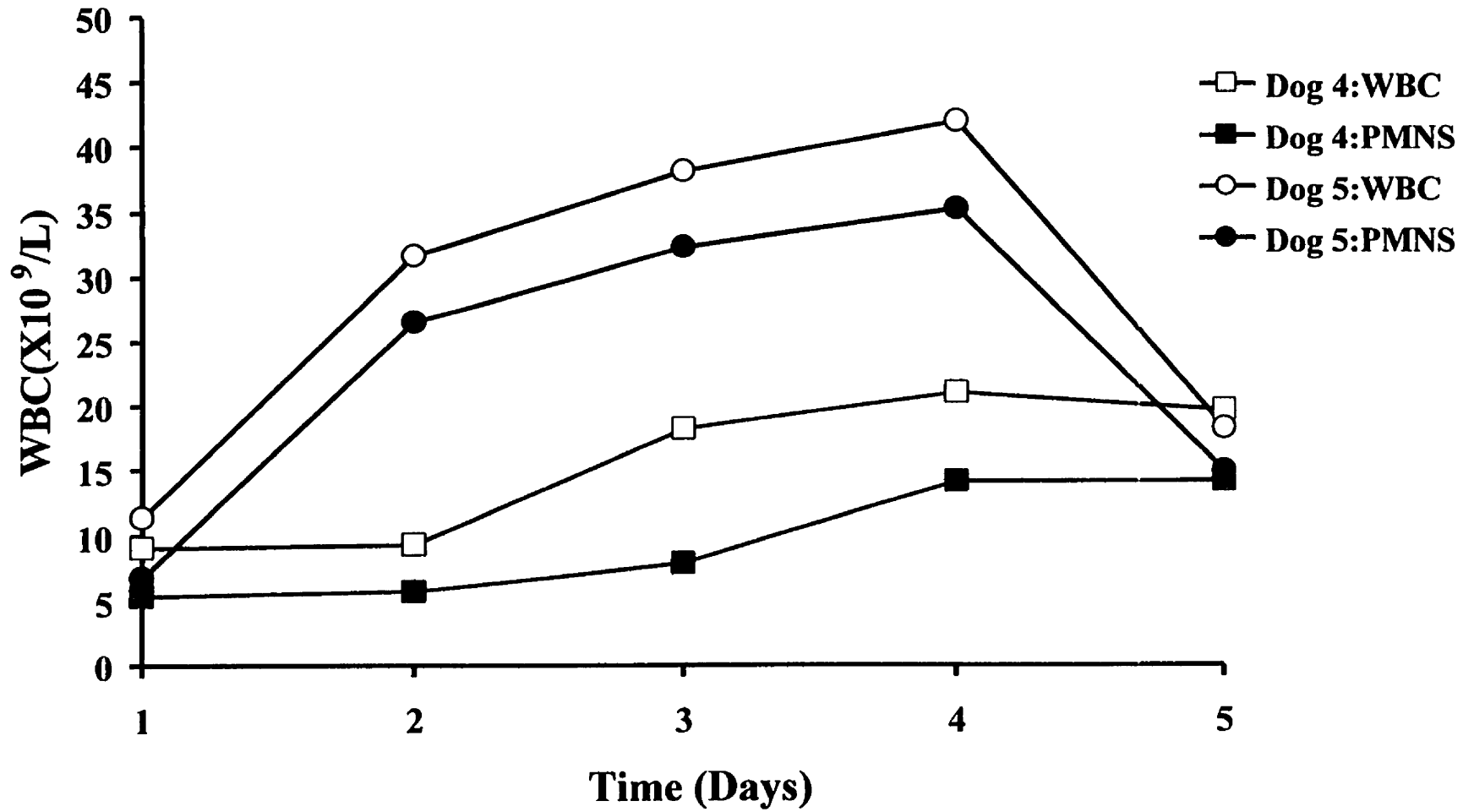
Dog	Sample	Total # of MNC	# of CD34⁺ cells (%)	Growth Factors Used	# of CD34⁺ After 24hr Culture	# of CD34⁺ After 72hr Culture
4 (Oregon)	130 ml BM	4.5×10^8	5.7×10^6 (1.3)	SCF, IL6, G-CSF, FLT3L	5.6×10^6	1.4×10^6
5(Boston)	210 ml PB	5.4×10^8	3.5×10^6 (0.7)	SCF, IL6, G-CSF, FLT3L, TPO	2.7×10^6	7.5×10^6

Table 10: Total number of CD34⁺ cells before and after prestimulation and transduction of bone marrow or peripheral blood of dogs 4 and 5.

3.3.3.2 Analysis of Gene Transfer Efficiency

Post-transduction, CFU assays were performed for dog #5 using the CD34⁻ fraction. CD34 negative cells were cultured at a cell concentration of 1×10^5 cell/ml in methylcellulose in the presence and absence of etoposide. 14 days later, plates were analyzed for colony formation. We previously determined that normal canine hematopoietic cells cannot survive in etoposide at concentrations above 0.2 µg/ml (Appendix A5). Colonies were considered to be drug resistant if they were capable of growing at concentrations higher than this level. No growth was evident at any concentration of etoposide (0.1-0.5 µg/ml) and colonies were not observed for unselected plates. Genomic DNA was also extracted from the CD34 negative cells for dog #5 and subjected to *MRP1* PCR. The post-transduction sample was PCR positive for proviral *MRP1* DNA (Figure 8). For technical reasons, post-transduction CFU assays were not

Figure 8: *MRP1* PCR analysis of genomic DNA extracted from dog #5 after transduction of CD34- cells with the MRP1-PG13 retroviral vector.

MRP1 PCR of Dog# 5 Transduced CD34- DNA Sample

1ng　100pg　50pg　10pg　-ve　　　　　CD34-

completed for dog #4. Since the entire CD34$^+$ fraction was infused into the dog, we were unable to assess post-transduction gene transfer efficiency. Similarly, all CD34$^+$ cells were infused into dog #5.

The following sections summarize the results of the *in vivo* drug challenges for both dogs. In addition to monitoring blood cell counts, peripheral blood and bone marrow were sampled at various time points and assayed to detect vector-containing cells. Clonogenic assays to detect drug resistant CFUs and *MRP1* PCR analysis of hematopoietic colonies and DNA samples were done to assess *MRP1* gene persistence *in vivo* after transfer of transduced cells into canine recipients. The sampling time points were as follows: 14 days post-infusion of transduced cells, after the 1st drug challenge, after the 3rd and 4th drug challenge (only dog #2). The analyses of each sampling interval are presented in sequential order.

3.3.3.3 Post-Infusion Analysis:

Fourteen days post-infusion of transduced cells and prior to chemotherapy challenge, blood and marrow were harvested from both dogs. CFU assays were performed in the presence and absence of etoposide using the mononuclear cells from each sample. The results are summarized in Table 11. Details are presented in Appendices A6 and A7. Colony formation was noted at drug concentrations of 0.1 and 0.2 μg/ml of etoposide for dog #4. Concentrations above 0.2 μg/ml were not used for this dog. For dog #5, bone marrow CFUs were noted for unselected plates and plates containing 0.1 μg/ml of etoposide. No colony formation was noted at higher etoposide concentrations (0.2, 0.3, 0.4 or 0.5 μg/ml). Although the peripheral blood sample for dog #5 was plated at a higher cell concentration (2 X 10^5 cells/ml) very few colonies were noted. Drug resistant CFUs were not detected for either dog in bone marrow or peripheral blood samples at 14 days post-infusion of transduced cells.

Dog	Sample	Drug [ ]	CFU-GM	BFU-E-E
Oregon (4)	BM	0	26	10
	BM	0.1	17	11
	BM	0.2	13	7
	PB	0	8	3
	PB	0.1	6	6
	PB	0.2	10	6
Boston (5)	BM	0	24	24
	BM	0.1	2	1
	PB	0	1	0

Table 11: Summary of CFU data for the 14 day post-infusion time point.
For each sample, cells were plated in triplicate and the results
were averaged. Data presented represent drug concentrations
where colony formation was evident.

To detect the presence of vector containing cells, colonies were plucked and analyzed by *MRP1* PCR. This PCR is able to detect at least 10 pg of DNA. For both dogs, *MRP1* PCR performed on selected colonies was negative. *MRP1* PCR was also performed on DNA extracted from peripheral blood and bone marrow mononuclear cells. For dogs 4 and 5, *MRP1* PCR was negative for all samples. 14 days after the infusion of transduced cells, *MRP1* containing cells were not observed in either the blood or bone marrow of dogs 4 and 5. Therefore, 14 days after the infusion of cells (prior to chemotherapy challenge), there was no evidence in either dog of engraftment of *MRP1* transduced cells in the bone marrow or peripheral blood. Vector containing cells were not detected.

3.3.3.3   Initial Chemotherapy Challenge

Dogs were challenged with VCR 14 days after infusion of genetically modified cells (day 1 of drug challenge). Dog #4 received one injection of VCR at a dose of 1 mg/m^2 I.V. For dog #5, the initial chemotherapy challenged was modified to try to apply heavier selection pressure. Dog #5 was challenged with VCR at a dose of 1.5 mg/m^2. A second dose of VCR (1.5 mg/m^2) was given on day four of chemotherapy challenge in order to maintain the selection pressure. Complete blood counts were monitored daily and total white blood cells, segmented neutrophils and platelet counts used to assess myelosuppression.

The results of VCR challenge for dog #4 are shown in Table 12 and Figure 9. Blood cell counts (white blood cells and neutrophils) started to decrease at day four and a nadir was reached on day five.   The recovery phase was seen by day nine. Chemoprotection was not observed for the initial drug challenge, as leukopenia and neutropenia were evident.

Dog #5 experienced a similar decrease in blood cell counts (white blood cells and neutrophils) as dog # 4. Cell counts started to drop at day four, and a neutrophil nadir was reached on day seven. Unlike dog #4, a decrease in platelets was observed for dog #5 (a platelet nadir was noted at day ten). White blood cell and neutrophil counts returned to normal by day ten and platelets by day 16. Dog #5 also experienced other chemotherapy related side effects not observed in dog #4, which precluded further dose escalation. Results are demonstrated in Table 13 and Figure 10. During the first week of chemotherapy, Boston (dog #5) experienced vomiting and diarrhea and became febrile around the time of his neutrophil nadir. He received supportive care consisting of fluids and broad-spectrum antibiotics. The dose of VCR used for Boston's drug challenge appeared to result in myelosuppression and gastrointestinal toxicity. No chemoprotection was observed as Boston suffered from neutropenia and thrombocytopenia.

Day	WBC X 10^9/L	Neutrophils X 10^9/L	Platelets X 10^9/L	Vincristine Challenge
1	8.0	4.64	175	1.0 mg/m^2
2	7.6	5.09	N/A	
3	7.2	5.69	152	
4	2.4	0.6	146	
5	3.9	0.12	135	
6	4.3	0.22	182	
7	5.1	0.66	244	
8	6.1	2.07	260	
9	12.4	7.1	309	
19	7.1	3.69	209	1.0 mg/m^2
20	5.8	3.83	148	
21	6.2	4.46	154	
22	6.5	3.25	162	
23	4.5	1.4	182	
24	4.2	0.38	190	
25	4.4	0.44	158	
26	4.4	0.62	196	0.5 mg/m^2
27	4.7	1.3	213	
28	9.3	5.49	224	
29	13.6	8.57	N/A	
30	11.8	7.67	N/A	

Table 12: Blood cell counts for dog # 4 (Oregon) *in vivo* drug challenges.

Figure 9: A graph illustrating the daily white blood cell and
segmented neutrophil counts for dog #4 (Oregon) during
chemotherapy challenges 1-3. Drug challenges are
represented by arrows on days 1, 19 and 26. The second
drug challenge was initiated after cell counts had
recovered.

WBC and Neutrophil Counts for Dog #4 Drug Challenge
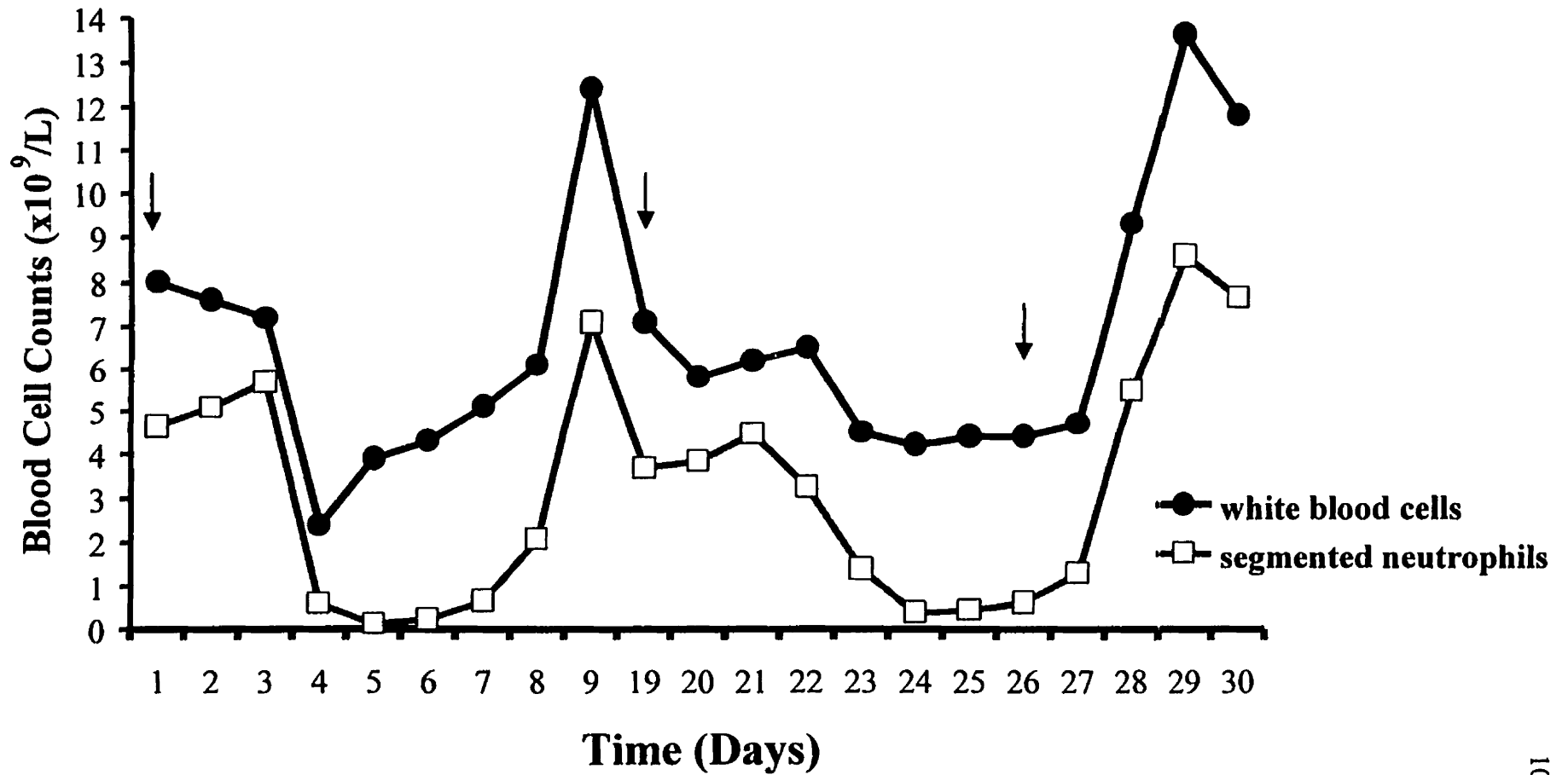
Day	WBC $X\ 10^9/L$	Neutrophils $X\ 10^9/L$	Platelets $X\ 10^9/L$	Vincristine Challenge
1	8.6	5.85	177	1.5 mg/m^2
2	10.9	9.81	168	
3	N/A	N/A	N/A	
4	3	2.13	144	1.5 mg/m^2
5	0.7	0.41	97	
6	0.4	0.07	75	
7	0.9	0.04	75	
8	1.8	0.04	N/A	
9	5.3	1.96	52	
10	13.7	7.95	42	
11	19	13.68	68	
16	13	9.62	382	
17	14.1	9.73	422	1.0 mg/m^2
18	11.6	8.93	315	
19	7.6	5.78	345	
20	7.9	5.69	217	
21	5.9	3.66	330	
22	4.5	1.35	327	
23	3	0.54	242	1.0 mg/m^2
24	2.1	0.67	305	
25	3.1	0.53	340	
26	3.5	0.81	319	
27	4.5	1.22	300	
28	6.2	2.91	300	

Table 13: Blood cell counts for dog # 5 (Boston) *in vivo* drug challenges.

Figure 10: A graph illustrating the daily white blood cell and segmented neutrophil counts for dog #5 (Boston) during chemotherapy challenges 1-4. Drug challenges are represented by arrows on days 1, 4,17 and 23. Drug challenge #3 was initiated after cell counts had recovered.

WBC and Neutrophil Counts for Dog #5 Drug Challenge
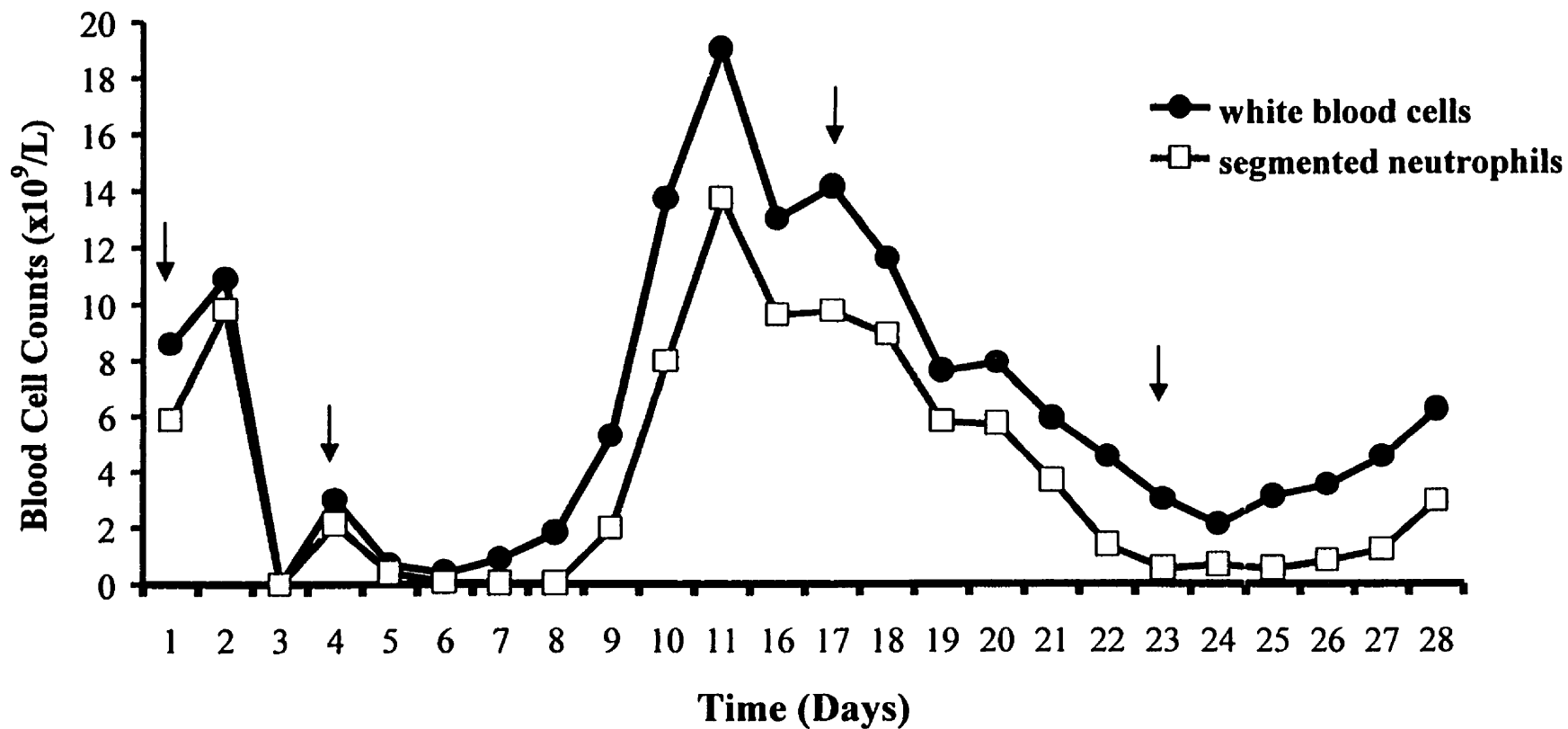
3.3.3.4 Post-Challenge Analysis

Peripheral blood and bone marrow were obtained 18 days after VCR challenge (32 days post-infusion of transduced cells) for dog #4 and 17 days after VCR challenge (31 days post-infusion of transduced cells) for dog #5. CFU assays were performed using blood and bone marrow mononuclear cells plated in the presence and absence of etoposide (range: 0-0.5 µg/ml). Peripheral blood mononuclear cells were plated at a cell concentration of 1 X10^5 cells/ml for dog # 4 and two cell concentrations (2 X 10^5 and 5 X10^5 cells/ml of methylcellulose) were used for dog #5. 14 days later colonies were counted and plucked for *MRP1* PCR.

For dog #4, colony formation was not evident for peripheral blood samples at any concentration of drug or for the plates without selection. Bone marrow CFUs were present at all concentrations of etoposide (0.1,0.2,0.3,0.4 µg/ml). A summary is presented in Table 14 and detailed results are in Appendix A8. Hematopoietic colonies were observed at cytotoxic doses of drug (0.3 and 0.4 µg/ml), however all colonies analyzed by *MRP1* PCR were negative.

Peripheral blood CFUs were observed for dog #5 at both cell plating concentrations. Colony formation was noted only for plates without selection. No growth was observed for plates containing etoposide (0.1-0.5 µg/ml). Bone marrow CFUs were detected for unselected plates and plates containing 0.1-0.3 µg/ml of etoposide. No growth was observed at etoposide concentrations of 0.4 or 0.5 µg/ml. A summary of results is presented in Table 14 and detailed results in Appendix A9. 130 colonies (from all drug concentrations) were analyzed by *MRP1* PCR. No positive colonies were noted. Vector containing CFUs were not evident any at concentration of etoposide for either dog (bone marrow or peripheral blood samples).

Dog	Sample	Drug [ ]	CFU-GM	BFU-E
Oregon (4)	BM	0	11	9
	BM	0.1	13	4
	BM	0.2	13	2
	BM	0.3	14	0
	BM	0.4	8	0
Boston (5)	BM	0	24	0
	BM	0.1	7	6
	BM	0.2	3	1
	BM	0.3	3	2
	PB	0	15	2

Table 14: Summary of CFUs for post-challenge samples. Samples were collected 18 days after the first VCR challenge for dog #4 and 17 days after drug challenge for dog #5 (after challenge 1 and 2). For each sample, cells were plated in triplicate and the results were averaged. Data presented represent drug concentrations where colony formation was evident.

MRP1 PCR was also performed on genomic DNA from blood and bone marrow mononuclear cells from both dogs. All samples were PCR negative. After the initial drug challenge, *MRP1* transduced cells were not detected for dogs 4 and 5 as assessed by *MRP1* PCR of bone marrow and peripheral blood CFUs and DNA samples. The engraftment of gene marked cells was not demonstrated.

3.3.3.5   Subsequent Chemotherapy Challenges

Subsequent chemotherapy challenges were performed for both dogs to assess bone marrow chemoresistance. A second VCR challenge (1 mg/m^2) was performed on

day 19 (18 days after the first chemotherapy challenge, 32 days post-infusion of transduced cells) for dog #4. The results are shown in Table 12 and Figure 9. The cell counts started to drop by day 23 and a neutrophil nadir was reached on day 24. To maintain selection pressure, a third dose of VCR was given (0.5 mg/m^2) on day 26. Cell counts began to increase and by day 28 they were within normal limits.

For dog #5, a third VCR challenge was performed on day 18 (17 days after the first dose and 31 days post-infusion of transduced cells). The original myelosuppressive dose of 1 mg/m^2 was used to avoid gastrointestinal toxicity. The results are shown in Table 13 and Figure 10. By day 22, white blood cell and neutrophil cell counts started to drop. To maintain selection pressure, a fourth dose of VCR was given (1.0 ug/m^2) on day 23, the day of the neutrophil nadir. White blood cell and neutrophil counts continued to drop, however they began to recover by day 28. Boston did not experience any gastrointestinal toxicity as a result of etoposide challenge three and four. Myelosuppression was observed as a result of drug challenge. For both dogs, bone marrow chemoresistance was not evident as leukopenia and neutropenia were observed after several chemotherapy challenges.

3.3.3.6   Detection of *MRP1* Positive Cells After Drug Challenge 3 and 4

For dog #4, bone marrow and blood were sampled during the recovery phase. Genomic DNA was extracted from mononuclear cells for both samples and subjected to *MRP1* PCR. All samples were PCR negative. Vector containing cells were not present. Post-drug challenge CFUs were not performed for dog #4. After three drug challenges, *MRP1* containing cells were not detected in either the blood or bone marrow of dog #4. A total of 378 peripheral blood and bone marrow CFUs from all sampling time points were analyzed for dog #4 by *MRP1* PCR. Engraftment of *MRP1* transduced cells was not demonstrated for this dog, as all colonies were negative for the provirus.

In contrast, vector-containing cells were evident in post-challenge blood and bone marrow samples of dog #5. Peripheral blood and bone marrow were obtained during dog #5's neutrophil nadir and just prior to initiating the fourth drug challenge. CFU assays were performed for both samples. Peripheral blood mononuclear cells were plated at a cell concentration of 5 X10^5 cells/ml of methylcellulose. 14 days later, colonies were counted and plucked. A summary of results is shown in Table 15, details are presented in Appendices A10 and A11. Peripheral blood CFUs were observed only for plates without selection. No growth was observed for plates containing etoposide (0.1-0.5 μg/ml). Bone marrow CFUs were detected for unselected plates and plates containing 0.1 μg/ml of etoposide. No growth was observed for plates containing the following etoposide concentrations 0.2, 0.3, 0.4 and 0.5 μg/ml.

Colonies were plucked and analyzed by *MRP1* PCR. Results are summarized in Table 16. 0.9% of unselected bone marrow CFUs were found to be *MRP1* positive. The percentage increased to 19% when bone marrow CFUs were grown in the presence of 0.1 μg/ml of etoposide. 4% of unselected peripheral blood CFUs were *MRP1* positive.

Peripheral blood and bone marrow were also sampled after the fourth drug challenge for dog #5. CFU assays were performed using blood and bone marrow mononuclear cells. Bone marrow CFUs were detected for unselected plates and plates containing 0.1 μg/ml of etoposide. No growth was observed at higher concentrations of etoposide (0.2-0.5 μg/ml). Peripheral blood CFUs were detected for unselected plates and all concentrations of etoposide used (0.1-0.5 μg/ml). Hematopoietic colonies were plucked and analyzed by *MRP1* PCR. Results are summarized in Table 16. 5.3% of unselected bone marrow CFUs were *MRP1* positive (Figure 11). All peripheral blood CFUs were PCR negative. *MRP1* PCR was also performed on genomic DNA from blood and bone marrow mononuclear cells after the third and fourth drug challenge for dog #5. All samples were *MRP1* negative.

Figure 11:   *MRP1* PCR was performed on unselected bone
            marrow CFUs from dog #5 after the fourth drug
            challenge. This figure illustrates three of the four
            positive colonies detected (noted by +). Seventy-
            five colonies in total were analyzed.

MRP1 PCR of Dog #5 Hematopoietic Colonies

Drug Challenge	Sample	Drug [ ]	CFU-GM	BFU-E
3	BM	0	31	26
	BM	0.1	5	2
	PB	0	12	5
4	BM	0	23	1
	BM	0.1	4	1
	PB	0	11	0
	PB	0.1	5	0
	PB	0.2	6	0
	PB	0.3	3	0
	PB	0.4	7	0
	PB	0.5	14	0

Table 15: Summary of CFUs for dog #5 (Boston) post-challenge samples. Samples were collected for drug challenge 3 on day 23 (37 days post infusion) and on day 30 for drug challenge #4 (44 days post-infusion). Cells were plated in triplicate for each sample and results were averaged. Drug concentrations where colony formation was evident are presented.

Though vector-containing cells were not detected for dog #4 they were present in clonogenic hematopoietic progenitors obtained from bone marrow and peripheral blood mononuclear cells of dog #5 after the three drug challenges. After a fourth drug challenge the percentage of *MRP1*-containing cells in bone marrow CFUs had increased. Vector-containing cells appeared to have engrafted and proliferated in response to drug challenge in one of two dogs studied

Sample/Drug Challenge	Etoposide [ ]	PCR⁺/Total Colonies	% Positive
BM Drug #3	0	1/111	0.9
PB Drug #3	0	1/25	4
BM Drug #3	0.1	3/16	19
BM Drug #4	0	4/75	5.3

Table 16: *MRP1* PCR results for Boston post-drug challenge CFUs. Data presented represent drug concentrations where positive colonies were detected.

In summary, myelosuppression was observed after multiple drug challenges for both dogs infused with MRP1-PG13 transduced CD34⁺ cells. Engraftment of *MRP1* positive cells was detected for one of the two dogs studied. Vector-containing cells were demonstrated after three drug challenges at a level of 0.9% in unselected bone marrow CFUs and 4% in peripheral blood CFUs. After a fourth drug challenge, 5% of bone marrow CFUs were *MRP1* positive. Bulk DNA samples of bone marrow and peripheral blood mononuclear cells were negative for proviral DNA at all sampling intervals.

Chapter 4

Discussion

4.1   Chemoprotection by *MRP1* Gene Transfer

4.1.1   Hematopoietic Chemoprotection by the Transfer of Drug Resistance Genes

Myelosuppression is one of the most serious side effects of cancer chemotherapy. Antineoplastic agents not only damage tumour cells, but may also target normal, highly proliferative hematopoietic cells[218]. Depression of hematopoietic lineages may have severe consequences, resulting in increased patient morbidity and mortality[173]. Although approaches exist to manage myelosuppression such as the administration of blood products, it is often necessary to reduce the dose or intensity of chemotherapy regimens. Such dose attenuation may result in suboptimal anti-neoplastic effects[182,183].

The transfer of drug resistance genes to hematopoietic stem cells is a novel approach to prevent chemotherapy-induced myelosuppression[193,200]. Typically, the acquisition of a drug resistance phenotype by neoplastic cells is a major obstacle in cancer chemotherapy, commonly resulting in ineffective cancer treatment[120]. The expression of drug resistance genes in normal hematopoietic cells could potentially be used as a strategy to overcome the acute hematologic toxicity associated with the use of many chemotherapeutics[193,200]. There are several promising candidate genes for hematopoietic chemoprotection including the *dihydrofolate reductase gene* (*DHFR*), conferring resistance to methotrexate and the *glutathione-S-transferase gene* conferring resistance to alkylating agents[20,193]. These genes and others such as the *multidrug resistance gene 1* (*MDR1*) have been studied for this purpose. *MDR1* has been extensively investigated both *in vitro* and in animal models[122].

The *MDR1* gene encodes P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) superfamily of transporters. Overexpression of this protein has been demonstrated to confer a broad drug resistance phenotype to naturally occurring drug products such as taxanes and anthracyclines[119]. *MDR1* retroviral-mediated gene transfer to hematopoietic cells has been evaluated in murine and human systems *in vitro*. Insertion of the *MDR1* gene in murine hematopoietic cells has provided chemoprotection

in vivo from chemotherapeutic agents such as taxol. Hanania et al. demonstrated in a murine model, that transfer of the *MDR1* gene to hematopoietic cells could confer chemoresistance[207]. Murine bone marrow cells were infused into lethally irradiated mice after transduction for 48 hours with ecotropic retroviral *MDR1* supernatant and hematopoietic growth factors. Mice were subsequently challenged with various doses of taxol that were determined to be equivalent to therapeutic doses in humans. The median white blood cell count after chemotherapy challenge was 83% (range 46-100%) in *MDR1* transduced mice and 41% (11-66%) in control mice. The transfer of *MDR1* to bone marrow cells was shown to confer chemoresistance. To demonstrate *MDR1* transduction of early progenitor cells, bone marrow cells from a taxol resistant mice were serially transplanted six times into recipient mice over a 17 month period. Each recipient was challenged with taxol to assess chemoresistance. Mice receiving *MDR1* transduced marrow were resistant to doses of taxol capable of inducing life-threatening myelosuppression, even after six successive rounds of transplantation. Results from this experiment provided evidence that retroviral-mediated gene transfer of *MDR1* to murine hematopoietic stem cells could prevent chemotherapy-induced myelosuppression.

The feasibility of transferring *MDR1* for hematopoietic chemoprotection has been established, however studies have revealed some problems. Aberrant splicing of vector-derived transcripts often occurs after *MDR1* gene transfer. In a study conducted by Sorrentino and colleagues, two different *MDR1* vectors (a Harvey murine sarcoma based vector and a MMLV based vector) were used to transfer the human *MDR1* cDNA to murine hematopoietic cells[205]. All cells transduced with *MDR1* vectors contained the spliced transcript, which averaged about 60% of the total vector derived message. Investigators detected a cryptic splice donor and acceptor site in the wildtype *MDR1* cDNA. Results from this study suggested that attenuation of P-gp expression and variability of *MDR1*-mediated chemoprotection might occur due to aberrant splicing of *MDR1* transcripts.

A more serious concern, recently reported by Bunting et al., is the development of a myeloproliferative syndrome in mice transplanted with expanded *MDR1*-transduced

hematopoietic stem cells[219]. In that study, murine bone marrow cells transduced with a Harvey murine sarcoma based vector containing the human *MDR1* cDNA were expanded in culture for at least 12 days with hematopoietic cytokines IL-6, IL-3 and SCF. Twenty-four mice transplanted with expanded *MDR1* transduced cells developed severe leukocytosis and splenomegaly. In most cases, the period of abnormal leukocyte counts was detected within the first few days and lasted three to fourth months. Eight mice demonstrated increased leukocyte counts at five to seven months after transplant. The most common phenotype seen in the abnormal cell population was consistent with a granulocytic morphology. Four of the 24 mice developed a leukemic blast phenotype. Despite the presence of abnormal cell counts, most mice appeared asymptomatic. Though investigators attributed the development of the myeloproliferative syndrome to the extended culture period, the possibility that transgene expression may play a pathological role was not ruled out. The authors also suggested that exposure to hematopoietic cytokines induced rapid cell division and may have lead to replication defects. This significant finding raises important issues about the safety of *MDR1* gene transfer. It is essential that these concerns are addressed promptly, as phase I human clinical trials have been initiated and are ongoing.

4.1.2 *MRP1* Gene Transfer for Chemoresistance

The *Multidrug Resistance-Associated Protein 1* (*MRP1*) may be a useful alternative to *MDR1* for hematopoietic chemoprotection. Like P-gp, *MRP1* is a member of the ABC superfamily of transport proteins and overexpression of the protein confers a similar pattern of drug resistance, though the range of drugs is not identical[133]. *MRP1* has potentially important clinical advantages over the use of *MDR1* for bone marrow chemoresistance. It has been documented that tumour cells may acquire a drug resistant phenotype by the upregulation of endogenous *MDR1*[120]. Agents such as verapamil and cyclosporine A have been shown to reverse P-gp mediated drug resistance *in vitro*[119]. The use of these agents would negate any beneficial effects of *MDR1* expressing hematopoietic cells. In contrast, *MDR1* reversal agents have little to no effect on *MRP1*

function[133,220]. *MRP1*-mediated hematopoietic chemoprotection would not be compromised by the use of agents that inhibit P-gp function. This would allow the use of chemosensitizers and antineoplastic drugs in the same patient, possibly facilitating increased tumour kill.

Transfer of the *MRP1* gene to various cell types *in vitro* has been demonstrated to confer drug resistance[170]. Researchers have also shown *in vivo* hematopoietic chemoprotection by retroviral-mediated gene transfer of *MRP1* to murine bone marrow cells[171]. Preclinical studies of *MRP1* gene transfer to prevent chemotherapy-induced myelosuppression have not yet been described in a large animal model. In this thesis the potential of *MRP1* to provide hematopoietic chemoprotection in a canine model was investigated.

We hypothesized that the transfer and expression of the *MRP1* gene to hematopoietic progenitor and stem cells would provide protection from the myelosuppressive effects of many cancer chemotherapy regimens and permit safer dose intensification. Our long-term goal is to provide hematopoietic chemoprotection in a canine model and develop optimal conditions for human trials of chemoprotection by *MRP1* gene transfer. Our objectives in this thesis were; 1) to determine if the MRP1-PG13 retroviral vector could transfer the *MRP1* gene to human hematopoietic cells and whether expression of the gene would give rise to drug resistance *in vitro*, 2) to determine the gene transfer efficiency of MRP1-PG13 for canine hematopoietic progenitor cells and 3) to determine in a canine model whether *MRP1* containing cells could provide protection *in vivo* from the myelosuppressive effects of chemotherapy.

4.1.3 MRP1-PG13 Viral Titer

A gibbon ape leukemia virus (GALV) pseudotyped retroviral vector producer cell line was developed and optimized in our laboratory for transfer of the human *MRP1* cDNA into hematopoietic cells. The MRP1-PG13 cell line was determined to produce

retroviral vectors bearing the *MRP1* cDNA at a titer of 1.7 X 10^5 viral particles/ml using K562 cells as a target and 2.8 X 10^5 viral particles/ml using the Jurkat cell line. The viral titer is an estimate of the amount of infectious viral particles present in one ml of supernatant generated by the producer cell line. The difference in viral titers could be attributed to a number of factors including increased cycling of one cell type (Jurkat) compared to the other or perhaps increased expression of the viral receptor on Jurkat cells. The titer of our MRP1-PG13 retroviral vector is fairly low compared to other PG13 based cell lines. Other investigators generally are using producer cell lines with a range of 5.0 X 10^5 to 1.0 X 10^6 viral particles/ml[42,43,71]. However, many of the human clinical trials utilize producer cell lines with a low titer, similar to that of our MRP1-PG13 producer[104,105]. Increasing the viral titer of the MRP1-PG13 producer cell line would be advantageous and may aid in increasing the gene transfer efficiency. Increasing the vector to cell ratio by using a higher titer producer cell line is one method to increase gene transfer however, there are other factors besides high viral titer that are significant. A correlation has been demonstrated between cell receptor expression and gene transfer efficiency[9,41,42]. Low gene transfer efficiencies may be observed if the viral receptor is expressed at low levels on the target cell surface. The cell cycle stage of the target cell is also important for retroviral-mediated gene transfer[8,37]. The cell must be cycling for stable integration of the transgene to occur. Although there are many strategies available to increase gene transfer, future studies in our laboratory will focus on increasing the existing viral titer of the MRP1-PG13 retroviral producer cell line.

4.1.4 Transfer and Expression of *MRP1* in Human Hematopoietic Cell Lines

The ability of the MRP1-PG13 retroviral vector to transfer *MRP1* to target cells was initially evaluated using the human hematopoietic cell lines K562 and Jurkat. As these cells give rise to individual colonies in methylcellulose, PCR analysis was used to determine the gene transfer efficiency into colony forming units (CFUs). For the Jurkat cell line the gene transfer efficiency was ~59% based on proviral DNA. The gene transfer efficiency for the K562 cell line was ~35% based on the presence of proviral

DNA and ~6% based on the resistance of clonogenic cells to etoposide. A significantly higher percentage of *MRP1* containing cells were present in the non-drug selected K562 plates compared to plates with etoposide. There are several possible explanations for this discrepancy. The initial gene transfer efficiency (35%) was based only on detection of the provirus by PCR analysis. It is possible that a significant proportion of transduced K562 cells may contain the *MRP1* cDNA, but are not expressing the protein at levels sufficient to confer drug resistance. Gene transfer and expression of the *MRP1* protein was evaluated using drug resistance clonogenic assays. The drug concentration used to select resistant clones may have been toxic for some *MRP1* expressing cells. As the drug concentration was increased, only a smaller percentage of *MRP1* expressing cells was capable of survival. Similar results were noted in a study conducted by Fruehauf et al., in which CD34+ peripheral blood progenitor cells were transduced with a retroviral vector containing the *MDR1* gene[221]. Proviral DNA was demonstrated in 22% of unselected primitive hematopoietic precursors however, only 1% were shown to be resistant to vincristine challenge. The investigators suggested that low expression of the retroviral vector or splicing of vector-derived transcripts could have contributed to the low levels of drug resistant cells. In our studies, the survival of only a small percentage (6%) of transduced cells in toxic doses of etoposide may have been the result of reduced *MRP1* gene expression.

Further analysis of the stability and expression of *MRP1* in transduced cells was evaluated using MRP1-PG13 transduced K562 clones. Southern blot analysis confirmed post-transduction proviral integration in expanded K562 clones, demonstrating stable and persistent transfer of the transgene after many replication cycles. Western blot analysis using a specific anti-human *MRP1* antibody revealed *MRP1* protein expression in transduced K562 cells. Successful gene expression in transduced cells was also demonstrated by flow cytometric analysis. A ~30 fold increase in *MRP1* expression was detected using this assay. Expression of *MRP1* in transduced cells was shown to confer drug resistance. *MRP1* positive K562 clones were able to form stable colonies in the presence 5 µg/ml of etoposide, 10 times the minimal cytotoxic dose for untransduced K562 cells.

The results of this set of experiments have confirmed that the MRP1-PG13 retroviral vector we developed is able to reliably transfer the *MRP1* gene to human hematopoietic cells and that expression of the gene gives rise to drug resistance. This is useful and novel information as studies of retroviral mediated gene transfer of *MRP1* are limited. Previously published reports have utilized ecotropic producer cell lines to target murine fibroblasts and murine bone marrow cells. *MRP1* gene transfer to human hematopoietic cells using an amphotrophic retroviral vector producer cell line has not yet been described. D'hondt and colleagues have demonstrated successful gene transfer and expression of *MRP1* to murine fibroblasts using an ecotropic retroviral vector. Expression of *MRP1* in transduced cells conferred drug resistance to a variety of agents including etoposide, vincristine and doxorubicin[170]. We have demonstrated similar results with our recently developed amphotrophic vector[217]. Transfer of *MRP1* conferred a drug resistant phenotype to human hematopoietic cells. The encouraging results from this initial series of experiments help pave the way for large animal and preclinical studies of chemoprotection by *MRP1* gene transfer.

4.1.5   *MRP1* Gene Transfer to Canine Hematopoietic Progenitor Cells

The MRP1-PG13 retroviral vector was further evaluated using a large animal model. To assess *MRP1* gene transfer into canine HSCs, bone marrow and/or peripheral blood were harvested from dogs after hematopoietic mobilization with recombinant human stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF). Total white blood cell and neutrophil counts were used to monitor hematopoietic mobilization. Based on neutrophil counts, an adequate mobilization was noted for three of the four dogs treated. The increase in neutrophil counts for these dogs ranged from a four to nine fold increase. Our current mobilization protocol differs slightly from those previously published, as we have used a much shorter mobilization period, administering hematopoietic cytokines once daily for five days[115]. Previous reports have illustrated an eight to ten fold increase in the number of circulating neutrophils after 14 days of rhG-CSF administration using 10 μg/kg/day[115]. A more accurate measure of successful

mobilization would have been the determination of the number of colony forming units (CFUs) in the peripheral blood before and during hematopoietic cytokine administration. A study performed by de Revel and colleagues has demonstrated that the number of CFUs in the peripheral blood increases dramatically by day seven of cytokine therapy[115]. However, these results were obtained using canine specific G-SCF and SCF. Future experiments in our laboratory will utilize canine hematopoietic cytokines for mobilization.

After five days of hematopoietic mobilization, primed marrow and mobilized peripheral blood samples were collected to obtain CD34$^+$ cells for transduction targets. A canine CD34 antibody was used in conjunction with an immunomagnetic column technique to separate CD34$^+$ cells from mononuclear cells[116]. The recovery of CD34$^+$ cells ranged from 0.4%-3.5% for fresh bone marrow samples and the average recovery was ~2.2%. These results are acceptable and were within the expected range, as they closely resemble those obtained by another research group using the same antibody[116]. McSweeny et al., documented a range of 0.7%-3.5% CD34$^+$ cells in unfractionated bone marrow cells[116]. They further demonstrated that the canine CD34 antibody recognizes approximately 2% of canine bone marrow cells. The average CD34$^+$ recovery that we achieved is comparable to previously published reports.

Our CFU data however, differ from results attained by McSweeny and colleagues. This group demonstrated an increase in the number of CFU-GM present in the CD34$^+$ population[116]. CFU-GM enrichment, though variable, was evident for all CD34$^+$ progenitor assays (range 1.8-55 fold). We did not detect a statistically significant increase in the number of progenitors present in CD34$^+$ CFU assays, although a 1.4 and a 4.5 fold increase was detected for two of the samples. The inconsistencies observed may be a result of several factors. The optimal conditions for canine CFU assays have not been determined. Our current CFU assay is more favourable for the growth of human hematopoietic progenitors. We utilize a commercially available methylcellulose product containing recombinant human cytokines SCF, IL-3, IL-6, G-CSF, GM-CSF and EPO. McSweeny et al., use canine cytokines such as G-CSF, GM-CSF and SCF in their CFU

assay, which favour the growth of canine hematopoietic progenitors. It may be necessary to supplement our methylcellulose with canine recombinant cytokines to create a more suitable CFU assay for canine hematopoietic progenitors. The purity of the $CD34^+$ sample may also influence the CFU assay results. Exogenous canine factors are not provided in our protocol and they may be necessary for hematopoietic colony formation. A less highly purified CD34 enriched population may receive growth support from any non-CD34 positive cells present in the culture. Additional studies are required to optimize culture conditions to support canine hematopoietic progenitors.

Utilizing our current transduction protocol, we have shown successful gene transfer of the human *MRP1* cDNA to canine hematopoietic cells. Isolated $CD34^+$ cells were incubated with MRP1-PG13 supernatants on fibronectin-coated culture flasks in the presence of hematopoietic cytokines. PCR analysis of individual hematopoietic colonies derived from transduced $CD34^+$ cells and expanded in methylcellulose cultures, demonstrated proviral DNA in ~13% of unselected canine hematopoietic progenitor cells. The average gene transfer efficiency for the CD34 negative fraction was ~18%. These results may actually be underestimated. The amount of amplifiable DNA in the colonies was not assessed, as the PCR (for the canine muscular dystrophin gene) was not optimized during the time frame allotted for these studies. As a result, colonies that may not have had amplifiable DNA were analyzed and considered negative by *MRP1* PCR. Thus, the gene transfer efficiency for the MRP1-PG13 retroviral vector may be higher than the current values. Typically, in our laboratory, an average ~80% of bone marrow CFUs analyzed contain amplifiable DNA (YongJun Zhao, personal communication). Our results are comparable to those obtained by Kiem and colleagues also utilizing a GALV pseudotyped retroviral vector[72]. Kiem et al. achieved gene transfer efficiencies in CFU-C ranging from 8.8%-29%, with an average of 20%. The slightly higher gene transfer efficiencies obtained by this group could be attributed to the use of different transduction conditions such as co-cultivation on producer cell lines, the addition of canine hematopoietic cytokines and higher vector titers. In the future, optimization of our transduction protocol, by the incorporation of higher titer vectors, may result in increased gene transfer efficiencies into canine hematopoietic cells.

Our results have shown that the MRP1-PG13 retroviral vector was able to transfer the *MRP1* gene to canine hematopoietic progenitor cells *in vitro*. It was important to demonstrate whether we could achieve gene transfer *in vitro* before proceeding with *in vivo* studies. Though the gene transfer efficiency could be improved, it was essential to continue with the next series of experiments to gain additional information. There are few *in vivo* studies of *MRP1* gene transfer and any results acquired would be new and useful. Future *in vitro* studies are planned to increase the gene transfer efficiency.

4.1.6 Expression of *MRP1* in Canine Hematopoietic Progenitor Cells

Difficulties were encountered in trying to demonstrate human *MRP1* expression in transduced canine samples. Currently, there is no information available regarding the canine *MRP1* protein or the canine *MRP1* gene, as it has not yet been cloned. Murine and human MRPs have been shown to have 88% amino acid identity[131,139]. Researchers have also demonstrated that the canine *MDR1* mRNA has high homology (~93%) with the human transcript[222]. Canine *MRP1* may have a comparable or higher homology and as a result, cross reactivity was expected when using assays to detect the human *MRP1* cDNA in canine bone marrow and peripheral blood samples.

As expected, the antihuman-*MRP1* antibody that was used in these studies appeared to interact with normal untransduced canine marrow when used for Western blot and flow cytometric analysis. The *MRP1* protein was detected in control samples using both assays. As an alternate method to assess proviral *MRP1* expression, reverse transcriptase PCR (RT-PCR) was performed using human *MRP1* specific primers. The CD34 negative fractions from two canine bone marrows were used to evaluate *MRP1* expression. *MRP1* expression was demonstrated for transduced CD34 negative cells, however *MRP1* expression was also detected in control samples. Densitometry was used to compare the levels of *MRP1* expression in transduced and untransduced samples. A 1.4 fold increase was detected for one sample and a 2.4 fold increase was noted for the

second transduced sample. Although it appears that *MRP1* expression increased as a result of transduction with the *MRP1*-PG13 retroviral vector, it is possible *MRP1* expression may be increased as a result of upregulation of endogenous canine *MRP1*. In future studies utilizing RT-PCR for *MRP1* expression, the RT-primers should be designed to incorporate viral sequences that would enable us to distinguish between endogenous canine *MRP1* and proviral *MRP1* expression. Sequences selected should be specific to the vector and should also be expressed.

Although *MRP1* expression was detected in transduced samples, demonstration of the ability of *MRP1* expression in canine samples to confer drug resistance *in vitro* was not evaluated. Further studies are planned to address this issue. The presence of *MRP1* transcripts in transduced cells supported the view that the studies completed fulfilled the following basic requirements of preclinical animal studies: gene transfer, gene expression and the acquisition of reasonable cell numbers, especially with the potential for *in vivo* selection.

4.1.7   *MRP1* and *In Vivo* Bone Marrow Chemoresistance

Evaluation of the ability of *MRP1* transduced cells to confer chemoresistance *in vivo* has not been evaluated in a large animal model. Recently, investigators utilizing a murine system have shown that retroviral mediated gene transfer of *MRP1* to murine hematopoietic cells provided chemoprotection *in vivo*[171]. Murine bone marrow cells were co-cultured with retroviral producer cell lines in the presence of IL-6, IL-3 and SCF. After transduction, cells were injected into lethally irradiated recipient mice. Engraftment of *MRP1* positive cells was demonstrated in the peripheral blood samples of 73% and 55% of mice at two months and six months post-transplant, respectively. Long-term engraftment (nine months) was detected in 33% of mice in the absence of chemotherapy challenge. Over a nine-month period, a decrease in the number of mice expressing *MRP1* in peripheral blood samples was noted (18 of 31 mice at two months and three of 15 mice at nine months post- transplant). In that study, the ability of *MRP1*

to confer drug resistance *in vivo* was evaluated by chemotherapy challenge with doxorubicin. Mice expressing high level of *MRP1* in hematopoietic cells experienced less severe doxorubicin-induced leukopenia and reduced mortality. 91% of mice expressing *MRP1* (as assessed by RT-PCR) were found to survive doxorubicin challenge as compared to 43% of control mice. Though these data are encouraging, results obtained in a murine model do not necessary reflect those seen in a large animal or human model. A canine model may provide more useful information as the canine hematopoietic system closely resembles the human system[117]. Using a canine model we next evaluated the ability of genetically modified hematopoietic progenitors carrying the *MRP1* gene to provide protection from the myelosuppressive effects of chemotherapy *in vivo*.

4.1.8 Canine *In Vivo* Studies of *MRP1* Mediated Hematopoietic Chemoprotection

For the initial *in vivo* chemoprotection studies, two dogs were treated with recombinant human G-CSF and SCF for hematopoietic mobilization. Peripheral blood and bone marrow were harvested and $CD34^+$ cells isolated for transduction. For both dogs, transduction of $CD34^+$ cells was performed in the presence of hematopoietic cytokines SCF, IL-6, G-CSF and FLT3L. TPO was included in the transduction protocol for dog #5. A significant difference was observed in the number of $CD34^+$ cells available for each dog after the transduction period. For dog #4 the number of $CD34^+$ cells were reduced almost four fold after transduction, whereas a three fold increase was detected for dog #5. It has been demonstrated that TPO can act directly to promote the proliferation and survival of primitive hematopoietic progenitor cells[79]. TPO can support the division of $CD34^+$ cells whether used alone, or in combination with other cytokines such as SCF^{80}. The expansion of $CD34^+$ cells for dog #5 could be attributed to the addition of thrombopoietin to the transduction protocol.

After transduction, 1×10^6 to 7.5×10^6 *MRP1* transduced autologous $CD34^+$ cells were infused into two non-myeloablated dogs. For one of the two dogs (dog #5),

PCR analysis was performed on DNA samples of bulk CD34 negative cells. The post-transduction CD34 negative sample was provirus positive. Unfortunately, gene transfer efficiency for the $CD34^+$ fraction post-transduction was not determined due to technical reasons. Transduction of hematopoietic progenitors was not evaluated for either dog. This is an important missing piece of information, as it is impossible to determine whether successful gene transfer occurred and whether *MRP1* positive cells were infused into the dogs. Without these results, it is difficult to accurately assess the ability of *MRP1* to confer hematopoietic chemoprotection.

Fourteen days after adoptive transfer, PCR analysis was used to assess the presence of provirus-containing cells in the peripheral blood and marrow cells. Samples obtained from both dogs (prior to drug challenge) were negative. Dogs were challenged on multiple occasions with the chemotherapeutic agent vincristine (VCR) at myelosuppressive doses. Blood cell counts were monitored daily for myelosuppression. Chemoprotection was not achieved in either dog as blood cell counts decreased as expected with VCR challenge. Leukopenia and neutropenia were observed as a result of drug administration.

After each drug challenge, blood and bone marrow were sampled and various assays performed, including clonogenic assays to detect drug resistant CFUs and *MRP1* PCR analysis of hematopoietic colonies and DNA samples. For each dog, hematopoietic colonies were observed at cytotoxic doses of etoposide at several time points. However, all of the drug resistant colonies were *MRP1* PCR negative. Drug resistance mechanisms other than *MRP1*, such as P-glycoprotein may have been induced as a result of the *in vivo* chemotherapy challenge, resulting in drug resistant hematopoietic progenitors. Future experiments will include a drug treated control dog to aid in assessing the contribution of other drug resistance mechanisms. Another explanation for these findings is the possibility that hematopoietic colonies analyzed by *MRP1* PCR did not contain amplifiable DNA. Determination of the amount of amplifiable DNA in CFU samples was attempted using PCR amplification of the canine muscular dystrophin gene. Difficulties were encountered in optimizing the PCR and as a result this assay was not

used. It is possible that the colonies contained proviral DNA, however after plucking and processing amplifiable DNA was not detected. Other PCR related possibilities include the sensitivity of the *MRP1* PCR. The *MRP1* PCR was capable of detecting a minimum of 10 pg of DNA. Our PCR may not be as sensitive as required to detect all *MRP1* positive colonies. Hematopoietic colonies growing at higher concentrations of etoposide tend to be very small and as a result, the amount of amplifiable DNA may also be reduced.

MRP1 containing cells were not detected in the peripheral blood or bone marrow after multiple drug challenges in one of the two dogs studied (dog #4). The logical conclusion is that *MRP1* containing hematopoietic cells did not engraft or engrafted at a level that was too low to be detected. Vector-containing cells were however, detected after multiple drug challenges in unselected CFUs of the second dog (dog #5). *MRP1* positive hematopoietic progenitors (CFUs) were detected by PCR in blood (4.0%) and bone marrow (0.9%) after three drug challenges. When bone marrow CFUs were cultured in the presence of 0.1 μg/ml of etoposide, 19% were *MRP1* positive by PCR. Although it appears that drug selection (0.1 μg/ml of etoposide) increased the number of *MRP1* containing CFUs, *MRP1* positive colonies were not detected at higher etoposide concentrations. No colony formation was observed at 0.2-0.5 μg/ml of etoposide. Since control CFU assays were not performed, it is possible that the drug concentration was not accurate and a higher dose of drug was used than desired. A more feasible explanation for the lack of colony growth at higher drug concentrations is that the transgene may not have been expressed sufficiently to confer the drug resistance phenotype. Sorrentino and colleagues have demonstrated that alternate splicing of *MDR1* vector transcripts occurs at a relatively high level (~60%) in murine bone marrow cells transduced with retroviral vectors containing the *MDR1* cDNA[205]. As a result, decreased expression of P-pg and a reduction in the ability of *MDR1* to confer drug resistance may occur. In our studies, the inability of *MRP1* to confer drug resistance at higher levels of etoposide may be the result of a similar phenomenon. However, this may not be a significant problem as it has been previously demonstrated in our laboratory by Northern blot analysis that the major transcript was expressed at higher

levels than the minor transcript[217]. Also, there was no evidence for alternate peptides by Western blot analysis[217]. Further investigations are required to determine if aberrant splicing of *MRP1* transcripts occurs in canine bone marrow cells after retroviral mediated gene transfer of the *MRP1* cDNA.

An alternate explanation for the failure of *MRP1* to confer drug resistance is *in vivo* repression of the viral promoter. Vector silencing has been identified as a universal problem with MMLV based gene transfer. Challita et al. have demonstrated in a murine model, reduced expression of a MMLV based vector in hematopoietic stem cells after transplant into recipient mice[223]. They observed inactivation of the MMLV-LTR in progeny of hematopoietic stem cells, resulting in failure of transcription and reduced expression of the transgene. Lack of expression of the transgene was associated with methylation of the vector LTR *in vivo*. As MRP1-PG13 is a MMLV based retroviral vector, reduced *MRP1* expression may be attributed to vector silencing.

Further analysis of peripheral blood and bone marrow samples were performed after the fourth drug challenge for dog #5. Surprisingly, the number of vector-containing cells present in unselected bone marrow CFUs increased to 5.3%. *MRP1* positive colonies were not detected at higher concentrations of etoposide. For dog #5, it appears that *MRP1* containing cells engrafted and increased in number as a result of *in vivo* selection with VCR.

It is interesting that vector-containing cells were evident for dog #5 in clonogenic hematopoietic progenitors (from blood and bone marrow), yet *MRP1* PCR of bulk DNA samples of peripheral blood and bone marrow samples were continually negative. Vector containing cells may have been present in bulk samples, but at a level too low to be detected by our PCR analysis. A discrepancy between the levels of gene marking present in hematopoietic progenitors (CFU-GM) and differentiated cells has been demonstrated in studies performed in our laboratory as well as by other investigators. In a study conducted by Lutzko et al., dogs with canine α-L-iduronidase deficiency received bone marrow mononuclear cells transduced in a long term culture system with a

retroviral vector containing the normal canine α-ID cDNA[224]. At the two to three year follow up sampling interval, the frequency of gene marked hematopoietic progenitors was ~6%, however only very low levels of the vector were detected in all other blood and bone marrow leukocytes (0.01-1%). Higher levels of gene marking were noted in hematopoietic progenitors compared to samples of total mononuclear cells. In a recent human clinical trial, Stewart et al. also documented discrepancies in the level of gene marking of differentiated hematopoietic cells as compared to hematopoietic progenitors[225]. Relatively low levels of gene marked cells were found in total blood and bone marrow DNA samples and high levels of gene marking in the CFU-GM population. Gene marked progenitors may not have been proliferating *in vivo* and as a result, differentiated cells containing the transgene were not detected. It is possible that *ex vivo* manipulation of hematopoietic cells results in a replication defect that does not allow progenitors to undergo the normal process of proliferation and differentiation. As a result, only gene marked hematopoietic progenitors were detected in our studies and not *MRP1* positive differentiated or mature cell types.

Engraftment and detection of *MRP1* containing cells was only noted for one of the two dogs (dog #5). There are several possible explanations for this finding. As transduction of hematopoietic progenitors was not evaluated for either dog, it was impossible to determine what percentage of infused cells, if any were *MRP1* positive. The dog that did not show evidence of engraftment of vector containing cells received a small cell graft (1 X 10^6 cells) and the levels of *MRP1* transduced cells in dog # 4 may have been too low to be detectable or to provide chemoprotection. Since the hematopoietic mobilization was inadequate for this dog (#4), steady state bone marrow may have been harvested rather than primed bone marrow. Retroviral-mediated gene transfer into steady state bone marrow CD34$^+$ cells may not be as efficient as transfer to primed bone marrow CD34$^+$ cells.

Vector containing cells were detected in dog #5 that received transduced CD34$^+$ cells from mobilized peripheral blood. Mobilized peripheral blood CD34$^+$ cells may be better targets for gene transfer than those from steady-state bone marrow. A study

conducted by Dunbar and colleagues demonstrated relatively high levels of retroviral gene transfer into nonhuman primate CD34^{+} cells collected from mobilized peripheral blood[43]. Levels of up to 5% gene transfer were obtained in this study. These investigators concluded that peripheral blood CD34^{+} cells collected after cytokine priming with SCF and G-CSF treatment were superior targets for retroviral gene transfer. Horwitz et al. recently provided evidence that may support the idea that mobilized peripheral blood CD34^{+} cells are ideal targets for retroviral gene transfer[41]. They examined the cell cycle status of G-CSF mobilized peripheral blood stem cells and concluded that a significant proportion of the cells moved from the quiescent phase of the cell cycle (G_0) to the G_1 phase. It has been previously shown that G_1 CD34^{+} cells are more prone to hematopoietic cytokine stimulation than G_0 CD34^{+} cells[41]. Cytokines are commonly used in retroviral gene transfer protocols to induce cell cycling and G_1 CD34^{+} cells may be more amenable to viral transduction, resulting in higher gene transfer efficiencies. If the gene transfer efficiency was increased for the mobilized peripheral blood CD34^{+} cells, a greater number of *MRP1* positive cells would have been infused into the dog. This dog also received a larger CD34^{+} cell dose (7.5×10^6 cells), which may have increased the number of *MRP1* positive cells available for engraftment.

In addition, the mobilized peripheral blood CD34^{+} cells were prestimulated and transduced in the presence of thrombopoietin (in combination with IL-6, SCF, FLT-3 and G-CSF). Thrombopoietin has been shown to accelerate primitive hematopoietic cell entry into the cell cycle alone or in combination with other cytokines such as SCF and FLT-3 ligand[79-81]. The addition of this cytokine to the protocol may have improved the survival, maintenance and possibly increased the cycling of hematopoietic progenitors, thereby enhancing the gene transfer efficiency and persistence of these cells.

Dog #5 exhibiting *MRP1* positive cells also received a higher chemotherapy dose and additional drug challenges. Several high dose drug challenges were required to stimulate gene modified cells to proliferate to a degree that cell numbers would be within detectable limits. Although obvious improvements in engraftment and endurance of vector containing cells were noted for this dog, functional chemoprotection was not

achieved. It appears that the levels of *MRP1* containing cells were too low to protect the dog from the myelosuppressive effects of chemotherapy.

Although chemoprotection was not achieved, the preliminary results from this experiment are encouraging. We demonstrated that engraftment of *MRP1* containing cells is possible in a non-conditioned animal and that these cells will proliferate in response to high doses of chemotherapy. Hematopoietic chemoprotection was most likely not observed because of the low levels of *MRP1* containing cells present *in vivo*. Multiple drug challenges were required for detection of gene marked cells to be evident. Increasing the number of vector containing cells may improve the ability of *MRP1* to provide chemoprotection.

Despite the fact that researchers have been studying *MRP1* for hematopoietic chemoprotection, there are currently are no published reports of *in vivo* studies of *MRP1* gene transfer in a large animal model. It is difficult to compare the results we have obtained to the sole murine study of *MRP1* gene transfer, as results obtained in a murine model do not necessarily reflect those seen in large animals. The *MDR1* gene has also been extensively studied as a potential candidate for the transfer of drug resistance genes, yet studies in large animals have been few. A recent study conducted by Hibino et al. investigated the ability of *MDR1* transduced peripheral blood progenitor cells to protect common marmosets (nonhuman primates) from chemotherapy-induced myelosuppression[226]. Though the investigators were able to obtain gene transfer efficiencies *in vitro* ranging from 5.9-13.7% as assessed by the percentage of drug resistant CFU-GM, investigators were only able to demonstrate low-level engraftment of *MDR1* positive cells (less than one percent). The low level of gene marking observed did not protect animals from docetaxel treatment, as neutropenia was observed in all marmosets after multiple (three) drug challenges. Investigators concluded that the number of *MDR1* positive cells was not high enough to prevent chemotherapy-induced myelosuppression. We observed similar results in our study, as *MRP1* transduced cells were unable to provide hematopoietic chemoprotection in dogs challenged with vincristine.

Results from human clinical trials of *MDR1* gene transfer have shown that engraftment of *MDR1* positive cells is possible however the levels obtained are low. In a study conducted by Moscow and colleagues, peripheral blood progenitor cells (CD34$^+$ cells) were transduced with a retroviral vector containing the *MDR1* cDNA[227]. Patients received an average of 1.9 X 10^3 vector-containing CD34$^+$ cells/kg and were subsequently challenged with several cycles of chemotherapy. *MDR1* positive cells were detected in the peripheral blood samples of all six patients. For three of the patients, vector-containing cells were not evident after hematopoietic reconstitution and only became apparent after multiple drug challenges. In contrast, vector-containing cells were present at the start of chemotherapy in the remaining three patients and were not detected shortly after the first drug challenge. The levels of gene marking observed for all six patients was low and ranged from 0.01-1%. *In vivo* expansion of *MDR1* positive cells was demonstrated, however the ability of *MDR1* to confer hematopoietic chemoresistance was not shown. Researchers suggested that low gene transfer efficiencies and low levels of engraftment of gene marked cells was a major limiting factor in their investigations. The results of this study closely resemble those obtained by our group. In our investigation, vector-containing cells were demonstrated after multiple drug challenges in one of the two dogs studied. Similar problems have been identified such as low levels of engraftment of gene marked cells and low gene transfer efficiencies. Strategies to overcome these limitations are currently being investigated in our laboratory.

In conclusion, we established that our GALV pseudotyped retroviral vector is capable of transferring *MRP1* to target human hematopoietic cells *in vitro* and that expression of *MRP1* in transduced cells confers drug resistance. *MRP1* gene transfer to canine hematopoietic progenitor cells *in vitro* was achieved. Furthermore, we demonstrated in a canine model, that vector-containing cells are able to engraft and proliferate in response to drug challenge, albeit at low levels. *MRP1* may be a promising candidate for hematopoietic chemoprotection in cancer treatment.

4.2   Further Investigations

It remains to be elucidated whether *MRP1* expression in canine hematopoietic progenitor cells confers drug resistance *in vitro*. Further studies are required to demonstrate the ability of transduced canine cells to survive at cytotoxic doses of antineoplastic agents

A logical and important progression of the study would be to increase the number of *MRP1* containing cells and enhance their engraftment. This may be accomplished by several means. Initially acquiring a larger cell graft could potentially increase the number of CD34$^+$ cells available for transduction. A greater number of CD34$^+$ cells may be obtained by optimizing canine hematopoietic mobilization with human cytokines or by using canine specific cytokines that have previously been shown to increase the number of circulating progenitors. A larger cell graft could also be acquired by harvesting both mobilized peripheral blood and primed bone marrow from the same animal. Enhancing the gene transfer efficiency into canine CD34$^+$ cells may also be another means to increase the number of *MRP1* containing cells. Future studies could focus on increasing the viral titer of the producer cell line or optimizing the transduction conditions for canine hematopoietic progenitors. Methods to improve the engraftment of gene marked cell present *in vivo* should also be pursued. Researchers have demonstrated that the use of sublethal irradiation or nonablative conditioning in gene therapy protocols could result in engraftment of gene marked cells at higher levels than those seen in non-conditioned animals[84,228]. The use of sublethal total body irradiation in our gene transfer protocol may augment engraftment of vector-containing cells.

Future studies should also incorporate a control dog that has been repopulated with transduced CD34$^+$ cells not containing *MRP1*. A drug treated control dog would facilitate a more accurate assessment of the ability of *MRP1* to provide hematopoietic chemoprotection.

Appendix Table: A1 CFU Data For Leslie

Source: Bone Marrow CD34 positive cells

Plate #	Cell #	CFU-GM	BFU-E
1	1 X 104	6	0
2	1 X 104	5	0
3	1 X 104	4	0
4	1 X 104	3	0
Average		5	0
1	5 X 104	13	0
2	5 X 104	12	1
3	5 X 104	13	0
4	5 X 104	16	2
Average		14	1
1	1 X 105	26	4
2	1 X 105	31	1
3	1 X 105	33	2
4	1 X 105	26	5
Average		29	3

Source: Bone Marrow CD 34 negative cells

Plate #	Cell #	CFU-GM	BFU-E
1	1 X 104	2	0
2	1 X 104	1	0
3	1 X 104	2	0
4	1 X 104	1	0
Average		2	0
1	5 X 104	10	0
2	5 X 104	10	1
3	5 X 104	11	0
4	5 X 104	13	2
Average		11	1
1	1 X 105	20	1
2	1 X 105	14	2
3	1 X 105	17	1
4	1 X 105	17	1
Average		17	1

CFU assays were performed for CD34 positive and negative fractions sorted using an unlabelled CD34 antibody. Colonies were counted at day fourteen. Cell number refers to number of cells plated /ml of methylcellulose. CFU-GM: Colony forming unit granulocyte-macrophage BFU-E: Burst forming unit erythroid

Appendix Table: A2 CFU Data For Sadie

Source: Bone Marrow CD34 positive cells

Plate #	Cell #	CFU-GM	BFU-E
1	1×10^5	3	2
2	1×10^5	3	1
3	1×10^5	3	1
Average		3	1
1	5×10^4	2	0
2	5×10^4	1	0
3	5×10^4	2	0
Average		2	0

Source: Bone Marrow CD 34 negative cells

Plate #	Cell #	CFU-GM	BFU-E
1	1×10^5	14	1
2	1×10^5	9	1
3	1×10^5	8	1
Average		10	1
1	5×10^4	6	2
2	5×10^4	5	1
3	5×10^4	5	0
Average		5	1

CFU assays were performed for CD34 positive and negative fractions sorted using an unlabelled CD34 antibody. Colonies were counted at day fourteen. Cell number refers to number of cells plated /ml of methylcellulose. CFU-GM:Colony forming unit granulocyte-macrophage. BFU-E:Burst forming unit erythroid.

Appendix Table: A3 CFU Data For Julia

CD34 Negative cells

Plate #	Cell #	CFU-GM	BFU-E
1	5×10^4	41	0
2	5×10^4	35	0
3	5×10^4	24	0
Average		33	0
1	1×10^5	40	0
2	1×10^5	32	0
3	1×10^5	38	0
Average		37	0

CD34 Positive cells

Plate #	Cell #	CFU-GM	BFU-E	Plate #	Cell #	CFU-GM	BFU-E
1	1×10^3	0	0	1	1×10^4	2	0
2	1×10^3	1	0	2	1×10^4	4	0
3	1×10^3	0	0	3	1×10^4	6	0
Average		0	0	Average		4	0
1	5×10^3	1	0	1	5×10^4	7	0
2	5×10^3	0	0	2	5×10^4	6	0
3	5×10^3	0	0	3	5×10^4	5	0
Average		0	0	Average		6	0

CFU assays were performed for CD34 positive and negative fractions sorted using an unlabelled CD34 antibody. Colonies were counted at day fourteen. Cell number refers to number of cells plated /ml of methylcellulose. CFU-GM: Colony forming unit granulocyte-macrophage, BFU-E: Burst-forming unit erythroid.

Appendix Table: A4 CFU Data For Kodiak

Source: Bone Marrow CD34 positive cells

Plate #	Cell #	CFU-GM	BFU-E
1	1×10^5	17	0
2	1×10^5	18	0
3	1×10^5	20	0
Average		18	0
1	5×10^4	5	0
2	5×10^4	5	0
3	5×10^4	5	0
Average		5	0

Source: Bone Marrow CD 34 negative cells

Plate #	Cell #	CFU-GM	BFU-E
1	1×10^5	7	0
2	1×10^5	3	0
3	1×10^5	3	0
Average		4	0
1	5×10^4	0	0
2	5×10^4	2	0
3	5×10^4	3	0
Average		2	0

CFU assays were performed for CD34 positive and negative fractions sorted using an unlabelled CD34 antibody. Colonies were counted at day fourteen. Cell number refers to number of cells plated /ml of methylcellulose. CFU-GM:Colony forming unit granulocyte-macrophage, BFU-E:Burst-forming unit erythroid.

Appendix Table: A5 Canine Dose Response for Normal Canine Marrow

Drug [ ]	CFU-GM	BFU-E
0	25	7
0.1	2	0
0.2	1	0
0.3	0	0
0.4	0	0
0.5	0	0

Dose Response for normal canine bone marrow mononuclear cells using the chemotherapeutic agent etoposide. The results from four experiments were averaged. Cells were plated in triplicate.

Appendix Table: A6 Oregon CFU data 14 days post infusion of transduced cells

Source: Bone Marrow					Source: Peripheral Blood			

Plate #	Drug [ ]	CFU-GM	BFU-E		Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	29	7		1	0	8	0
2	0	32	10		2	0	10	5
3	0	16	10		3	0	6	5
4	0	27	12		4	0	6	3
Average		26	10		Average		8	3
1	0.1	22	14		1	0.1	7	7
2	0.1	12	7		2	0.1	2	2
3	0.1	18	9		3	0.1	6	6
4	0.1	15	12		4	0.1	7	7
Average		17	11		Average		6	6
1	0.2	14	6		1	0.2	14	10
2	0.2	16	7		2	0.2	12	5
3	0.2	15	10		3	0.2	7	4
4	0.2	8	4		4	0.2	8	6
Average		13	7		Average		10	6

Bone marrow and peripheral blood were harvested 14 days post-infusion of transduced cells. CFU assays were performed for both samples. Cells were plated in methylcellulose containing various concentrations of etoposide. Drug concentrationis in ug/ml of methylcelluose. Colonies were counted at day fourteen. CFU-GM; Colony forming unit granulocyte-macrophage. BFU-E: Burst forming unit erythroid.

Appendix Table: A7 Boston CFU data 14 days post-infusion of transduced cells

Source: Bone Marrow

Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	31	25
2	0	17	16
3	0	24	31
Average		24	24
1	0.1	2	1
2	0.1	2	2
3	0.1	3	0
Average		2	1

* No colonies detected at 0.2, 0.3, 0.4 or 0.5 ug/ml.

Source: Peripheral Blood

Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	2	0
2	0	0	0
3	0	1	0
Average		1	0

* No colonies detected at 0.1, 0.2, 0.3, 0.4 or 0.5 ug/ml.

Bone marrow and peripheral blood were harvested at fourteen days post-infusion of transduced cells. CFU assays were performed. Cells were plated in methylcellulose contaning various concentrations of etoposide Drug concentration is in ug/ml of methylcelluose. Colonies were counted at day fourteen.

Appendix Table: A8 Oregon bone marrow CFU data for drug challenge #1

Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	4	18
2	0	15	5
3	0	13	4
Average		11	9
1	0.1	11	5
2	0.1	10	3
3	0.1	18	5
Average		13	4
1	0.2	14	0
2	0.2	7	1
3	0.2	17	4
Average		13	2
1	0.3	12	0
2	0.3	15	1
3	0.3	15	0
Average		14	0
1	0.4	17	0
2	0.4	2	0
3	0.4	6	0
Average		8	0

Bone marrow was harvested after drug challenge with vincristine. CFU assays were performed. Cells were plated in methylcellulose contaning various concentrations of etoposide. Drug concentration is in ug/ml of methylcelluose. Colonies were counted at day fourteen.

Appendix Table: A9 Boston CFU data for drug challenge #1.

Challenge #1: Bone Marrow CFUs

Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	25	0
2	0	27	0
3	0	19	0
Average		24	0
1	0.1	4	3
2	0.1	5	7
3	0.1	13	8
Average		7	6
1	0.2	3	1
2	0.2	2	0
3	0.2	4	1
Average		3	1
1	0.3	4	0
2	0.3	3	0
3	0.3	1	6
Average		3	2

* No colonies were detected at 0.4
  or 0.5 ug/ml

Challenge #1: Peripheral Blood CFUs

Plate #	Cell #	Drug [ ]	CFU-GM	BFU-E
1	2 X 105	0	10	0
2	2 X 105	0	11	0
Average			11	0
1	5 X 105	0	12	1
2	5 X 105	0	18	3
Average			15	2

* No colonies detected at 0.1, 0.2, 0.3, 0.4
  or 0.5 ug/ml

Bone marrow and peripheral blood were harvested after drug challenge # 1. CFU assays were performed. Cells were plated in methylcellulose contaning various concentrations of etoposide. Drug concentration is in ug/ml of methylcelluose. Colonies were counted at day fourteen.

Appendix Table: A 10 Boston CFU data for drug challenge # 3.

Challenge # 3: Bone Marrow CFUs

Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	47	13
2	0	20	29
3	0	25	36
Average		31	26
1	0.1	5	2
2	0.1	6	1
3	0.1	5	3
Average		5	2

* No colonies detected at 0.2, 0.3, 0.4 or
0.5 ug/ml

Challenge # 3: Peripheral Blood CFUs

Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	12	3
2	0	13	1
3	0	12	5
Average		12	5

* No colonies detected at 0.1, 0.2, 0.3,
0.4 or 0.5 ug/ml

Bone marrow and peripheral blood were harvested after drug challenge # 3. CFU assays were performed. Cells were plated in methylcellulose contaning various concentrations of etoposide. Drug concentration is in ug/ml of methylcellulose. Colonies were counted at day fourteen.

Appendix Table: A 11   Boston CFU Data for Drug Challenge # 4

Bone Marrow CFUs

Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	29	2
2	0	19	0
3	0	22	1
Average		23	1
1	0.1	3	0
2	0.1	7	0
3	0.1	3	2
Average		4	1

* No colonies detected at 0.2, 0.3, 0.4 or 0.5 ug/ml

Peripheral Blood CFUs

Plate #	Drug [ ]	CFU-GM	BFU-E	Plate #	Drug [ ]	CFU-GM	BFU-E
1	0	11	0	1	0.3	3	0
2	0	10	0	2	0.3	2	0
3	0	11	0	3	0.3	4	0
Average		11	0	Average		3	0
1	0.1	6	0	1	0.4	4	0
2	0.1	3	0	2	0.4	8	0
3	0.1	7	0	3	0.4	8	0
Average		5	0	Average		7	0
1	0.2	5	0	1	0.5	18	0
2	0.2	6	0	2	0.5	8	0
3	0.2	7	0	3	0.5	16	0
Average		6	0	Average		14	0

Bone marrow and peripheral blood were harvested after drug challenge # 3. CFU assays were performed.
Cells were plated in methylcellulose containing various concentrations of etoposide. Drug concentration is in

References

1.   Wolff JA, Lederberg J: An early history of gene transfer and therapy. Hum Gene Ther 5:469, 1994

2.   Anderson WF: Human gene therapy. Science 256:808, 1992

3.   Cournoyer D, Caskey CT: Gene therapy of the immune system. Annu Rev Immunol 11:297, 1993

4.   Friedmann T, Roblin R: Gene therapy for human genetic disease? Science 175:949, 1972

5.   Morsy MA, Mitani K, Clemens P, Caskey CT: Progress toward human gene therapy. Jama 270:2338, 1993

6.   Mulligan RC: The basic science of gene therapy. Science 260:926, 1993

7.   Morgan RA, Anderson WF: Human gene therapy. Annu Rev Biochem 62:191, 1993

8.   Miller AD: Retroviral vectors. Curr Top Microbiol Immunol 158:1, 1992

9.   Miller AD, Garcia JV, von Suhr N, Lynch CM, Wilson C, Eiden MV: Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. J Virol 65:2220, 1991

10.   Turner ML: Human haematopoietic progenitors as a target for gene therapy. Scott Med J 38:131, 1993

11.   Dunbar CE, Emmons RV: Gene transfer into hematopoietic progenitor and stem cells: progress and problems. Stem Cells 12:563, 1994

12.   Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, et al.: T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. Science 270:475, 1995

13.   Dube ID, Cournoyer D: Gene therapy: here to stay. Cmaj 152:1605, 1995

14.   Wivel NA, Wilson JM: Methods of gene delivery. Hematol Oncol Clin North Am 12:483, 1998

15.   Lee JH, Klein HG: Cellular gene therapy. Hematol Oncol Clin North Am 9:91, 1995

16.   Vile RG, Russell SJ: Retroviruses as vectors. Br Med Bull 51:12, 1995

17.   Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM: Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation 97:1114, 1998

18.   Szilvassy SJ, Hoffman R: Enriched hematopoietic stem cells: basic biology and clinical utility. Biol Blood Marrow Transplant 1:3, 1995

19.   Fraser CC, Hoffman R: Hematopoietic stem cell behavior: potential implications for gene therapy. J Lab Clin Med 125:692, 1995

20.   Allay JA, Persons DA, Galipeau J, Riberdy JM, Ashmun RA, Blakley RL, Sorrentino BP: In vivo selection of retrovirally transduced hematopoietic stem cells. Nat Med 4:1136, 1998

21.   Kerr WG: Genetic modification of the hematolymphoid compartment for therapeutic purposes. Hematol Oncol Clin North Am 12:503, 1998

22.   Dunbar CE: Gene transfer to hematopoietic stem cells: implications for gene therapy of human disease. Annu Rev Med 47:11, 1996

23.   Emerson SG: Ex vivo expansion of hematopoietic precursors, progenitors, and stem cells: the next generation of cellular therapeutics. Blood 87:3082, 1996

24.   Schneidkraut MJ, Hangoc G, Bender JG, Huntenburg CC: The contribution of animal models to the development of treatments for hematologic recovery following myeloablative therapy: a review. J Hematother 5:631, 1996

25.   Janssen GJEaWE: Mobilization of Peripheral Blood Stem Cells: Are All Regimens Created Equal?, in Mertelsmann D (ed): Hematopoietic Stem Cells: Biology and Therapeutic Applications. New York, Marcel Dekker Inc., 1995, p 403

26.   Krause DS, Fackler MJ, Civin CI, May WS: CD34: structure, biology, and clinical utility. Blood 87:1, 1996

27.   Verfaillie CM: Can human hematopoietic stem cells be cultured ex vivo? Stem Cells 12:466, 1994

28.   McSweeney PA, Rouleau KA, Storb R, Bolles L, Wallace PM, Beauchamp M, Krizanac-Bengez L, Moore P, Sale G, Sandmaier B, de Revel T, Appelbaum FR, Nash RA: Canine CD34: cloning of the cDNA and evaluation of an antiserum to recombinant protein. Blood 88:1992, 1996

29.   Huss R: CD34- stem cells as the earliest precursors of hematopoietic progeny. Exp Hematol 26:1022, 1998

30.   Shimizu Y, Ogawa M, Kobayashi M, Almeida-Porada G, Zanjani ED: Engraftment of cultured human hematopoietic cells in sheep. Blood 91:3688, 1998

31.   Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, Grupp SA, Sieff CA, Mulligan RC, Johnson RP: Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3:1337, 1997

32.   Zanjani ED, Almeida-Porada G, Livingston AG, Flake AW, Ogawa M: Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Exp Hematol 26:353, 1998

33.   Israels L: The Regulation of Hematopoiesis, in Israels L (ed): Mechanisms in Hematology. Toronto, Bayer Healthcare Division, 1996, p 4

34.   Suda YGaT: Characterization and Enrichment of Human Hematopoietic Stem Cells, in Mertelsmann D (ed): Hematopoietic Stem Cells. New York, Marcel Dekker Inc, 1995, p 1

35.   Chu P, Lutzko C, Stewart AK, Dube ID: Retrovirus-mediated gene transfer into human hematopoietic stem cells. J Mol Med 76:184, 1998

36.   Einerhand DVaMPW: Viral Vector Systems for Bone Marrow Gene Therapy, in Mertelsmann D (ed): Hematopoietic Stem Cells. New York, Marcel Dekker Inc., 1995

37.   Buchschacher GL, Jr., Wong-Staal F: Development of lentiviral vectors for gene therapy for human diseases. Blood 95:2499, 2000

38.   Miller AD, Buttimore C: Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 6:2895, 1986

39.   Markowitz D, Goff S, Bank A: A safe packaging line for gene transfer: separating viral genes on two different plasmids. J Virol 62:1120, 1988

40.   Lemischka IR, Raulet DH, Mulligan RC: Developmental potential and dynamic behavior of hematopoietic stem cells. Cell 45:917, 1986

41.   Horwitz ME, Malech HL, Anderson SM, Girard LJ, Bodine DM, Orlic D: Granulocyte colony-stimulating factor mobilized peripheral blood stem cells enter into G1 of the cell cycle and express higher levels of amphotropic retrovirus receptor mRNA. Exp Hematol 27:1160, 1999

42.   Kiem HP, Heyward S, Winkler A, Potter J, Allen JM, Miller AD, Andrews RG: Gene transfer into marrow repopulating cells: comparison between amphotropic and gibbon ape leukemia virus pseudotyped retroviral vectors in a competitive repopulation assay in baboons. Blood 90:4638, 1997

43.   Dunbar CE, Seidel NE, Doren S, Sellers S, Cline AP, Metzger ME, Agricola BA, Donahue RE, Bodine DM: Improved retroviral gene transfer into murine and Rhesus peripheral blood or bone marrow repopulating cells primed in vivo with stem cell factor and granulocyte colony-stimulating factor. Proc Natl Acad Sci U S A 93:11871, 1996

44.   Kremer EJ, Perricaudet M: Adenovirus and adeno-associated virus mediated gene transfer. Br Med Bull 51:31, 1995

45.   Frey BM, Hackett NR, Bergelson JM, Finberg R, Crystal RG, Moore MA, Rafii S: High-efficiency gene transfer into ex vivo expanded human hematopoietic progenitors and precursor cells by adenovirus vectors. Blood 91:2781, 1998

46.   Watanabe T, Kawano Y, Kanamaru S, Onishi T, Kaneko S, Wakata Y, Nakagawa R, Makimoto A, Kuroda Y, Takaue Y, Talmadge JE: Endogenous interleukin-8 (IL-8)

surge in granulocyte colony-stimulating factor-induced peripheral blood stem cell mobilization. Blood 93:1157, 1999

47.   Neering SJ, Hardy SF, Minamoto D, Spratt SK, Jordan CT: Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. Blood 88:1147, 1996

48.   Bregni M, Shammah S, Malaffo F, Di Nicola M, Milanesi M, Magni M, Matteucci P, Ravagnani F, Jordan CT, Siena S, Gianni AM: Adenovirus vectors for gene transduction into mobilized blood CD34+ cells. Gene Ther 5:465, 1998

49.   Brenner M: Gene transfer by adenovectors. Blood 94:3965, 1999

50.   Hollon T: Researchers and regulators reflect on first gene therapy death. Nat 6:6, Med 2000

51.   Lehrman S: Virus treatment questioned after gene therapy death. Nature 401:517, 1999

52.   Marshall E: Gene therapy death prompts review of adenovirus vector. Science 286:2244, 1999

53.   Miller HI: Gene therapy on trial. Science 287:591, 2000

54.   Smaglik P: Tighter watch urged on adenoviral vectors...with proposal to report all 'adverse events'. Nature 402:707, 1999

55.   Carter PJ, Samulski RJ: Adeno-associated viral vectors as gene delivery vehicles. Int 6:17, J Mol Med 2000

56.   Monahan PE, Samulski RJ: AAV vectors: is clinical success on the horizon? Gene 7:24, Ther 2000

57.   Russell DW, Kay MA: Adeno-associated virus vectors and hematology. Blood 94:864, 1999

58.   Nathwani AC, Hanawa H, Vandergriff J, Kelly P, Vanin EF, Nienhuis AW: Efficient gene transfer into human cord blood CD34+ cells and the CD34+CD38- subset using highly purified recombinant adeno-associated viral vector preparations that are free of helper virus and wild-type AAV. Gene 7:183, Ther 2000

59.   Poeschla EM, Wong-Staal F, Looney DJ: Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. Nat Med 4:354, 1998

60.   Amado RG, Chen IS: Lentiviral vectors--the promise of gene therapy within reach? Science 285:674, 1999

61.   Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE: Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science 283:682, 1999

62.   Li S, Huang L: Nonviral gene therapy: promises and challenges. Gene 7:31, Ther 2000

63.   Gao X, Huang L: Cationic liposome-mediated gene transfer. Gene Ther 2:710, 1995

64.   Coonrod A, Li FQ, Horwitz M: On the mechanism of DNA transfection: efficient gene transfer without viruses. Gene Ther 4:1313, 1997

65.   Eglitis MA, Kantoff P, Gilboa E, Anderson WF: Gene expression in mice after high efficiency retroviral-mediated gene transfer. Science 230:1395, 1985

66.   Dick JE, Magli MC, Huszar D, Phillips RA, Bernstein A: Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. Cell 42:71, 1985

67.   Williams DA, Lemischka IR, Nathan DG, Mulligan RC: Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature 310:476, 1984

68.   Jordan CT, Lemischka IR: Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev 4:220, 1990

69.   Dubé ID, Stewart AK: Adoptive Transfer of Genetically Modified Hematopoietic Stem Cells. Transfusion Science 17:137, 1996

70.   Goerner M, Bruno B, McSweeney PA, Buron G, Storb R, Kiem HP: The use of granulocyte colony-stimulating factor during retroviral transduction on fibronectin fragment CH-296 enhances gene transfer into hematopoietic repopulating cells in dogs. Blood 94:2287, 1999

71.   Kiem HP, Andrews RG, Morris J, Peterson L, Heyward S, Allen JM, Rasko JE, Potter J, Miller AD: Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. Blood 92:1878, 1998

72.   Kiem HP, McSweeney PA, Bruno B, Goerner M, Buron G, Morris J, Storb R, Miller A: Improved gene transfer into canine hematopoietic repopulating cells using

CD34-enriched marrow cells in combination with a gibbon ape leukemia virus-pseudotype retroviral vector. Gene Therapy 6:966, 1999

73.   Kwok WW, Schuening F, Stead RB, Miller AD: Retroviral transfer of genes into canine hemopoietic progenitor cells in culture: a model for human gene therapy. Proc Natl Acad Sci U S A 83:4552, 1986

74.   Stead RB, Kwok WW, Storb R, Miller AD: Canine model for gene therapy: inefficient gene expression in dogs reconstituted with autologous marrow infected with retroviral vectors. Blood 71:742, 1988

75.   Kantoff PW, Gillio AP, McLachlin JR, Bordignon C, Eglitis MA, Kernan NA, Moen RC, Kohn DB, Yu SF, Karson E, et al.: Expression of human adenosine deaminase in nonhuman primates after retrovirus-mediated gene transfer. J Exp Med 166:219, 1987

76.   Bodine DM, McDonagh KT, Brandt SJ, Ney PA, Agricola B, Byrne E, Nienhuis AW: Development of a high-titer retrovirus producer cell line capable of gene transfer into rhesus monkey hematopoietic stem cells. Proc Natl Acad Sci U S A 87:3738, 1990

77.   Kantoff PW, Flake AW, Eglitis MA, Scharf S, Bond S, Gilboa E, Erlich H, Harrison MR, Zanjani ED, Anderson WF: In utero gene transfer and expression: a sheep transplantation model. Blood 73:1066, 1989

78.   Kotani H, Newton PB, 3rd, Zhang S, Chiang YL, Otto E, Weaver L, Blaese RM, Anderson WF, McGarrity GJ: Improved methods of retroviral vector transduction and production for gene therapy. Hum Gene Ther 5:19, 1994

79.   Murray LJ, Young JC, Osborne LJ, Luens KM, Scollay R, Hill BL: Thrombopoietin, flt3, and kit ligands together suppress apoptosis of human mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division. Exp Hematol 27:1019, 1999

80.   Kim HJ, Okamoto Y, Ito M, Takaue Y, Kawano Y, Watanabe T, Yamaue T,

Tohda Y, Ogose T, Shimada T, Shimosaka A, Kuroda Y: Evaluation of a cytokine

combination including thrombopoietin for improved transduction of a retroviral gene into

G-CSF-mobilized CD34+ human blood cells. Stem Cells 15:347, 1997

81.   Yagi M, Ritchie KA, Sitnicka E, Storey C, Roth GJ, Bartelmez S: Sustained ex

vivo expansion of hematopoietic stem cells mediated by thrombopoietin. Proc Natl Acad

Sci U S A 96:8126, 1999

82.   Palsson B, Andreadis S: The physico-chemical factors that govern retrovirus-

mediated gene transfer. Exp Hematol 25:94, 1997

83.   Movassagh M, Desmyter C, Baillou C, Chapel-Fernandes S, Guigon M,

Klatzmann D, Lemoine FM: High-level gene transfer to cord blood progenitors using

gibbon ape leukemia virus pseudotype retroviral vectors and an improved clinically

applicable protocol. Hum Gene Ther 9:225, 1998

84.   Rosenzweig M, MacVittie TJ, Harper D, Hempel D, Glickman RL, Johnson RP,

Farese AM, Whiting-Theobald N, Linton GF, Yamasaki G, Jordan CT, Malech HL:

Efficient and durable gene marking of hematopoietic progenitor cells in nonhuman

primates after nonablative conditioning. Blood 94:2271, 1999

85.   Hanenberg H, Hashino K, Konishi H, Hock RA, Kato I, Williams DA:

Optimization of fibronectin-assisted retroviral gene transfer into human CD34+

hematopoietic cells. Hum Gene Ther 8:2193, 1997

86.   Verfaillie CM, McCarthy JB, McGlave PB: Differentiation of primitive human

multipotent hematopoietic progenitors into single lineage clonogenic progenitors is

accompanied by alterations in their interaction with fibronectin. J Exp Med 174:693,

1991

87.   Dao MA, Yu XJ, Nolta JA: Clonal diversity of primitive human hematopoietic progenitors following retroviral marking and long-term engraftment in immune-deficient mice. Exp Hematol 25:1357, 1997

88.   Friedmann T, Yee JK: Pseudotyped retroviral vectors for studies of human gene therapy. Nat Med 1:275, 1995

89.   Salmons B, Gunzburg WH: Targeting of retroviral vectors for gene therapy. Hum Gene Ther 4:129, 1993

90.   Barquinero J, Kiem HP, von Kalle C, Darovsky B, Goehle S, Graham T, Seidel K, Storb R, Schuening FG: Myelosuppressive conditioning improves autologous engraftment of genetically marked hematopoietic repopulating cells in dogs. Blood 85:1195, 1995

91.   Bienzle D, Abrams-Ogg AC, Kruth SA, Ackland-Snow J, Carter RF, Dick JE, Jacobs RM, Kamel-Reid S, Dube ID: Gene transfer into hematopoietic stem cells: long-term maintenance of in vitro activated progenitors without marrow ablation. Proc Natl Acad Sci U S A 91:350, 1994

92.   Carter RF, Abrams-Ogg AC, Dick JE, Kruth SA, Valli VE, Kamel-Reid S, Dube ID: Autologous transplantation of canine long-term marrow culture cells genetically marked by retroviral vectors. Blood 79:356, 1992

93.   Kiem HP, Darovsky B, von Kalle C, Goehle S, Stewart D, Graham T, Hackman R, Appelbaum FR, Deeg HJ, Miller AD, et al.: Retrovirus-mediated gene transduction into canine peripheral blood repopulating cells. Blood 83:1467, 1994

94.   Schuening FG, Storb R, Stead RB, Goehle S, Nash R, Miller AD: Improved retroviral transfer of genes into canine hematopoietic progenitor cells kept in long-term marrow culture. Blood 74:152, 1989

95.   Schuening FG, Kawahara K, Miller AD, To R, Goehle S, Stewart D, Mullally K, Fisher L, Graham TC, Appelbaum FR, et al.: Retrovirus-mediated gene transduction into long-term repopulating marrow cells of dogs. Blood 78:2568, 1991

96.   Bodine DM, Moritz T, Donahue RE, Luskey BD, Kessler SW, Martin DI, Orkin SH, Nienhuis AW, Williams DA: Long-term in vivo expression of a murine adenosine deaminase gene in rhesus monkey hematopoietic cells of multiple lineages after retroviral mediated gene transfer into CD34+ bone marrow cells. Blood 82:1975, 1993

97.   Van Beusechem VW, Valerio D: Gene transfer into hematopoietic stem cells of nonhuman primates. Hum Gene Ther 7:1649, 1996

98.   Xu LC, Karlsson S, Byrne ER, Kluepfel-Stahl S, Kessler SW, Agricola BA, Sellers S, Kirby M, Dunbar CE, Brady RO, et al.: Long-term in vivo expression of the human glucocerebrosidase gene in nonhuman primates after CD34+ hematopoietic cell transduction with cell- free retroviral vector preparations. Proc Natl Acad Sci U S A 92:4372, 1995

99.   Lutzko C CP, Dube ID: Gene Transfer into Hematopoietic Stem Cells, in Dube ID (ed): Transfusion Science: Gene Therapy: Current Status, Future Prospects, vol. 17. Newark, Elsevier Science Lts, 1996, p 129

100.   Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, et al.: Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med 323:570, 1990

101.   Brenner MK: Gene marking. Gene Ther 3:278, 1996

102.   Brenner MK, Rill DR, Moen RC, Krance RA, Mirro J, Jr., Anderson WF, Ihle JN: Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. Lancet 341:85, 1993

103.   Brenner MK, Rill DR, Holladay MS, Heslop HE, Moen RC, Buschle M, Krance RA, Santana VM, Anderson WF, Ihle JN: Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. Lancet 342:1134, 1993

104.   Dunbar CE, Kohn DB, Schiffmann R, Barton NW, Nolta JA, Esplin JA, Pensiero M, Long Z, Lockey C, Emmons RV, Csik S, Leitman S, Krebs CB, Carter C, Brady RO, Karlsson S: Retroviral transfer of the glucocerebrosidase gene into CD34+ cells from patients with Gaucher disease: in vivo detection of transduced cells without myeloablation. Hum Gene Ther 9:2629, 1998

105.   Liu JM, Kim S, Read EJ, Futaki M, Dokal I, Carter CS, Leitman SF, Pensiero M, Young NS, Walsh CE: Engraftment of hematopoietic progenitor cells transduced with the Fanconi anemia group C gene (FANCC). Hum Gene Ther 10:2337, 1999

106.   Kruth S: Canine Models for Gene Therapy. Transfusion Science 17:71, 1996

107.   Mannick JA, Lochte HL, Ashley CA, Thomas ED, J.W. F: Autografts of bone marrow in dogs after lethal total-body radiation. Blood 15:255266, 1960

108.   Thomas ED, Collins JA, E.C. H, Ferrebee JW: Marrow transplants in lethally irradiated dogs given methotrexate. Blood 19:217, 1962

109. Cavins JA, Scheer SC, Thomas ET, Ferrebee JW: The recovery of lethally irradiated dogs given infusion of autologous leukocytes preserved at -80 C. Blood 23:38, 1964

110. Schuening FG, Storb R, Goehle S, Graham TC, Hackman R, Mori M, Souza LM, Appelbaum FR: Recombinant human granulocyte colony-stimulating factor accelerates hematopoietic recovery after DLA-identical littermate marrow transplants in dogs. Blood 76:636, 1990

111. Schuening FG, Appelbaum FR, Deeg HJ, Sullivan-Pepe M, Graham TC, Hackman R, Zsebo KM, Storb R: Effects of recombinant canine stem cell factor, a c-kit ligand, and recombinant granulocyte colony-stimulating factor on hematopoietic recovery after otherwise lethal total body irradiation. Blood 81:20, 1993

112. Storb R, Yu C, Barnett T, Wagner JL, Deeg HJ, Nash RA, Kiem HP, McSweeney P, Seidel K, Georges G, Zaucha JM: Stable mixed hematopoietic chimerism in dog leukocyte antigen-identical littermate dogs given lymph node irradiation before and pharmacologic immunosuppression after marrow transplantation. Blood 94:1131, 1999

113. Storb R, Yu C, Zaucha JM, Deeg HJ, Georges G, Kiem HP, Nash RA, McSweeney PA, Wagner JL: Stable mixed hematopoietic chimerism in dogs given donor antigen, CTLA4Ig, and 100 cGy total body irradiation before and pharmacologic immunosuppression after marrow transplant. Blood 94:2523, 1999

114. Storb R, Yu C, Wagner JL, Deeg HJ, Nash RA, Kiem HP, Leisenring W, Shulman H: Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. Blood 89:3048, 1997

115. de Revel T, Appelbaum FR, Storb R, Schuening F, Nash R, Deeg J, McNiece I, Andrews R, Graham T: Effects of granulocyte colony-stimulating factor and stem cell

factor, alone and in combination, on the mobilization of peripheral blood cells that engraft lethally irradiated dogs. Blood 83:3795, 1994

116.   McSweeney PA, Rouleau KA, Wallace PM, Bruno B, Andrews RG, Krizanac-Bengez L, Sandmaier BM, Storb R, Wayner E, Nash RA: Characterization of monoclonal antibodies that recognize canine CD34. Blood 91:1977, 1998

117.   Bruno B, Nash RA, Wallace PM, Gass MJ, Thompson J, Storb R, McSweeney PA: CD34+ selected bone marrow grafts are radioprotective and establish mixed chimerism in dogs given high dose total body irradiation. Transplantation 68:338, 1999

118.   Volm M: Multidrug resistance and its reversal. Anticancer Res 18:2905, 1998

119.   Ling V: Multidrug resistance: molecular mechanisms and clinical relevance. Cancer Chemother Pharmacol 40:S3, 1997

120.   Nooter K, Stoter G: Molecular mechanisms of multidrug resistance in cancer chemotherapy. Pathol Res Pract 192:768, 1996

121.   Kartner N, Riordan JR, Ling V: Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 221:1285, 1983

122.   Licht T, Pastan I, Gottesman MM, Herrmann F: The multidrug-resistance gene in gene therapy of cancer and hematopoietic disorders. Ann Hematol 72:184, 1996

123.   Ford JM: Modulators of multidrug resistance. Preclinical studies. Hematol Oncol Clin North Am 9:337, 1995

124.   Hegewisch-Becker S: MDR1 reversal: criteria for clinical trials designed to overcome the multidrug resistance phenotype. Leukemia 10:S32, 1996

125.   Hickman JA: Apoptosis and chemotherapy resistance. Eur J Cancer 32A:921, 1996

126.   Reed JC: Double identity for proteins of the Bcl-2 family. Nature 387:773, 1997

127.   Simonian PL, Grillot DA, Nunez G: Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. Blood 90:1208, 1997

128.   Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG: Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258:1650, 1992

129.   Loe DW, Deeley RG, Cole SP: Biology of the multidrug resistance-associated protein, MRP. Eur J Cancer 32A:945, 1996

130.   Barrand MA, Bagrij T, Neo SY: Multidrug resistance-associated protein: a protein distinct from P- glycoprotein involved in cytotoxic drug expulsion. Gen Pharmacol 28:639, 1997

131.   Borst P, Evers R, Kool M, Wijnholds J: The multidrug resistance protein family. Biochim Biophys Acta 1461:347, 1999

132.   Krishnamachary N, Center MS: The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. Cancer Res 53:3658, 1993

133.   Kavallaris M: The role of multidrug resistance-associated protein (MRP) expression in multidrug resistance. Anticancer Drugs 8:17, 1997

134.   Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G: Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res 59:2532, 1999

135.   Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P: MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A 96:6914, 1999

136.   Kool M, van der Linden M, de Haas M, Baas F, Borst P: Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. Cancer Res 59:175, 1999

137.   Hipfner DR, Deeley RG, Cole SP: Structural, mechanistic and clinical aspects of MRP1. Biochim Biophys Acta 1461:359, 1999

138.   Rappa G, Finch RA, Sartorelli AC, Lorico A: New insights into the biology and pharmacology of the multidrug resistance protein (MRP) from gene knockout models. Biochem Pharmacol 58:557, 1999

139.   Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, Borst P: Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. Nat Med 3:1275, 1997

140.   Wang Q, Beck WT: Transcriptional suppression of multidrug resistance-associated protein (MRP) gene expression by wild-type p53. Cancer Res 58:5762, 1998

141.   Deeley RG, Cole SP: Function, evolution and structure of multidrug resistance protein (MRP). Semin Cancer Biol 8:193, 1997

142. Breuninger LM, Paul S, Gaughan K, Miki T, Chan A, Aaronson SA, Kruh GD: Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. Cancer Res 55:5342, 1995

143. Benderra Z, Trussardi A, Morjani H, Villa AM, Doglia SM, Manfait M: Regulation of cellular glutathione modulates nuclear accumulation of daunorubicin in human MCF7 cells overexpressing multidrug resistance associated protein. Eur 36:428, J Cancer 2000

144. Hooijberg JH, Pinedo HM, Vrasdonk C, Priebe W, Lankelma J, Broxterman HJ: The effect of glutathione on the ATPase activity of MRP1 in its natural membranes. Febs 469:47, Lett 2000 Mar 3

145. Loe DW, Deeley RG, Cole SP: Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res 58:5130, 1998

146. Lautier D, Canitrot Y, Deeley RG, Cole SP: Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. Biochem Pharmacol 52:967, 1996

147. Nooter K, Westerman AM, Flens MJ, Zaman GJ, Scheper RJ, van Wingerden KE, Burger H, Oostrum R, Boersma T, Sonneveld P, et al.: Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. Clin Cancer Res 1:1301, 1995

148. Barrand MA, Robertson KJ, Neo SY, Rhodes T, Wright KA, Twentyman PR, Scheper RJ: Localisation of the multidrug resistance-associated protein, MRP, in resistant large-cell lung tumour cells. Biochem Pharmacol 50:1725, 1995

149. Berger W, Elbling L, Hauptmann E, Micksche M: Expression of the multidrug resistance-associated protein (MRP) and chemoresistance of human non-small-cell lung cancer cells. Int J Cancer 73:84, 1997

150. Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, Norris MD: Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. Cancer Res 54:5036, 1994

151. Filipits M, Suchomel RW, Dekan G, Haider K, Valdimarsson G, Depisch D, Pirker R: MRP and MDR1 gene expression in primary breast carcinomas. Clin Cancer Res 2:1231, 1996

152. Filipits M, Malayeri R, Suchomel RW, Pohl G, Stranzl T, Dekan G, Kaider A, Stiglbauer W, Depisch D, Pirker R: Expression of the multidrug resistance protein (MRP1) in breast cancer. Anticancer Res 19:5043, 1999

153. Norris MD, Bordow SB, Marshall GM, Haber PS, Cohn SL, Haber M: Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. N Engl J Med 334:231, 1996

154. Canitrot Y, Bichat F, Cole SP, Deeley RG, Gerlach JH, Bastian G, Arvelo F, Poupon MF: Multidrug resistance genes (MRP) and MDR1 expression in small cell lung cancer xenografts: relationship with response to chemotherapy. Cancer Lett 130:133, 1998

155. Arts HJ, Katsaros D, de Vries EG, Massobrio M, Genta F, Danese S, Arisio R, Scheper RJ, Kool M, Scheffer GL, Willemse PH, van der Zee AG, Suurmeijer AJ: Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. Clin Cancer Res 5:2798, 1999

156. Schummer B, Siegsmund M, Steidler A, Toktomambetova L, Kohrmann KU, Alken P: Expression of the gene for the multidrug resistance-associated protein in human prostate tissue. Urol Res 27:164, 1999

157. Kuss BJ, Deeley RG, Cole SP, Willman CL, Kopecky KJ, Wolman SR, Eyre HJ, Lane SA, Nancarrow JK, Whitmore SA, et al.: Deletion of gene for multidrug resistance in acute myeloid leukaemia with inversion in chromosome 16: prognostic implications. Lancet 343:1531, 1994

158. Ross DD, Doyle LA, Schiffer CA, Lee EJ, Grant CE, Cole SP, Deeley RG, Yang W, Tong Y: Expression of multidrug resistance-associated protein (MRP) mRNA in blast cells from acute myeloid leukemia (AML) patients. Leukemia 10:48, 1996

159. Schneider E, Cowan KH, Bader H, Toomey S, Schwartz GN, Karp JE, Burke PJ, Kaufmann SH: Increased expression of the multidrug resistance-associated protein gene in relapsed acute leukemia. Blood 85:186, 1995

160. Legrand O, Simonin G, Beauchamp-Nicoud A, Zittoun R, Marie JP: Simultaneous activity of MRP1 and Pgp is correlated with in vitro resistance to daunorubicin and with in vivo resistance in adult acute myeloid leukemia. Blood 94:1046, 1999

161. Legrand O, Zittoun R, Marie JP: Role of MRP1 in multidrug resistance in acute myeloid leukemia. Leukemia 13:578, 1999

162. Leith CP, Kopecky KJ, Chen IM, Eijdems L, Slovak ML, McConnell TS, Head DR, Weick J, Grever MR, Appelbaum FR, Willman CL: Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood 94:1086, 1999

163. Filipits M, Suchomel RW, Zochbauer S, Brunner R, Lechner K, Pirker R: Multidrug resistance-associated protein in acute myeloid leukemia: No impact on treatment outcome. Clin Cancer Res 3:1419, 1997

164. Burger H, Nooter K, Sonneveld P, Van Wingerden KE, Zaman GJ, Stoter G: High expression of the multidrug resistance-associated protein (MRP) in chronic and prolymphocytic leukaemia. Br J Haematol 88:348, 1994

165. Webb M, Brun M, McNiven M, Le Couteur D, Craft P: MDR1 and MRP expression in chronic B-cell lymphoproliferative disorders. Br J Haematol 102:710, 1998

166. Lorico A, Rappa G, Flavell RA, Sartorelli AC: Double knockout of the MRP gene leads to increased drug sensitivity in vitro. Cancer Res 56:5351, 1996

167. Twentyman PR, Versantvoort CH: Experimental modulation of MRP (multidrug resistance-associated protein)-mediated resistance. Eur J Cancer 32A:1002, 1996

168. Courtois A, Payen L, Vernhet L, de Vries EG, Guillouzo A, Fardel O: Inhibition of multidrug resistance-associated protein (MRP) activity by rifampicin in human multidrug-resistant lung tumor cells. Cancer Lett 139:97, 1999

169. Gollapudi S, Kim CH, Tran BN, Sangha S, Gupta S: Probenecid reverses multidrug resistance in multidrug resistance-associated protein-overexpressing HL60/AR and H69/AR cells but not in P-glycoprotein-overexpressing HL60/Tax and P388/ADR cells. Cancer Chemother Pharmacol 40:150, 1997

170. D' Hondt V, Caruso M, Bank A: Retrovirus-mediated gene transfer of the multidrug resistance- associated protein (MRP) cDNA protects cells from chemotherapeutic agents. Hum Gene Ther 8:1745, 1997

171. Machiels JP, Govaerts AS, Guillaume T, Bayat B, Feyens AM, Lenoir E, Goeminne JC, Cole S, Deeley R, Caruso M, Bank A, Symann M, V DH: Retrovirus-mediated gene transfer of the human multidrug resistance- associated protein into hematopoietic cells protects mice from chemotherapy-induced leukopenia. Hum Gene Ther 10:801, 1999

172. Mauch P, Constine L, Greenberger J, Knospe W, Sullivan J, Liesveld JL, Deeg HJ: Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. Int J Radiat Oncol Biol Phys 31:1319, 1995

173. Kim SK, Demetri GD: Chemotherapy and neutropenia. Hematol Oncol Clin North Am 10:377, 1996

174. Israels L: Chemotherapeutic Antineoplastics, in Israels L (ed): Mechanisms in Hematology. Toronto, Bayer Healthcare Division, 1996, p 16

175. Guest I, Uetrecht J: Drugs toxic to the bone marrow that target the stromal cells. Immunopharmacology 46:103, 2000

176. Mitsuyasu RT, Golde DW: Clinical role of granulocyte-macrophage colony-stimulating factor. Hematol Oncol Clin North Am 3:411, 1989

177. Haas R, Murea S: The role of granulocyte colony-stimulating factor in mobilization and transplantation of peripheral blood progenitor and stem cells. Cytokines Mol Ther 1:249, 1995

178. George JN: Platelets. Lancet 355:1531, 2000

179. McCullough J: Current issues with platelet transfusion in patients with cancer. Semin 37:3, Hematol 2000

180. Brugger W, Scheding S, Ziegler B, Buhring HJ, Kanz L: Ex vivo manipulation of hematopoietic stem and progenitor cells. Semin 37:42, Hematol 2000

181. Stiff P, Chen B, Franklin W, Oldenberg D, Hsi E, Bayer R, Shpall E, Douville J, Mandalam R, Malhotra D, Muller T, Armstrong RD, Smith A: Autologous transplantation of ex vivo expanded bone marrow cells grown from small aliquots after high-dose chemotherapy for breast cancer. Blood 95:2169, 2000

182. Levin L, Simon R, Hryniuk W: Importance of multiagent chemotherapy regimens in ovarian carcinoma: dose intensity analysis. J Natl Cancer Inst 85:1732, 1993

183. Levin L, Hryniuk WM: Dose intensity analysis of chemotherapy regimens in ovarian carcinoma. J Clin Oncol 5:756, 1987

184. Murphy D, Crowther D, Renninson J, Prendiville J, Ranson M, Lind M, Patel U, Dougal M, Buckley CH, Tindall VR: A randomised dose intensity study in ovarian carcinoma comparing chemotherapy given at four week intervals for six cycles with half dose chemotherapy given for twelve cycles. Ann Oncol 4:377, 1993

185. Tokuda Y, Ohta M, Okumura A, Kuge S, Kubota M, Tajima T, Mitomi T: High-dose chemotherapy with autologous hematopoietic stem-cell transplantation in breast cancer. Cancer Chemother Pharmacol 40:S94, 1997

186. Steward WP: High dose chemotherapy with haemopoietic support for breast cancer. Eur J Cancer 31A:S21, 1995

187. Murray N: Importance of dose and dose intensity in the treatment of small-cell lung cancer. Cancer Chemother Pharmacol 40:S58, 1997

188. Elias A: Dose-intensive therapy in lung cancer. Cancer Chemother Pharmacol 40:S64, 1997

189. Womer RB, Daller RT, Fenton JG, Miser JS: Granulocyte colony stimulating factor permits dose intensification by interval compression in the treatment of Ewing's sarcomas and soft tissue sarcomas in children. Eur 36:87, J Cancer 2000

190. Fibbe WE, Pruijt JF, Velders GA, Opdenakker G, van Kooyk Y, Figdor CG, Willemze R: Biology of IL-8-induced stem cell mobilization. Ann N Y Acad Sci 872:71, 1999

191. Laterveer L, Lindley IJ, Heemskerk DP, Camps JA, Pauwels EK, Willemze R, Fibbe WE: Rapid mobilization of hematopoietic progenitor cells in rhesus monkeys by a single intravenous injection of interleukin-8. Blood 87:781, 1996

192. Pruijt JF, Willemze R, Fibbe WE: Mechanisms underlying hematopoietic stem cell mobilization induced by the CXC chemokine interleukin-8. Curr Opin Hematol 6:152, 1999

193. Licht T, Herrmann F, Gottesman MM, Pastan I: In vivo drug-selectable genes: a new concept in gene therapy. Stem Cells 15:104, 1997

194. Aran JM, Pastan I, Gottesman MM: Therapeutic strategies involving the multidrug resistance phenotype: the MDR1 gene as target, chemoprotectant, and selectable marker in gene therapy. Adv Pharmacol 46:1, 1999

195. Letourneau Sylvain GM, Cournoyer Denis: Hematopoietic Chemoprotection by Drug Resistance Gene Transfer, in Dube ID (ed): Transfusion Science: Gene Therapy:Current Status Future Prospects, vol. 17. Newark, Elsevier Science Ltd, 1996, p 147

196.	Licht T, Gottesman MM, Pastan I: Transfer of the MDR1 (multidrug resistance) gene: protection of hematopoietic cells from cytotoxic chemotherapy, and selection of transduced cells in vivo. Cytokines Mol Ther 1:11, 1995

197.	Baum C EHG, Ostertag W. and Stocking C.: Retroviral Transfer of Resistance Genes for Cancer Therapy: Implications for Vector Design, in Development OfEC-oa (ed): Gene Delivery Systems , A State of the Art Review. Paris, OECD, 1996, p 281

198.	Spencer HT, Sleep SE, Rehg JE, Blakley RL, Sorrentino BP: A gene transfer strategy for making bone marrow cells resistant to trimetrexate. Blood 87:2579, 1996

199.	Sorrentino BP, Brandt SJ, Bodine D, Gottesman M, Pastan I, Cline A, Nienhuis AW: Selection of drug-resistant bone marrow cells in vivo after retroviral transfer of human MDR1. Science 257:99, 1992

200.	Rafferty JA, Hickson I, Chinnasamy N, Lashford LS, Margison GP, Dexter TM, Fairbaim LJ: Chemoprotection of normal tissues by transfer of drug resistance genes. Cancer Metastasis Rev 15:365, 1996

201.	Galski H, Sullivan M, Willingham MC, Chin KV, Gottesman MM, Pastan I, Merlino GT: Expression of a human multidrug resistance cDNA (MDR1) in the bone marrow of transgenic mice: resistance to daunomycin-induced leukopenia. Mol Cell Biol 9:4357, 1989

202.	Galski H, Merlino GT, Gottesman MM, Pastan I: Expression of a human multidrug-resistance cDNA (MDR1) under the control of a beta-actin promoter in transgenic mice. Biotechnology 16:103, 1991

203.	Mickisch GH, Merlino GT, Galski H, Gottesman MM, Pastan I: Transgenic mice that express the human multidrug-resistance gene in bone marrow enable a rapid

identification of agents that reverse drug resistance. Proc Natl Acad Sci U S A 88:547, 1991

204. Mickisch GH, Aksentijevich I, Schoenlein PV, Goldstein LJ, Galski H, Stahle C, Sachs DH, Pastan I, Gottesman MM: Transplantation of bone marrow cells from transgenic mice expressing the human MDR1 gene results in long-term protection against the myelosuppressive effect of chemotherapy in mice. Blood 79:1087, 1992

205. Sorrentino BP, McDonagh KT, Woods D, Orlic D: Expression of retroviral vectors containing the human multidrug resistance 1 cDNA in hematopoietic cells of transplanted mice. Blood 86:491, 1995

206. Fruehauf S, Wermann K, Buss EC, Hundsdoerfer P, Veldwijk MR, Haas R, Zeller WJ: Protection of hematopoietic stem cells from chemotherapy-induced toxicity by multidrug-resistance 1 gene transfer. Recent Results Cancer Res 144:93, 1998

207. Hanania EG, Fu S, Roninson I, Zu Z, Deisseroth AB: Resistance to taxol chemotherapy produced in mouse marrow cells by safety-modified retroviruses containing a human MDR-1 transcription unit. Gene Ther 2:279, 1995

208. Corey CA, DeSilva AD, Holland CA, Williams DA: Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells. Blood 75:337, 1990

209. Deeg HJ, Socie G: Malignancies after hematopoietic stem cell transplantation: many questions, some answers. Blood 91:1833, 1998

210. Kruger W, Gruber M, Hennings S, Fehse N, Fehse B, Gutensohn K, Kroger N, Zander AR: Purging and haemopoietic progenitor cell selection by CD34+ cell separation. Bone Marrow Transplant 21:665, 1998

211. Baum C, Eckert HG, Stockschlader M, Just U, Hegewisch-Becker S, Hildinger M, Uhde A, John J, Ostertag W: Improved retroviral vectors for hematopoietic stem cell protection and in vivo selection. J Hematother 5:323, 1996

212. Beck-Engeser G, Stocking C, Just U, Albritton L, Dexter M, Spooncer E, Ostertag W: Retroviral vectors related to the myeloproliferative sarcoma virus allow efficient expression in hematopoietic stem and precursor cell lines, but retroviral infection is reduced in more primitive cells. Hum Gene Ther 2:61, 1991

213. Lozzio CB, Lozzio BB: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45:321, 1975

214. Gillis S, Watson J: Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2-producing human leukemia T cell line. J Exp Med 152:1709, 1980

215. Limon A, Briones J, Puig T, Carmona M, Fornas O, Cancelas JA, Nadal M, Garcia J, Rueda F, Barquinero J: High-titer retroviral vectors containing the enhanced green fluorescent protein gene for efficient expression in hematopoietic cells. Blood 90:3316, 1997

216. Dybing J, Lynch CM, Hara P, Jurus L, Kiem HP, Anklesaria P: GaLV pseudotyped vectors and cationic lipids transduce human CD34+ cells. Hum Gene Ther 8:1685, 1997

217. Omori F, Juopperi T, Chan CK, Chang YN, Phipps S, Nanji S, Zhao Y, Stewart AK, Dube ID: Retroviral-mediated transfer and expression of the multidrug resistance protein 1 gene (MRP1) protect human hematopoietic cells from antineoplastic drugs. J Hematother Stem Cell Res 8:503, 1999

218. Jakubowski A: Myelosuppression, in John M. Kirkwood MTL, Joyce M. Yasko (ed): Current Cancer Therapeutics. New York, Churchill Brothers Inc., 1996, p 317

219. Bunting KD, Galipeau J, Topham D, Benaim E, Sorrentino BP: Effects of retroviral-mediated MDR1 expression on hematopoietic stem cell self-renewal and differentiation in culture. Ann N Y Acad Sci 872:125, 1999

220. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG: Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 54:5902, 1994

221. Fruehauf S, Breems DA, Knaan-Shanzer S, Brouwer KB, Haas R, Lowenberg B, Nooter K, Ploemacher RE, Valerio D, Boesen JJ: Frequency analysis of multidrug resistance-1 gene transfer into human primitive hematopoietic progenitor cells using the cobblestone area-forming cell assay and detection of vector-mediated P-glycoprotein expression by rhodamine-123. Hum Gene Ther 7:1219, 1996

222. Steingold SF, Sharp NJ, McGahan MC, Hughes CS, Dunn SE, Page RL: Characterization of canine MDR1 mRNA: its abundance in drug resistant cell lines and in vivo. Anticancer Res 18:393, 1998

223. Challita PM, Kohn DB: Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. Proc Natl Acad Sci U S A 91:2567, 1994

224. Lutzko C, Kruth S, Abrams-Ogg AC, Lau K, Li L, Clark BR, Ruedy C, Nanji S, Foster R, Kohn D, Shull R, Dube ID: Genetically corrected autologous stem cells engraft, but host immune responses limit their utility in canine alpha-L-iduronidase deficiency. Blood 93:1895, 1999

225.   Stewart AK, Sutherland DR, Nanji S, Zhao Y, Lutzko C, Nayar R, Peck B, Ruedy
C, McGarrity G, Tisdale J, Dube ID: Engraftment of gene-marked hematopoietic
progenitors in myeloma patients after transplant of autologous long-term marrow
cultures. Hum Gene Ther 10:1953, 1999

226.   Hibino H, Tani K, Ikebuchi K, Wu MS, Sugiyama H, Nakazaki Y, Tanabe T,
Takahashi S, Tojo A, Suzuki S, Tanioka Y, Sugimoto Y, Nakahata T, Asano S: The
common marmoset as a target preclinical primate model for cytokine and gene therapy
studies. Blood 93:2839, 1999

227.   Moscow JA, Huang H, Carter C, Hines K, Zujewski J, Cusack G, Chow C,
Venzon D, Sorrentino B, Chiang Y, Goldspiel B, Leitman S, Read EJ, Abati A,
Gottesman MM, Pastan I, Sellers S, Dunbar C, Cowan KH: Engraftment of MDR1 and
NeoR gene-transduced hematopoietic cells after breast cancer chemotherapy. Blood
94:52, 1999

228.   Quesenberry PJ, Stewart FM, Zhong S, Habibian H, McAuliffe C, Reilly J,
Carlson J, Dooner M, Nilsson S, Peters S, Stein G, Stein J, Emmons R, Benoit B,
Bertoncello I, Becker P: Lymphohematopoietic stem cell engraftment. Ann N Y Acad Sci
872:40, 1999