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Allison Marie Lytle

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Date

Examination of the Immunological Aspects of Gene Therapy for the Treatment of Hemophilia A

By

Allison Marie Lytle  
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science  
Molecular and Systems Pharmacology

---

Chris Doering, Ph.D.  
Advisor

---

Mandy Ford, Ph.D.  
Committee Member

---

Nael McCarty, Ph.D.  
Committee Member

---

Trent Spencer, Ph.D.  
Committee Member

---

Roy Sutliff, Ph.D.  
Committee Member

Accepted:

---

Lisa A. Tedesco Ph.D.  
Dean of the James T. Laney School of Graduate Studies

---

Date

Examination of the Immunological Aspects of Gene Therapy for the Treatment of Hemophilia A

By

Allison Marie Lytle  
B.A., Emory University, 2008

Advisor: Chris Doering, Ph.D.

An abstract of a dissertation submitted to the Faculty of the  
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## Abstract

### Examining the Immunological Aspects of Gene Therapy for the Treatment of Hemophilia A

By Allison Marie Lytle

The 1950's and 1960's gave rise to the era of recombinant DNA and the notion that "good DNA" could be transferred into individuals with heritable diseases as a curative therapy. Decades were spent developing methods to produce high titer virus and modifying recombinant vectors to improve infectivity. Finally, in 1990, the Food and Drug Administration approved their first clinical gene therapy trial for the treatment of adenosine deaminase severe combined immunodeficiency (ADA-SCID). Gene therapy was on the rise and the entire country had high hopes that this kind of therapy would revolutionize modern medicine and provide a cure for over 1500 diseases recently shown to be genetically determined. Unfortunately, several clinical trials resulted in severe and in some cases fatal adverse events, sending the field back into the laboratory to focus on improving the efficacy and safety of viral mediated gene transfer.

Since these events, the field of gene therapy has made significant progress improving the efficacy and safety of both *ex vivo* and *in vivo* gene transfer methods. Clinical trials have been initiated covering over 8 different indications with at least 100 trials beginning in 2016 alone. While a significant amount of effort has been dedicated to vector development and safety, very few preclinical studies have examined the immunological implications of gene transfer. Hemophilia A (HA), an X-linked heritable bleeding disorder caused by a deficiency in coagulation factor VIII (FVIII), offers a unique opportunity to compare the immunomodulatory capabilities of both *ex vivo* and *in vivo* methods of gene transfer in the context of the same disease.

Both methods of gene transfer have been described to facilitate a tolerogenic immune state in which transgene specific immunoregulatory cells are generated to provide surveillance and prevent immune responses. However, neither of these mechanisms have been studied in the context of FVIII gene transfer, a coagulation factor known to be immunogenic and cause serious complications in 20-30% of patients with severe HA. Current clinical gene therapy trials only include patients that have previously received protein replacement therapy and are considered tolerized to recombinant FVIII protein. However, we must consider what the immunological implications of gene therapy would be in previously untreated patients, or patients with suboptimal gene therapy outcomes that would require some level of exogenous FVIII infusions. This dissertation will focus on the immunological aspects of FVIII gene transfer for two different gene therapy paradigms demonstrated to be curative in preclinical models of HA. We hypothesize that *in vivo* gene transfer methods targeting hematopoietic stem cells result in transplantation tolerance of FVIII gene-modified cells, and is a more durable mechanism of tolerance induction compared to *in vivo* methods targeting hepatocytes. Furthermore, this dissertation will discuss the development of novel pharmacological agents used to improve the safety of hematopoietic stem cell transplantation gene therapy.

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AAV	adeno-associated virus
Ad	adenovirus
ADA-SCID	adenosine deaminase severe combined immunodeficiency
AdHP	adenovirus helper plasmid
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
AT	antithrombin
ATG	anti-thymocyte globulin
BDD-FVIII	B-domain deleted coagulation factor VIII
BSA	bovine serum albumin
BU	bethesda unit
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
Ef1 $\alpha$	elongation factor 1 alpha
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FDA	US Food and Drug Administration
FIX/ FIXa	activated coagulation factor IX
FV/ FVa	activated coagulation factor V
FVII/ FVIIa	activated coagulation factor VII
FVIII/ FVIIIa	activated coagulation factor VIII
FX/ FXa	activated coagulation factor X
FXII/ FXIIa	activated coagulation factor XII
GFP	green fluorescent protein
HA	hemophilia A
hAAT	human $\alpha$ 1 antitrypsin
HCR	ApoE hepatic control region
HEK	human embryonic kidney
hFVIII	human coagulation factor VIII
HIV	human immunodeficiency virus
HLP	hybrid liver specific promoter
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
IgE	immunoglobulin E
IgG	immunoglobulin G
IPTG	isopropyl- $\beta$ -D-1-thiogalactopyranoside
ITI	immune tolerance induction
ITR	inverted terminal repeat
LMO2	LIM domain only 2

LTR	long terminal repeat
LV	lentivirus
MPL	myeloproliferative leukemia protein
mRNA	messenger ribonucleic acid
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIC	pre-integration complex
polyA	polyadenylation
qPCR	quantitative polymerase chain reaction
RIP	ribosomal inhibitory protein
RNA	ribonucleic acid
rpFVIII	recombinant porcine coagulation factorVIII
Sca-1	stem cell antigen-1
SCID-X1	X-linked severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SIN	self-inactivating
SPDP	succinimidyl-3-(2-pyridyldithio)propionate
ssDNA	single stranded DNA
T-ALL	T cell acute lymphoblastic leukemia
TBI	total body irradiation
Teff	T effector cells
TF	tissue factor
TIL	tumor infiltrating lymphocytes
Tregs	regulatory T cells
USD	united states dollar
VCN	vector copy number
VLR	variable lymphocyte receptor
VSVG	vesicular stomatitis virus
vWF	Von Willebrand factor
WAS	Wiskott-Aldrich syndrome
$\gamma$ -RV	gamma-retrovirus

## **Chapter 1**

### **Introduction**



## 1.1 A History of Clinical Gene Therapy

Between the late 1940's and early 1950's Dr. Joshua Lederberg and colleagues made a series of significant contributions to bacterial genetics that helped launch the classical era of molecular biology. Most notably, in 1952, Dr. Lederberg and his graduate student Norton Zinder published a mechanism of bacterial gene transfer in which they termed transduction, from the Latin word meaning to lead across.[1] In this series of experiments, Lederberg and Zinder used a U-shaped glass tube fitted with a very fine filter that could prevent mixing of bacterial cells from each side of the device. The glass tube contained two different *Salmonella* nutritional mutants on each side of the filter suspended in nutrient depleted broth that would require genetic recombination for survival. However, the filter prevented physical contact between the *Salmonella* mutants and therefore the expected outcome was to observe no growth of *Salmonella* recombinants.

On the contrary, the experiment led to new *Salmonella* mutants capable of expanding in nutrient depleted broth, suggesting genetic recombination had taken place. After purification and careful biochemical analysis of the culture media, Lederberg had discovered the agent of gene transfer to be a bacteriophage that had previously infected his strain of *Salmonella*. While in culture, the *Salmonella* bacterial cells were lysogenic and released multiple virions carrying fragments of host DNA, including genes that provide nutritional resistance. The bacteriophage progeny could travel back and forth between the filter and infect each strain of *Salmonella* on either side of the U-shaped glass device. The resultant gene transfer, which Lederberg described to be the process of transduction, led to the survival and growth of recombinant *Salmonella* in nutrient depleted broth. These early experiments demonstrated that viral gene transfer could confer a phenotype to infected cells thus formulating the very basis of gene therapy, using viral vectors packaged with an engineered transgene designed to confer a healthy phenotype to cells expressing a defective gene.

The next twenty years were marked by several monumental contributions to molecular biology. In 1953 Watson and Crick described the alpha helical structure of DNA and inferred the existence of a "copying mechanism" based on the structure and complementarity of the molecule.[2]

Scientists went on to discover the mechanism of DNA replication and described phosphodiester oligonucleotide synthesis. In 1958, Francis Crick described the Central Dogma establishing that the flow of genetic information begins with transcription of DNA into an RNA intermediary molecule followed by translation into a protein product. Crick suggested the information embedded in the sequences of nucleic acids and amino acids to be unidirectional, meaning that proteins lack the heritable nature of DNA and are unable to provide sequence information that will result in the synthesis of new proteins. [3] By the end of the decade, messenger RNA had been discovered and confirmed to be the intermediary between DNA and proteins.[4, 5]

The 1960's were marked by rapid developments in the field of biochemistry including in vitro protein synthesis described by Marshall Nirenberg and his postdoctoral fellow J. Heinrich Matthei, which led to the discovery of the first known codon for the amino acid phenylalanine.[6] Nirenberg went on to win the Nobel Prize in Physiology and Medicine for cracking the full genetic code and deciphering the sequences and identity of 64 RNA codons for all 20 amino acids.

The ground-breaking discoveries that defined the 1950's and 1960's essentially launched the era of recombinant DNA technology eventually leading to major breakthroughs like the Human Genome Project and clinical gene therapy. Scientists now had the capability to generate physical maps of DNA using restriction enzymes demonstrated to cleave DNA at specific short sequences present throughout the genome. Using bacterial cloning techniques and site-directed mutagenesis, molecules of DNA could be designed and synthesized to direct bacterial expression of proteins in vitro and manipulate their biosynthetic properties.[7] By 1971, over 1500 diseases had been identified and catalogued to be genetically determined.[8] These advances provided the intellectual landscape from which Friedman and Roblin hypothesized that viruses could be engineered to express synthetic genes that are otherwise mutated or absent in the host genome. These bioengineered viruses could transfer "good DNA" to defective cells resulting in long-term expression of the functional protein, thus conferring a therapeutic effect and correcting the phenotype in patients suffering from a genetic disease.[9] The ideas of Friedman and Roblin

published in 1972 led to the development of both *ex vivo* and *in vivo* gene transfer platforms and the development of viral vectors that can genetically modify multiple cells types in order to maximize the number of targeted diseases.

#### A. Developing *Ex Vivo* Gene Transfer Methods

Scientists had begun to focus on two main approaches for targeting disease, each with their own strengths and challenges. These strategies largely influenced vector development within the field. The first approach involved an *ex vivo* transduction paradigm that would later become the gene transfer platform for the first FDA approved clinical gene therapy trial. This strategy typically involves isolation of autologous cells from a patient, followed by genetic modification within the laboratory using bioengineered viral vectors. After transduction, these cells are re-introduced into the patient avoiding an immune response against the viral proteins. Scientists believed *ex vivo* gene transfer methods would eliminate off-target effects by avoiding transduction of off-target organs. Additionally, this method prevented genetic modification of germ line cells and was potentially more efficacious by eliminating renal and liver clearance of vector.

Beginning in 1981, three different laboratories had reported independently the effective gene transfer and integration of recombinant DNA into eukaryotic cell genomes using naturally occurring murine retroviruses.[10-12] These viruses efficiently integrate into their host genome as part of their natural life cycle to reproduce viral progeny using the host cell's DNA replication machinery. Shortly thereafter, groups developed retrovirus packaging cells lines that could generate recombinant virus pseudotyped with engineered envelope proteins to increase the breadth of transducible cells.[13] With an integrative gene transfer profile and exemplary transduction efficiency, retroviruses quickly became an excellent vector candidate for *ex vivo* gene therapies that depend on persistence and expansion of genetically modified cells. Finally, in 1990, Rosenberg et al. published in the *New England Journal of Medicine* the safety of peripherally infused gene-modified autologous TILs in patients with metastatic melanoma. Retrovirally transduced TILs were

shown to persist for months in the tumors and peripheral blood of patients, and demonstrated no additional side effects when compared to unmodified TILs upon infusion. [14]

These series of events led to a monumental leap in clinical gene therapy beginning with the first approved clinical trial in 1990 for the treatment of severe combined immunodeficiency caused by a mutation in the adenosine deaminase gene. ADA is a ubiquitous metabolic enzyme responsible for the deamination of adenosine and deoxyadenosine. The absence of functional ADA leads to intracellular accumulation of these toxic metabolites, causing a multitude of problems most notably in the immune compartment. Patients with ADA-SCID suffer from repeated life threatening infections due to the lack of functional lymphocytes and have a shortened life expectancy (less than 2 years) without treatment. On September 14, 1990, 4 year old Ashanthi DeSilva became the first individual to receive gene therapy under the supervision of Dr. French Anderson and colleagues at the National Institute of Health in Bethesda Maryland. The treatment involved isolation of mature autologous lymphocytes from peripheral blood by apheresis and followed by *ex vivo* expansion in the laboratory. Once actively dividing, expanded lymphocytes were genetically modified using a retroviral vector packaged with a non-defective copy of the human ADA gene and then reinfused back into the patient. DeSilva's treatment was a success facilitating stable immune function and ADA enzyme activity in peripheral lymphocytes. However, further trial enrollment confirmed variability in efficacy among patients using this approach. Obtaining adequate numbers of autologous lymphocytes is dependent on the severity of the patient's phenotype as well as their responsiveness to enzyme replacement therapy. Furthermore, modified lymphocytes appeared to be short-lived requiring repeated infusions to maintain immune function.[15]

Dr. Anderson and collaborators imagined a more curative gene therapy that involved a single infusion of genetically-modified cells with the ability to completely correct the immune defect. To that end, gene therapists would need to target a population of cells that would lead to ADA gene correction in all terminally differentiated cells of the immune compartment, including peripheral lymphocytes. Their vision quickly became a reality as scientists and physicians developed efficient

methods to isolate HSCs, multipotent stem cells with the potential to undergo self-renewal and differentiate into the myeloid and lymphoid lineages of the hematopoietic compartment.[16] By the early 90's, the stem cell transplantation paradigm had been revolutionized and physicians could isolate mobilized autologous HSCs from the peripheral blood of patients in as little as one day.[17]

These advancements facilitated rapid development in the field of gene therapy. HSCs were an ideal target cell for *ex vivo* gene therapy methods as these cells could now be easily isolated and could differentiate into the blood and immune cells that are defective in a wide variety of hematological and immunological diseases. Between 1992 and 2006, clinical trials using HSCT gene therapy had been initiated for the treatment of ADA- SCID, SCID-X1, chronic granulomatous disease, and Wiskott-Aldrich syndrome.[18] The excitement was palpable and the scientific community believed HSCT gene therapy was on the brink of curing any genetic disease that was targetable through HSC differentiation.

However, the HSCT gene therapy movement quickly came to a halt when 5 out of 20 patients enrolled in the SCID-X1 clinical trials were diagnosed with T-cell acute lymphoblastic leukemia (T-ALL) several years after treatment.[19] Gene expression analysis determined that patients had elevated levels of several genes, most notably the *LMO2* proto-oncogene when compared to 104 different pediatric leukemia samples. After extensive analysis of the malignant cells, it was determined that *LMO2* gene activation was caused by insertional mutagenesis by the gamma-retroviral vector genome inserted 35 kilobases upstream of the transcription start site. While several other acquired somatic mutations in these patients ultimately led to the development T-ALL, it became clear that insertional mutagenesis played a significant role in the development of T-cell leukemia, and a better understanding of vector biology was necessary for HSCT gene therapy to move forward.[20] In the past decade, the field has made significant advances to reduce the risks of insertional mutagenesis and improve safety profiles of integrating vectors. These efforts have resulted in over 60 ongoing clinical gene therapy trials world-wide using retroviruses as the method of gene transfer.

## B. Current Integrating Vectors

Using gene therapy to treat monogenic diseases requires stable and long-term expression of the healthy gene. Depending on the target cell type, different approaches to gene transfer are required to achieve persistent therapeutic transgene expression. Integrating vectors are paramount for HSCT gene therapy paradigms in which genetically modified stem cells will undergo proliferation and differentiation into mature blood and immune cells. Stable integration of the vector genome into the transplanted stem cell must take place to avoid loss of the transgene in terminally differentiated cells.

Retroviruses are enveloped viruses with single stranded RNA genomes capable of integrating into the host genome after infection. These viruses gain access to a cell through the binding of their surface glycoproteins to target cell surface receptors as well as fusion of the viral envelope to the cell surface membrane. Viral entry is highly specific and the composition of surface glycoproteins largely determines the range of cells of which retroviruses are able to infect. Once inside the cell, the RNA genome is uncoated from the protein core and reverse-transcribed into the double stranded proviral genome by reverse transcriptase already packaged within the virus. After reverse transcription, the proviral genome complexes with viral proteins to form the PIC to facilitate transport into the nucleus. Once inside the nucleus, the viral genetic material is integrated into the host genome as the provirus with the help of the viral protein integrase.[21]

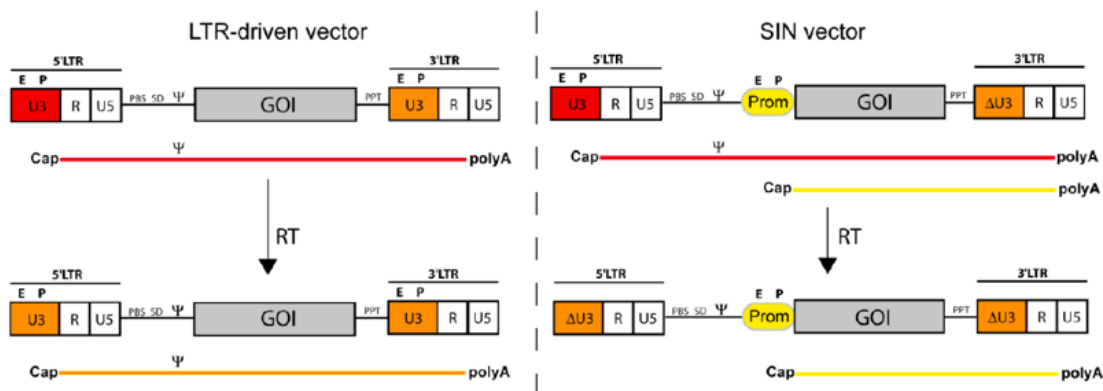
$\gamma$ -RVs were among the first retroviral vectors to be tested in clinical trials using *in vivo* gene therapy methods. Wildtype  $\gamma$ -RVs are classified as simple retroviruses because their RNA genome contains only three major coding domains, *gag*, *pol* and *env*. These genes contain the coding information for structural proteins including the capsid and the pre-integration complex, reverse transcriptase and integrase, and the surface and transmembrane proteins respectively. Additionally, integration of these genes into the host genome provides all the necessary information for the provirus to generate infectious progeny that will eventually leave the cell and go on to infect

new cells. In addition to the coding regions, the RNA genome is flanked by non-coding sequences termed LTRs. LTRs contain strong promoter sequences that drive proviral gene expression as well as potent transcriptional enhancer elements that can trans-activate nearby genes within the cell. **(Figure 1.1)**[22] Manufacturing recombinant viral gene therapy vectors requires the presence of these viral proteins in order to generate packaged virus capable of transferring genetic material, specifically the therapeutic transgene. However, certain safety measures must be taken to generate replication incompetent virus incapable of producing infectious virions.

First and second generation gene therapy  $\gamma$ -RV vectors could be manufactured by the transient transfection of a multi-plasmid system or the use of packaging cells that stably express viral proteins. In each of these approaches, necessary viral genes were present over several DNA elements to prevent the packaging of a complete viral genome capable of producing infectious viral particles. The expression plasmid, which is ultimately transcribed and packaged as the viral genome, contained the therapeutic transgene flanked by each viral LTR. Integration of the provirus resulted in transgene expression driven by the strong enhancer and promoter functions of the  $\gamma$ -RV LTR. However, as was observed in the SCID-X1 trial, the integration profile of  $\gamma$ -RVs and the trans-activating capabilities of the viral LTRs led to insertional mutagenesis and leukemogenesis in some patients.

In order to improve the safety profile of integrating viruses, next generation retroviral vectors adopted a SIN design to eliminate the trans-activating potential of the LTR viral promoter. SIN vectors maintain a truncation in the 3' U3 region eliminating the promoter/enhancer activity of the 3'LTR. **(Figure 1.1)** During the process of reverse transcription, this deletion is replicated into the 5'LTR eliminating the necessary transcriptional elements required for viral gene expression. Instead, an autonomous internal promoter is included downstream of the 5'LTR providing the ability to regulate gene expression at the cellular and tissue level. Moreover,

Figure 1.1



**Figure 1.1 Schematic of gamma-retroviral vectors using either an LTR or an internal promoter/self-inactivating design to drive transgene expression.** The gamma-retroviral genome has flanking LTRs that harbor strong promoter activity. The left panel describes the plasmid design of a recombinant gamma-retroviral vector using the LTR to drive expression of the gene of interest. On the contrary, the right panel depicts the plasmid design of a self-inactivating (SIN) vector that requires the use of an internal promoter due to a truncation within the U3 region of the 5'LTR. The lower half of each panel depicts the proviral genome after reverse transcription of the packaged genome. On the left panel, the U3 region of the 5' LTR remains intact and is capable of driving transgene expression once integrated into the host genome. However, the right panel shows that reverse transcription of a SIN plasmid results in a transcription defective 5'LTR. Instead, the SIN design requires the inclusion of an internal promoter to facilitate gene expression after integration into the host genome. By incorporating cell and tissue-specific promoters, this vector design offers the ability to modulate ectopic expression of the gene of interest. This figure was adapted from the journal *Viruses*, an open-access journal of virology. It was originally designed by Maetzig et al. and is licensed under the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>). [23] Abbreviations: LTR: long-terminal repeat, U3: unique 3 region, U5: unique 5 region, R: repeated sequence, PBS: primer binding site, SD: splice



donor, PPT: polypurine tract, Prom: internal promoter, GOI: gene of interest, SIN: self-inactivating,  
E: enhancer, P: promoter,  $\Psi$ : packaging signal

elimination of viral promoter activity within the LTR's provides an added level of safety by further reducing the risks of forming replication competent virus through recombination events.[23]

Eliminating the trans-activating potential of the viral promoter was only the first-step towards improving the safety profile of integrating vectors. LVs are complex retroviruses with a more comprehensive genome compared to  $\gamma$ -RVs. In addition to *gag*, *pol*, and *env*, the LV genome contains the genes *tat* and *rev* which are responsible for regulating gene expression at the transcriptional and post-transcriptional levels, as well as 5 additional accessory proteins *nef*, *vpr*, *vif*, *vpu*, and *p6*. Similarly to  $\gamma$ -RVs, LV gene therapy vectors utilize a multi-plasmid transfection system to decrease the risk of recombination and production of replication competent virus. Additionally, multiple groups have determined that elimination of the 5 accessory proteins adds an additional level of safety without decreasing viral titer or efficiency of gene transfer.[24, 25] LV's can also interact with the cell's nuclear import machinery providing the ability to infect either dividing or non-dividing cells and thus broadening its utility in a gene therapy setting. However, most significantly, LVs have a safer integration profile compared to  $\gamma$ -RVs and exhibit a broader distribution in transcriptional units rather than regulatory sequences upstream of active transcriptional sites.[26]

Lentiviruses have become the vector of choice for many *ex vivo* transduction platforms including HSCT gene therapy. In 2013, Aiuti et al. reported stable clinical improvement after HSCT gene transfer using a SIN-LV in three patients treated for WAS. Contrary to  $\gamma$ -RV WAS clinical trials, no genotoxicity was observed in any of the patients at 20 to 32 months of follow up.[27] Clinical efficacy and safety of SIN-LVs was further substantiated in a clinical trial for the treatment of metachromatic leukodystrophy.[28] However,  $\gamma$ -RVs are still a major contributor to clinical gene therapy. In 2016, Strimvelis became the first HSCT gene therapy product approved by the European Commission for the treatment of ADA-SCID, the only primary immunodeficiency in which no

genotoxic adverse events were observed in the initial  $\gamma$ -RV clinical trials. However, insertional mutagenesis remains a concern, and LV vectors will be the future of HSCT gene therapy.[29]

### **C. Developing *In Vivo* Gene Transfer Methods**

Unlike primary immune deficiencies or hematological diseases, there are monogenic diseases that affect cell types within solid tissue organs. An *ex vivo* transduction paradigm has little efficacy when targeting these diseases as the therapeutic outcome relies on the infusion and persistence of a cell type that will engraft into the parenchyma. Instead, *in vivo* gene transfer methods rely on the direct infusion of a vector packaged with the therapeutic transgene. This approach to gene therapy was pioneered by Crystal and colleagues in 1994 using a replication-deficient Ad based vector designed to target the respiratory epithelium of patients with cystic fibrosis.[30] Very quickly Dr. Crystal and other groups demonstrated the versatility of Ad and its ability to transduce multiple organs. However, these studies also confirmed the immunogenic nature of the vector and the subsequent loss of expression as well as dose-related adverse events due to cellular and humoral mediated immunity.[31] These claims were further substantiated by the death of an 18-year old boy named Jesse Gelsinger in 1999 who participated in a phase 1 dose escalation study for the treatment of ornithine transcarbamylase deficiency. A single infusion of liver-targeted adenoviral vector resulted in a fulminate acute inflammatory response that ultimately led to severe systemic inflammation and multi-organ failure.

After 23 years of research, many groups have learned to use the immunogenicity of Ad to their advantage. Most notably, Ad has been used as an immune potentiator through the gene transfer of various immune-activating cytokines or vaccine-related antigens. However, *in vivo* Ad transduction results in short-term high expression of the transgene and therefore limits its utility in a gene therapy setting.[31] In order for *in vivo* gene therapy to advance, researchers would need to develop a different viral vector with equivalent gene transfer efficiency but significantly reduced immunogenicity.

In 1965 AAV particles were discovered and reported as contaminants in several preparations of Ad.[32] In the following years, researchers elucidated the viral structure and genome design of the novel parvovirus. Additionally, AAV was shown to be non-pathogenic in humans, could be produced in excess of 100,000 viral particles per cell, and could persist long-term within infected cells.[33] In 1996 two landmark papers were published establishing AAV as an excellent candidate for *in vivo* gene transfer.[34, 35] In both studies, AAV vector infusion did not trigger immune-mediated clearance, as was the case for Ads and retroviral vectors, and demonstrated long-term stable gene expression from muscle tissue in mice. To their surprise, investigators also learned that the AAV proviral genome primarily exists as an extra-chromosomal episome within infected cells, eliminating the risks of insertional mutagenesis from integration into the host genome. In addition to these studies, Dr. Samulski and colleagues provided a significant body of work characterizing the tissue tropism of multiple naturally occurring serotypes. His laboratory pioneered the concept of AAV tropism modification and engineering hybrid vectors to increase tissue specificity and enhance transduction.[33] The perception of AAV gene therapy was promising, and from here on, all *in vivo* gene transfer studies targeting post-mitotic cells revolved primarily around AAV vectors.

Within ten years of these publications, clinical trials using recombinant AAV vectors were approved for the treatment of cystic fibrosis, hemophilia B, Batten's disease, Canavan's disease, and  $\alpha_1$ -antitrypsin deficiency.[36] Each of these trials demonstrated measurable low level expression of the transgene that closely followed the lifespan of the target cell. For a disease like cystic fibrosis in which target airway epithelial cells turnover approximately every 60 days, patients would require repeated AAV infusions triggering anti-vector immune responses. However, other target tissues such as liver and muscle demonstrated transgene expression for the duration of several years. Very quickly it became clear that *in vivo* AAV gene transfer would not be a one size fits all approach, and that each target cell type or tissue had its own unique set of obstacles in terms of gene expression and immune responses. Nonetheless, these early trials provided over a decade of safety data and solidified the study of AAV-based vectors in humans.

Finally, in 2008, *in vivo* gene therapy had a major breakthrough. Three clinical trials reported clinical efficacy using AAV based gene transfer for the treatment of Leber's congenital amaurosis, an autosomal recessive disease that causes functional blindness from a lack of retinoid recycling. Each of these studies involved a single subretinal injection at the site of action and reported significant improvement in patients for several years follow-up. Success continued to follow with the publication of another AAV-based clinical trial in the New England Journal of Medicine in 2011 for the treatment of hemophilia B.[37] In this study, Nathwani et al. reported significant clinical improvement in all 10 treated patients and prolonged transgene expression within 3 years after gene transfer.[38] With almost 30 years of research and clinical testing in humans, AAV became the first clinically approved gene therapy product in the European Union with several others projected to be approved in the United States.

#### **D. Current Non-Integrating Vectors**

Gene therapies targeting solid tissues and organs require the direct injection or intravenous infusion of viral vectors engineered to transduce cells *in vivo*. Non-integrating vectors are particularly attractive for this gene transfer paradigm as integrating vectors pose a risk of insertional mutagenesis, and intravenous infusions offer systemic exposure to the virus. However, the utility of current non-integrating vectors are dependent on their target tissues and therapeutic design. AAV has been the lead candidate for *in vivo* gene therapy development due to its reduced immunogenicity and decades of clinical safety data showing minimal adverse events and persistent transgene expression. However, Ad has proven successful in several cancer gene therapies by facilitating transduction of target tumor cells with suicide genes or genes that compensate for mutations in tumor suppressor genes and oncogenes. Ad has also been used to potentiate the immune system against tumor cells or facilitate target tumor cell lysis.[39]

Adenoviruses are non-enveloped viruses with an icosahedral capsid approximately 80nm in size and a double stranded DNA genome approximately 36 kilobases long. With a packaging capacity approximately 8 times the size of AAV, adenoviruses were particularly attractive for gene

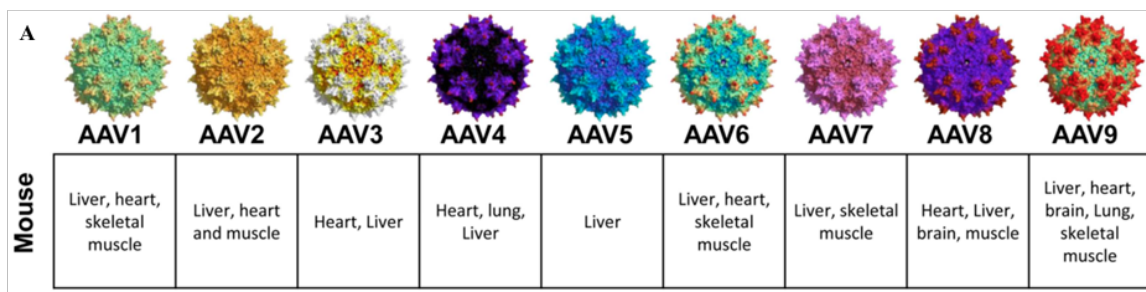
therapy development as they could accommodate the average size of a human gene. Additionally, Ad can efficiently transduce both dividing and quiescent cells offering a more diverse repertoire of cellular targets.[39] However, as previously mentioned, adenoviruses proved to be highly immunogenic, causing life-threatening systemic inflammation and short-lived gene expression.

On the other hand, AAV has become the preferred vector for *in vivo* gene transfer, demonstrating both robust gene expression and long-term persistence. While no known pathology has been reported, clinical data has shown mild engagement of the immune response that varies depending on vector type due to pre-existing immunity, packaged transgene, and route of administration.[40] AAV has many naturally occurring serotypes showing both broad and tissue-specific tropism. **(Figure 1.2)** Tissue tropism is multi-factorial including binding affinity to the cell surface, entry into the cell, uncoating of the protein capsid, and nuclear import of the viral genome. However, characterization of the viral capsid structure as well as amino acid sequence analysis of different serotypes has led to the development of biosynthetic AAV vectors with modified tissue tropisms and enhanced infectivity.[41, 42]

Wildtype AAV contains a single-stranded DNA genome that is 4.7kb in length. Similar to LTRs, the AAV viral genome is flanked by ITRs that facilitate replication, integration into the host genome, rescue from the site of integration, and packaging into nascent capsids. The viral genome encodes 4 nonstructural proteins (Rep40, Rep52, Rep68, and Rep78) and 3 capsid proteins (VP1, VP2, and VP3). In addition to co-infection with helper virus, these proteins are necessary for the completion of the AAV life cycle.[43] As with LV gene therapy, engineering of AAV vectors involves the removal of these essential proteins and replacement with the therapeutic transgene cDNA. Instead, these proteins are provided in auxiliary DNA plasmids through transfection or viral transduction of producer cell lines during the manufacturing process.

Contrary to LV vectors, recombinant AAV provirus exists as stable extrachromosomal episomes in transduced cells. However, despite the absence of proteins necessary for integration, recombinant AAV has been shown to integrate into the host genome at a low frequency. These

Figure 1.2



**Figure 1.2 Different serotypes demonstrate variable tissue-tropism in mice.** The tissue tropism of each naturally occurring serotype was observed in mice after tail vein injection. This figure was adapted from In Tech Open, an open-access book publishing company. It was originally designed by Melisa Vance, Angela Mitchell, and Richard J. Samulski and is licensed under the CC Attribution 3.0 (<https://creativecommons.org/licenses/by/3.0/legalcode>).[44] Abbreviations: HSPG: heparin sulfate proteoglycan, FGFR1: Fibroblast growth factor receptor 1, HGFR: hepatocyte growth factor receptor, PDGFR: platelet derived growth factor receptor, EGFR: endothelium growth factor receptor.

integration events have not been associated with genotoxicity and tumorigenesis in both nonhuman primate studies and human patients.[45] The packaging capacity of the AAV genome is limited to 4.7kb. These size restrictions make AAV vector design more complex for larger transgenes required of diseases such as cystic fibrosis, muscular dystrophy, and HA. Packaging of oversized transgenes results in genome truncations or packaged viral particles with incomplete transgene cDNA fragments. These incompletely packaged AAV viral particles rely on host cell DNA repair mechanisms to assemble the transgene based on complimentary overlapping ends of each cDNA fragment. While this mechanism of assembly results in stable episomal proviral genomes, oversized transgenes cause a significant loss of vector potency.[46] However, AAV has been shown to overcome these limitations by using biosynthetic high-expressing transgenes to drive gene expression, specifically in the case of HA.[47]

Both *ex-vivo* and *in-vivo* gene transfer platforms have had tremendous clinical success. While not without their own unique disadvantages and obstacles, each method has resulted in the development and implementation of a clinical gene therapy product. However, despite the method of vector transduction, gene therapy results in the long-term expression of a gene product that is absent from the host proteome. Although therapeutic in nature, these proteins have the potential to be immune-activating, resulting in neutralization of the gene product and/or immune-mediated clearance of the transduced cells. The field of gene therapy has spent decades developing safer viral vectors that maintain efficient gene transfer. However, only a few groups have studied the immunological aspects of gene therapy, and none of these studies have made any comparisons between methods of gene transfer. Given that both AAV and LV based gene therapies have demonstrated therapeutic correction in a murine model of HA, this disease model provides a unique opportunity to compare the immunological consequences of both *in vivo* and *ex vivo* gene transfer.[47-51]



## 1.2 Hemophilia A

Hemophilia refers to a group of bleeding disorders caused by deficiencies in circulating plasma proteins called coagulation factors. These proteins play a key role in the body's ability to regulate thrombosis and hemostasis. HA is an X-linked recessive inherited disease caused by a deficiency in functional FVIII. Defects in FVIII expression can cause spontaneous and prolonged bleeding in the joints and soft tissues, with severe phenotypes resulting in intracranial hemorrhage. The incidence of HA in the United States is approximately 1:5000 males, with roughly one third of infant diagnoses resulting from spontaneous mutations in the *F8* gene. [52, 53] According to the World Federation of Hemophilia, HA can be subdivided into three categories based on plasma levels of circulating FVIII. Persons with severe HA have less than 1% of normal FVIII levels (with normal levels being defined as the amount of FVIII activity present in pooled normal human plasma and designated as 1 International Unit per milliliter) and experience spontaneous weekly bleeding, while people with moderate HA have an improved phenotype and rarely experience spontaneous bleeding. These patients have FVIII levels between 1-5% of normal and typically experience prolonged bleeding after surgery, major injury, or dental work. Finally, patients with mild HA have FVIII levels between 5-40% of normal and in many cases, do not experience any bleeding episodes. Therefore, minimal increases in FVIII levels could dramatically improve clinical outcomes for patients with severe HA.

In newborns, circumcision is the most common cause of bleeding, however, iatrogenic heel-stick bleeds and intracranial hemorrhage from the birthing process closely follow. The hallmark signs of HA, repeated joint bleeding leading to chronic arthropathy, do not begin until the toddler age when the child becomes mobile and will persist into adulthood.[54] In patients with severe hemophilia, joint bleeding can easily occur in weight-bearing joints in the absence of trauma. Repeated episodes of hemarthroses led to severe inflammation and joint damage, including erosion of cartilage, a decrease in range of motion, and muscle atrophy. In addition to arthropathy, muscle hematomas are another kind of bleeding episode in patients with hemophilia. Typically due to

trauma or injections, bleeding in the muscle tissue can lead to blood loss, muscle wasting, infection, and nerve damage. Depending on the location of the bleed, these episodes can be life-threatening and result in amputation.[52] Lastly, close to 30% of treated patients with severe hemophilia develop neutralizing inhibitory antibodies against FVIII. While certain risk factors have been identified, prediction models have not been able to adequately identify inhibitor patients and improve clinical outcomes.[55]

The general consensus of hematologists is that prophylactic treatment should begin around 1-2 years of age and be tailored to the patient's bleeding phenotype and individual experiences to prevent joint damage and increase lifespan. Typically, this course of treatment requires 3,000-4,000 IU of FVIII concentrate/year for an average sized child with severe HA and has an annual cost of \$36,000/year. The cost of treatment becomes challenging for patients living in emerging markets where the median per capital household income is roughly \$3,000 annually.[56, 57] The annual price tag of FVIII concentrates leaves 75% of the global hemophilia population without adequate treatment and at a higher risk for mortality and co-morbidities.[58]

As previously mentioned, the prevalence of HA is estimated to be approximately 1 in 5000 live births, with 80% of persons with hemophilia living in the developing world. These countries are lacking in health care infrastructure including clinicians, lab technicians, and physical treatment centers. Additionally, the burden of high cost hemophilia care falls on the individual due to limited availability of insurance coverage. Furthermore, governments are unable to provide adequate support and funding as rare genetic disorders must compete with infectious and malignant diseases for health finance. These problems are compounded by the fact that 70-80% of patients do not have access to FVIII concentrates, 30% of treated patients developed anti-FVIII inhibitory antibodies, and many people suffer comorbidities from viral contamination of plasma-derived FVIII products.[59]

### **A. Physiology of Coagulation**

The concept of blood coagulation was first described in 1960 as a “waterfall sequence” of proenzymes that are proteolytically cleaved and activate other proenzymes downstream within this signaling cascade.[60, 61] Hemostasis is the process by which the body inhibits bleeding, however, unchecked pro-coagulant activity can also lead to thrombosis. Thrombohemorrhagic balance is maintained at the intersection of platelet activation and interaction with vessel walls, followed by the interface of the fibrinolytic system and the coagulation cascade. A variety of things can insult this balance including trauma, infection, inflammation, as well as pharmacological agents that modulate antithrombotic or thrombogenic components of the coagulation system.[62]

The endothelial layer of vessel walls is characterized as anti-thrombotic because it is comprised of neutrally charged phospholipids that don't promote binding of pro-coagulant proteins. Additionally, the endothelium synthesizes and secretes activators of fibrinolysis as well as inhibitors of platelet activation and coagulation. However, in the presence of injury, the subendothelium is a potent activator of coagulation through exposure of collagen, von Willebrand factor, and proteins involved in platelet adhesion. Vascular injury activates primary hemostasis facilitating the formation of the platelet plug. Exposure of collagen and von Willebrand factor from the subendothelium provide a binding surface for platelet adhesion.[63] Platelets then undergo degranulation, releasing coagulation factors and calcium. Calcium coats the surface of phospholipids and facilitates binding of coagulation proteins post-transcriptionally modified with gamma-carboxyglutamic acid-rich domains. Lastly, platelets begin to aggregate causing the formation of the platelet plug.[63]

Secondary hemostasis, which takes place simultaneously with primary hemostasis, involves activation of the clotting cascade and the formation of fibrin to stabilize platelet aggregates. The coagulation cascade is comprised of zymogens, generally serine proteases, and procofactors that require cleavage for activation. Most procoagulants and anticoagulants are synthesized by liver hepatocytes, however, FVIII has been shown to be synthesized in the liver sinusoidal endothelial

cells. Secondary hemostasis is typically divided into two different pathways, the intrinsic and extrinsic pathway. The intrinsic pathway, otherwise known as the contact-activation pathway, has classically been used to describe *in vitro* coagulation. FXII, which initiates the intrinsic pathway, can undergo self-activation in the presence of negatively charged surfaces. It is this mechanism that is utilized by certain clinical assays, including the activated partial thromboplastin time to determine bleeding pathologies. Understanding the physiological significance of the contact activation pathway and the importance of FXII has been under investigation. It is now accepted that collagen exposure from vessel damage, protein complexes on membrane surfaces, protein aggregates, infectious pathogens, or the presence of pathophysiological materials can activate this pathway *in vivo*, and the series cleavage steps take place on the platelet surface.[64]

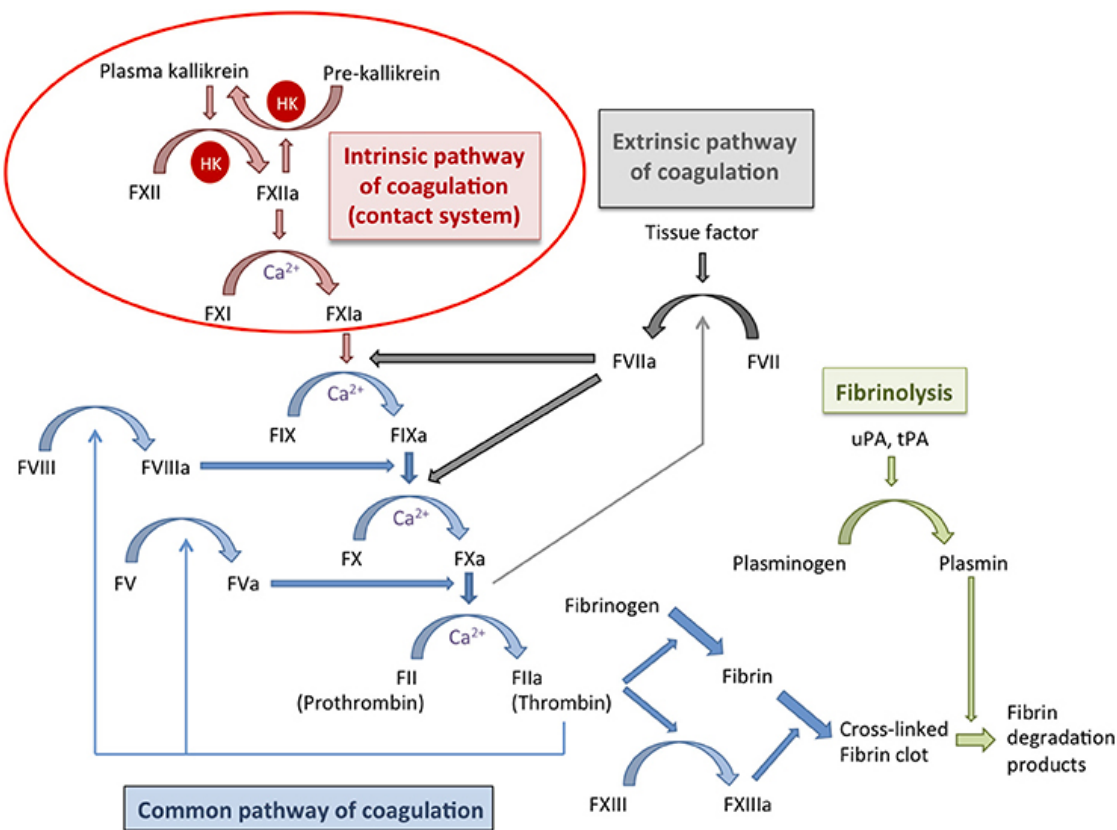
On the other hand, the extrinsic pathway is thought to be the primary contributor to physiological activation of coagulation. The extrinsic pathway is activated by tissue factor exposure from extravascular tissues and fibroblasts in the presence of tissue and vessel injury. Tissue factor is a cofactor for the serine protease coagulation factor VIIa, and together they form the extrinsic Xase complex. This complex activates both coagulation factors X and IX into their activated forms FXa and FIXa respectively. The presence of FXa, albeit limited, results in the conversion of a small amount prothrombin into activated thrombin, with the capacity to activate platelets and convert coagulation factors V and VIII into their activated forms respectively. This process results in propagation of coagulation through the formation of the intrinsic Xase complex comprised of FVIIIa:FIXa on the surfaces of activated platelets. The intrinsic Xase complex can convert FX into FXa with a higher efficiency than the extrinsic complex.[65, 66] Generation of FXa in conjunction with FVa results in the formation of the prothrombinase complex (FXa:FVa), a potent activator of prothrombin. This process, which also takes place on the phospholipid surface, very quickly generates increasing levels of thrombin, resulting in the formation of the fibrin network and a feed-forward loop into the intrinsic pathway that amplifies coagulation.[67]

Both the intrinsic and extrinsic pathways of the coagulation cascade converge into the final common pathway, generation of the prothrombinase complex which converts prothrombin into thrombin. (**Figure 1.3**) The endpoint of the coagulation cascade is to generate a fibrin network that stabilizes platelet aggregates. This is achieved through thrombin generation which cleaves fibrinogen into fibrin. These fibrin molecules polymerize into a protein network that is stabilized by the cross-linking action of activated coagulation factor XIII.[68] Once bleeding has subsided and blood vessels have been repaired, the fibrin stabilized platelet plug must be removed through enzymatic processes. This is typically activated by either tissue-type plasminogen activator or urokinase-type plasminogen activator secreted by endothelial cells in the vasculature. These proteins, in the presence of fibrin, convert plasminogen into plasmin, the primary enzyme responsible for fibrin degradation.[69, 70]

In order to maintain a thrombohemorrhagic balance, mechanisms must exist to turn off hemostasis and avoid thrombotic injury. While thrombin participates in feed-forward loops to amplify procoagulant activity, it also engages in negative-feedback loops to regulate and turn off the coagulation cascade. Thrombin activates protein C on the surface of endothelial cells in complex with thrombomodulin. Activated protein C in turn will cleave and inactivate FVIIIa and Va to inhibit the intrinsic Xase complex and prothrombinase complex.[71] Tissue factor pathway inhibitor also exists in circulation and can inhibit both FXa and the TF:VIIa extrinsic Xase complex.[72] Several serine protease inhibitors of the serpin family circulate in plasma to inhibit coagulation factors through a covalent interaction of the active site, most notably AT.

AT is the primary inhibitor of coagulation but has also been shown to play a role in modulating inflammation and tumor cell migration.[73] AT can inhibit several coagulation factors but the primary mechanism of anticoagulation is inhibition of thrombin. The kinetics of inhibition are further enhanced by the presence of heparin.[74] In addition to inhibition of the coagulation cascade, intact endothelium secretes anti-platelet agents including nitric oxide which inhibits platelet aggregation.[75]

Figure 1.3



**Figure 1.3 The extrinsic and intrinsic pathways converge into the common pathway of the coagulation cascade.** While the extrinsic pathway is the primary activating pathway of coagulation *in vivo*, both pathways are necessary for efficient secondary hemostasis. Tissue factor driven coagulation results in minimal thrombin generation capable of feeding into the intrinsic pathway. This feedforward mechanism results in further thrombin generation and convergence into the common pathway of coagulation, ultimately leading to the formation of a fibrin clot. This figure was adapted from *Frontiers in Cellular and Infection Microbiology*, an open access journal. It was designed by Torsten G Loof, Christin Deicke, and Eva Medina and is licensed under the Creative Commons Attribution license CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>). Abbreviations: HK: high molecular weight kininogen, FXII/FXIIa: activated coagulation factor XII, FXI/FXIa: activated coagulation factor XI, FIX/FIXa: activated coagulation factor FIX,

FVIII/FVIIIa: activated coagulation factor FVIII, FV/FVa: activated coagulation factor FV, FX/FXa: activated coagulation factor FX, FII/FIIa: activated coagulation factor FII, FVII/FVIIa: activated coagulation FVII, FXIII/FXIIIa: activated coagulation factor XIII, uPA: urokinase-type plasminogen, tPA: tissue plasminogen activator

## **B. Coagulation Factor VIII**

FVIIIa is a procofactor of the zymogen serine protease FIX within the intrinsic pathway of the coagulation cascade. It is primarily synthesized by liver sinusoidal endothelial cells, although auxiliary sites of protein production have been reported.[76-79] FVIII is synthesized as a 300kDa single chain protein organized into discrete domains: A1-A2-B-A3-C1-C2. The A domains are approximately 30% homologous with each other, and display some homology with the copper binding proteins ceruloplasmin. The C domains have a similar structure to FV and mediate plasma-membrane binding. Lastly, the heavily glycosylated B domain has no known homology to any existing protein and removal of the B domain does not impede procoagulant activity.[80]

As human FVIII is translated and translocated into the endoplasmic reticulum, the primary translated product undergoes intracellular proteolysis to form a heterodimer of the heavy chain (A1-A2-B) and the light chain (A3-C1-C2). The heterodimer is associated through a noncovalent metal ion interaction at the A1 and A3 domains, and upon secretion from the cell, the light chain immediately binds to von Willebrand factor to protect FVIII from degradation. Human FVIII has demonstrated poor expression compared to other proteins of similar size in heterologous expression systems due to low level steady state mRNA transcripts and reduced secretion.[81] These observations are partly due to the engagement of the unfolded protein response during post-translational modification, inhibiting transport of FVIII from the endoplasmic reticulum to the Golgi apparatus. This phenomenon seems specific to human FVIII, as similar analysis using porcine FVIII has no effect on secretion.[82]

Activation of FVIII by either thrombin or FXa results in the enhanced catalysis of FX cleavage by FIXa. Cleavage takes place at the A1-A2 junction, the A2-B domain junction, near the N-terminus of the light chain, and within the C2 domain. Proteolytic cleavage by both thrombin and FXa results in the release of von Willebrand factor and the formation of a heterotrimer. While the metal-ion dependent interactions remain intact, the A2 domain stays associated in the heterotrimer through electrostatic interactions. Dissociation of the A2 domain is believed to be the primary



mechanism of FVIIIa inactivation, however, Protein C is also a potent anticoagulant and inactivates both FVIIIa and FVa.[83]

In the late 1950's and 1960's, treatment for hemophilia was highly inefficient as it required infusions of large volumes of freshly frozen plasma to stop bleeding episodes. In 1964 Judith Pool discovered that slowly thawing freshly frozen plasma under cold temperatures resulted in a precipitate highly concentrated in FVIII. This product, also known as cryoprecipitate, could be stored frozen and allowed physicians to infuse patients with much smaller volumes. It was not until the late 1960's that manufacturers could separate coagulation factors from pooled plasma and package these proteins into lyophilized concentrates. This development revolutionized the standard of care for hemophilia, as patients could now perform self-injections in the comfort of their own home. Unfortunately, as the AIDS epidemic arose in the 1980s, thousands of people with hemophilia died from FVIII precipitates contaminated with HIV, in addition to other infectious agents. However, the 1980s was also a period of rapid development in molecular biology and biochemistry, resulting in the manufacturing and licensing of recombinant FVIII products. These developments launched the new golden era; a time when men with hemophilia had a life expectancy similar to that of the general population.

Prophylactic FVIII protein replacement therapy is highly invasive, requiring weekly infusions of recombinant product. Poor expression in heterologous systems results in high manufacturing costs and treatment costs. The average expenditure of treatment for HA is roughly \$160,000 annually, with 80% of that being spent on replacement therapy.[84] Additionally, 20-30% of treated patients develop an immune response against recombinant FVIII, requiring alternative replacement products or ITI. Typically, ITI requires daily high dose infusions of recombinant FVIII to tolerize the immune system and eliminate anti-FVIII inhibitory antibodies.[85] Course of treatment has been estimated to cost upwards of \$1 million and is economically not feasible in countries with emerging markets.[84] Because of these deficiencies, researchers have focused a considerable amount of effort developing gene therapy strategies for HA.

### 1.3 Clinical Gene Therapy for Hemophilia A

As previous trials have shown, developing a high expression transgene is an important component to clinically efficacious gene therapy. It is widely accepted that truncation or deletion of the B-domain from recombinant FVIII increases the number of mRNA transcripts and secreted protein in both heterologous expression systems and *in vivo* gene transfer platforms.[86, 87] Additionally, it has been reported that codon optimization of the transgene resulted in enhanced FVIII expression in F8<sup>-/-</sup> mice.[88] However, replacement of the human A1 and A3 domains with the corresponding porcine FVIII sequences has resulted in some of the highest FVIII expressing constructs reported to date.[89] Incorporation of these porcine sequences results in 10-100 fold greater expression than human FVIII due to enhanced secretion.[90, 91] Strategic bioengineering of recombinant FVIII molecules has had a significant impact on transgene expression profiles and launched HA to the forefront of gene therapy development.

AAV-based gene transfer has had significant clinical success in patients with hemophilia B. As of 2016, there have been several approved clinical trials using various serotypes and high expressing FIX transgenes.[92] All treated patients have demonstrated mild to moderate circulating FIX levels varying between 12 weeks and 16 months follow up. The success of AAV based hemophilia B trials have paved the way for HA, leading to the initiation of the first AAV clinical trial for the treatment of HA. Thus far, 6/7 patients who received the highest dose of vector have maintained circulating FVIII levels in the normal range between 34 and 50 weeks of follow up. However, as expected, these patients experienced acute liver toxicity due to immune-mediated clearance of the vector, and one of the seven high dose patients has maintained liver enzyme levels approximately 5% higher than the normal range.[93]

Acute liver toxicity is one of the few limitations that could hinder clinical AAV gene therapy development for transgenes with notoriously low expression, or monogenetic diseases that require large increases in gene expression in order to see therapeutic improvement. Previous studies have shown that therapeutic levels of gene expression from intramuscular injections of AAV vectors is

largely dependent on the transgene. While effective for both  $\beta$ -galactosidase and erythropoietin expression, intramuscular directed expression of FIX resulted in transient expression and an anti-FIX immune response.[94] In the case of hemophilia, liver directed expression due to AAV transduction of hepatocytes has had superior efficacy in terms of systemic levels of protein expression and reduced immune responses against the transgene product. However, pre-existing immunity to the viral capsid limits the number of patients eligible for treatment, as the prevalence of anti-AAV antibody titers may exceed 60% of the population.[40] Additionally, the non-integrating nature of AAV causes a huge unknown in terms of life-long liver expression in the presence of either normal hepatocyte turnover or disease-mediated stresses. Lastly, anti-capsid immune responses causing acute liver toxicity has placed a dosing limitation on AAV vectors.[37] However, early data from a more recent clinical study demonstrated therapeutic efficacy at lower doses of vector. Thus far, no adverse events related to liver toxicity have been reported suggesting that high-expressing transgenes can overcome the limitations of anti-capsid immunity in AAV gene transfer.[95]

Clinical trials using LV- based HSCT gene therapy for HA have lagged compared to AAV-based gene therapy platforms. Several preclinical studies have shown that targeting HSCs for FVIII gene transfer results in phenotypic correction of HA in both murine and canine animal models.[51, 96, 97] Additionally, *ex vivo* transduction reduces the risk of anti-vector immune responses observed in AAV gene transfer. However, stem cell transplantation requires the use of conditioning agents to make space in the hematopoietic compartment for newly transplanted cells to engraft and undergo hematopoiesis. Conditioning regimens generally include genotoxic agents such as chemotherapeutics and radiation, which are highly effective at killing cells. These drugs also have significant side effects such as thrombocytopenia, sterility, risk of infection, and can cause secondary malignancies. A strong correlation exists between the stringency of conditioning regimens and levels of engraftment. In turn, therapeutic levels of FVIII expression are directly dependent on levels of engrafted therapeutic cells. As such, higher doses of conditioning agents

will increase the therapeutic effects of HSCT gene therapy, while simultaneously increasing the side effects associated with chemotherapeutics and radiation. For diseases like X-linked SCID, the risks of conditioning and stem cell transplantation are minimal compared to the clinical outcomes of patients treated with the standard of care. However, the risk-to-benefit ratio is diminished when considering diseases such as HA in which protein replacement therapy is available and effective. While a case can be made for people living with HA in countries that cannot afford to provide or subsidize the cost of FVIII concentrates for prophylactic care, developing non-genotoxic conditioning agents will increase the safety of stem cell transplantation and broaden the utility of HSCT gene therapy.

Lastly, several studies have been published examining the use of gene therapy to treat HA in the presence of anti-FVIII inhibitory antibodies. One approach has been to use platelet-specific promoters to drive FVIII expression so that FVIII can remain shielded from the immune system and released only in the event of bleeding.[98] This method of gene transfer relies on the transplantation and engraftment of genetically-modified stem cells as well as the risks associated with conditioning regimens. In addition to HSCT gene therapy, others have shown inhibitor eradication in a canine model of HA using AAV mediated liver-directed FVIII expression.[99] However, as with many gene therapy studies using large animals, the sample size only included two dogs and the vector doses exceeded what was considered clinically safe for humans.

#### **1.4 Immunogenicity of FVIII**

Beyond efficient gene transfer, gene therapy approaches must overcome the immune barrier in order to achieve persistent high levels of transgene expression. The human immune system is responsible for protecting the body from invading pathogens by continually recognizing “non-self” peptides from “self” peptides. In the context of gene therapy, functional “healthy” proteins that are otherwise absent from the host’s proteome are being expressed at high levels to correct the disease state. Additionally, many gene therapy platforms redirect gene expression to cellular compartments that are ectopic sources of the transgene product. Overcoming the immune

barrier and developing immunological tolerance to FVIII are important aspects of gene therapy design for HA.

Many factors indicate that FVIII is a highly immunogenic protein. Patients with severe HA have a higher incidence of inhibitory antibody development compared to patients with hemophilia B.[100] Acquired hemophilia, an auto-immune form of the bleeding disease, typically results from autoantibodies against FVIII rather than FIX.[101] FVIII has also proven to be a formidable immunological challenge in gene therapy designs.[48, 102] AAV liver-directed expression of recombinant FVIII induced a strong humoral immune response that attenuated FVIII expression in nonhuman primates. However, repeated infusions of immunosuppressants rescued FVIII activity and eradicated anti-FVIII inhibitory antibodies, suggesting the initial immune response did not result in cytotoxicity of transduced hepatocytes. Similarly, Ide et al. have shown that varying the stringency of conditioning agents for transplantation in HSCT gene therapy studies can also result in transient FVIII activity followed by immune-mediated clearance of the transgene product. Nonetheless, clinical trials using AAV-based gene transfer systems are underway and LV-HSCT approaches are not far behind. While many groups have spent considerable effort increasing the efficacy of HA gene therapy through vector and transgene design, understanding the mechanisms of tolerance for both AAV and LV-HSCT mediated FVIII gene transfer is the next step to optimizing clinical gene therapy for HA.

### **1.5 Hypothesis**

AAV and LV-HSCT are two vastly different gene-transfer systems that differ in both vector design and transduction, as well as mechanisms of tolerance induction to the transgene product. Current ongoing clinical studies are enrolling patients that have had repeated FVIII protein replacement injections with no documented adverse events, including anti-FVIII antibody development. These patients are considered tolerized to exogenous FVIII infusions, given their medical history. However, we must consider what the immunological implications of gene therapy would be in previously untreated patients, or patients with suboptimal gene therapy outcomes that

would require some level of protein replacement therapy. We hypothesize, that LV-HSCT gene therapy results in post-transplantation tolerance under conditions of unimolecular microchimerism, low-level engraftment of genetically-modified transplanted cells (microchimerism with <10% genetically-modified peripheral blood mononuclear cells) expressing a single transgene product (unimolecular neoantigen) that is absent from the host proteome. Post-transplantation tolerance is an immunological state in which the host immune cells are not activated by the neo-antigen but maintain the ability to launch a pathogen-specific immune response.[103] Bone marrow chimerism can result in life long central tolerance in which donor-derived antigen presenting cells can facilitate deletion of auto-reactive T-cells. In addition to central tolerance, bone marrow chimerism induces regulatory T-cells (Tregs) in the periphery that provide surveillance for immune-activation against donor neo-antigens. Lastly, post-transplantation tolerance achieved through bone marrow chimerism is robust and will facilitate transplantation of donor solid organ tissues that would otherwise undergo immunological rejection.[104]

On the other hand, AAV transduction of hepatocytes has been suggested to induce peripheral tolerance, or the induction of antigen specific regulatory T-cells to inhibit anti-transgene immune responses.[105, 106] However, demonstration of this immune non-responsive state in the context of HA gene therapy is questionable considering the difficulties experienced in preclinical models. We hypothesize that LV-HSCT gene transfer of FVIII results in more robust tolerance induction to exogenous FVIII compared to an AAV liver-based gene transfer system. This dissertation will focus on the immunological aspects of FVIII gene transfer for both AAV and HSCT gene therapy. Initial discussion will focus on the efficacy of incorporating a bioengineered high-expressing FVIII transgene into a liver-directed AAV gene therapy platform. It will then move on to evaluate induction of FVIII-specific tolerance in the context of both liver-directed AAV and LV-HSCT gene transfer. It will also investigate novel approaches toward developing non-genotoxic conditioning agents for HSCT gene therapy. Finally, this dissertation will conclude with a discussion on the future of gene therapy for HA.

## Chapter 2

### **Bioengineered Coagulation Factor VIII Enables Long-Term Correction of Murine Hemophilia A Following Liver-Directed Adeno-Associated Viral Vector Delivery**

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Brown HC, Wright JF, Zhou S, Lytle AM, Shields JE, Spencer HT, Doering CB.

### **[Bioengineered Coagulation Factor VIII Enables Long-Term Correction of Murine Hemophilia A Following Liver-Directed Adeno-Associated Viral Vector Delivery](#)**

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Harrison Brown designed and cloned expression cassettes, performed and designed the experiments, and wrote and edited the paper

Fraser Wright and Shangzhen Zhou produced and characterized AAV virus

Alison Lytle and Jordan Shields performed experiments

Trent Spencer and Chris Doering conceived the experiments and edited the paper

Note: The experimental contributions of Allison M Lytle are specific to Figure 3.4 panel F. The data and conclusions from this publication offer an explanation for the observations and conclusions reported in chapter 3 and were therefore included in this dissertation. Dr. Harrison Brown, who was responsible for the majority of the data presented in this chapter and is the first author on this publication, is a second author on the manuscript presented in chapter 3. Allison M Lytle was the primary contributor to the design and performance of experiments presented in chapter 3.

## 2.1 ABSTRACT

Clinical data support the feasibility and safety of AAV vectors in gene therapy applications. Despite several clinical trials of AAV-based gene transfer for hemophilia B, a unique set of obstacles impede the development of a similar approach for HA. These include 1) size of the FVIII transgene, 2) humoral immune responses to FVIII, 3) inefficient biosynthesis of FVIII, and 4) AAV vector immunity. Through bioengineering approaches, a novel FVIII molecule, designated ET3, was generated and shown to demonstrate 10-100 fold improvement in biosynthetic efficiency. In the current study, the utility of ET3 was assessed in the context of liver-directed, AAV-mediated gene transfer into HA mice. Due to the large transgene size and use of proven, but not minimized regulatory elements, the AAV-ET3 genomes packaged into viral particles as partial genome fragments. Despite this potential limitation, a single peripheral vein administration of AAV-ET3 into immune-competent HA mice resulted in correction of the FVIII deficiency at lower vector doses than previously reported for similarly oversized AAV-FVIII vectors. Therefore, ET3 appears to improve vector potency and mitigate several of the critical barriers to AAV-based clinical gene therapy for HA.

## 2.2 INTRODUCTION

HA is an X-linked congenital bleeding disorder characterized by a deficiency in functional FVIII in the blood compartment. Recently, clinical advancements have been made using recombinant AAV-based gene transfer for hemophilia B. However, a unique set of obstacles impede the development of a similar approach for the related and more common bleeding disorder HA [107]. These obstacles include 1) inefficient biosynthesis of hFVIII [81], 2) limited packaging capacity of recombinant AAV (4.7 kb) [108, 109] which is exceeded by FVIII expression cassettes as BDD-FVIII cDNAs alone are approximately 4.4 kb, 3) humoral immune responses to the transgene product (*i.e.* FVIII) [110], and 4) capsid-mediated cytotoxicity of the virus itself, which has been proposed to be as low as  $2 \times 10^{12}$  vector particles (vp)/kg [40].



FVIII is a large glycoprotein containing a domain structure (A1-A2-B-*ap*-A3-C1-C2) that is secreted at levels up to 3 orders of magnitude lower than other similarly sized secreted glycoproteins in vivo and in vitro. This low secretory rate has been spatially and temporally linked to inefficient posttranslational trafficking from the endoplasmic reticulum (ER) to the Golgi [81, 111-115]. The primary determinants of this biosynthetic limitation are specific amino acid sequences within the A1 and A3 domains of the molecule itself that cannot be overcome by standard transgene expression technologies such as more efficient DNA regulatory elements (e.g. promoters/enhancers), transgene copy number, targeted integration at genomic “hotspots” for transcription, and codon optimization. Instead, bioengineering of the FVIII molecule itself appears to be required to overcome inefficient secretion. We have developed such a bioengineered FVIII molecule (herein designated ET3 and previously referred to as HP47) using knowledge gained from the characterization of B-domain deleted BDD rpFVIII [116, 117]. Both rpFVIII and ET3 are secreted 10-100 fold more efficiently than other FVIII constructs through diminished interactions with ER resident chaperones and attenuated induction of the unfolded protein response [115-118]. The enhanced secretory capacity of ET3 is enabled through the substitution of “high expression” rpFVIII sequences in the A1 and *ap*-A3 domains into rhFVIII, which, despite retaining 91 percent overall amino acid identity, have been shown to be necessary and sufficient to confer 10 – 100-fold improved biosynthesis [117]. In addition to the A1 and *ap*-A3 domain substitutions, the BDD hfVIII protein, designated HSQ, substitutes the 14 amino acid human-derived SQ linker sequence for the B-domain, whereas ET3 utilizes the 24 amino acid porcine-derived OL linker sequence, which have been previously described and shown to produce similar levels of FVIII expression when substituted into porcine FVIII [116].

In the present study, proof of concept of a liver-directed rAAV vector encoding ET3 to confer long-term expression of therapeutic levels of FVIII in immune-competent HA mice was investigated. The vector design was based on an rAAV vector previously shown to effectively deliver and express the human FIX transgene [119]. This current vector, designated rAAV-HCR-

ET3, has a total genome size of 5.9 kb, which is 125% of the 4.7 kb endogenous rAAV genome and therefore is oversized. Recently published data from Samulski and colleagues suggest that oversized AAV genomes are packaged into preformed viral capsids as incomplete ss DNA fragments with either strand polarity likely containing overlapping portions of the transgene, which are heterogeneously truncated during the packaging process. Intracellular reassembly of these transgene fragments was suggested to confer transgene product expression levels 25-37 fold lower than that attained from smaller, non-fragmented transgenes [108, 109, 120, 121]. The standard mechanism of AAV reassembly and second strand synthesis has been previously reviewed [122]. Given the limitations of generating complete rAAV vectors for large transgenes such as FVIII, understanding the mechanisms and efficiencies of oversized rAAV genome packaging and transgene cassette reassembly are critical to the development of the next generation of rAAV vectors.

## 2.3 MATERIALS AND METHODS

*Vector Cloning* - The previously described HP47/ET3 transgene [117] was released from the ReNeo vector backbone by digesting with NotI followed by Klenow fragment fill-in and subsequent *SpeI* digestion. The previously described AAV2 vector backbone AAV-HCR-hAAT-FIX [119] was digested with *BglII* followed by Klenow fragment fill in and subsequent *NheI* digest. The AAV-HCR-hAAT vector backbone was ligated to the ET3 transgene, generating the rAAV-HCR-ET3 viral vector expression plasmid. An equivalent AAV expression vector encoding a previously described non-bioengineered BDD hFVIII construct (designated HSQ) [123] was generated by releasing HSQ with *NotI* followed by Klenow fragment fill in and subsequent *XhoI* digestion. AAV-HCR-hAAT-FIX was digested with *BglII* followed by Klenow fragment fill in and subsequent *Sall* digestion. AAV-HCR-hAAT was ligated to the HSQ transgene, generating the rAAV-HCR-HSQ viral vector expression plasmid.

*Transient Transfection* - Naïve human hepatocellular liver carcinoma (HepG2) cells were grown to 75% confluency in 6 well plates. Cells were transfected with 2µg of rAAV-HCR-ET3 or

rAAV-HCR-HSQ viral expression plasmid using 6  $\mu$ L Lipofectamine 2000 (Life Technologies, Carlsbad, CA) in DMEM supplemented with 10% fetal bovine serum. Twenty-four and 48 hours post transfection, media was replaced with 1 mL AIM-V serum free medium (Life Technologies). Seventy-two hours after transfection, conditioned media was collected and analyzed for FVIII activity by one stage clot assay as previously described [123].

*Vector Production* - The recombinant AAV8 vector encoding ET3 was produced as described previously [124]. Briefly, HEK 293 cells were transfected by calcium phosphate using the following plasmids: 1) the adenovirus helper plasmid AdHP encoding adenovirus helper proteins E2 and E4, 2) the AAV8 packaging plasmid AAV8PK encoding AAV2 Rep and AAV8 Cap, and 3) the vector plasmid encoding the transgene expression cassette. The transfected cells were harvested 72 hours after transfection, lysed by homogenization, clarified by centrifugation, and vectors purified by precipitation with 8% polyethylene glycol and two rounds of cesium chloride gradient ultracentrifugation. Purified vectors were dialyzed into phosphate buffered saline (PBS) and supplemented with Pluronic F68 (0.001% final concentration). The empty capsid free vector preparations were quantified by SDS- PAGE with silver staining by comparing VP1, 2 and 3 staining intensity by scanning densitometry with an established AAV reference vector.

*Molecular Studies* - Viral ssDNA was isolated from virus stocks using the QIAmp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Preparations for size analysis were prepared free of carrier RNA. Size analysis was performed by alkaline gel electrophoresis and subsequent Southern blot. DNA was probed using a cocktail of biotinylated probes directed against the A2, C2, and bovine polyadenylation sequences (**Table S1**) and detected using the North2South Chemiluminescent Detection kit (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions. Sizing analysis was performed by interpolating from the distances migrated by a DNA standard run in the alkaline gel to that of a single-stranded DNA ladder. Cellular DNA was extracted from liver tissue following perfusion for 10 minutes with 0.9% saline solution using the DNeasy Blood and Tissue Kit (Qiagen) according to the

manufacturer's instructions. All qPCR was performed using a StepOne real-time PCR platform (Life Technologies) and analyzed on the device's software. QPCR quantitation was performed using a standard curve generated from dilutions of rAAV-HCR-ET3 viral expression plasmid. For determination of FVIII mRNA quantity in HepG2 cells transfected with AAV-FVIII expression plasmids, mRNA was extracted from cells using the Qiagen RNEasy kit, and quantitative reverse-transcription PCR was performed as previously described [116]. Quantitation of regions of the viral genome was performed in separate experiments using sets of primers directed against regions that spanned the length of the rAAV-HCR-ET3 transgene. (**Table 1.S2**) To control for any differences in primer efficiency, each reaction was quantitated separately against plasmid standard curves generated using each region-specific primer pair. (**Figure 1.S1**) Isolated viral ssDNA was added to 2x Power SYBR green PCR master mix (Life Technologies) with final concentrations of 0.3125  $\mu$ M each of forward and reverse primers to a final volume of 20  $\mu$ L. The data shown is the mean of two qPCR reactions. As all virus used in this study was generated in a single production run, error bars are not shown to avoid confusion between the inter-assay variability and variability due to independent virus preparations. For determination of liver transgene copy numbers, 40 ng of cellular DNA was used as template in 2x Power SYBR Green PCR master mix with final primer concentrations of 0.3125  $\mu$ M each. The primer set directed against the A2 domain (**Table 1.S2**) was used for determination of transgene copy number in transduced livers. Quantitation was performed using a standard curve generated from rAAV-HCR-ET3 viral expression plasmid. Forty ng of naïve liver genomic DNA was added to each standard curve reaction to mimic the cellular DNA environment of the experimental samples. To determine the presence of full-length mRNA in the livers of treated mice, RNA was extracted from liver tissue using the RNeasy kit (Qiagen) according the manufacturer's instructions. One  $\mu$ g of RNA was subject to DNA removal and reverse transcribed using the Quantitect reverse transcription kit (Qiagen) according to the manufacturer's instructions. PCR was performed using the primer set directed against the C2 region of ET3. (**Table S2**)

*Animal Studies* - All animal studies were performed under the guidelines set by the Emory University Institutional Animal Care and Use Committee. Immune competent, exon 16 disrupted C57BL/6 backcrossed mice were used as a murine model of HA [125] and C57BL6 background mice used as healthy control. Hydrodynamic plasmid injections were performed in unanesthetized four to six week old HA mice. For these studies, 10ug of plasmid was diluted into 2.1mL Transit-EE hydrodynamic delivery solution (Mirus) and delivered by tail vein injection over the course of seven to ten seconds. Blood plasma was collected 24 hours after injection. AAV administration studies were performed in eight to twelve week old mice, which were administered a single tail vein injection of AAV viral vector diluted to a volume of 200  $\mu$ L in sterile filtered DPBS containing Pluronic F68 (0.001%). For both *in vivo* studies, citrated plasma was collected by retro-orbital bleed into one-tenth volume 3.8% trisodium citrate. FVIII activity was measured using the commercially available COATEST SP fVIII assay (Coatest SP, Diapharma, West Chester, OH, USA) according to the manufacturer's instructions using a standard curve generated from pooled citrated human plasma (George King, Overland Park, KS). Using this standard curve, the limit of detection for the assay was empirically determined to be 0.015 units (U) of fVIII/mL. Baseline determinations of fVIII activity in untreated HA mice were determined to be below the limit of detection of the assay. Circulating antibodies against fVIII were detected by ELISA [126]. Antibodies to fVIII were captured using immobilized recombinant hfVIII (Kogenate, Bayer, Leverkusen, Germany) bound to microtiter plates and detected using alkaline phosphatase conjugate of goat anti-mouse IgG as previously described [123, 127]. Absorbance at 405 nm was measured from dilutions of test plasma and plotted against the logarithm of the plasma dilution. The resulting sigmoidal curves were fit to the 4-parameter logistic equation by non-linear regression using the Levenberg-Marquardt algorithm. ELISA titer was defined empirically as a dimensionless value calculated from the reciprocal plasma dilution resulting in an optical density of 0.3 from the fitted curves [127]. The presence of inhibitory antibodies was confirmed by Bethesda assay, performed as previously

described [128, 129]. Bethesda titer was calculated as the average of dilutions of inhibitor found to produce between 25% and 75% residual activity.

*In vivo specific activity of rAAV-derived FVIII* - Antigen concentrations were determined using a capture ELISA designed to detect the heavy chain of FVIII. Monoclonal antibody (421C) directed towards a porcine A1 domain epitope was immobilized on 96 well high binding polystyrene plates (Corning, Corning, NY) and used to capture FVIII antigen from plasma samples diluted in HBS Tween-80. Samples were then incubated with a biotinylated secondary monoclonal antibody (4C7) directed towards a human A2 domain epitope for one hour in blocking buffer containing 1% BSA. Full length FVIII was measured using goat anti-mouse IgG antibody conjugated to alkaline phosphatase and *p*-nitrophenylphosphate. Absorbance at 405nm was read 30 minutes after the addition of the chromogenic substrate. Antigen concentrations were extrapolated from a standard curve derived from purified FVIII protein serially diluted in E-16 disrupted HA mouse plasma. Specific activity of mouse plasma samples were determined by plotting antigen concentration against FVIII activity measured by COATEST SP FVIII assay kit. Linear regression analysis was performed, comparing AAV treated mouse plasma samples to the standard curve.

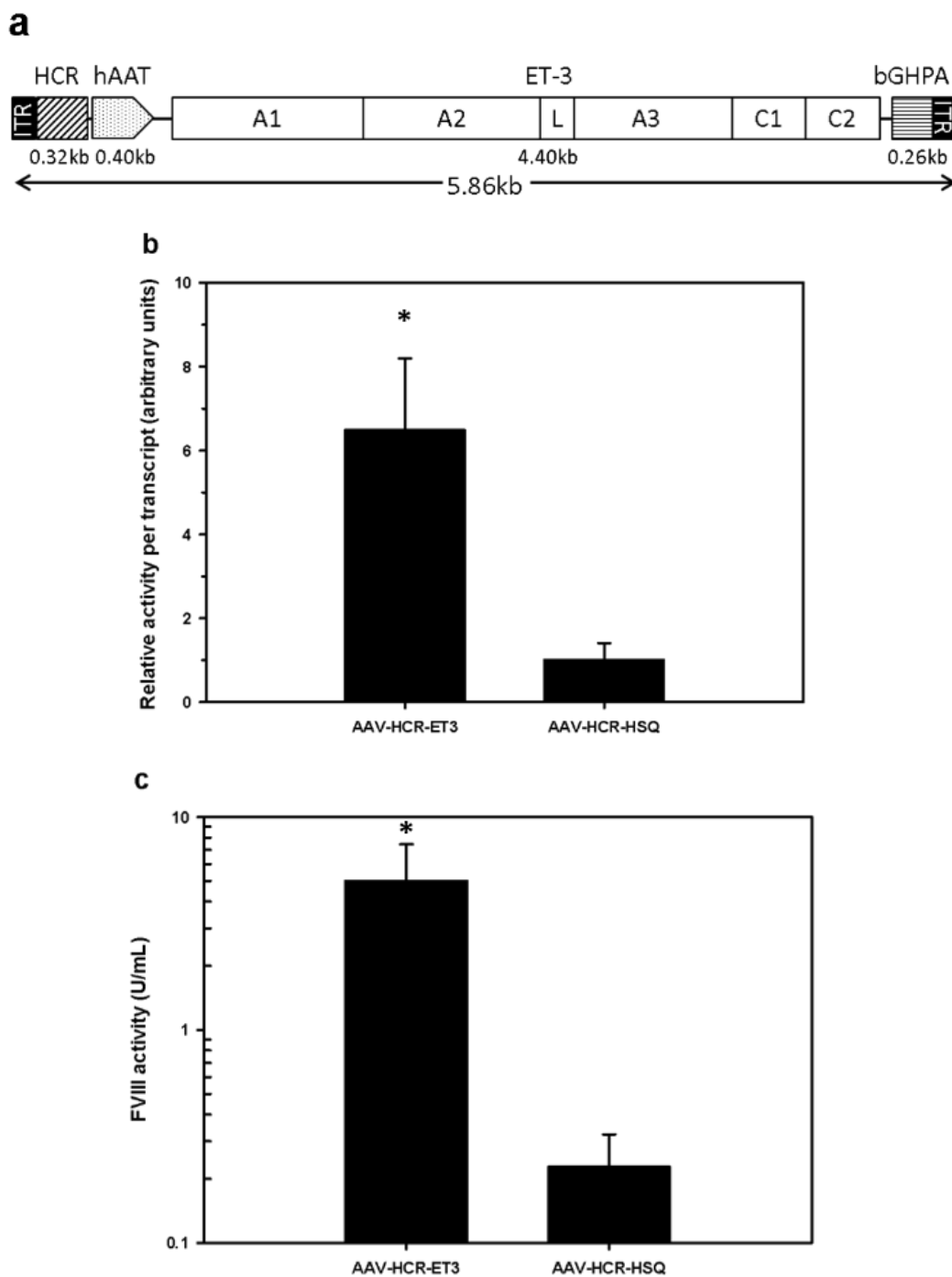
*Tail Transection Assay* - Tail transection bleeding assay was performed as previously described [128]. Briefly, mice were anesthetized under isoflurane and their tails were warmed for 10 minutes in a 37 °C water bath. Tails then were transected 4 mm from the tip using a scalpel and immediately placed into 14 mL vials containing 0.9% saline solution held at 37 °C. Tails were allowed to bleed freely for 45 minutes, at which time they were removed from the vials. Blood loss during 45 minutes was measured by mass of blood accumulated in the tube and normalized to body weight while controlling for evaporative loss.

## **2.4 RESULTS**

*Viral vector construction and in vitro comparison of BDD hFVIII and ET3 expression.* The rAAV vector design was based on constructs previously used to express the human coagulation factor IX transgene from liver tissue [119]. The ET3 transgene, which consists of human FVIII

sequences in the A2, C1, and C2 domains and porcine FVIII sequences in the A1 and *ap*-A3 domains, or alternatively, a BDD hFVIII transgene with a 14 amino acid linker (SQ), designated HSQ, were cloned into an AAV expression cassette controlled by a liver-specific ApoE (HCR)/ (hAAT) enhancer/promoter and flanked by AAV2 inverted terminal repeats (ITRs). (**Figure 2.1a**) Alignment of HSQ and ET3 amino acid sequences reveals 91 percent identity. (**Figure 2.S2**) We have previously shown that the increased expression of ET3 is conferred through enhanced post-translational processing of the nascent FVIII peptide [117]. To determine if the post-translational biosynthetic efficiency of ET3 expression compared to HSQ is recapitulated by the two AAV expression plasmid constructs in the context of the liver-directed transcriptional control elements in the rAAV-HCR-hAAT vector backbone, an *in vitro* transfection experiment utilizing the human hepatocellular carcinoma HepG2 cell line was performed. rAAV-HCR-ET3 and rAAV-HCR-HSQ expression plasmids were transiently transfected into HepG2 cells, and FVIII activity in the conditioned medium and quantity of FVIII mRNA transcripts were determined. Interestingly, a greater number of ET3 mRNA transcripts per cell (850, +/- 39), were determined compared to HSQ transcripts per cell (284, +/- 69). However, this 3-fold increase in mRNA resulted in over a 20-fold increase in FVIII secretion into the medium (0.70 units/mL +/- 0.24 ET3, and 0.034 units/mL +/- 0.010 HSQ). Together, these findings show that the rAAV-HCR-ET3 transfected HepG2 cells demonstrated 7-fold higher levels of FVIII activity per mRNA transcript into the conditioned medium than the rAAV-HCR-HSQ transfected cells (**Figure 2.1b**), suggesting that the post-translational biosynthetic efficiency of ET3 expression is the primary determinant of its enhanced secretion in the context of liver-directed expression. We cannot rule out, however, that increased transcriptional efficiency or mRNA stability may further contribute to the enhanced expression of ET3 compared to HSQ. To further examine the finding of enhanced expression of ET3, an *in vivo* comparison of the two vector-transgene designs by hydrodynamic injection of the expression plasmids was performed. In this experimental system, rAAV-HCR-ET3 expression plasmid conferred 20-fold higher plasma levels of FVIII activity than rAAV-HCR-HSQ expression plasmid

Figure 2.1



**Figure 2.1: Viral Vector Design** The 5.86 kb rAAV-HCR-ET3 genome encodes the high expression bioengineered FVIII molecule ET3, which consists of porcine FVIII sequences in the A1 and *ap*-A3 domains and human sequence in the A2, C1, and C2 domains. The ET3 transgene is under the control of a liver-specific hAAT promoter/ApoE HCR enhancer sequence, and



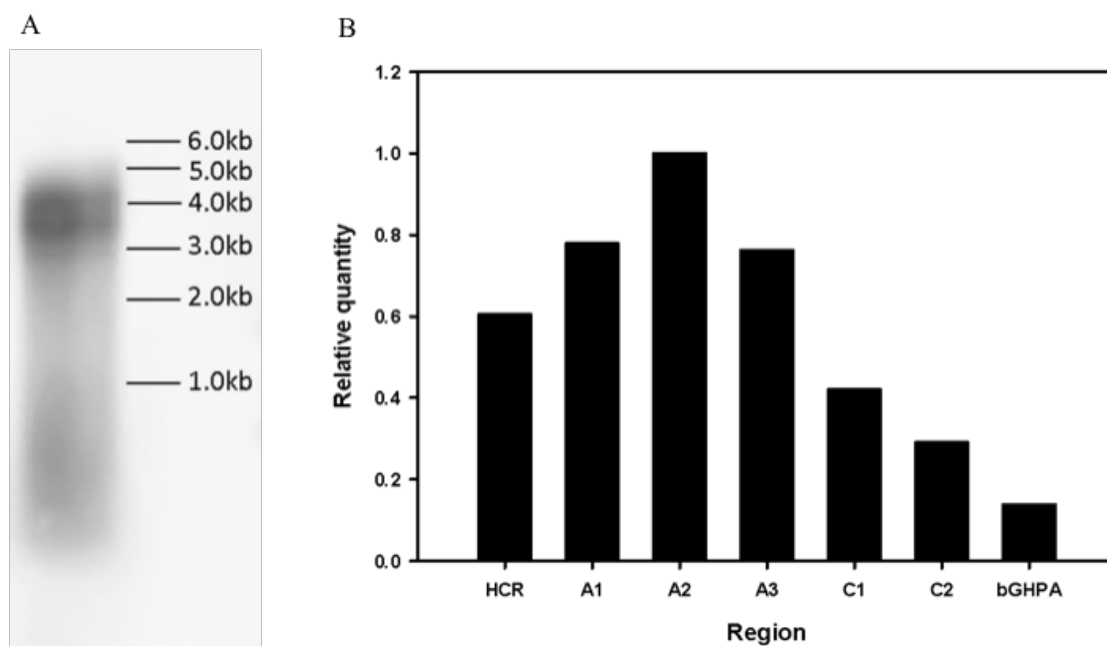
termination is governed by a bGHPA. The genome is flanked by AAV2 ITRs on both the 5' and 3' ends (**A**). FVIII activity was measured in conditioned medium of HepG2 cells that were transiently transfected with rAAV-HCR viral expression vectors encoding ET3 or the non-bioengineered BDD hFVIII construct HSQ, and activity normalized to quantity of FVIII mRNA transcripts per cell (**B**). Plasma FVIII activity was measured in HA mice that were hydrodynamically injected with rAAV-HCR viral expression plasmid encoding ET3 or HSQ (**C**). Baseline FVIII levels were determined to be below the limit of detection for all mice (data not shown). All error bars show one sample standard deviation, N=3 for *in vitro* studies and 3-4 for *in vivo* studies. (\* =  $p < 0.05$  by 2-way t-test)

again supporting the claim of enhanced expression of ET3 compared to HSQ. (**Figure. 2.1c, Table 2.S3**)

*rAAV vector production and characterization* - AAV particles encoding the rAAV-HCR-ET3 transgene were generated by transient transfection of HEK293 cells and subsequent purification of the vector particles from supernatants and cell lysates as previously described [124]. rAAV-HCR-ET3 was designed with a vector genome of 5.9 kb from ITR to ITR, which exceeds the endogenous rAAV genome size by 25%. Despite its oversized design, production of approximately  $1.2 \times 10^{13}$  total rAAV-HCR-ET3 vp at concentrations of  $5.3 \times 10^{12}$  vp per mL was achieved.

To assess the effect of the oversized genome on rAAV packaging, viral ssDNA obtained from cesium chloride gradient purified rAAV-HCR-ET3 was subjected to alkaline gel electrophoresis followed by Southern blot analysis using probes directed to the A2 and C2 domain sequences of FVIII and the bovine polyadenylation signal sequence. (**Figure 2.2a**) The rAAV-HCR-ET3 vector preparation did not contain detectable genetic material at the position expected for full-length genomes (5.9 kb). Rather, a heterogeneous smear of viral ssDNA approaching 5.0kb was observed suggesting that the majority of viral genomic DNA was packaged as truncated fragments. It has been suggested previously that oversized transgenes, such as that encoded by rAAV-HCR-ET3, may extend beyond the capsid, exposing free 5' ends of ssDNA on the outside of the viral particles. A comparison of Southern blot analysis of viral particles treated with DNase prior to disruption of the viral capsid to those that were not DNase treated failed to detect a difference in genome size, suggesting that vector genomic ssDNA does not extend beyond the viral capsid (data not shown).

The composition of the truncated rAAV-HCR-ET3 genome fragments was assessed by qPCR using primer sets directed against different genome regions spanning from ITR to ITR. (**Figure 2.2b**) Although this analysis could not distinguish strand polarity, it did show that sequences corresponding to the A2 domain of FVIII, which are located near the center of the

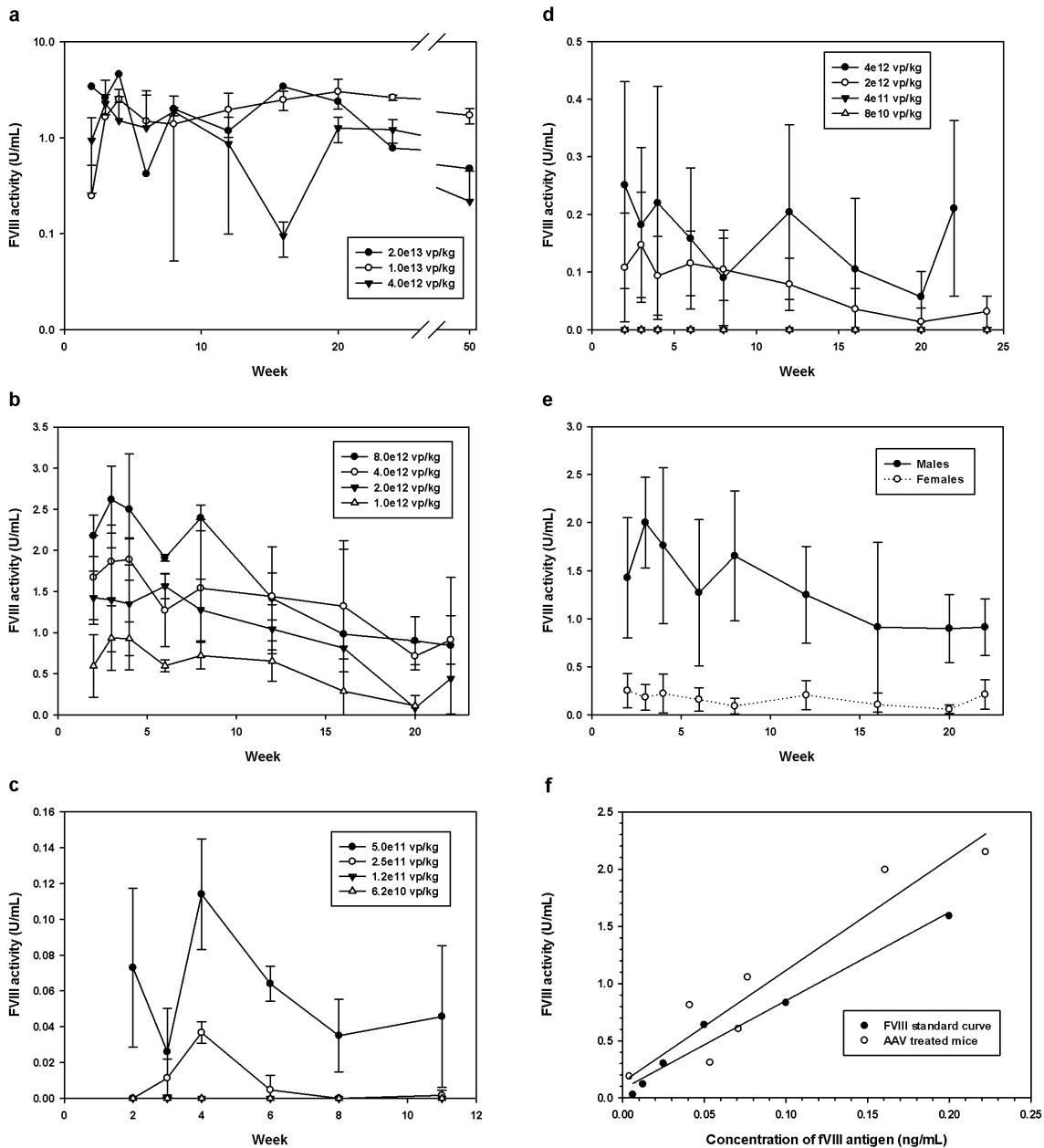
**Figure 2.2**

**Figure 2.2: Molecular assembly of rAAV-HCR-ET3 vector particles.** ssDNA from rAAV-HCR-ET3 viral particles were purified, subjected to alkaline gel electrophoresis, and detected by Southern blot (a). The molecular composition of the packaged viral genomes was determined by quantitative PCR directed against specific regions of the vector genome and normalized to the quantity of the A2 region (b).

designed viral genome, were the most prevalent. Comparatively, the terminal sequences, both encompassing either the promoter or poly A signal sequence, were up to 7.3-fold less prevalent than the central A2 sequences. This result is consistent with the current theory that oversized AAV viral genomes are packaged from one of the 3' ITRs of either polarity and truncated prematurely before reaching the other ITR.

*In vivo expression of FVIII rAAV-HCR-ET3* - A dose finding study was performed to determine the ability of rAAV-HCR-ET3 to provide therapeutic levels of circulating fVIII activity *in vivo*. Immune-competent 8-12 week old male mice were administered a single peripheral vein injection of the vector at doses ranging from  $6.2 \times 10^{10}$  to  $2.0 \times 10^{13}$  vp/kg. A long term (50 week) study was performed on a first cohort of mice receiving high vector doses ( $4 \times 10^{12}$  –  $2 \times 10^{13}$  vp/kg), while follow-up on the second cohort that received lower doses ( $1 \times 10^{12}$  –  $8 \times 10^{12}$  vp/kg) was shorter (24 weeks). Lastly, as the lower dose limit was not attained in the second cohort, a third cohort of mice was dosed with rAAV-HCR-ET3 at even lower levels ( $6.2 \times 10^{10}$  –  $5 \times 10^{11}$  vp/kg) and was followed out to 11 weeks post-AAV injection. Starting two weeks after vector administration, circulating plasma was assayed for FVIII activity at scheduled time points. Male mice receiving rAAV-HCR-ET3 showed dose responsive increases in circulating FVIII activity. **(Figures 2.3a-c)** Supraphysiologic FVIII levels, at over  $>3$  units (U)/mL, were achieved at the dose of  $1 \times 10^{13}$  vp/kg. For reference, the FVIII activity level observed in pooled normal human plasma is defined as 1 U/mL, while clinical HA disease classifications are severe –  $<1\%$ , moderate 1 – 5%, and mild 5 – 40% normal FVIII activity. Correction to curative levels ( $>0.4$  U/mL or 40% normal human fVIII activity level) was achieved at doses as low as  $1.0 \times 10^{12}$  vp/kg, and partial correction ( $>0.05$  U/mL) was seen at doses as low as  $5 \times 10^{11}$  vp/kg. FVIII expression was maintained throughout the duration of the experiment in all but the lowest doses of vector administered. However, there was a trend toward activity loss over the duration of the experiment, which is consistent with gradual liver tissue turnover and loss of the episomal AAV genomes.

Figure 2.3



**Figure 2.3: Dose finding of rAAV-HCR-ET3 in a murine model of HA.** Dose finding studies were performed in male and female immune-competent HA mice. FVIII activity is presented from mice that did not form neutralizing antibodies. In male mice, a long term, high dose study (a), a medium duration, mid-dose extension study (b) and a short term, minimum effective dose study (c) were performed. In female mice, a shorter duration dose finding study was performed (d). A comparison between male and female mice receiving 4e12 vp/kg (e) shows the intersex difference

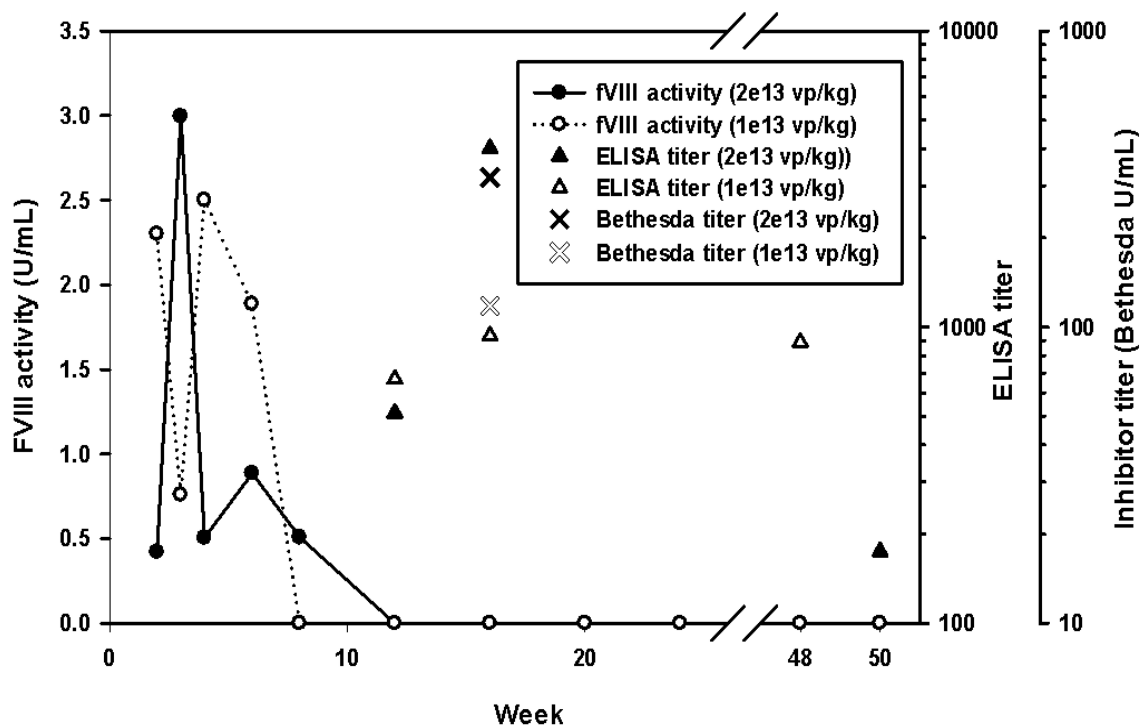
in FVIII levels. Baseline determination of FVIII activity in all mice tested was below the limit of detection of the assay (data not shown). N=2-4 for all doses, error bars show one sample standard deviation. To determine the specific activity of in vivo rAAV derived FVIII, the specific activity of a panel of plasmas from rAAV-HCR-ET3 treated mice (open circles) was compared to a standard curve generated from purified ET3 protein serially diluted in HA mouse plasma (closed circles) (f). Antigen concentrations were determined using a capture ELISA and FVIII activity was determined using a chromogenic substrate activity assay.

*Formation of anti-FVIII inhibitors* - Two mice that received high doses of rAAV-HCR-ET3 (2e13 and 1e13 vp/kg) displayed sudden and sustained losses of circulating FVIII activity at weeks 8 and 12, respectively. **(Figure 2.4)** Prior to the losses in plasma FVIII activity, both mice were expressing ET3 at supraphysiologic levels (>250% of normal). As repeated intravenous administration of recombinant rFVIII (rFVIII) to naïve HA mice is known to be immunogenic, all mice were tested for inhibitory antibodies to FVIII by ELISA. In the two mice that lost FVIII activity, positive anti-FVIII ELISA titers of 512 and 668 were observed at week 12 post rAAV-HCR-ET3 infusion, suggesting that these animals had mounted neutralizing humoral immune responses against the transgene product, which persisted for the duration of the study. The antibodies were confirmed to be inhibitory by Bethesda assay at week 16, which showed inhibitor titer of 320 and 118 BU per mL. Development of inhibitors, as detected either by loss of FVIII activity or ELISA titer, was not detected in any of the other experimental mice throughout the course of the long term follow-up (data not shown).

*Expression of FVIII rAAV-HCR-ET3 in female mice* - Previously, it has been reported that rAAV has reduced ability to mediate liver gene transfer in female mice [130]. Therefore, a limited dose finding study for rAAV-HCR-ET3 was performed in 8 – 12 week old immune-competent female mice. At doses ranging from 8e10 to 4e12 vp/kg, female mice showed an attenuated response to rAAV-HCR-ET3 gene transfer, with doses as low as 2e12 vp/kg resulting in partial correction of FVIII activity, which was maintained for the duration of the study. **(Figure 2.3d)** No female mice developed neutralizing antibodies to fVIII at the vector doses tested. With respect to the results obtained in male HA mice, circulating fVIII activity levels in female mice were approximately 8-fold lower at all doses tested, which is consistent with previous reports. **(Figure 2.3e)**

*Molecular composition of rAAV-derived FVIII* - Our molecular studies of the rAAV viral vectors suggests that the majority of viral transgenes package as sub-5.0kb fragments, which lack either the necessary 5' transcriptional control region or 3' FVIII coding sequence and poly(A)

Figure 2.4



**Figure 2.4: Formation of FVIII inhibitors following rAAV-HCR-ET3 administration.** Two mice that received one of the high doses of rAAV-HCR-ET3 (2e13 vp/kg and 1e13 vp/kg) experienced sudden and persistent loss of FVIII activity at between 8 and 12 weeks after vector administration. Anti-ET3 ELISA assay confirmed these mice had developed IgG antibodies to FVIII, which persisted for the duration of the study. A Bethesda assay was performed at week 16 to confirm the presence of neutralizing antibodies against FVIII.

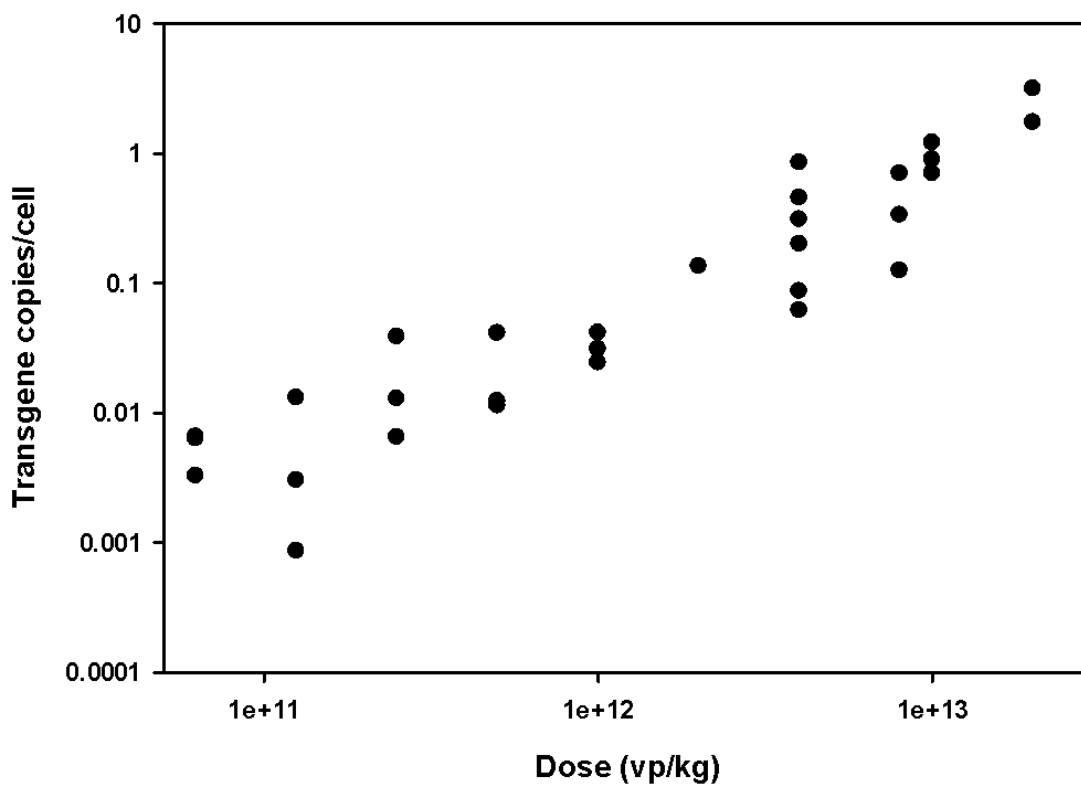


signal necessary to confer expression of full-length FVIII protein. To confirm the expression of complete ET3 mRNA sequences in mice treated with viral vector, RNA extracted from the livers of treated and untreated mice were subjected to reverse transcription PCR analysis for the presence of ET3 C2 domain RNA sequence, a region spanning position 5036 to 5205 at the 3' end of the transgene predicted to be truncated during the packaging process. **(Figure 2.S3)** Truncation at the 5' end, which includes the promoter region, would preclude transcription of ET3 mRNA, whereas truncation at the 3' end would result in loss of template for the PCR reaction. The detection of this mRNA region therefore supports the presence of full-length mRNA from the truncated viral ssDNA genomes.

While this analysis supports the presence of full length FVIII mRNA transcripts, it does not exclude the possibility that only a small proportion of mRNA transcripts in treated animals contain the complete FVIII coding sequence. The presence of mRNA transcripts coding a truncated FVIII sequence could lead to the production of a heterogeneous population of dysfunctional FVIII molecules. To interrogate the presence of dysfunctional FVIII protein in rAAV-treated mice, we determined the *in vivo* specific activity of rAAV-derived FVIII in vector-treated HA mice. Antigen concentrations, measured using a capture ELISA designed to detect the heavy chain of FVIII, a region not predicted to be truncated from the promoter region of the antisense-coding vector genomes, were plotted against the FVIII activity of each sample and compared to a standard curve of purified FVIII protein serially diluted in HA mouse plasma. The specific activity of the AAV treated mouse samples was found to be similar to that of the standard curve, indicating that the majority of mRNA transcripts encode a fully functional FVIII molecule.

*Viral genome copy number in transduced livers* - QPCR analysis of viral genome target sequences within the centrally-located A2 domain of FVIII was performed using DNA extracted from livers of all transduced male mice. **(Figure 2.5)** rAAV-HCR-ET3 proviral genomes containing the A2 domain sequence persisted as long as 1 year after treatment. There were approximately 2 viral transgenes per cell in mice receiving  $2 \times 10^{13}$  vp/kg, and a dose responsive

Figure 2.5



**Figure 2.5: *In vivo* viral genome copy number.** Quantitative PCR was used to determine the number of viral genomes in transduced livers. Primers directed against the FVIII A2 domain were used to determine the number of viral genome copies of this domain per cell for all mice receiving rAAV-HCR-ET3.

decrease in transgene number at the lower doses, with mice receiving the lowest doses ( $6.4 \times 10^{10}$  vp/kg) approaching the limit of detection.

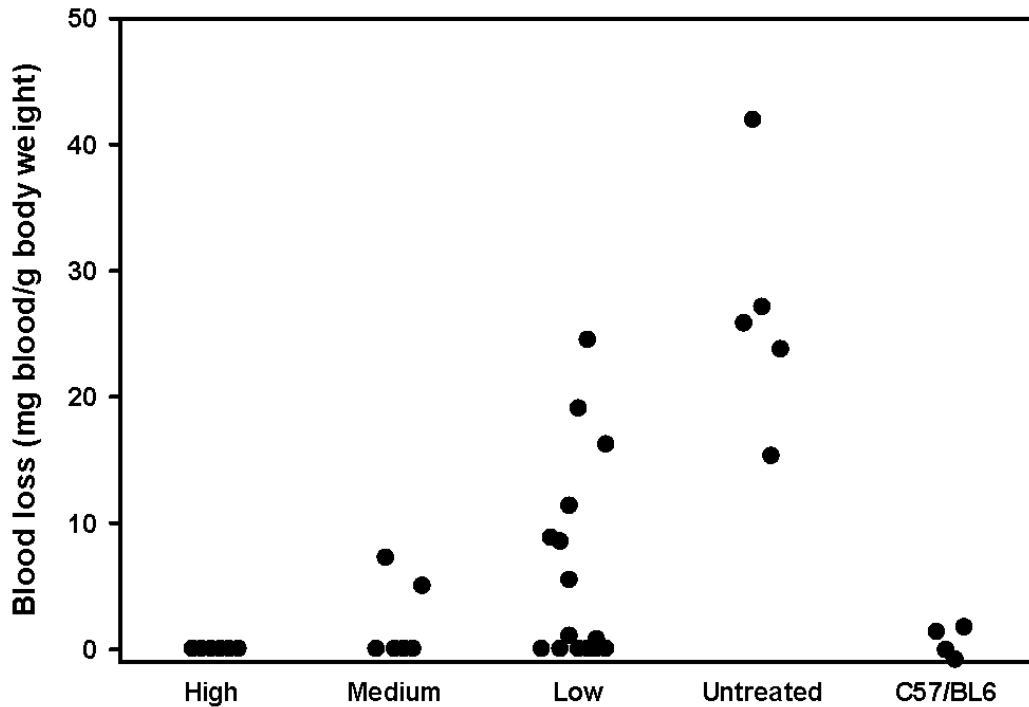
*Correction of bleeding phenotype* - Correction of the HA bleeding phenotype was assessed by tail transection bleeding assay on animals that received varying doses of rAAV-HCR-ET3. **(Figure 2.6)** Overall, mice that received rAAV-HCR-ET3 treatment demonstrated less blood loss than saline-treated control mice. Specifically, all mice that received one of the high vector doses ( $8 \times 10^{12}$ –  $2 \times 10^{13}$  vp/kg) showed no detectable blood loss while mice that received medium ( $1 \times 10^{12}$  –  $4 \times 10^{12}$  vp/kg) or low ( $6.2 \times 10^{10}$  –  $5.0 \times 10^{11}$  vp/kg) doses demonstrated dose responsive decreases in blood loss that, both as individuals groups and as a whole, were significantly less than saline treated controls ( $p < 0.005$ , Mann-Whitney U test).

## 2.5 DISCUSSION

The favorable safety profile, ease of delivery, durable expression and lack of innate immune stimulation position rAAV as a promising vector for clinical gene therapy of HA. However, large transgene size, potential humoral immunogenicity and poor biosynthetic efficiency of human FVIII present as significant obstacles hindering clinical translation. This point is supported by the observation that five clinical trials of AAV-factor IX vectors for the treatment of hemophilia B have been conducted or currently are in progress, while none have been initiated for rAAV-FVIII vectors for testing in persons with HA.

To overcome these obstacles, several advancements in AAV-FVIII vector design have been reported. For example, Lu *et al* described an rAAV-FVIII construct incorporating a  $\beta$ -actin promoter with a cytomegalovirus enhancer and a bovine growth hormone poly(A) sequence that expressed FVIII at levels 3-5 fold higher than a comparable AAV construct utilizing a mini-transthyretin promoter with a synthetic poly(A) sequence [131]. The use of these large regulatory control elements resulted in a final transgene size of 5.8kb, which, similar to rAAV-HCR-ET3, was substantially oversized. Despite having no detectable vector genomes larger than 5.0kb, the optimized vector developed by Lu *et al* provided correction of FVIII levels in HA mice at a dose

Figure 2.6



**Figure 2.6: Phenotypic correction of the bleeding diathesis.** HA mice treated with rAAV-HCR-ET3 were subjected to a tail transection bleeding assay to test for phenotypic correction. For clarity, mice are grouped by vector dose: High ( $8 \times 10^{12} - 2 \times 10^{13}$  vp/kg), medium ( $2 \times 10^{12} - 8 \times 10^{12}$  vp/kg) low ( $6.3 \times 10^{10} - 1 \times 10^{12}$  vp/kg) and no treatment control.

of  $2 \times 10^{11}$  vector genomes (vg)/mouse, or approximately  $8 \times 10^{12}$  vg/kg. Additionally, Arruda and colleagues recently described a minimally bioengineered hFVIII transgene delivered via an oversized (5.5 kb) rAAV vector achieving circulating FVIII expression up to 60% of normal at a dose of  $8 \times 10^{12}$  vg/kg [132]. This bioengineering approach incorporates a single amino acid substitution which eliminates a furin cleavage site within human FVIII to mimic what occurs naturally in the B domain of canine FVIII. This substitution conferred a 2.5-fold increase in secreted single chain FVIII form, greater specific activity and improved hemostatic potency. Lastly, Nathwani and colleagues have developed a novel rAAV-FVIII vector that is reduced in size to 5.2 kb through promoter and polyadenylation sequence engineering and encodes a codon-optimized BDD hFVIII transgene that also incorporates a bioengineered linker containing additional N-glycan sequence motifs [133]. The combination of these engineered elements resulted in a vector that mediated 10 to 20 fold higher expression of FVIII compared to the non-engineered vector. However, the exact mechanism(s) responsible for the improved expression were not determined.

In the present study, we sought to enhance the potency of rAAV-FVIII vectors through the integration of a fVIII molecule that has been bioengineered for more efficient cellular secretion, which is the known rate-limiting step in recombinant FVIII biosynthesis and presumably also exists in gene therapy settings. This novel approach may be complimentary to the advancements described above by others in the field and combinations of the technologies, such as 1) reduction in AAV genome size through the use of smaller gene regulation elements like the ones described by Nathwani and colleagues or 2) the incorporation of the canine B-domain substitution described by Arruda and colleagues or 3) the insertion of N-glycan motifs into the FVIII B-domain linker sequence, which could potentially result in additive or synergistic improvements in vector performance. Regardless of these speculations, the present study demonstrates that rAAV-HCR-ET3 can achieve curative levels of FVIII activity *in vivo* using a minimal dose of  $1 \times 10^{12}$  vp/kg, which represents an 8-fold reduction in vector dose needed to achieve curative levels of circulating FVIII compared to the results obtained by the groups of Lu and Arruda discussed above, and doses

comparable to that achieved with the vector developed by Nathwani and colleagues despite the substantially oversized nature of rAAV-HCR-ET3. As production of sustained therapeutic levels of FVIII remains a critical barrier to clinical gene therapy of HA and no recent transformational advances have been made in vector manufacturing or potency, transgene engineering approaches, such as that demonstrated by ET3, may provide the key innovation necessary to achieve clinical success. Furthermore, the reduced viral vector dose needed to achieve curative levels of FVIII represents a significant improvement in safety over other AAV-FVIII constructs through reduction in the viral antigen load delivered to the recipient, which previous and ongoing clinical trials have shown to initiate a cytotoxic immune response against transduced cells at vector doses as low as  $2 \times 10^{12}$  vp/kg [40].

Our laboratory has spent considerable effort studying the differential efficiency of FVIII biosynthesis, as well as other biochemical properties, of orthologous FVIII molecules. The knowledge gained has been applied to the bioengineering of enhanced FVIII molecules such as ET3/HP47 with markedly improved biosynthetic efficiency and increased stability following thrombin activation [117]. Previously, we have described the improved performance of ET3 and other similar high expression FVIII sequence element containing constructs over standard human BDD FVIII constructs in heterologous recombinant protein production systems as well as gene therapy applications involving  $\gamma$ -retroviral and lentiviral vector gene transfer into HSCs [116-118, 134-136]. However, the current study is the first report utilizing the high expression sequence elements of porcine FVIII to boost FVIII levels in a liver-directed AAV-mediated gene therapy application. Consistent with data we have reported previously, the ET3 transgene enabled 20-fold higher expression compared to that achieved using the BDD human FVIII transgene in transiently transfected HepG2 cells. Furthermore, the enhanced expression conferred by the ET3 transgene compared to the BDD human FVIII transgene was recapitulated *in vivo* via hydrodynamic plasmid injection, where ET3 achieved 20-fold higher levels of circulating FVIII activity.

The bioengineered design of ET3 can be compared in some regards to a strategy currently being pursued in AAV clinical gene therapy of hemophilia B where constructs encoding a naturally occurring human coagulation factor IX molecule termed factor IX-Padua exhibit greater specific procoagulant activity than normal human FIX [137], and thus also increased vector potency. In the current study, the construct modifications implemented were designed to enhance expression/secretion efficiency, a bioengineering strategy which similarly increases vector potency. However, unlike the factor IX-Padua mutation, which is a naturally occurring mutation, ET3 is a non-naturally occurring FVIII chimera that substitutes the high-expression sequence identified in rpFVIII into BDD hFVIII [117].

When inserted into the rAAV-HCR-hAAT vector backbone, the expected genome size was 5.9 kb, which substantially exceeds the 4.7 kb size of endogenous AAV genomes. It seems likely that further improvement in both titer and potency could be garnered by reducing the vector genome size similar to what was described previously by Nathwani and colleagues [133]. Unlike other, smaller, bioengineered vectors, such as that reported by the Nathwani group, molecular analysis of rAAV-HCR-ET3 revealed that there was no detectable full length transgene packaged into the vector [133]. Rather, consistent with previous reports of oversized vectors, molecular analysis of the rAAV-HCR-ET3 vector revealed that it packaged primarily as truncated transgene fragments, with no detectable viral transgenes larger than 5.0kb [108, 109] PCR analysis of the purified viral genomes revealed a packaging bias favoring the central portion of the transgene. This result is consistent with the current model of the AAV ssDNA transgene packaging from the 3' end of both sense and antisense strands, and, in the case of oversized transgenes, being truncated before packaging completely through a presently unknown mechanism. This model predicts that most viral particles will carry either a sense or antisense template both of which will contain A2-A3 domain transgene sequences, while the HCR-A1 transgene regions will only be carried by antisense coding viral particles, and the C1-C2-bGHPA transgene regions carried only by the sense coding viral particles. This differential prevalence of transgene region frequency, combined with the sub-

5.0kb smear of viral ssDNA detected by Southern blot analysis, suggests a heterogeneous population of partially encoding viral transgenes. It is possible, therefore, that intracellular reassembly of the anti-sense viral transgenes containing the complete HCR promoter region and truncated FVIII template may lead to the production of dysfunctional FVIII protein products, presenting a potential safety concern by exposing treated individuals to novel immunological epitopes.

Previous work by the Nathwani lab with a similarly sized AAV-FVIII vector failed to detect either persistent truncated FVIII transgene or truncated FVIII protein in mice treated with the oversized vector [133]. To alternatively investigate the presence of full-length FVIII mRNA in AAV treated mice, we performed RT-PCR analysis of mRNA transcripts (**Figure 2.3S**) in the livers of treated mice. This analysis showed the presence of the C2 domain of FVIII, an area found to be preferentially truncated in the packaged viral transgenes, which is only possible if the complete 5' sequence upstream of the interrogated 3' sequence region is present. These findings, coupled with the high specific activity of circulating FVIII in AAV-treated mice (**Figure 2.3f**), supports a model wherein, despite the truncated nature of the vector transgene, the majority of translated protein product contains the complete FVIII peptide sequence.

The differential prevalence of viral ssDNA transgene sequences in the product preparations necessitates considerations that must be taken into account when titering oversized rAAV vectors using PCR-based methods. The heterogeneous nature of the viral particles suggests that the quantity of a single region of ssDNA within the transgene may not be representative of entire viral genome. This brings to light an important safety consideration, as it has been shown in clinical trials that rAAV vector doses greater than  $2 \times 10^{12}$  vg/kg can induce acute liver toxicity due to capsid-targeted cytotoxic T cell-mediated destruction of transduced cells [40, 138, 139]. In the present study, rAAV-HCR-ET3 was titered by quantitative protein analysis of DNA-containing viral particles. This approach provides an accurate estimation of the number of viral particles that contain DNA in the preparation, but does not account for the regions of transgene DNA that has been packaged. As



there were no detectable full-length transgenes packaged within the rAAV capsids, titering oversized rAAV vectors by either determination of viral particle number by mass or optical density, or by use of qPCR amplicons directed to a single viral genome segment, does not describe the heterogeneous nature of the vector genomes in the preparation. This presents a significant challenge to vector product manufacturing, characterization, and quality metrics for clinical product development comprising oversized AAV vector genomes.

Proviral rAAV-HCR-ET3 genomes persisted in the livers of treated mice for the duration of the study, between 11 and 50 weeks, as determined directly by qPCR and indirectly by the continuous detection of circulating FVIII activity. While the vector copy numbers obtained in the present study are substantially lower than those seen in studies using similar vectors [132, 133], in the previous studies, cellular DNA was collected soon after AAV administration, between 6 and 17 weeks. In the present study, vector doses that were comparable to those used in the previous studies were collected as long as 48 to 50 weeks after vector administration. The low vector copy numbers in the present study may be explained by loss of episomal AAV DNA over this extensive time period.

Despite its fragmented packaging, rAAV-HCR-ET3 provided complete ( $\geq 100\%$  normal) correction of the FVIII deficiency at doses as low as  $2 \times 10^{12}$  vp/kg. Additionally, doses as low as  $1 \times 10^{12}$  vp/kg provided lower, but still curative FVIII activity levels. Finally, a minimally effective dose of  $5 \times 10^{11}$  vp/kg was identified based on the criteria of achieving a sustained therapeutic levels of FVIII between 0.02 – 0.12 units/ml for more than 10 weeks post-vector administration. In addition to measurement of plasma FVIII activity, phenotypic correction was demonstrated by hemostatic challenge using the tail clip bleeding assay, which demonstrated dose responsive correction of bleeding diathesis. Although an overall reduction in bleeding was observed that trended toward significance in terms of a quantitative correlation between blood loss and vector dose or plasma FVIII activity level, previous experience with this assay in our laboratory has shown that blood loss does not directly correlate to FVIII activity levels in plasma. Furthermore, as others have shown, it

frequently is observed that mice with levels of FVIII activity below the threshold of detection (1.6% of normal) fail to lose detectable amounts of blood. It is possible, therefore, that sub-detectable levels of FVIII activity may be sufficient for the correction of bleeding diathesis in this murine model.

Initial exposure to FVIII by persons with HA represents an immunologic challenge that can lead to the development of neutralizing humoral immunity in approximately 25% of persons with severe HA [110, 140]. Since recombinant AAV gene therapies are designed to provide a continuous source of the exogenous protein in treated individuals, this response presents a considerable safety and efficacy concern as these vectors are translated into human trials. Indeed, when administered to immune-competent macaques, recombinant AAV encoding a codon optimized BDD human FVIII molecule resulted in the formation of neutralizing antibodies to FVIII [133]. Furthermore, the incidence of anti-FVIII inhibitor formation in persons with HA is significantly greater than that of anti-FIX inhibitors in persons with hemophilia B [110]. Thus, immunogenicity remains among the most significant safety concerns for all new HA therapeutics including gene therapies [141, 142]. Despite the demonstrated safety in the ongoing clinical trial of scAAV2/8-LP1-hFIXco for the treatment of hemophilia B, these results may not be predictive of safety in a similarly designed clinical trial for AAV-FVIII vectors for the treatment of HA [143]. As there has not been a clinical trial of AAV gene therapy for HA completed to date, this issue can only be discussed at this time using preclinical data. In the present study, we utilized the C57BL/6 background exon-16 disrupted murine model of HA, a genetic background previously shown to produce higher anti-FVIII inhibitory antibodies than the Balb/c model of HA [144]. Using this model, 2 animals developed inhibitory inhibitors to ET3, as evidenced by loss of FVIII activity and confirmed by ELISA and Bethesda assays. The two mice that developed anti-FVIII immune responses received the highest doses of virus ( $2 \times 10^{13}$  and  $1 \times 10^{13}$  vp/mouse) and exhibited supraphysiological levels of circulating FVIII prior to inhibitor development. This finding suggests that in immune-competent individuals, there may be an upper vector dose limit imposed by both

the viral capsid antigen load and the plasma FVIII load (potency). The possibility remains that the unique porcine sequences known to be necessary for high-level expression in ET3 also conferred increased immunogenic risk and were the target of the anti-FVIII immune response observed. However, Lollar and colleagues recently performed a rigorous comparison of BDD rhFVIII and rpFVIII in the murine HA model, which revealed that the overall immunogenicities were similar with anti-A2 and anti-C2 constituting the majority of the inhibitors detected against each molecule [145]. An important safety consideration in light of the high expression porcine sequences present in ET3 is that both plasma-derived and now rpFVIII products have been used clinically, the former for more than two decades, to treat acute bleeding following the development of anti-hFVIII inhibitors in the settings of congenital and acquired HA [146, 147]. Therefore, there may be sufficient data available to support the risk/benefit case for transition to clinical safety (including immunogenicity) testing of ET3 delivered by AAV vector, lentiviral vector or intravenous infusion of the recombinant molecule.

As predicted by all previous gene transfer and recombinant protein expression studies, the ET3 transgene demonstrates high-level transgene product biosynthesis following liver-directed recombinant AAV delivery. The current data directly support the benefit of incorporating the high expression FVIII sequence elements encoded within ET3 into AAV gene transfer systems for treatment of HA. The recombinant AAV-HCR-ET3 vector provided a long-term source circulating FVIII activity and correction of bleeding diathesis at vector doses lower than those of other bioengineered FVIII variants despite its larger size, making ET3 an ideal candidate transgene for incorporation into smaller AAV vector systems. However, due to the immune response exhibited by some mice against FVIII, additional immune modulation may be necessary to prevent the development of humoral immune response to FVIII. Previous data from our lab has shown that *ex vivo* genetic modification of HSC using a lentivector gene transfer platform promotes immune non-responsiveness to FVIII [148]. While the immune nonresponsive profile induced by *ex vivo* HSC gene modification is desirable and this approach is predicted to be curative, the overall approach is

clinically more complex than a single peripheral vein infusion of recombinant AAV. Furthermore, the pre-conditioning regimens needed to attain therapeutic levels of cellular engraftment and protein expression pose additional risks. Therefore, it seems logical that different gene therapy strategies may be more amenable than others to application in distinct geographical, economically developed and clinically-advanced populations.

## **2.6 Acknowledgements**

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## 2.7 Supplemental Information

**Supplemental Table 2.1**

A2	GAAGATGGGCCAACTAAATCAGATCCGCGGTGCCTGACCCGCTATTACT CTAGTTTC
C2	CACCTCCAAGGGAGGAGTAATGCCTGGAGACCTCAGGTGAATAATCCA AAAGAGTG
bGHPA	GTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCCTGTCCCTTCCTAATA AAATG

**Supplemental Table 2.1: Biotinylated probes used for detection of AAV-HCR-ET3 viral genomes** A cocktail of biotinylated probes was used for detection of AAV-HCR-ET3 viral genomes during Southern blot analysis.

**Supplemental Table 2.2**

Name	Sequence	Amplicon Length (bases)
HCR +	TTCGGTAAGTGCAGTGGAAG	
HCR -	GTCCTCGTCCGTATTTAAGC	191
Porcine A1 +	CCTGAAGAACATGGCTTCTC	
Porcine A1 -	TACCGGGAAGGACTTTATCG	133
Human A2 +	CTCACGGAATCACTGATGTC	
Human A2 -	TTGGCCCATCTTCTACAGTC	136
Porcine A3 +	TGGAGCAGCTCTGGGATTAC	
Porcine A3 -	GCAAATTCCTGGGAAGACCAC	109
Human C1 +	TCAATGCCTGGAGACCAAG	
Human C1 -	AGATGTAGAGGCTGGAGAAC	119
Human C2 +	CCTCCAAGGGAGGAGTAATG	
Human C2 -	CATCTTGACTGCTGGAGATG	169
bGHPA +	CCTTCTAGTTGCCAGCCATC	
bGHPA -	CCAGCATGCCTGCTATTGTC	199

**Supplemental Table 2.2: Primers used for regional transgene analysis** Primer sets spanning the length of the AAV-HCR-ET3 viral transgene were used for quantitative PCR analysis of the packaged ssDNA content of AAV-HCR-ET3 viral particles.

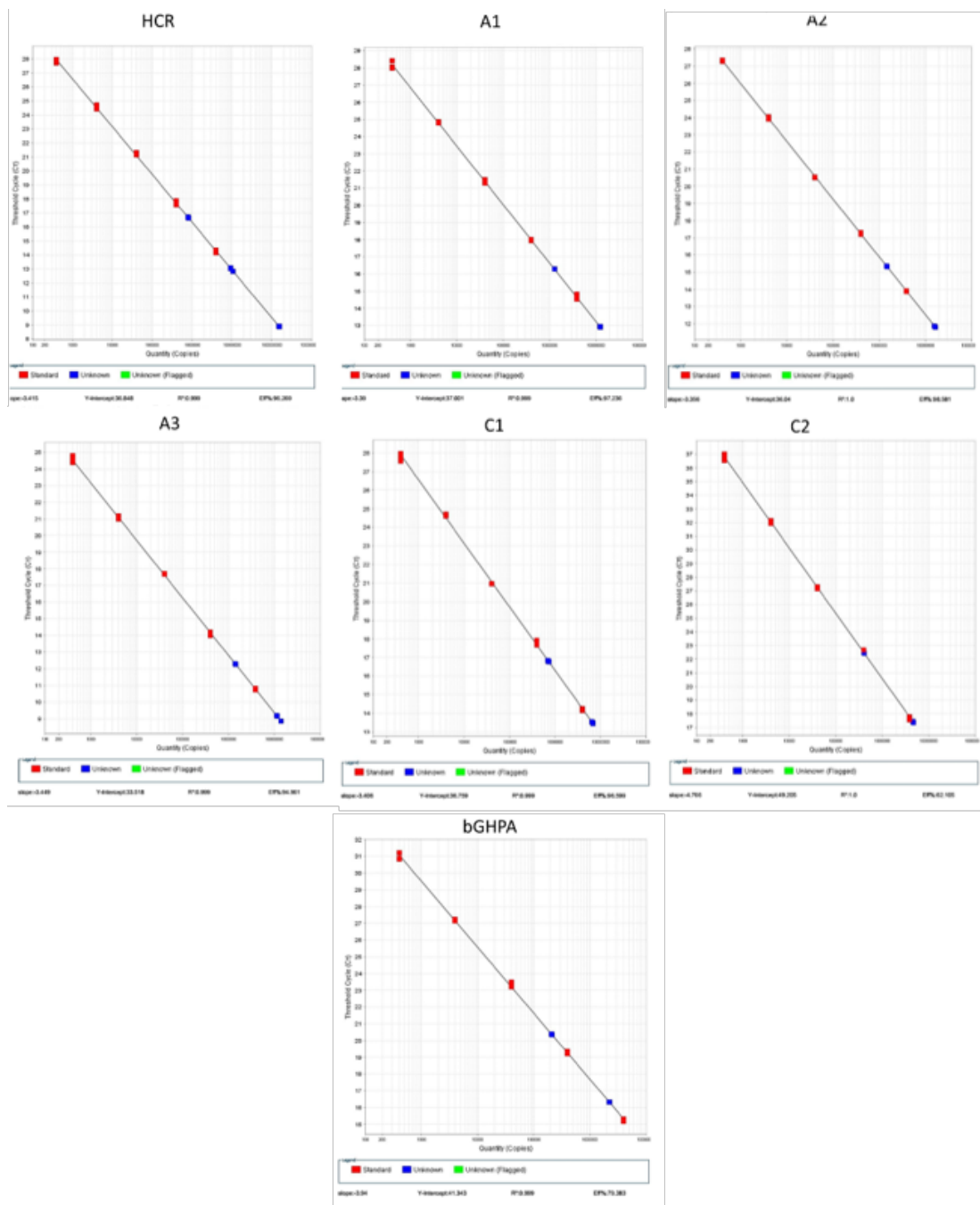
**Supplemental Table 2.3**

Mouse ID	Plasmid	Injection Time (seconds)	fVIII activity (U/mL)
355N	AAV-HCR-ET3	8	3.74
356R	AAV-HCR-ET3	10	7.80
357R	AAV-HCR-ET3	10	3.46
356L	AAV-HCR-HSQ	8	0.333
357N	AAV-HCR-HSQ	10	0.146
358L	AAV-HCR-HSQ	7	0.202

**Supplemental Table 2.3: Hydrodynamic injection of FVIII encoding AAV**

**expression plasmids** Mice were hydrodynamically injected with either AAV-HCR-ET3 or AAV-HCR-HSQ encoding expression plasmids. Individual injection times and resulting FVIII activity at 24 hours are shown.

## Supplemental Figure 2.1

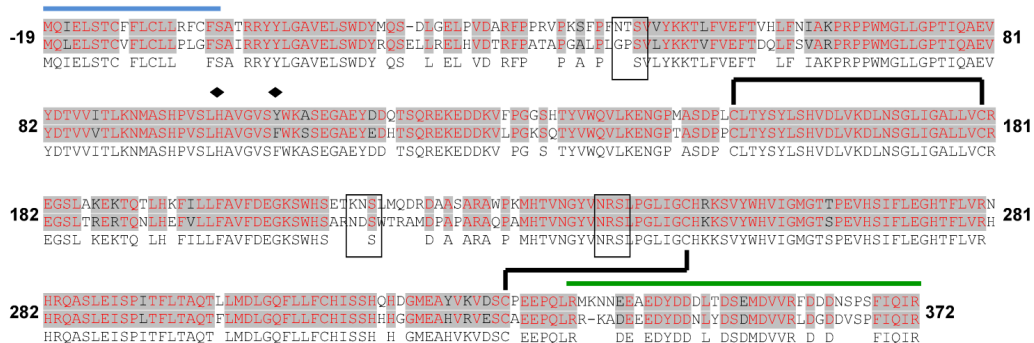


**Supplemental Figure 2.1: Standard curves for quantitative PCR analysis** To control for variation in primer efficiency during quantitative PCR analysis, standard curves of AAV-HCR-ET3 viral expression plasmid were generated for each primer set spanning the length of the AAV-HCR-ET3 transgene.

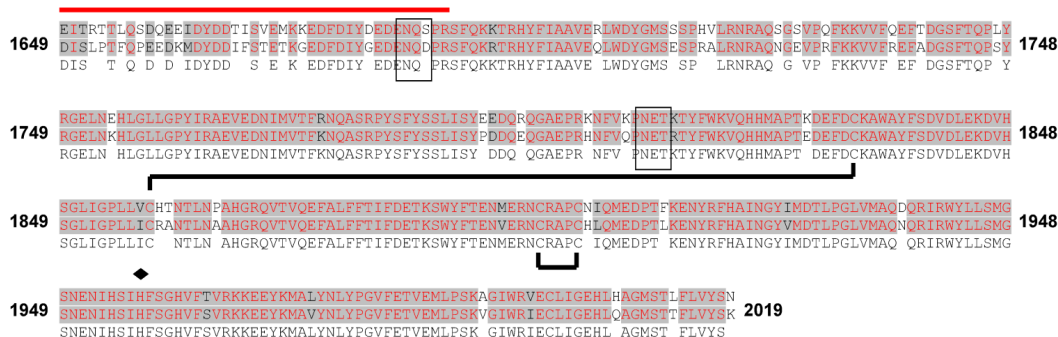


## Supplemental Figure 2.2

## A1 domain



## A3 domain



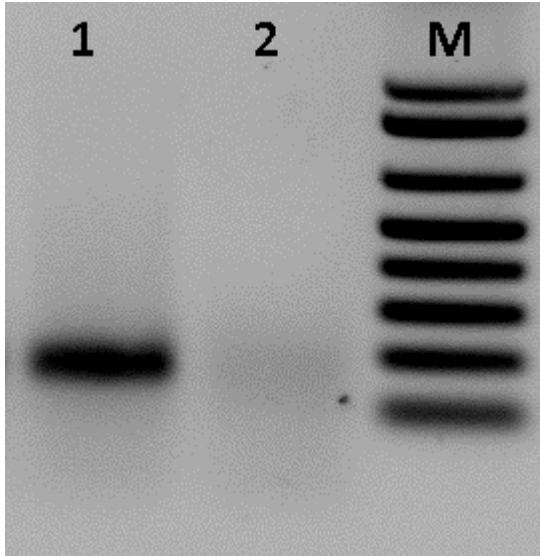
## Legend:

- signal peptide
- heavy chain acidic domain
- activation peptide
- disulfide linkages
- ◆ metal ion binding residues
- predicted N-linked glycan attachment sites

FVIII	Identity
Human	100%
ET3	91%

**Supplemental Figure 2.2: Sequence alignment of porcine-substituted domains of ET3 and HSQ** Amino acid sequence alignments for the signal peptide (black bar), A1 domain, heavy chain acidic domain (green bar), activation peptide (red bar) and A3 domain of human (top) and ET3 (bottom) fVIII are shown. Identical residues are distinguished by red type with gray background, similar residues are shown black type with gray background and all other residues are displayed in black type with transparent background. Disulfide

linkages are noted by the black lines connecting cysteine residues. Places where either human, ET3 or both sequences encode a N-linked glycosylation attachment site (N-X-S/T) are outlined with a black box. Additional amino acid differences in the synthetic linker sequence in ET3 and HSQ are not shown.

**Supplemental figure 2.3**

**Supplemental figure 2.3: ET3 C2 domain sequence RNA is present in liver of treated mice** Reverse transcription PCR analysis of RNA isolated from livers of treated and untreated mice shows ET3 C2 domain sequence in a mouse treated with AAV-HCR-ET3 (lane 1) and no detectable ET3 C2 domain sequence in untreated control (lane 2).

### Chapter 3

#### **Immunogenicity is a Barrier to Liver-Directed AAV Gene Therapy of Hemophilia A**

This research was published in *Molecular Therapy Methods & Clinical Development*.

Lytle AM, Brown HC, Paik N, Knight K, Wright JF, Spencer HT, Doering CB.

#### **Immunogenicity as a Barrier to Liver-Directed AAV Gene Therapy of Hemophilia A**

*Molecular Therapy Methods & Clinical Development*. 2016. 3: 15056.

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Allison Lytle performed and designed the experiments, and wrote and edited the paper

Harrison Brown performed and designed experiments

Fraser Wright and Shangzhen Zhou produced and characterized AAV virus

NaYoon Paik and Kristopher Knight performed experiments

Trent Spencer and Chris Doering conceived the experiments and edited the paper

### 3.1 Abstract

Immune responses to FVIII and FIX present as obstacles to clinical intervention of HA and B, respectively. Previously, we showed that HSC retroviral vector gene therapy induces immune tolerance to FVIII in both naïve and pre-immunized settings. Liver-directed AAV-FIX vector gene transfer has achieved similar results in preclinical hemophilia B models. However as clinical immune responses to FVIII and FIX differ, we investigated the ability of liver-directed AAV-FVIII gene therapy to affect FVIII immunity. Both FVIII naïve and pre-immunized animals were administered recombinant AAV8 encoding a liver-directed bioengineered FVIII expression cassette. Naïve animals receiving high or mid doses subsequently achieved near normal FVIII activity levels. However, challenge with adjuvant-free recombinant FVIII induced loss of FVIII activity and anti-FVIII antibodies in mid dose AAV, but not high dose AAV or comparator HSC lentiviral vector (LV) gene therapy cohorts. Furthermore, unlike what was shown previously for HSC LV gene therapy, AAV-FVIII administration to HA inhibitor mice conferred no effect on anti-FVIII antibody or inhibitory activity titers. These data suggest that functional differences exist in the immune modulation achieved by gene therapy approaches incorporating liver-directed AAV vectors as compared to HSC-directed LV.

### 3.2 Introduction

HA and B are X-linked recessive bleeding disorders that result from decreased synthesis or functionality of FVIII and FIX, respectively. They are clinically characterized by prolonged and at times spontaneous bleeding in the joints and soft tissues resulting in significant hemarthropic morbidity. Left untreated, severe hemophilia (A or B), as defined by <1% circulating FVIII or FIX activity, is uniformly lethal. The primary therapeutic option is protein replacement therapy with optimal results being obtained through prophylaxis consisting of 2 – 3 injections per week of 20 – 50 International Units (IU)/kg of plasma-derived or recombinant (r) human (h) FVIII or FIX. Although nearly equivalent on a unit activity basis, the mass equivalents are very different with a typical FVIII product dose containing 2 – 5 µg/kg FVIII while a FIX product dose contains 100 –

250 µg/kg FIX. HA and B differ clinically in terms of the immune responses observed against protein replacement products. Pathogenic inhibitors against FVIII develop in up to 33% of persons with severe HA while anti-FIX inhibitors occur in only 3% of individuals with severe hemophilia B. [149] The immune responses also differ in the observation of anaphylactoid reactions and nephrotic syndrome in the setting of FIX, but not FVIII inhibitors. ITI has been shown to successfully eradicate inhibitory antibodies in 63-100% of treated HA patients, however, the patient inclusion criteria for this treatment are limiting and the cost easily can exceed \$1,000,000 USD per patient.[150] Although the exact mechanism of action of ITI is not well understood, preclinical studies suggest administration of high doses of FVIII inhibits the restimulation of FVIII-specific memory B cells and prevents their differentiation into antibody secreting plasma cells.[151] Depletion of FVIII-specific memory B cells may deplete efficient antigen presenting cells and shift re-stimulation of T<sub>eff</sub> cells to induction of T<sub>regs</sub>. T cell anergy, anti-idiotypic antibodies and suppressor T cells are other possible mechanisms suggested to play a role in successful ITI.[152-154] Gene therapy offers not only the potential for a cure of FVIII and FIX deficiencies, but also the opportunity to modulate both naïve and primed immune systems through both central and peripheral tolerance mechanisms.[105, 155-158] For example, in vivo production of FVIII and FIX achieved through gene transfer approaches could be an efficient and cost effective modality for ITI.

AAV and lentiviral LV vectors have become leading strategies for clinical gene transfer due to their target cell transduction capabilities, limited toxicity, and ability to confer high level therapeutic transgene expression. Safety and clinical efficacy are being demonstrated in the setting of several blood cell disorders using autologous transplantation of ex vivo LV vector transduced HSC.[27, 28] HSCT gene therapy involves the *ex vivo* transduction of autologous HSCs followed by transplantation into an HSC depleted (i.e. 'conditioned') recipient. HSCs are efficiently transduced using integrating viruses such as  $\gamma$ -retroviruses and HIV-1 based LV vectors allowing for replication of the proviral genome during cell division. As genetically modified HSCs undergo both self-renewal and differentiation, they create a reservoir of transgene-expressing cells that

persist in the bone marrow compartment for the lifetime of the individual and amplify the transgene sequence within the recipient up to  $10^6$ -fold. Additionally, as the HSC progeny undergo cellular differentiation, the immune system is repopulated with genetically modified cells of the myeloid and lymphoid lineages that now promote recognition of the therapeutic transgene product as a “self” protein within the host proteome.

In addition to clinical trials of HSCT gene therapy, multiple trials are underway for *in vivo* delivery of liver-directed AAV vectors encoding FIX.[37, 159] AAV gene therapy is less invasive in that it requires a single peripheral infusion of vector into an unconditioned patient. As the packaged viral vector reaches its target tissue, cells undergo *in vivo* transduction resulting in episomal transgene expression and secretion of transgene product into the periphery. AAV gene transfer is being developed for the treatment of a variety of diseases including hemophilia B, human alpha-1 antitrypsin deficiency, lysosomal storage disease and certain forms of congenital blindness.[160-164] Several groups currently are in the late-preclinical stage of both LV and AAV vector-based approaches for the treatment of HA and clinical trials are anticipated. [165] As clinical gene therapy trials designed to evaluate the efficacy and toxicity associated with FVIII gene transfer are eminent, understanding the immunological consequences ranging from establishment of tolerance to the development of pathogenic inhibitors is of practical concern.

Previously, we demonstrated correction of the bleeding phenotype in FVIII naïve HA mice using either *in vivo* liver-directed AAV gene transfer or *ex vivo* HSC-targeted LV gene transfer incorporating bioengineered high expression FVIII transgenes. [47, 48, 89, 166] Furthermore, *ex vivo* HSC-directed LV gene therapy was shown to both eradicate anti-FVIII inhibitors and restore circulating FVIII levels to HA mice with pre-existing immunity to FVIII.[49, 166] Recently, two groups showed that liver-directed expression of FIX, achieved using either LV or AAV vectors, could promote the disappearance of pathological inhibitory antibodies in mouse models of hemophilia B with pre-existing immunity to human FIX. [157, 158] Both IgG and IgE anti-FIX antibodies were eliminated following AAV-FIX gene therapy and this correlated with the

establishment of therapeutic levels of circulating FIX antigen. Additionally, treated animals demonstrated therapeutic correction of the bleeding phenotype as well as inhibition and elimination of anti-FIX reactive B cells and plasma cells. Given that the immune responses to FVIII and FIX differ in several aspects, herein, we sought to investigate the durability of the apparent immune tolerance achieved in the FVIII naïve gene therapy setting, assess the capability of liver-directed gene therapy for HA to modulate pre-existing immunity to FVIII, and draw comparisons between the immune consequences of liver-directed versus HSC-directed gene therapy.

### 3.3 Materials and Methods

*Animals Experiments.* All animal studies were performed under the guidelines set by the Emory University Institutional Animal Care and Use Committee. Exon 16 disrupted mice backcrossed onto a C57BL/6 background were used as a model of HA [167]. In the HSCT gene therapy experiments, 6 – 8 week old transgenic mice expressing GFP from the  $\beta$ -actin promoter on a C57BL/6 background were used as congenic donors of HSCs [168] TBI was administered using a vented chamber within Gammacell 40 Extractor. Immunizations and immunological challenges using purified FVIII were performed by retro-orbital injection. Lastly, AAV administration was delivered intravenously to 8-10 week old mice by tail-vein injection.

*Whole Bone Marrow Transplants.* Transplant recipients received 11Gy TBI split over 2 doses 4 hours apart. Whole bone marrow was isolated from 6-8 week old congenic GFP positive transgenic mice. Cells were flushed from the femurs and tibias and filtered to eliminate debris. After 10 minutes of red cell lysis, whole bone marrow cells were resuspended in sterile PBS, counted, and 5 million cells per mouse were immediately infused, via retro-orbital injection, into transplant recipients.

*Sca-1<sup>+</sup> Enriched Bone Marrow Transplants.* Whole bone marrow was enriched for Sca-1<sup>+</sup> antigen as previously described using positive immunomagnetic bead selection [50]. Cells were then stimulated for 24 hours in media containing murine stem cell factor (100ng/mL), murine interleukin-3 (20ng/mL), human interleukin-11 (100ng/mL) and human Flt-3 ligand (100ng/mL).



After stimulation, cells were transduced twice in half volume (MOI between 16 and 70) virus at a density of 2 million Sca-1<sup>+</sup> cells/mL over the course of 24 hours. Cells were then resuspended in PBS and one million transduced cells were injected retro-orbitally into lethally irradiated (11Gy TBI) naïve HA mice.

*LV-FVIII Production.* Viral accessory plasmids along with the bioengineered high expression FVIII transgene (designated ET3) encoding expression plasmid were transiently transfected in 293T-17 cells using a calcium phosphate transfection method to generate LV vectors pseudotyped with the VSVG envelope similar to the method described previously except for the utilization of the calcium phosphate transfection reagent (Sigma Aldrich, St. Louis, MO). [169] Conditioned media was collected for three days beginning at 48 hours post transfection and passed through a 0.45µm filter. Virus was concentrated by overnight centrifugation at 10,000 x g, followed by filtration using a .22 µm filter. Viral concentrate titers were determined using quantitative real time PCR analysis.

*AAV-FVIII Production.* DNA fragments encoding either the 5' AAV ITR proximal to the hybrid liver specific promoter (HLP) or a synthetic rabbit beta-globin polyadenylation signal and 3' ITR were synthesized de novo (Integrated DNA Technologies and Genescript) and introduced to the flanking ends of the ET3 transgene by restriction enzyme digestion/ligation-based molecular cloning techniques to generate AAV8-HLP-ET3 (5,111 base pairs). (**Supplementary Figure 3.1**) [102]. The complete sequence is available as Supplementary Figure 1. Recombinant AAV8/2 vector particles carrying the HLP-ET3 cassette were produced as previously described [170]. Briefly, 293T cells were triple transfected by the calcium phosphate method with the AAV8-HLP-ET3 cassette, adenovirus helper plasmid AdHP, and AAV8 packaging plasmid AAV8PK [171, 172]. 72 hours after transfection, the cells were harvested, lysed, and clarified by centrifugation. Vector particles were precipitated using 8% polyethylene glycol, re-suspended and purified using two round of cesium chloride gradient ultracentrifugation. Vector particles were dialyzed into PBS

supplemented with 0.001% pluronic F68 and quantified by quantitative SDS-PAGE densitometry against a standard reference vector.

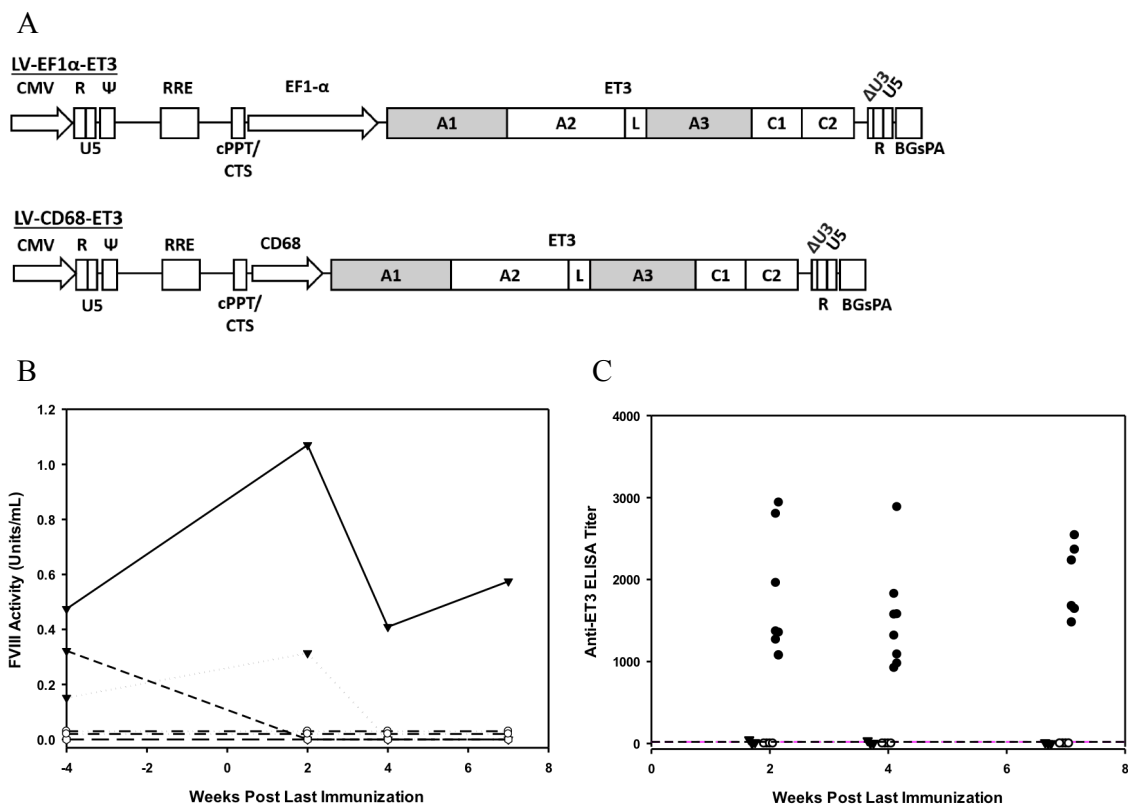
*FVIII and Immune Response Analyses.* Anti-FVIII antibody titers were determined using an ELISA. Immobilized hFVIII (Recombinant) or ET3 was bound to microtiter plates and then incubated with mouse plasma. Anti-fVIII antibodies were detected using a pan goat-anti mouse IgG secondary antibody conjugated to alkaline phosphatase and absorbance was read at 405nm. The absorbance values of test plasmas were plotted against the logarithm of the plasma dilution, and the titers were determined to be the reciprocal of the dilution in which the OD value was three times that of the background, or an OD reading of 0.3. [173] Inhibitory antibody titers were determined using a Bethesda assay as previously described. [174, 175] Titers were determined as the average dilution of test plasmas in which 50% of the FVIII activity was inhibited. Lastly, FVIII activity was determined using a commercially available COATEST SP FVIII assay (Coatest SP, Diapharma) according to the manufacturer's instruction. Activity was quantified using a standard curve generated from pooled citrated human plasma (Georgia King, Overland Park, KS).

*Molecular Studies.* DNA and RNA were isolated from livers perfused for 15 minutes with saline using the DNeasy Blood and Tissue Kit (Qiagen) and the RNeasy mini kit (Qiagen) respectively, according to the manufacturer's instructions. All qPCR and data analysis was performed on a Step-One real time PCR platform using the device's software. Copy number was determined using a standard curve generated from dilutions of AAV8-HLP-ET3 plasmid. Standards or 100ng of sample DNA was added to 1x SYBR Green PCR Master Mix (Life Technologies) containing 300 nM concentrations of forward and reverse primers. Copy number was normalized to the number of mammalian diploid genomes present in 100 ng of DNA. Similarly, mRNA transcript levels were determined on AAV treated liver tissues as described previously using 100 ng of sample RNA. [90]

### **3.4 Results**

*Immune tolerance to FVIII achieved through LV HSCT gene therapy.* Previously, our group showed that retroviral transduction and transplantation of HSCs under both myeloablative and non-myeloablative conditioning using a high expressing FVIII transgene produced sustained, curative plasma FVIII levels in a murine model of HA. [48-50] Furthermore, HSCT gene therapy recipient animals were shown to be immunologically non-responsive to repeated weekly injections of recombinant FVIII as evidenced by long-term FVIII expression, the absence of anti-FVIII antibody production, and apparent T-cell tolerance in co-culture activation assays. [49]. However, most of these studies were performed using  $\gamma$ -retroviral vectors with intact LTRs driving expression of a porcine FVIII transgene. Due to risk of insertional mutagenesis by  $\gamma$ -retroviruses with intact LTRs and the potential immunogenicity of the porcine FVIII transgene, our recent studies were modified to incorporate a SIN HIV-1-based LV vector encoding the bioengineered ET3 FVIII transgene. **(Figure 3.1a)** We further addressed immunologic tolerance induced under transplantation conditions resulting in a mix of modified and non-modified cells, or under a condition of unimolecular microchimerism, which we define by low-level engraftment of genetically-modified autologous cells (microchimerism with <10% genetically-modified peripheral blood mononuclear cells) expressing a single transgene product (unimolecular neoantigen) that is absent from the host proteome. For the current study, HA mice were transplanted with genetically modified *sca-1*<sup>+</sup> cells isolated from congenic C57Bl/6 animals. Donor *sca-1*<sup>+</sup> cells were transduced with SIN-LV-ET3 vector containing either a cell type-independent, constitutively active promoter derived from the human *EEF1A1* gene locus, designated SIN-LV-EF1 $\alpha$ -ET3, or a myeloid-restricted promoter generated from the human *CD68* locus, designated SIN-LV-CD68-ET3. One million *sca-1*<sup>+</sup> enriched transduced cells were injected retro-orbitally into lethally irradiated HA mice to ensure engraftment. Modeling unimolecular microchimerism, all transplanted animals displayed <0.1 transgene copy per diploid genome equivalent (N = 6; mean VCN (peripheral blood) less than the level of detection; < 0.06 proviral genomes per diploid genome equivalent). At these low levels of engraftment, animals transplanted with SIN-LV-CD68-ET3 transduced cells displayed measurable

Figure 3.1



**Figure 3.1 Immune tolerance to FVIII achieved through LV HSCT gene therapy:** A) Schematic of vector design. B) Baseline FVIII activity was determined in (▼) LV-CD68-ET3 and (◦) LV-eF1 $\alpha$ -ET3 HSCT treated mice prior to immunological challenges and subsequently measured after 4 weekly immunizations of 1 $\mu$ g recombinant ET3. C) Anti-FVIII IgG titers were measured by ELISA and are shown for (▼) LV-CD68-ET3 and (◦) LV-eF1 $\alpha$ -ET3 HSCT treated mice following the final immunization as well as (●) the immunized controls. All mice received 4 weekly injections of 1 $\mu$ g of recombinant ET3 post-transplantation. The data represent 2, 4, and 7 weeks post final immunization.

levels of circulating FVIII prior to immunological challenge. **(Figure 3.1b)** At 10 weeks, a series of weekly immunological challenges consisting of intravenous injections of 1 $\mu$ g of highly purified recombinant FVIII, were initiated. Under these conditions, none of the transplanted animals developed measurable ELISA titers 7 weeks after immunization. Conversely, all of the control mice developed robust immune responses to FVIII that persisted beyond 7 weeks post immunization. **(Figure 3.1c)** Despite low to undetectable VCN or levels of plasma FVIII activity, transplanted animals displayed an attenuated immune response compared to immunized controls. These results extend our previous studies showing unimolecular chimerism of genetically-modified cells, even at very low levels, is adequate for establishment of transplantation tolerance.

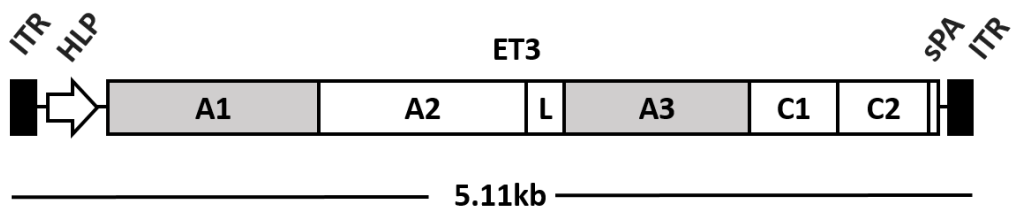
*Dose dependent tolerance achieved through AAV-FVIII in the naive HA setting.* We then wanted to compare the level of tolerance achieved with HSCT to that achieved through AAV-directed gene transfer. Liver-directed AAV-FVIII vectors have shown preclinical efficacy in several animal models of HA. [102, 176, 177] However, these studies have been limited in sample size (e.g. canine and non-human primate studies), duration (< 8 weeks), or have utilized immune deficient (e.g. CD4-deficient) animals to obviate immune complications. Therefore, the risk of inhibitor development and the durability of immune tolerance achieved in the absence of inhibitors has yet to be established in any preclinical model. In an earlier study, we demonstrated that clinically-relevant doses of AAV8 vector encoding a liver-directed, APO E hepatic control region enhancer-human  $\alpha$ -1 anti-trypsin promoter, expression cassette containing a bioengineered FVIII transgene, designated ET3, conferred correction of the FVIII deficiency and bleeding phenotype in HA mice despite being oversized, i.e. larger than the estimated packaging capacity of recombinant AAV8.[47] However, we also observed that a small fraction of experimental animals developed inhibitory antibodies to FVIII 8 – 12 weeks after vector administration. This occurred only in the highest dose cohorts and occurred despite the presence of supraphysiological FVIII levels immediately prior to inhibitor development. To investigate this observation further in the current study, naïve HA mice were treated with either a mid ( $4 \times 10^{12}$  vp/kg) or a high ( $2 \times 10^{13}$  v/kg)

dose of a promoter shortened, liver-directed AAV8-HLP-ET3 vector. **(Figure 3.2a)** Plasma FVIII activity was monitored for 24 weeks. AAV dose dependent expression was observed with the mid dose cohort averaging 70% normal human levels (0.7 IU/ml) and the high dose cohort averaging 200% normal levels (2 IU/ml) that was sustained for the 5 month observation period. **(Figure 3.2b and c)** Subsequently, the animals were challenged with a series of intravenous injections of recombinant ET3 (1 µg each) in the absence of adjuvant. FVIII activity in the mid dose cohort declined to undetectable levels and corresponded with the appearance of robust anti-ET3 humoral immune responses as detected by anti-FVIII IgG ELISA. **(Figure 3.2d)** Overall, animals treated with high dose AAV8-ET3 displayed a trend toward a transient decline in FVIII activity during the immunization period. However, FVIII levels did not completely decrease to baseline and subsequently increased following termination of the recombinant FVIII infusion challenge protocol. These data suggest that higher doses of AAV8-ET3 are capable of establishing and maintaining active tolerance, while lesser doses may be insufficient.

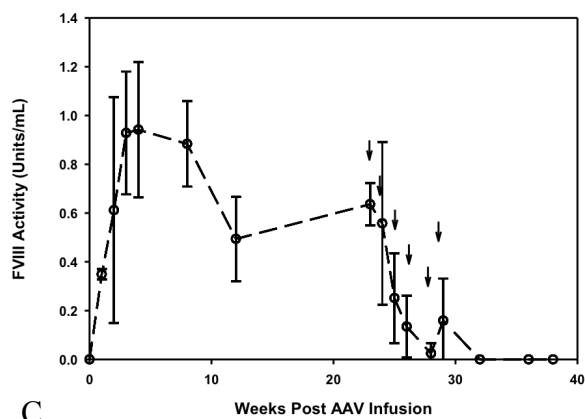
*Efficacy of AAV-FVIII in the setting of pre-existing immunity.* Liver-directed expression of FIX following AAV or LV gene transfer has been shown to eliminate pre-existing high-titer inhibitory antibodies in a mouse model of hemophilia B.[157, 158] While two different viral vector systems were utilized, similar results were obtained with both approaches affecting a decrease in anti-FIX ELISA and Bethesda titers after vector administration. Additionally, each group showed that liver-directed expression resulted in the depletion of FIX-specific memory B cells and plasma cells. In an attempt to extend these findings to HA, we evaluated the therapeutic potential of the AAV8-HLP-ET3 vector in HA mice with pre-existing immunity to FVIII. In the current study, HA mice were pre-immunized with commercially available recombinant hFVIII via 4 weekly injections of 1µg FVIII each. Baseline humoral immune responses were characterized by both total anti-FVIII IgG ELISA titers and anti-FVIII inhibitory (Bethesda) titers. Upon confirmation of anti-FVIII immunity in 100% of the animals, they were administered AAV8-HLP-ET3 at a dose ( $4 \times 10^{12}$  vp/kg) previously shown to restore FVIII to near normal human levels in circulation. Subsequently,

Figure 3.2

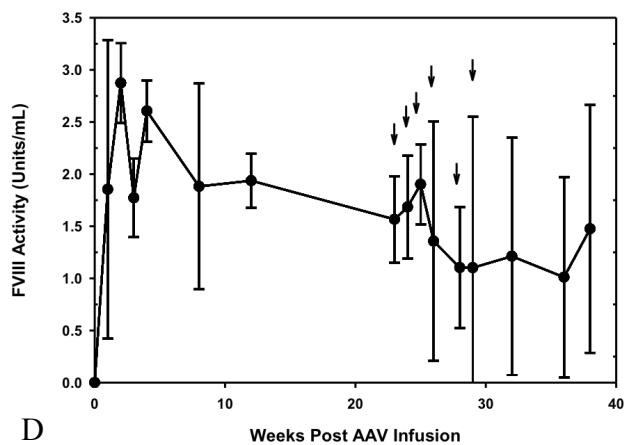
A



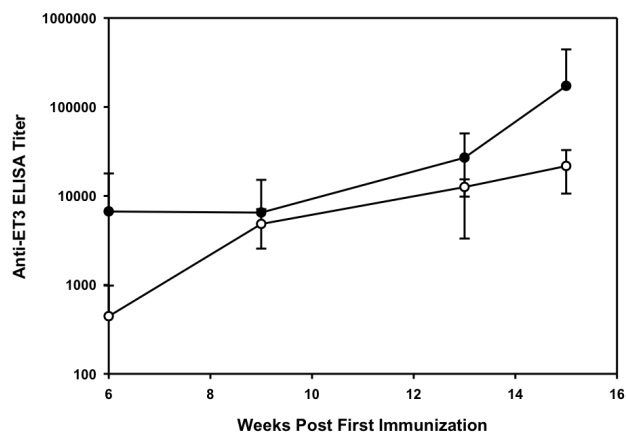
B



C



D



**Figure 3.2 Dose dependent tolerance achieved through AAV-FVIII** A) Schematic of vector design. B and C) FVIII activity was measured beginning at two weeks post AAV8-HLP-ET3 ( $4 \times 10^{12}$  vp/kg or  $2 \times 10^{13}$  vp/kg) infusion. Arrows indicate weekly immunizations of 1ug recombinant ET3. D) Anti-ET3 ELISA titers are shown beginning at one week post initial immunization. (●) Naïve HA mice were treated with  $4 \times 10^{12}$  vp/kg AAV8-HLP-ET3 followed by weekly immunological challenges of 1ug recombinant ET3 while (◦) the immunized controls did not receive vector.

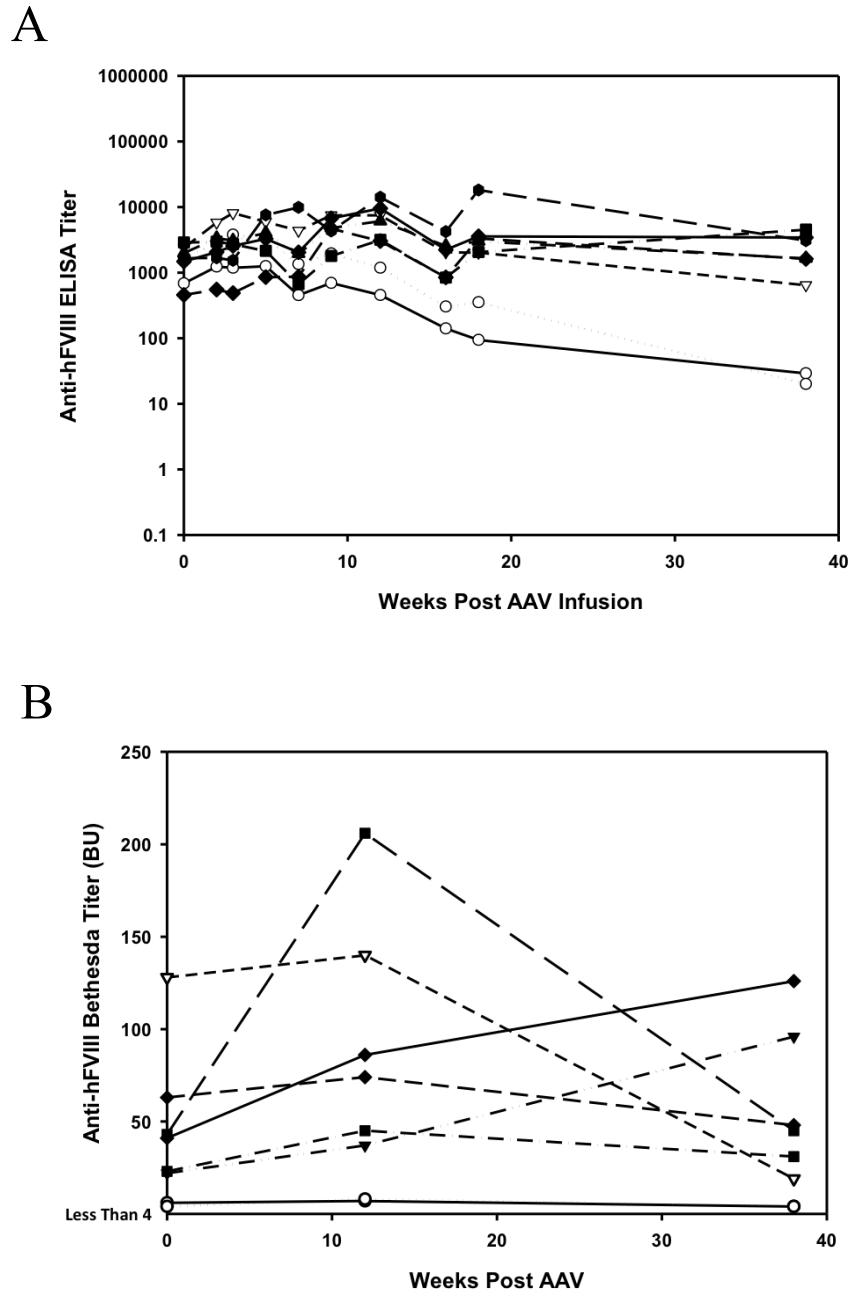


the animals were monitored for 16 weeks during which time no significant changes in anti-FVIII ELISA or Bethesda titer were observed. **(Figure 3.3a and b, Supplementary Figure 3.2)**

A confounding variable of the above experiment was the sequence disparity between the FVIII immunogen and the FVIII transgene product. The immunogen consisted of full-length recombinant hFVIII product possessing B domain sequence of varying length established by heterogeneous intracellular processing by PACE/furin. Conversely, the transgene product is B domain-deleted ET3 that has approximately 9% amino acid sequence disparity to B domain-deleted hFVIII present in the A1, activation peptide and A3 domains of the protein. The immune consequences of this sequence disparity in the setting of pre-existing immunity are unknown and may have limited the immune modulation possible through this approach. For example, it is possible that the immunogenic determinants of hFVIII may differ significantly from ET3, and therefore liver synthesized ET3 is not capable of inhibiting or eradicating hFVIII immunogen specific memory B cells or plasma cells. Therefore to test this possibility, the experiment was repeated using animals pre-immunized with recombinant ET3. However again, no change in anti-FVIII ELISA or Bethesda titers was observed in animals pre-immunized with ET3. **(Figure 3. 4a and b, Supplementary Figure 3.3)**

Given that AAV8-ET3 administration did not modulate pre-existing anti-hFVIII or ET3 immunity at the total IgG or inhibitor levels and no circulating ET3 antigen or activity was observed at any time point, three alternative explanations appeared possible. The first is that the pre-existing immunity to FVIII somehow prevented AAV-FVIII transduction of hepatocytes. The second is that the adaptive immune system eliminated the transduced cells through a cytotoxic immune response. Similar observations to these have been made in clinical trials of AAV-FIX for hemophilia B where it has been shown that plasma from individuals containing anti-AAV antibodies can impair transduction of mouse liver through AAV neutralization and that CD8<sup>+</sup> T-cell mediated clearance of transduced hepatocytes frequently occurs 8 – 12 weeks post vector administration when AAV-FIX doses equal to or exceeding  $2 \times 10^{12}$  vp/kg are tested.[38, 178]

Figure 3.3

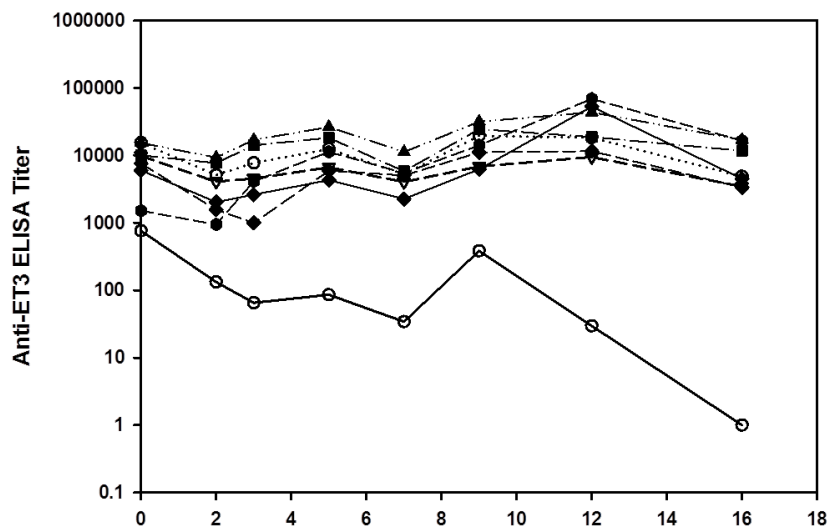


**Figure 3.3 Efficacy of AAV-ET3 in the context of pre-existing immunity to hFVIII: A)** Individual anti-hFVIII ELISA titers for (closed shapes) the naïve HA preimmunized with hFVIII and treated with  $4 \times 10^{12}$  vp/kg of AAV8-HLP-ET3 vector and (open shapes) the preimmunized

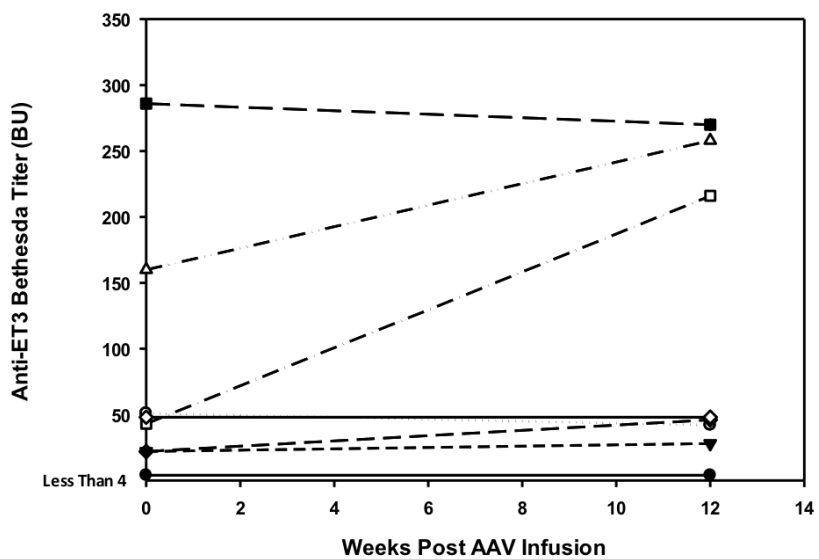
controls. B) Anti-hFVIII Bethesda titers in (closed shapes) the naïve HA mice preimmunized with hFVIII and treated with  $4 \times 10^{12}$  vp/kg of AAV-HLP-ET3 vector and (open shapes) the preimmunized controls.

Figure 3.4

A



B

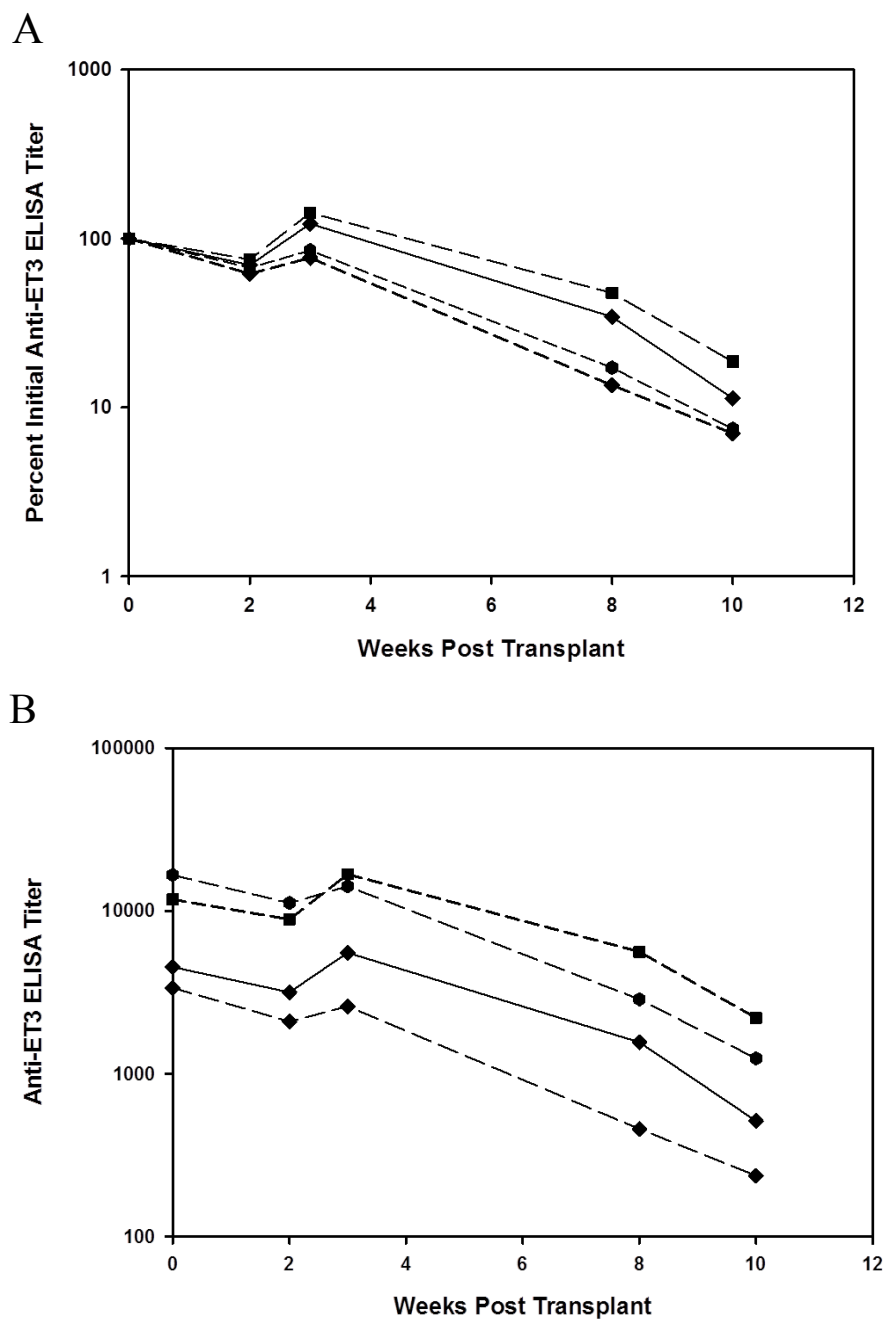


**Figure 3.4: Efficacy of AAV-ET3 in the context of pre-existing immunity to ET3** A) Individual anti-ET3 ELISA titers for (closed shapes) the naïve HA preimmunized with ET3 and treated with  $4 \times 10^{12}$  vp/kg of AAV8-HLP-ET3 vector and (open shapes) the preimmunized controls. B) Anti-

ET3 Bethesda titers in (closed shapes) the naïve HA mice preimmunized with ET3 and treated with  $4 \times 10^{12}$  vp/kg of AAV-HLP-ET3 vector and (open shapes) the preimmunized controls.

Since laboratory mice generally do not possess anti-AAV neutralizing antibodies and CD8<sup>+</sup> T cells responses against transduced hepatocytes also are not observed in murine models, we hypothesized a third scenario wherein hepatocytes were effectively transduced by AAV-FVIII and subsequently biosynthesized ET3 molecules that were rapidly neutralized upon cellular secretion by the saturating levels of circulating anti-FVIII inhibitory antibodies. In order to test this hypothesis, AAV treated animals harboring pre-existing and ongoing humoral immunity to ET3 received lethal doses of radiation (11Gy TBI) to pharmacologically eliminate antigen specific effector T-cells and B-cells, as well as possibly some antibody secreting plasma cells which are known to be more radiation resistant.[179] Previously, we demonstrated that HSCT gene therapy in the setting of 11Gy TBI eradicated pre-existing anti-FVIII humoral immunity, provided sustained circulating FVIII activity and established robust immune non-responsiveness to both the immunogen, hFVIII, and the transgene product, porcine FVIII or ET3.[49, 166] In the current study we sought to detect genetically-modified hepatocytes and circulating transgene product (ET3) in the context of humoral immunity. A similar approach was used but instead incorporated transplantation of non-genetically modified donor HSCs to help ‘reset’ or minimally dampen the pre-existing anti-FVIII immune status. Successful transplantation and engraftment of donor HSCs was confirmed by flow cytometry and determined to be approximately 90% donor cell engrafted (data not shown). Animals were monitored for 10 weeks post-transplantation during which time a gradual decrease in anti-FVIII antibody titers was observed by ELISA. **(Figure 3.5a and b)** However despite the decrease in anti-FVIII IgG titer, plasma FVIII activity remained below the level of detection by chromogenic substrate assay in all animals. As a more direct approach to identify genetically modified hepatocytes expressing ET3, the experimental animals were euthanized and primary liver cells were assayed for ET3 transgene DNA copy number as well as ET3 RNA transcript levels. **(Figure 3.6)** The positive detection and quantitation of ET3 transgene DNA and mRNA transcripts in liver tissue supports the conclusion that primary liver hepatocytes were transduced in vivo by administration of the AAV vector and that the transgene cassette

Figure 3.5



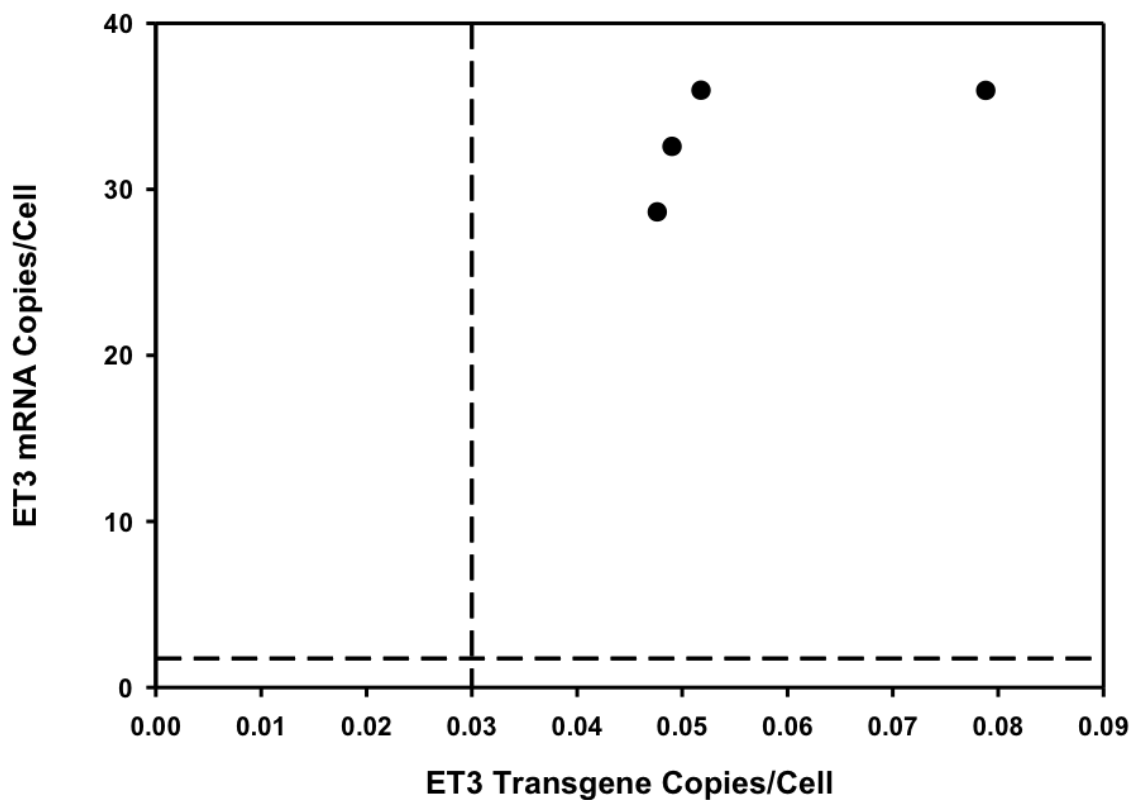
**Figure 3.5: Rescue HSCT of AAV-ET3 treated mice with anti-ET3 inhibitory antibodies A)**

Anti-ET3 IgG titers were measured by ELISA in naïve HA mice pre-immunized with ET3 and then treated with  $4 \times 10^{12}$  vp/kg AAV8-HLP-ET3 vector post-transplantation. Mice were condition with

11Gy TBI and then transplanted with 5 million whole bone marrow cells. B) Depicted are the individual anti-ET3 IgG titers measured by ELISA.



Figure 3.6



**Figure 3.6 Quantification of transgene and mRNA copies per cell in the livers of AAV treated mice** Copy number and mRNA levels were determined in hepatocytes isolated from the perfused livers of naïve HA mice pre-immunized with ET3 and treated with  $4 \times 10^{12}$  vp/kg AAV8-HLP-ET3 vector.

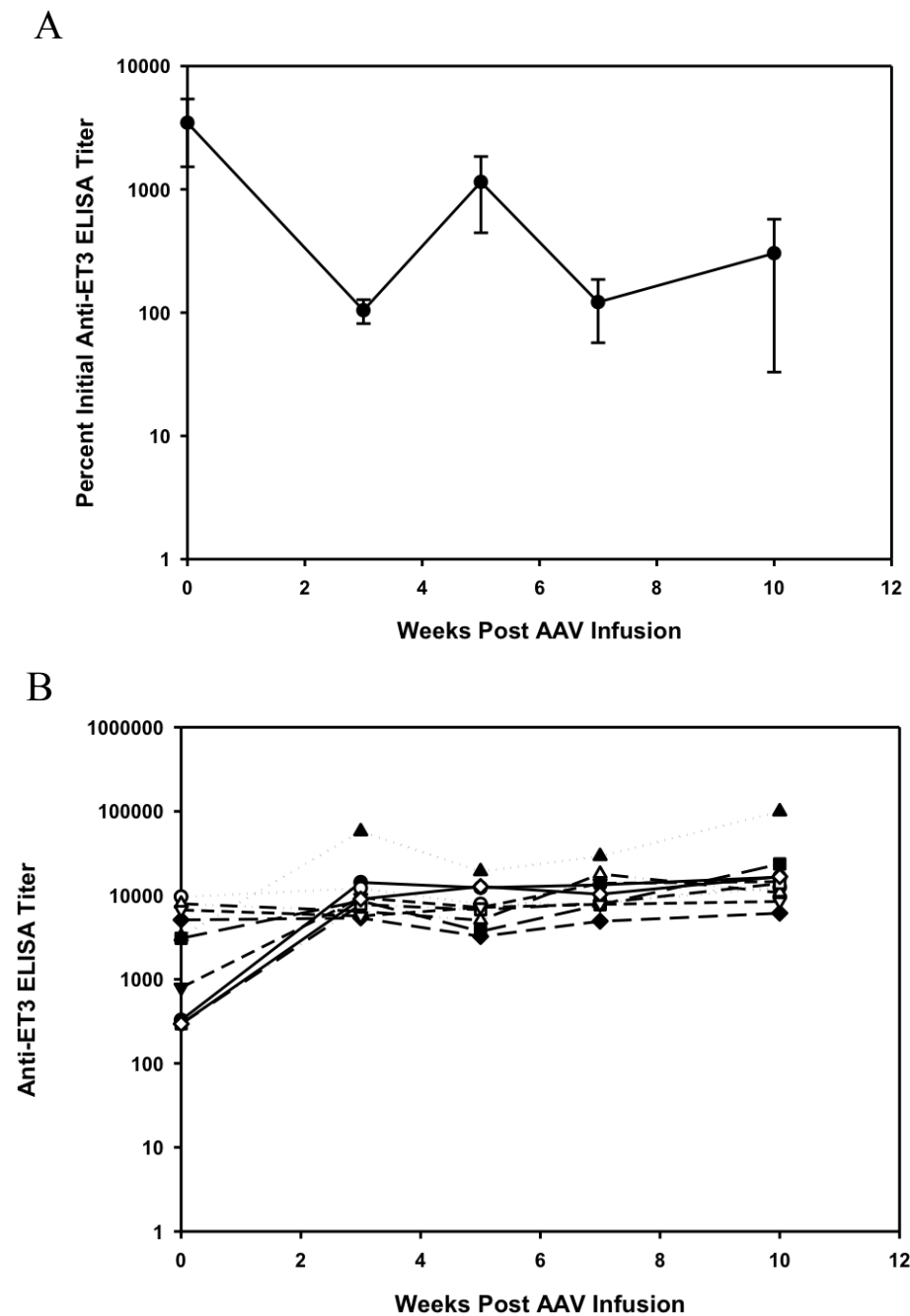
delivered remained functional throughout the experiment despite the presence of ongoing FVIII immunity.

*Efficacy of AAV-FVIII in the setting of low titer anti-FVIII immunity.* Liver-directed AAV-FVIII also has been shown to overcome pre-existing anti-FVIII antibodies in a canine model of HA harboring low anti-FVIII inhibitory titers ( $\geq 3$  BU).[99] As the pre-existing inhibitory titers to ET3 in our earlier experiment ranged from 46 – 270 BU and would be considered in the high range, it is possible that an immune threshold exists linking inhibitor titer and the ability of AAV8-HLP-ET3 to modulate the anti-FVIII immune response. In order to investigate this possibility, HA mice (n=10) were immunized by weekly injection of recombinant ET3 with concomitant monitoring of the anti-FVIII humoral immune response. After 3 immunizations, all mice had measurable ELISA titers and Bethesda titers ranging from 0 – 25 BU. Subsequently, the animals were administered  $4 \times 10^{12}$  vp/kg of AAV8-HLP-ET3 and monitored biweekly for changes in ELISA titer. (**Figure 3.7a and b**) Again, however, no significant differences were observed between the low titer cohort compared to the two previous high titer cohorts suggesting that a pre-existing immune threshold does not exist in HA mice.

### 3.5 Discussion

HA remains a candidate disease for clinical gene therapy. The therapeutic threshold of achieving 10 – 50 pM circulating levels of transgene product (i.e. FVIII) is generally predicted to be achievable with current gene transfer technology. Although FVIII replacement therapy certainly is effective at maintaining hemostasis in most cases, the products are short acting, challenging to administer prophylactically and prohibitively expensive for the majority of patients. Gene therapies have the potential to overcome these challenges and transform the care of persons with HA. Of the available clinical gene transfer technologies, LV and AAV vectors appear to be at the forefront. [165, 180-182] Possibly the most significant unresolved issue for these approaches, other than demonstration of therapeutic success, is the effect FVIII gene transfer-based therapy will have on anti-FVIII immunity as anti-FVIII immune responses represent the most significant

Figure 3.7



**Figure 3.7 Efficacy of AAV-FVIII in the setting of low-titer anti-FVIII immunity** A) Anti-ET3 IgG titers are represented as the percent of the initial titer determined by ELISA in mice with low initial anti-ET3 Bethesda titers (0-25 BU). Naïve HA mice were pre-immunized with ET3 and treated with  $4 \times 10^{12}$  vp/kg of AAV8-HLP-ET3 vector. B) Depicts the individual anti-ET3 IgG titers

measured by ELISA in mice with low initial anti-ET3 Bethesda titers. Mice were pre-immunized with ET3 and treated with  $4 \times 10^{12}$  vp/kg of AAV8-HLP-ET3 vector.

challenge to state of the art clinical care. The patient population targeted in all gene therapy of HA clinical trials to date has been those previously treated with FVIII replacement products without evidence of existing or prior anti-FVIII immunity. This patient exclusion criteria may preclude the acquisition of data regarding the ability of FVIII gene transfer to modulate tolerance and/or immune activation as well as the prediction of outcomes for non-tolerized patients. It has been shown in several model systems, as well as clinically for other diseases, that gene transfer can either elicit new immune responses or alternatively induce a tolerogenic state depending on the platform used and disease setting. HSCT gene transfer typically involves the transplantation of LV transduced HSCs genetically modified to express a missing or defective protein, which may present as a neo-antigen depending on the underlying causal mutation. As these cells repopulate the immune system, the resulting chimerism facilitates transplantation tolerance, an immunological state in which the host immune cells are not activated by the neo-antigen but maintain the ability to launch a pathogen-specific immune response. [103] Contrary to HSCT gene therapy, AAV vectors are more efficient at in vivo targeting of non-hematopoietic tissues such as liver parenchyma and then have the ability to take advantage of the liver's role in immune homeostasis to facilitate tolerance. This hypothesis has been substantiated in preclinical hemophilia B studies in which AAV vectors incorporating a FIX transgene have been shown to induce transgene specific regulatory T-cells and inhibit reactive T-cell responses. [106] Although clinical deficiencies in FVIII and FIX are indistinguishable, the proteins themselves are not homologous and the respective immune responses to them are quite dissimilar with FVIII demonstrating more potent immunogenicity at lower concentrations. These confounding differences suggest that gene transfer facilitated tolerance may differ in terms of durability when incorporating a FVIII transgene in the context of HA gene therapy.

We previously showed and demonstrate again in the current study, that both HSCT LV gene therapy and liver-directed AAV gene therapy incorporating a high-expression FVIII transgene can elevate plasma FVIII activity levels into the normal range. [47, 89] However in both studies, anti-FVIII inhibitor formation occurred under certain experimental conditions. For HSCT LV gene

therapy, we discovered that pre-transplantation radiation or other pharmacological-based T cell suppression is critical to the avoidance of an immune response to FVIII post-transplantation. [48] Furthermore, we demonstrated that these same animals are immunologically non-responsive to intravenous challenge with recombinant FVIII. In these studies, we utilized earlier generation  $\gamma$ -retroviral vectors encoding a high expression porcine FVIII transgene. Therefore to evaluate tolerance in the context of a state of the art clinical HSCT LV gene therapy platform, naïve HA mice were transplanted with sca-1<sup>+</sup> enriched bone marrow cells transduced using an LV vector expressing ET3 via a universal (SIN-LV-EF1 $\alpha$ -ET3) or myeloid specific (SIN-LV-CD68-ET3) promoter. All transplanted animals engrafted with >80% total donor cells and less than 10% genetically-modified cells in the peripheral blood mononuclear cell population. Interestingly, baseline FVIII activity prior to immunological challenges was only detectable in SIN-LV-CD68-ET3 transduced sca-1<sup>+</sup> cell treated animals and apparently was not a determinant of transplantation induced FVIII tolerance. After four weekly intravenous injections of recombinant FVIII, transplanted animals were assayed for the development of anti-FVIII immune responses. SIN-LV-EF1 $\alpha$ -ET3 transduced sca-1<sup>+</sup> cell treated mice had no measurable ELISA titer at any time for the duration of the experiment. Although transient, near baseline titers were observed in two of the SIN-LV-CD68-ET3 transduced sca-1<sup>+</sup> cell treated animals, the immune responses observed in the HSCT gene therapy cohorts spontaneously resolved. Cell mediated clearance of genetically-modified cells is unlikely as transgene copies were detected in several tissues at 7 weeks with higher transgene copy numbers observed in the SIN-LV-CD68-ET3 cohort which is consistent with the higher MOI used for transduction of the sca-1<sup>+</sup> HSC stem and progenitor cells. These data suggest that unimolecular chimerism of genetically modified cells in the myeloid compartment is sufficient to facilitate FVIII specific transplantation tolerance following HSCT LV-FVIII gene therapy. Specifically, CD68 expression has been confirmed in both thymic and liver tissues, both of which are known for their roles in T-cell education and T<sub>reg</sub> induction, respectively, suggesting a potential mechanism for CD68 mediated transplantation tolerance.[183]

In the previous AAV-FVIII gene transfer studies, we did not evaluate specifically the durability of tolerance induction and/or whether reintroduction of FVIII protein could break tolerance, which is a potential clinical outcome. Additional studies demonstrating that AAV liver-directed expression of coagulation factor IX prevents inhibitor formation after peripheral infusions of FIX concentrate in multiple animal models of hemophilia B suggest that a similar beneficial outcome may result from AAV-FVIII gene therapy. [184, 185] In the FIX studies, tolerance was induced using a dose observed to evade an anti-capsid CD8<sup>+</sup> T-cell response in previous clinical trials. However in our experiments, this dose of AAV8-HLP-ET3 (4e12 vg/kg) was not shown to be tolerogenic as evident by the observation of rapid, high titer FVIII-specific inhibitory antibody formation following serial challenges with adjuvant-free recombinant FVIII. Even at relatively high doses (2 x 10<sup>13</sup> vp/kg), AAV8-HLP-ET3 was unable to completely inhibit a humoral immune response against FVIII during recombinant FVIII challenge, reinforcing the hypothesis that significant differences in immunogenicity exist between FVIII and FIX. These observations may be associated with the tendency of FVIII to engage in the unfolded protein response, perhaps contributing to its immunogenicity.[82] Furthermore, there is a significant difference between the specific activities of FIX versus FVIII. A single unit of FIX has a plasma concentration of approximately 5000 ng/mL while a single unit of FVIII only has a plasma concentration between 100-200 ng/mL. At steady state, liver-directed expression of FIX results in antigen molar concentrations order of magnitude greater than FVIII and could therefore modulate the response of antigen presenting cells and immune effector cells differently. These effects may be amplified with AAV-FVIII compared to AAV-FIX as both expression levels and liver-directed tolerance are shown to be vector-dose-dependent. [105] Our results parallel these conclusions in that only the very high dose of AAV8-HLP-ET3 (2 x 10<sup>13</sup> vp/kg) was able to attenuate inhibitor formation during rigorous, but clinically modelled, immunological challenges with recombinant FVIII. *Herzog et al.* have shown that adoptive transfer of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T-cells from AAV-FIX treated mice can suppress anti-FIX antibody formation in naïve and subsequently immunologically challenged

mice. [105, 106] However, it is important to note that for all of these studies, analysis of Treg responses, adoptive transfers experiments, and analysis of Foxp3 induction were performed in immune-competent F9 ( $+/+$ ) mice, in which self-tolerance exists against murine FIX. As our knowledge of Treg biology expands, the literature has established that natural Tregs (nTregs) originating in the thymus and responsible for maintaining self-tolerance are selected for through the interaction of peptides bound to MHC and T-cell receptors expressed on the surface of nTregs. [186] Given that CD4<sup>+</sup> T-cell epitopes have been demonstrated to be conserved between species, it is possible that nTreg-specific epitopes could be conserved between orthologs. [187] Therefore, nTregs responsible for maintaining self-tolerance to murine FIX may also contribute to CD4<sup>+</sup> CD25<sup>+</sup> Treg responses observed in AAV-FIX studies using F9 ( $+/+$ ) immune-competent mice. Subsequently, it is plausible that the AAV-Treg induction paradigm may not be applicable to mouse models of hemophilia in which self-tolerance to the murine ortholog may not be present.

In contrast to reducing product immunogenicity, elimination of pre-existing immune responses is a distinct therapeutic objective. Since, both liver-directed AAV and LV vector-based gene therapy have been shown to eliminate circulating inhibitory antibodies and transgene specific memory B-cells in pre-immunized hemophilia B mice [157, 158], a logical extension is that liver-directed FVIII gene transfer may provide therapeutic benefit to HA patients with inhibitors. Furthermore, this represents a critical un-met clinical need as long term treatment options are limited or not available to the vast majority of the HA inhibitor patient population. Therefore in addition to studying liver-directed AAV gene therapy in the FVIII naïve state, we also analyzed the therapeutic potential of AAV8-HLP-ET3 in pre-immunized HA mice harboring both low and high FVIII inhibitory titers. We observed no significant changes in either ELISA titer or Bethesda titers over the course of 16 weeks suggesting no modulation of anti-FVIII antibody secreting cells or FVIII-specific T-cell responses. Both copy number and mRNA analysis confirmed transduction of hepatocytes and expression of the transgene, which likely was being instantly neutralized by the circulating inhibitors once secreted from the cell.



Overall, the results of the current study again highlight the differences in both the immune response to FVIII and FIX and the immune consequences of liver-directed as compared to HSC-directed gene transfer. Unlike what has been shown for liver-directed FIX expression in hemophilia B mice, liver-directed FVIII gene therapy in both the naïve and pre-immunized setting did not tolerize HA mice to exogenous infused recombinant FVIII. However, similar to our previous results, in the current study HSC-directed LV-ET3 gene therapy again was effective at promoting immune non-responsiveness to serial challenges with exogenous recombinant FVIII. Therefore, despite both ET3 gene transfer approaches delivering sustained, curative levels of plasma FVIII activity in HA mice without the initial provocation of anti-FVIII inhibitors, only the HSC-LV approach appears to have a major effect on FVIII immunity. Although HSC-LV gene therapy does require pre-conditioning for HSC engraftment, this component also has clear suppressive effects on the immune system that likely contribute to its success. However, even with stringent pre-conditioning AAV8-HLP-ET3 gene transfer was unable to efficiently eradicate an anti-FVIII humoral immune response. Therefore, there clearly are differences in therapy related immune consequences. Another caveat of the current non-clinical studies is that the line of HA mice used demonstrate uniform inhibitor responses following 6 or less intravenous infusions of exogenous FVIII. This is in stark contrast to the human clinical situation where inhibitor frequency is less than 0.3 in severe HA and significantly less in moderate and mild cases. As all clinical hemophilia gene therapy studies to date have been conducted in the previously treated population without inhibitors, the current studies cannot model this situation. However, as clinical trials move forward towards the treatment of FIX or FVIII naïve patients or patients with pre-existing immunity, the current results suggest that differences may be observed between the HA and B setting as well as the success of HSCT LV compared to AAV gene therapy.

### **3.6 Acknowledgements**

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### 3.7 Supplemental Information

**Figure 3.1**

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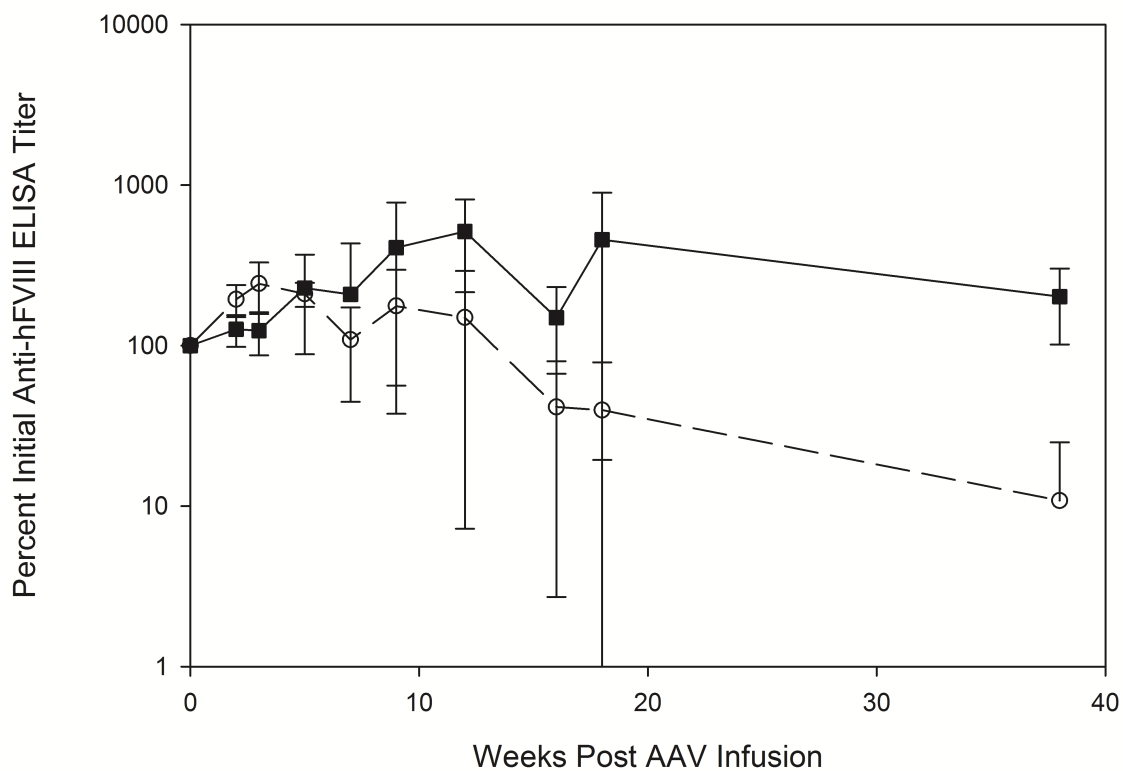
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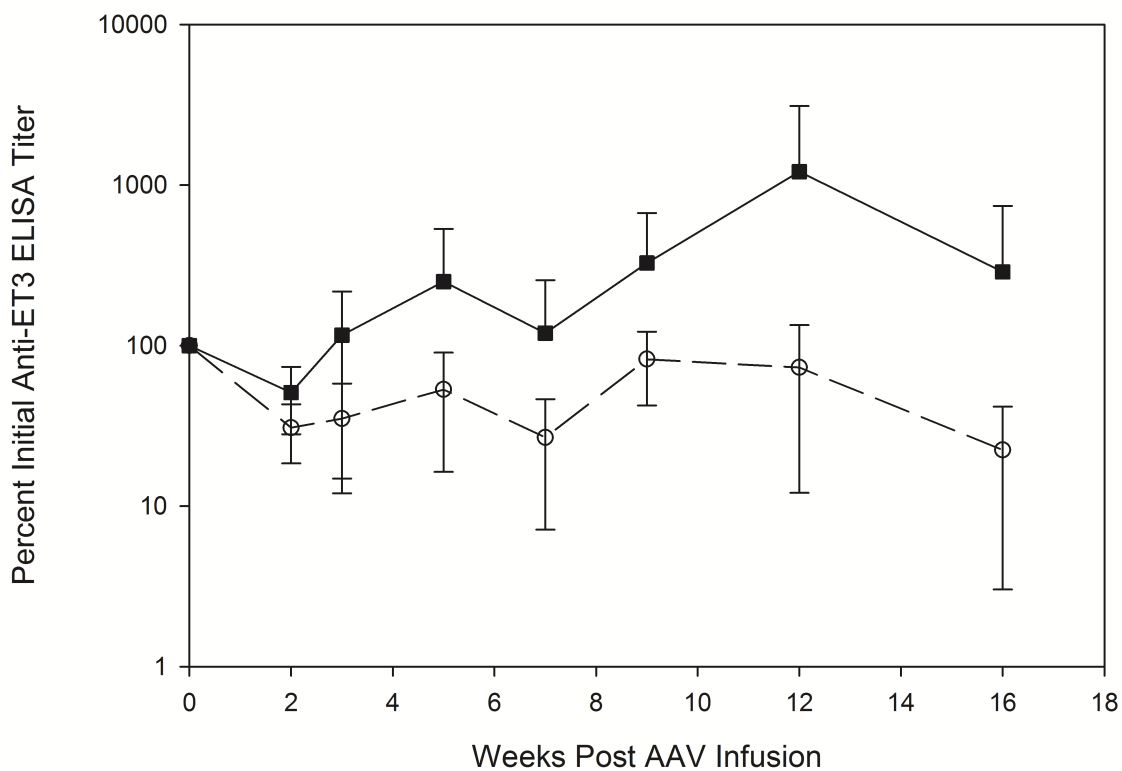
**Figure 3.1 : AAV-HLP-ET3 expression cassette sequence**

Figure 3.2



**Figure 3.2 Mean percent of initial titers in mice treated with AAV-ET3 with pre-existing immunity to hFVIII.** Anti-hFVIII IgG titers are represented as the percent of the initial titer determined by ELISA. (Closed shapes) Naïve HA mice were pre-immunized with hFVIII and treated with  $4 \times 10^{12}$  vp/kg of AAV8-HLP-ET3 vector while (open shapes) the pre-immunized controls did not receive vector.

Figure 3.3



**Figure 3.2 Mean percent of initial titers in mice treated with AAV-ET3 with pre-existing immunity to ET3.** Anti-ET3 IgG titers are represented as the percent of the initial titer determined by ELISA. (Closed shapes) Naïve HA mice were pre-immunized with hFVIII and treated with  $4 \times 10^{12}$  vp/kg of AAV8-HLP-ET3 vector while (open shapes) the pre-immunized controls did not receive vector.



## Chapter 4

### **Developing an Immunotoxin Based Preparative Regimen for HSCT Gene Therapy for the Treatment of Hemophilia A**

Allison Lytle performed and designed the experiments, and wrote and edited the chapter

Kristopher Knight performed experiments

Shanmuganatham Chandrakasan conceived and designed experiments

Trent Spencer and Chris Doering conceived the experiments and edited the paper

#### 4.1 Abstract

Transplantation of genetically-modified stem cells is becoming a new paradigm of treatment for rare genetic disorders in which patients do not survive adolescence. ADA-SCID is the first monogenic immune disease curable by an approved gene therapy product shown to be efficacious in pediatric patients. This treatment represents a major milestone for HSCT gene therapy not only because of its curative potential but also because it reduces the risks of the current standard of care. HSCT gene therapy involves the transplantation of autologous cells eliminating the risks of graft versus host disease. However, this method of gene transfer requires the depletion of bone marrow cells prior to transplantation in order to facilitate engraftment of gene-modified cells. Chemotherapeutic alkylating agents are profoundly ablative in the bone marrow compartment and used clinically as a preparative regimen for stem cell transplantation. However, these drugs are also associated with toxicities including hepatic veno-occlusive disease, thrombocytopenia, secondary malignancies, and sterility. Developing non-genotoxic drugs that can effectively ablate HSCs is the single most important obstacle to overcome as the field pushes to expand this therapy to other less severe monogenic diseases. In this study, we evaluated the use of a saporin based immunotoxin targeting the CD45 cell surface receptor present on hematopoietic cells as a conditioning agent in a mouse model of HSCT gene therapy for HA.

#### 4.2 Introduction

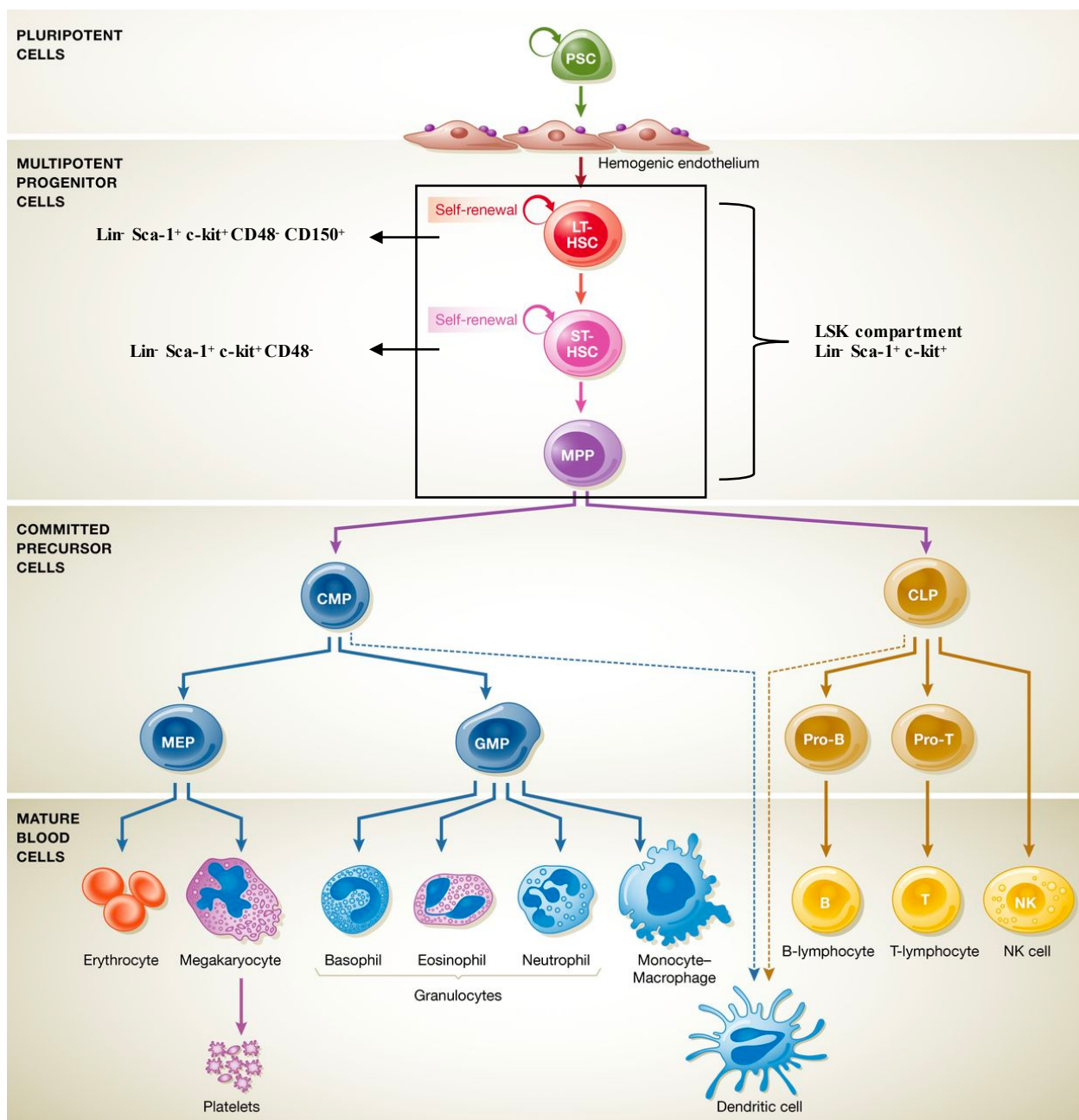
The concept of HSCT was born out of necessity during the era of the Atomic Bomb. Scientists and physicians were looking for a way to protect people from the ionizing lethal effects of radiation exposure. In 1955, Main and Prehn reported that skin allograft rejection could be overcome with high doses of X-radiation in conjunction with a bone marrow transplant.[188] This landmark publication was one of two studies confirming that cells within bone marrow compartment could confer protective properties against radiation. These cells would become known as HSCs and are defined by their ability to undergo self-renewal in addition to hematopoiesis, the process of differentiating into mature blood and immune cells. (**Figure 4.1**) The

observations of Main and Prehn would revolutionize treatment for hematological and oncological diseases, giving physicians the ability to treat patients more aggressively or provide cell therapy options for lethal hematological malignancies.[189]

HSCT is used to treat hematopoietic failure in patients with a variety of diseases. Once transplanted into a patient, HSCs can hone to their hematopoietic niches and re-establish hematopoiesis, a process referred to as engraftment. However, successful engraftment in patients requires the use of a preparative (conditioning) regimen in order to make space for new cells within the hematopoietic compartment and in some cases provide immune suppression or anti-cancer therapy. Typical conditioning agents include total body irradiation or a cocktail of chemotherapeutics that are genotoxic and can provide cytotoxic effects against non-dividing cells. Intensity of conditioning is largely dependent on the donor source, autologous (self) or allogeneic (non-self), as well as the underlying disease. Furthermore, the overall health status of the patient must also be taken into consideration as preparative regimens are associated with both acute and chronic toxicities, most notably viral infections, sinusoidal obstruction syndrome, sterility, and secondary malignancies.[190]

HSCT gene therapy has curative therapeutic potential for inherited non-malignant diseases such as hemoglobinopathies, metabolic disorders, and immune deficiencies by providing a lifelong reserve of genetically-modified stem cells that can compensate for the existing genetic defect. Furthermore, this gene therapy platform involves gene transfer into autologous stem cells isolated from the patient avoiding the risks of graft versus host disease. Despite these safety measures, HSCT gene therapy still requires the use of highly toxic preparative regimens to provide physical space within the hematopoietic niche. The risks of these drugs have limited the use of HSCT gene therapy to severe inherited disorders with limited available treatments and/or poor patient outcomes, as is the case for adrenoleukodystrophy and metachromatic leukodystrophy.[27, 28]

Figure 4.1



**Figure 4.1 Hematopoiesis of stem and progenitor cells** During embryonic development, pluripotent stem cells give rise to HSCs capable of differentiating into the myeloid and lymphoid lineages of the blood and immune system. HSCs can undergo self-renewal or differentiate into multi-potent progenitors (MPPs). Based on environment cues, MPPs commit to either myeloid or lymphoid progenitors and further differentiate into their respective lineages. This figure was

adapted from EMBO Molecular Medicine, an open access journal in the field of molecular medicine. It was originally designed by Mania Ackermann, Steffi Liebhaber, Jan-Henning Klusmann, and Nico Lachmann and is licensed under the Attribution 3.0 Unported license (CC BY 3.0). (<https://creativecommons.org/licenses/by/3.0/>)

Abbreviations: PSC: pluripotent stem cell, LT-HSC: long-term hematopoietic stem cell, ST-HSC: short-term hematopoietic stem cell, MPP: multi-potent progenitor, CMP: common myeloid progenitor, MEP: megakaryocyte-erythroid progenitor, GMP: granulocyte-macrophage progenitor, CLP: common lymphoid progenitor, Pro-B: pro-B cell, Pro-T: pro-T cell, Lin<sup>-</sup>: lineage negative, Sca-1<sup>+</sup>: stem cell antigen-1, CD: cluster of differentiation

There is a clear and unmet need for both gene and cell therapies to develop non-genotoxic conditioning agents capable of creating adequate space in the hematopoietic niche.

Initial attempts to develop these agents utilized an antibody-based clearance approach to specifically target bone marrow cells and reduce off-target effects. In 2007, Czechowicz *et al.* published a study in *Science* demonstrating that an antibody targeting the c-kit receptors could facilitate robust engraftment in Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice.[191] The c-kit receptor has been established to participate in hematopoiesis and is expressed on the majority of hematopoietic progenitor cells in mice.[192] However, this model was shown to be ineffective in immune-competent mice limiting its use to Phase 1 clinical studies examining SCID-based stem cell transplants.

Low dose TBI has demonstrated efficacy in some allogeneic transplantations such as sickle cell disease in which host cells have a selective disadvantage, however, this level of conditioning has routinely shown poor engraftment in other gene therapy settings.[193-195] Furthermore, Uchida *et al.* 2014 has shown that deep depletion in the bone marrow compartment is necessary for engraftment of genetically-modified cells in a nonhuman primate animal model, even when using a highly optimized and efficient gene transfer system.[196] A systematic approach to developing antibody-based preparative regimens would be to start with a pan-hematopoietic cell surface receptor in order to achieve maximum depletion.

CD45, also known as protein tyrosine phosphatase receptor type C, is a cell surface receptor present on all blood and immune cells of hematopoietic origin. While the exact mechanisms remain unknown, CD45 is recognized for participating in lymphocytic cell signaling. It has also been shown that levels of CD45 expression may vary depending on both cell type and maturation.[197] Many groups have investigated targeting CD45 using lytic monoclonal antibodies or radiolabeled anti-CD45 antibody conjugates to achieve bone marrow depletion. The mechanism of action of these antibodies, in addition to radiation cytotoxicity, is to utilize host immunity through the complement system or mechanisms of antibody-dependent cellular toxicity, each resulting in targeted cell lysis by host effector cells. However, these studies either

resulted in poor engraftment under allogeneic conditions or minimal to no depletion in the stem cell compartment.[198-200]

Recently *Palchaudhuri* et al. reported a novel approach to the CD45 story by creating an anti-CD45 immunotoxin capable of cellular internalization.[201] Biotinylated anti-CD45 antibody was mixed with recombinant saporin, a ribosome inactivating protein, conjugated to streptavidin in equimolar concentrations. A single peripheral infusion of the CD45-immunotoxin (CD45-SAP) resulted in deep depletion of the bone marrow compartment and over 70% donor cell engraftment in immune-competent mice. These results demonstrated the first successful bone marrow transplantations in immune-competent mice not requiring the use of radiation or chemotherapeutics. This method of conditioning was also shown to result in phenotypic correction of murine sickle cell chimeras in which wildtype mice were first lethally irradiated and transplanted with the bone marrow of sickle mice harboring the human beta globin mutation for sickle disease. At 8 weeks post-transplantation, mice were treated with CD45-immunotoxin followed by transplantation of healthy donor cells.

Safer conditioning regimens that maintain a favorable risk to benefit ratio are crucial to the advancement of HSCT gene therapy for HA. In these studies, we investigated our own anti-CD45-saporin based immunotoxin capable of depleting stem and progenitor cells in immune-competent mice. Furthermore, we evaluated this toxin as part of a preparative regimen in a murine model of HSCT gene therapy for HA.

#### **4.3 Materials and Methods**

*Animals Experiments.* All animal studies were performed under the guidelines set by the Emory University Institutional Animal Care and Use Committee. Exon 16 disrupted mice backcrossed onto a C57BL/6 background were used as a model of HA [167]. In the HSCT gene therapy experiments, 6 – 8 week old transgenic mice expressing the enhanced green fluorescent protein (GFP) from the  $\beta$ -actin promoter on a C57BL/6 background were used as congenic donors of HSCs [168] TBI) was administered using a vented chamber within Gammacell 40 Extractor. CD45-

Sap255 was administered by retro-orbital injection. All donor cell recipient mice were between the ages of 8-10 weeks. Mice were bled retro-orbitally to monitor engraftment, complete blood counts, FVIII activity, and anti-FVIII immune responses.

*Purification of Sap-25.5* The purification of recombinant Sap255 was done in collaboration with the laboratory of Pete Lollar, with significant contributions from John Healey and Ernie Parker. The cloning of recombinant Sap255 was adapted from the methods of Günhan *et al.* 2008.[202] Plasmids were propagated in the *E.coli* strain BL21 (DE3) in fresh LB broth containing ampicillin (100ug/mL) and chloramphenicol (34ug/mL). Overnight cultures were induced using 100mM IPTG and grown to an OD value of approximately 0.8. Culture was spun down at room temperature and lysed in a solution of BugBuster with 2mM DTT and 5mM EDTA and benzonase. Lysates were frozen overnight and then spun the following day at approximately 16,000 rpm at 4 degrees Celsius to clarify the solution of any precipitates. Lysates were diluted 5x in phosphate buffer and purified using an HP CM FF Sepharose ion exchange column.

*Generation of CD45-Sap255.* Purified saporin was conjugated to anti-CD45.2 IgG Clone 104 using an SPDP linker system. All the conjugation steps were performed phosphate buffer pH=8 according to the manufacturer's instructions. Briefly, 40ug of SPDP linker was resuspended in DMSO and incubated with 0.5mg of antibody at room temperature for one hour. Using an Amicon Ultra 2mL 30K filter, excess SPDP linker was removed and then incubated with 0.5mg of saporin for 18 hours at 4 degrees Celsius. Overnight conjugation was filtered using an Amicon Ultra 0.5ml 50K filter at 14,000g for 30 mins. Mice were doses based on the original concentration of IgG.

*Whole Bone Marrow Transplants.* Transplantation recipients received CD45-Sap255 at a dose of 3mg/kg. Whole bone marrow was isolated from 6-8 week old congenic GFP positive transgenic mice. Cells were flushed from the femurs and tibias and filtered to eliminate debris. After 10 minutes of red cell lysis, whole bone marrow cells were resuspended in sterile PBS,



counted, and 20 million cells per mouse were immediately infused, via retro-orbital injection, into transplantation recipients.

*Sca-1<sup>+</sup> Enriched Bone Marrow Transplants.* Whole bone marrow was enriched for Sca-1<sup>+</sup> antigen as previously described using positive immunomagnetic bead selection [50]. Cells were then stimulated for 24 hours in media containing murine stem cell factor (100ng/mL), murine interleukin-3 (20ng/mL), human interleukin-11 (100ng/mL) and human Flt-3 ligand (100ng/mL). After stimulation, cells were transduced twice in half volume virus at a density of 2 million Sca-1<sup>+</sup> cells/mL over the course of 24 hours. Cells were then resuspended in PBS and one million transduced cells were injected retro-orbitally into CD45-Sap255 conditioned HA mice.

*LV-FVIII Production.* Viral accessory plasmids along with the bioengineered high expression FVIII transgene (designated ET3) encoding expression plasmid were transiently transfected in 293T-17 cells using a calcium phosphate transfection method to generate LV vectors pseudotyped with the VSVG envelope similar to the method described previously except for the utilization of the calcium phosphate transfection reagent (Sigma Aldrich, St. Louis, MO). [169] Conditioned media was collected for three days beginning at 48 hours post transfection and passed through a 0.45µm filter. Virus was concentrated by overnight centrifugation at 10,000 x g overnight, followed by filtration using a .22 µm filter. Viral concentrate titers were determined using quantitative real time PCR analysis.

*FVIII and Immune Response Analyses.* Anti-FVIII antibody titers were determined using an ELISA. Immobilized ET3 was bound to microtiter plates and then incubated with mouse plasma. Anti-fVIII antibodies were detected using a pan goat-anti mouse IgG secondary antibody conjugated to alkaline phosphatase and absorbance was read at 405nm. The absorbance values of test plasmas were plotted against the logarithm of the plasma dilution, and the titers were determined to be the reciprocal of the dilution in which the optical density (OD) value was three times that of the background, or an OD reading of 0.3. [173] FVIII activity was determined using a commercially available COATEST SP FVIII assay (Coatest SP, Diapharma) according the

manufacturer's instruction. Activity was quantified using a standard curve was generated using pooled citrated human plasma (Georgia King, Overland Park, KS).

*Depletion and Engraftment Analysis* Depletion analysis was performed using flow cytometric analysis. Bone marrow was flushed from the femurs of CD45-Sap255 treated mice and lysed with red cell lysis buffer for 10 minutes at room temperature. Cells were then washed in FACS buffer (PBS + 1% FBS) and kept on ice for the remaining of the staining process. Stem cells were defined using a lineage cocktail of monoclonal antibodies in addition to Sca-1+ antigen, CD117, CD48, and CD150. Engraftment was monitored by detection of GFP<sup>+</sup> cells in peripheral blood obtained retro-orbitally. Blood was spun at 5000g for 15 minutes at 4°C and the plasma removed. The cellular component was resuspended in 50uL of PBS and lysed 3 times at room temperature in 3mLs of red cell lysis buffer with a 10 min spin in between each lysis step. Samples were resuspended in FACS buffer after the final wash for staining.

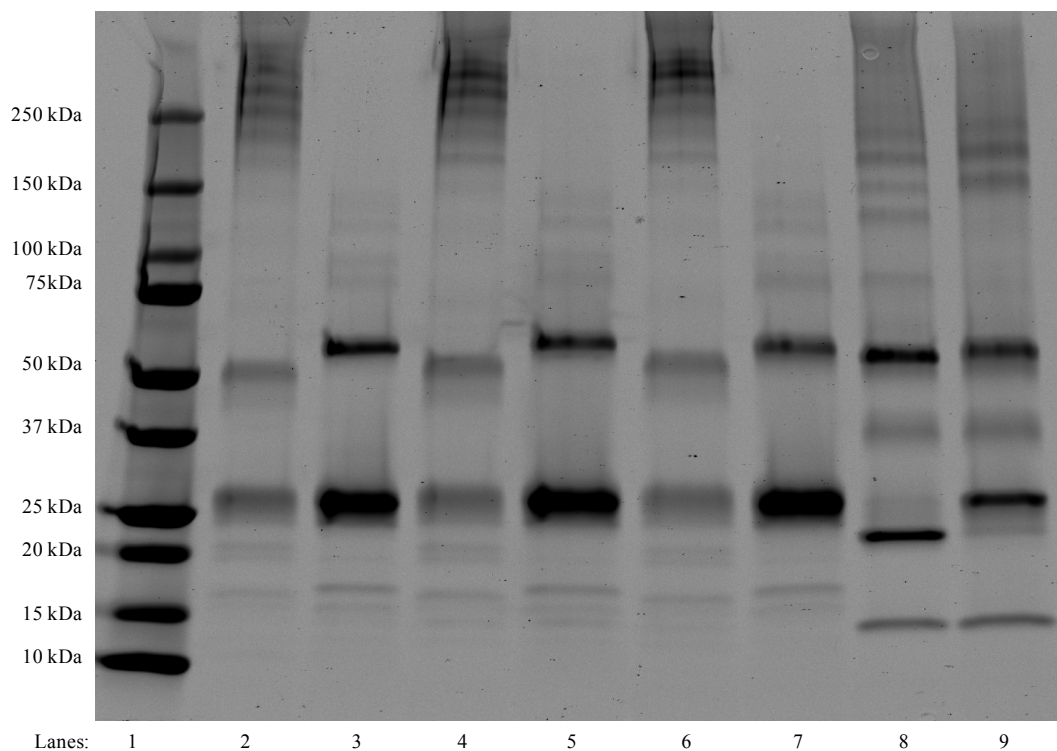
#### **4.4 Results**

*Conjugation of an anti-CD45-saporin based immunotoxin.* The monoclonal antibody clone 104 that recognizes the isoform CD45.2 was used in this study, as this clone was demonstrated to have higher persistence in vivo, measurable levels of cellular internalization, and greater efficacy in stem cell depletion compared to other isoforms.[201] While there are several commercially available monoclonal antibodies that recognize 3 isoforms of the CD45 receptor, for the purposes of this publication, CD45 refers to clone 104. Using a heterobifunctional linker system, SPDP (N-succinimidyl-3-(2-pyridyl-dithiopropionate), CD45 was conjugated to a recombinant saporin variant engineered to express a free cysteine at residue 255 (Sap255) of the amino acid sequence using a method adapted from Günhan *et al.* 2008.[202] Saporin is a type-1 ribosomal inhibitor protein (RIP) capable of inhibiting ribosomal translation and facilitate cell death in eukaryotes. Despite its potent toxicity, saporin has low binding affinity for cell surface receptors and therefore cannot enter the cell independently or cause significant off-target side effects. To generate an effective saporin-based immunotoxin, the type-1 RIP must be conjugated to a protein that binds

with high affinity to a cell-surface receptor on the target cell and is efficiently internalized in order to facilitate cytotoxicity.

SPDP is a bifunctional linker molecule with an amine reactive group capable of forming an amide bond with primary amines on the surface of proteins. Once CD45 antibody has reacted with the SPDP linker, the crosslinked complex underwent site-specific conjugation by forming a disulfide bond with the free cysteine on Sap255 and the pyridyldithiol group present on the unbound side of the SPDP linker. Because the process of conjugation happens randomly, directing saporin conjugation to a specific amino acid residue increases the probability of maintaining the cytotoxic function of the RIP. This conjugation reaction repeatedly resulted in high molecular weight products  $\geq 250\text{kDa}$ . Given the molecular weight of saporin to be approximately  $30\text{kDa}$ , we estimated that each molecule of IgG has at least 3 molecules of saporin. **(Figure 4.2)**

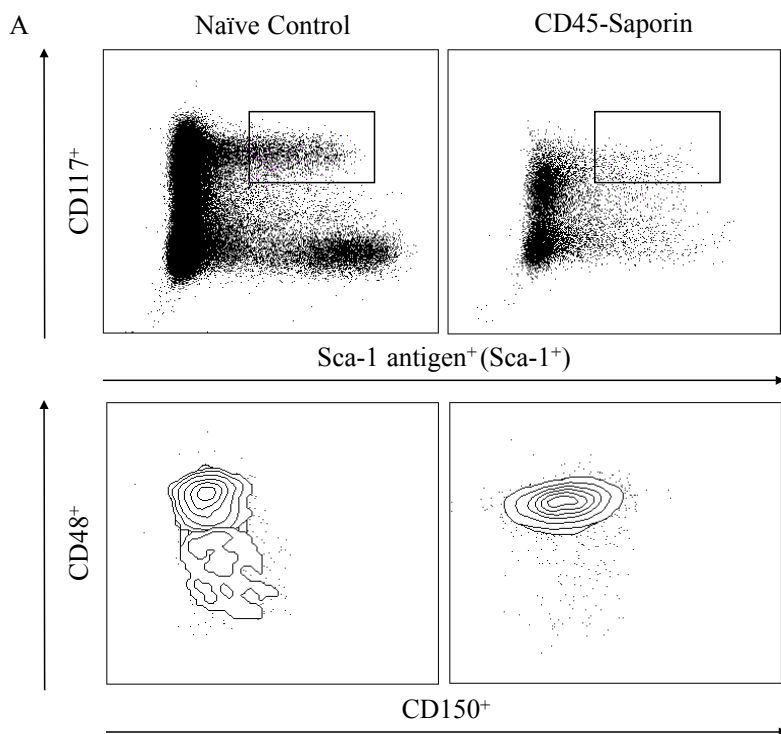
*Stem and progenitor cell depletion using an anti-CD45-saporin immunotoxin* CD45 is a cell surface receptor present on all cells differentiated from HSCs. While the biology of CD45 is still largely unknown, it exemplifies high antigen density with over 200,000 receptors estimated on the surface of lymphocytes.[203] Therefore, targeting CD45<sup>+</sup> cells should result in deep depletion within the stem and progenitor compartment. We examined the depletion potential of CD45-Sap255 in our mouse model of HA mice. Mice received a single intravenous injection of immunotoxin and were examined for bone marrow depletion 6 days later using flow cytometric analysis. **(Figure 4.3a-c)** CD45-Sap255 demonstrated dose-responsive depletion in both the progenitor and stem cell compartments. While close to 80% depletion was observed in all progenitor ( $\text{Lin}^-$ ,  $\text{L}^-\text{K}^+$ ,  $\text{L}^-\text{S}^+\text{K}^+$ ) and stem cell ( $\text{CD48}^-$ ) compartments, quiescent and short-term stem cells exhibited greater sensitivity to CD45-Sap255 depletion at the lower dose compared to progenitor cells. Nonetheless,  $\text{Lin}^-$  and  $\text{L}^-\text{K}^+$  compartments exhibited significant depletion at the lower dose of Sap255. This depletion may be due to common lymphoid progenitors

**Figure 4.2**

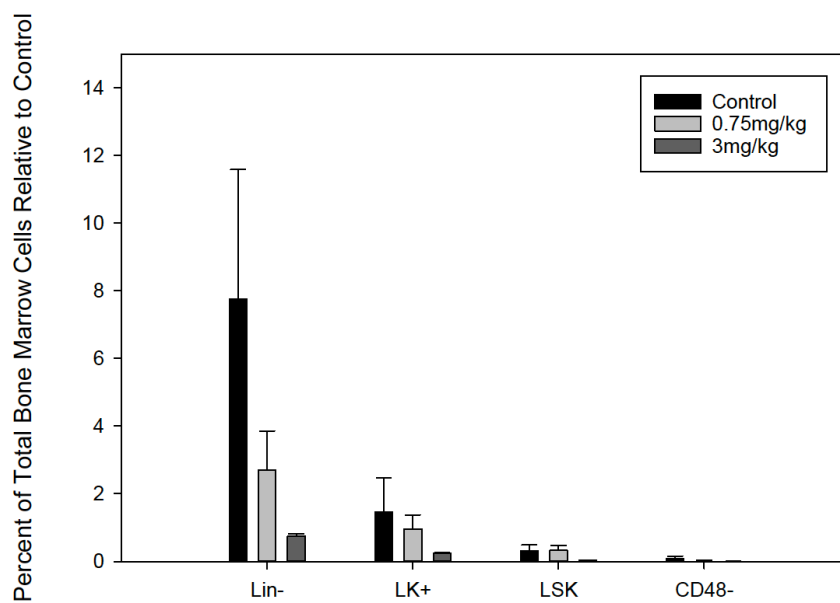
**Figure 4.2 Conjugation of Sap-255 with CD45 antibody** Saporin-255 has been modified to introduce a free cysteine residue at position 255 of the amino acid sequence. Using an SPDP (succinimidyl 3-(2-pyridyldithio) propionate) linker, anti-CD45 antibody was conjugated to Sap255 forming a site-specific disulfide linkage between saporin and the SPDP linker. Lanes 2, 4 and 6 show non-reduced product of the three triplicate conjugation experiments. The reaction resulted in generation of high molecular weight products  $\geq 250$  kDa. The band present at  $\sim 28$  kDa is free Sap255 and the band present between 50-60 kDa is most likely a dimer of Sap-255 as this band was previously shown to be detected in a Western blot under non-reducing conditions using a commercially available anti-saporin antibody.[202] Lanes 3, 5, and 7 show conjugation product under reducing conditions. Light chain IgG2a is present at approximately 25 kDa and most likely masking free Sap255 at  $\sim 28$  kDa. Heavy chain IgG2a is present at  $\sim 55$  kDa. Lanes 8 and 9 show

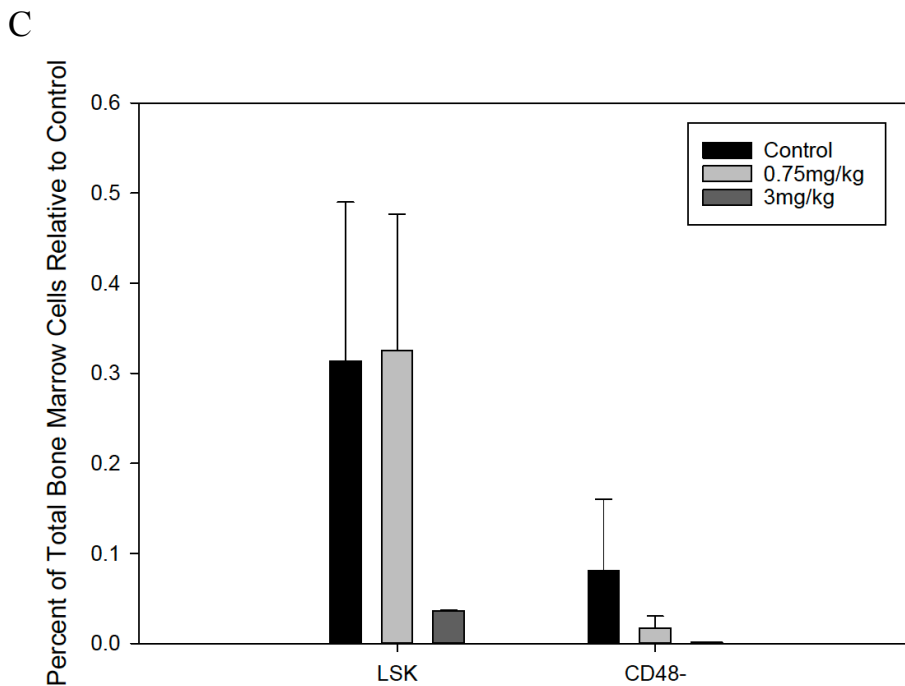
reduced and non-reduced CD45-Sap using reagents described in Palchaudhuri *et al.* 2016. 5ug of protein was loaded in each lane.

Figure 4.3



B





**Figure 4.3 CD45-Sap255 mediated depletion of stem and progenitor cells in HA mice**

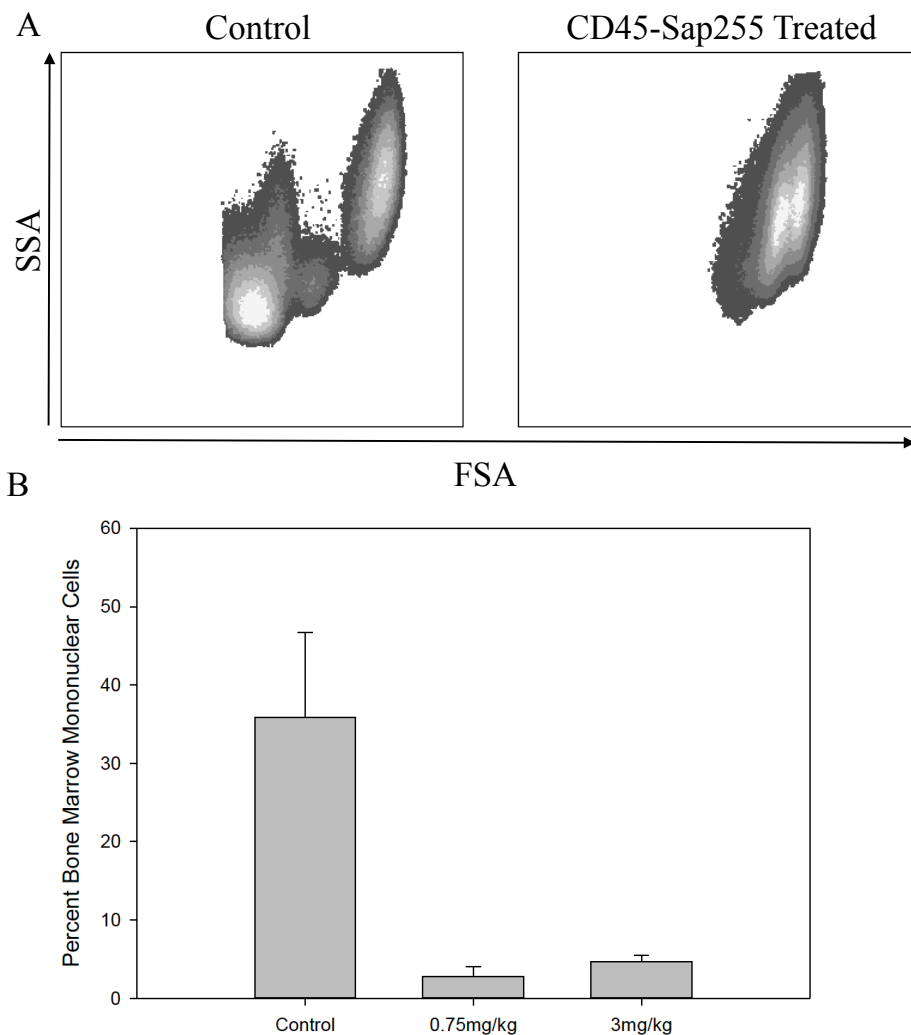
Quiescent murine stem cells can be analyzed by flow cytometry and are identified by the cell surface markers Lineage<sup>-</sup> CD117<sup>+</sup> Sca-1<sup>+</sup> CD48<sup>-</sup> CD150<sup>+</sup>. Figure 2A is representative flow cytometric analysis used to quantify depletion of CD45-Sap255 6 days post injection. The following gating schematic is based off a lineage negative gate determined by negative staining of a lineage cocktail. CD45-Sap255 treatment results in near complete depletion of the CD48<sup>-</sup> compartment and thus depletion of short-term and quiescent stem cells. Panel B depicts the percent of bone marrow cells within each compartment normalized to the total number of cells present in aged matched controls 6 days after CD45-Sap255 injections. Each dose represents an individual depletion experiment. The data supports the conclusion that CD45-Sap255 facilitates dose-response depletion of bone marrow cells. Panel C shows the data from Panel B zoomed in on the LSK and CD48<sup>-</sup> compartments. The error bars indicate the standard deviation for n=3 mice for each immunotoxin dose.

as mononuclear cells in the bone marrow were depleted based on flow cytometric side scatter (SSC) analysis. (**Figure 4.4a and b**)

*A preparative regimen using CD45-Sap255 in a murine model of HSCT gene therapy for HA.* While Uchida *et al.* 2014 established that deep depletion is necessary for engraftment of transplanted cells, understanding the percent of depletion required of each progenitor sub-compartment is largely unknown. Previous work has shown that depletion of T-cells is critical to achieving engraftment of gene-modified cells, therefore anti-thymocyte globulin (ATG) was incorporated into our preparative regimen.[48] Furthermore, we hypothesized that incorporation of ATG prior to CD45-Sap255 treatment would deplete stem cells and therefore increase the apparent dose of CD45-Sap255 by decreasing the pool of CD45<sup>+</sup> cells. In previously published data, robust engraftment with little variation was achievable if cells were transplanted anywhere between 2 and 12 days post immunotoxin injection.[201] Given these observations, we designed a transplantation protocol that involved two days of conditioning followed by transplantation of FVIII gene-modified cells 3 days later. (**Figure 4.5a**) Cells were transduced using an LV-CD68-FVIII vector previously described in Chapter 3 that incorporates a myeloid-specific promoter shown to drive therapeutic levels of FVIII expression. In addition to CD45-Sap255 conditioned mice +/- ATG, a control group of 3 mice treated with a lethal dose of TBI (11Gy) was included to confirm the viability of the transduced cells after transplantation.

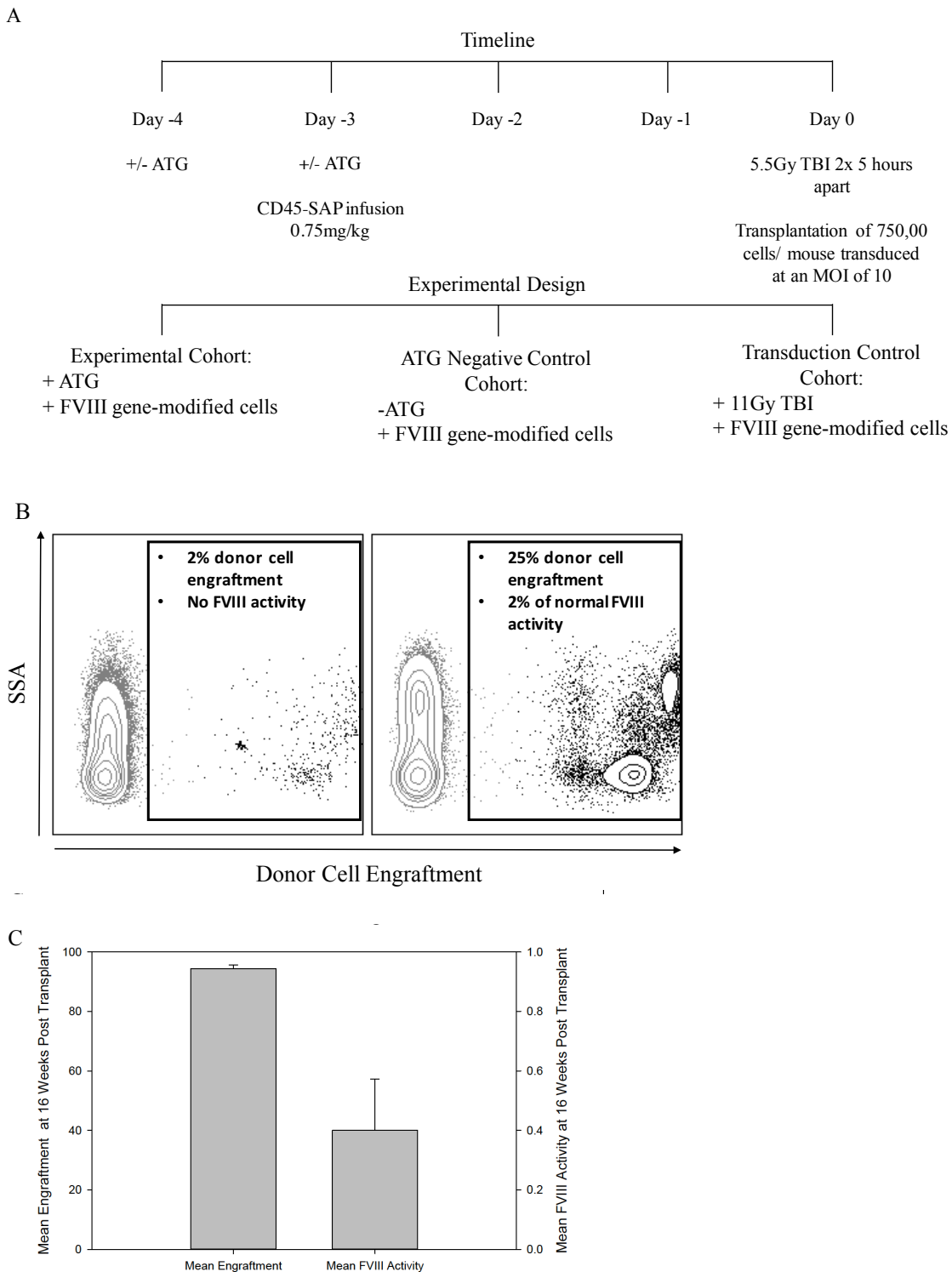
Therapeutic levels of FVIII activity are a measure of efficacy in HSCT gene therapy for HA. Initial monitoring of transplanted mice was performed by measuring FVIII activity in peripheral blood. At 2 weeks and 4 weeks, no circulating FVIII activity could be measured in peripheral blood of CD45-Sap255 conditioned mice, thus indicating no presence of engrafted cells, while robust FVIII activity could be detected in the radiation control group. However, because more significant depletion was observed in the short-term and quiescent stem cell compartment, the kinetics of engraftment and hematopoiesis may be much slower compared to other published studies in which substantial progenitor and lineage positive cells are also depleted by



**Figure 4.4**

**Figure 4.4 CD45-Sap255 mediated depletion of bone marrow mononuclear cells** Bone marrow mononuclear cells are lineage positive cells that reside within the bone marrow compartment and include lymphocytes, monocytes, and progenitors. Panel A demonstrates representative flow of bone marrow cells stained with a lineage positive cocktails. Mononuclear cells are characterized by low side scatter quantities on the y-axis. Panel B quantifies the percent of bone marrow mononuclear cells relative to control 6 days after CD45-Sap255 depletion. The error bars indicate the standard deviation for n=3 mice.

**Figure 4.5**



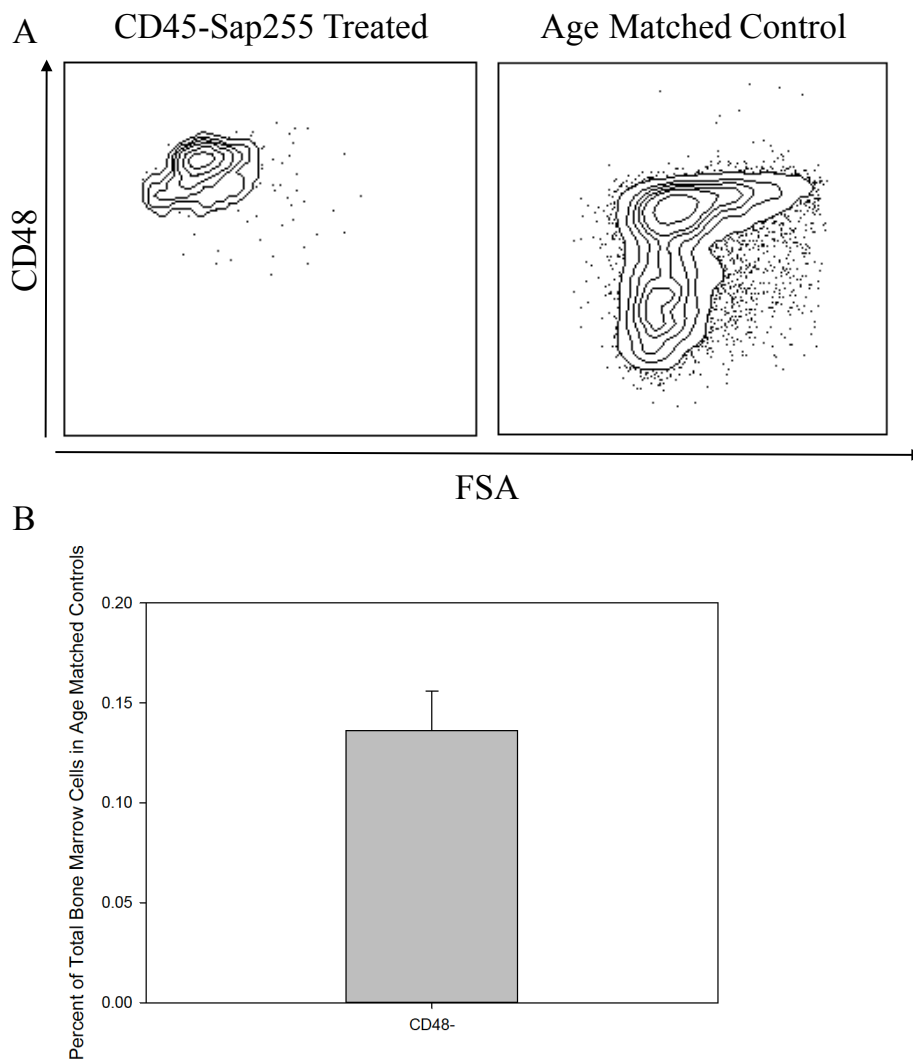
**Figure 4.5 HSCT gene therapy for HA using a CD45-Sap255 conditioning regimen** CD45-Sap255 was used as part of a conditioning regimen for HSCT gene therapy in a mouse model of

HA. Panel A outlines the transplantation design including the timing of drug infusions as well as the multiplicity of infection (MOI) used to transduce stem cells and the number of cells transplanted per mouse. Both the experimental and negative control cohorts each contained 9 mice, while the transduction or radiation control cohort contained 3 mice. Panel B shows the flow cytometric analysis of peripheral blood from both mice that demonstrated measurable levels of engraftment. Additionally, each graph notes the levels of FVIII activity for each mouse with engrafted donor cells. Panel C shows mean engraftment and FVIII activity for control mice treated with 11Gy TBI. Error bars represent standard deviation for all experiments.

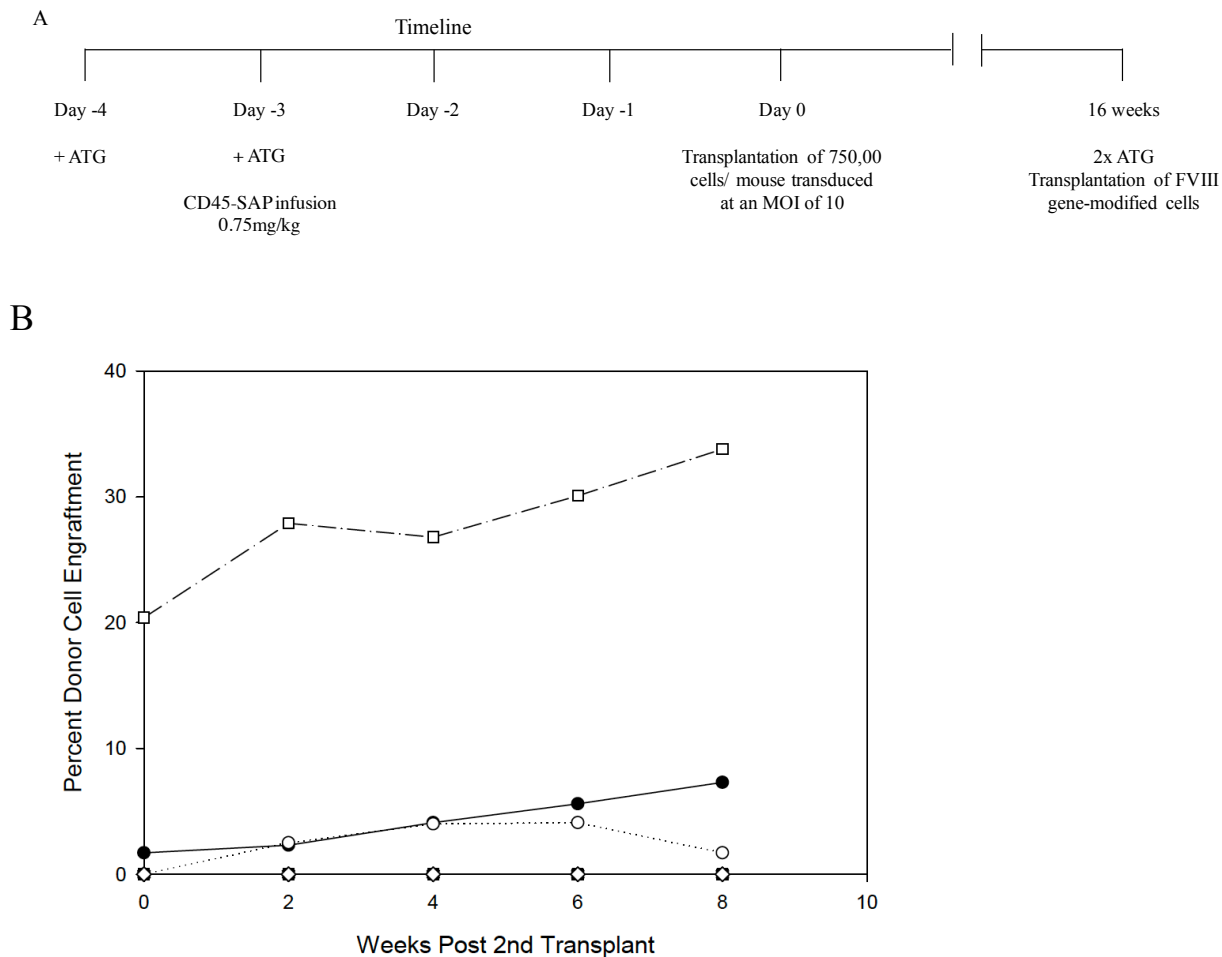
chemotherapeutics or radiation. However, it is important to note that Palchauduri *et al.* 2016 reported roughly 30% engraftment at 2 weeks post-transplantation using CD45-SAP.

At 16 weeks post-transplantation, 2 of the 8 treated mice showed signs of engraftment by flow cytometric analysis. Additionally, one of these mice demonstrated measurable levels of FVIII activity. **(Figure 4.5b)** While not statistically significant, the data suggested that optimization of CD45-Sap255 conditioning may result in curable engraftment levels of FVIII gene-modified cells in a mouse model of HA. In order to optimize an immunotoxin-based preparative regimen, several questions must be addressed. First, were the cells viable at the time of transplantation? Analysis of radiation controls at 16 weeks indicates that FVIII gene-modified cells were capable of robust engraftment and secretion of therapeutic levels of FVIII. **(Figure 4.5c)** Next, is there an immune-mediated component preventing engraftment of FVIII-gene modified cells? Inhibitor analysis showed no circulating anti-FVIII inhibitory antibodies in mice treated with ATG, however, one mouse in the no ATG cohort tested positive for anti-FVIII IgG. Lastly, if the 0.75mg/kg dose of CD45-Sap255 depleted the CD48<sup>-</sup> short-term and quiescent stem cell compartment, are these mice still depleted of stem cells? Bone marrow depletion analysis of a single mouse from the experimental cohort indicated that the CD48<sup>-</sup> compartment was still depleted relative to age matched controls. **(Figure 4.6a and b)** Therefore, we hypothesized that a CD48<sup>-</sup> compartment could still be engrafted by FVIII gene-modified cells 16 weeks post CD45-Sap255 conditioning. The remaining mice within the experimental cohort were treated with another regimen of ATG and transplanted a second time with gene-modified cells. **(Figure 4.7a)** However, only marginal increases in engraftment were observed in 3 of the treated mice. **(Figure 4.7b)**

*Estimating clearance of CD45-Sap255.* In addition to anti-FVIII inhibitory IgG facilitating clearance of gene-modified cells, it is plausible that excess CD45-Sap255 is bound to the surface of cells resistant to cytotoxicity mediated through internalization of the CD45 receptor. Given that there are approximately 200,000 CD45 cell surface receptors on the surface of lymphocytes, and approximately  $5 \times 10^8$  bone marrow cells in an 8week old mouse, one can estimate that there are

**Figure 4.6****Figure 4.6 CD45-Sap255 conditioned mice maintain CD48<sup>-</sup> depletion 16 weeks post injection**

A single mouse from the experimental cohort showed CD48<sup>-</sup> cell depletion in the bone marrow compartment 16 weeks post infusion of 0.75mg/kg CD45-Sap255. Panel A shows representative flow of an age-matched control compared to the single mouse of the experimental cohort. Contour plots show CD48 staining from cells gates from the LSK population of whole bone marrow. Panel B shows the quantification of the percent of CD48<sup>-</sup> cells of the total bone marrow compartment in age matched controls. Error bars represent the standard deviation of n=3 mice.

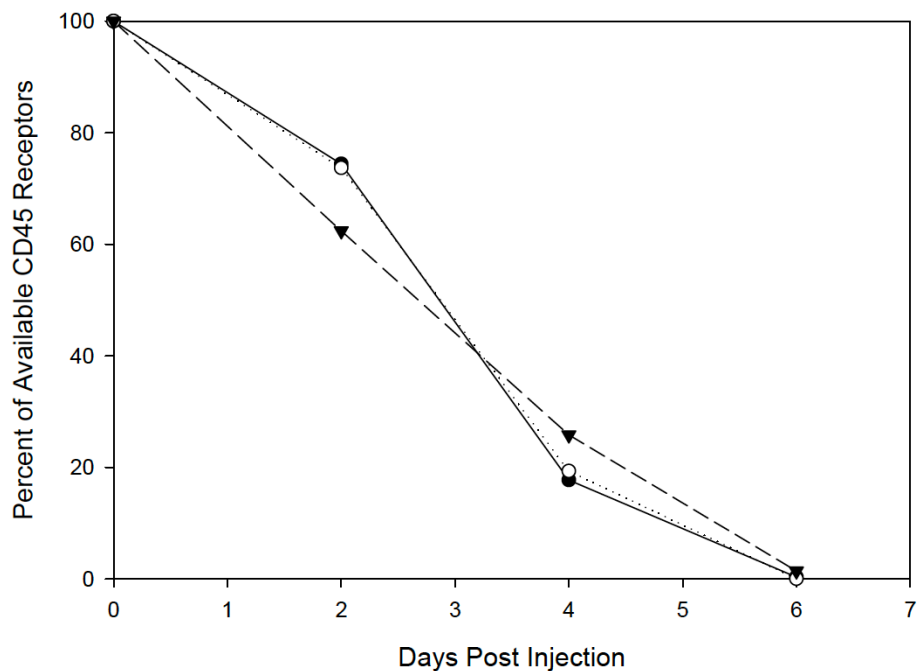
**Figure 4.7**

**Figure 4.7 Engraftment of experimental cohort after 2<sup>nd</sup> transplantation of FVIII gene-modified cells** In order to determine if a CD48<sup>-</sup> depleted compartment is engraftable by FVIII gene-modified cells, mice from the previous experimental cohort were transplanted a second time with FVIII gene-modified cells. Panel A shows a transplantation timeline for experimental mice that received a second transplant, and panel B shows levels of engraftment over a 2 month time frame from the time of re-transplantation (n= 8 mice).

approximately  $9 \times 10^{13}$  CD45 receptors available for binding.[203, 204] While the 0.75mg/kg dose of CD45-Sap255 provides a number of circulating IgG molecules in the same order of magnitude as the estimated number of total CD45 receptors within the bone marrow compartment, the higher dose of 3mg/kg is in excess of this estimation. Furthermore, Clone 104 is indicated to be a high affinity antibody and therefore it is likely that the majority of excess CD45-Sap255 will remain bound to the surface of cells resistant to CD45 internalization.

In order to design a transplantation timeline using a higher dose of CD45-Sap255, it is important that we model clearance of the toxin to prevent cytotoxicity of our transplanted cells. To simulate clearance of the saporin-based toxin, biotinylated Clone 104 IgG was injected retro-orbitally into naïve HA mice. Persistence of bound IgG was determined by flow cytometry using a streptavidin molecule conjugated to a fluorophore. In addition to Clone 104 staining, a second clone of anti-CD45 (clone 30F-11) was used to stain for total CD45 to show the percent of CD45 receptors bound by Clone 104. An isotype control was injected to assess non-specific binding. Pilot studies indicated that 100% of the CD45<sup>+</sup> compartment was bound 30 minutes after injection by both the streptavidin fluorophore and clone 30F-11. Flow cytometric analysis indicated that by day 6 anti-CD45 clone 104 was not bound to peripheral blood cells. **(Figure 4.8)** Day 6 analysis also showed no cell surface binding of clone 104 in bone marrow cells (data not shown). Taken together, this data indicates that by day 6 after CD45-Sap255 infusion, no toxin will be available in the peripheral blood or bone marrow of conditioned mice to facilitate cell killing of transplanted gene-modified cells.

To confirm these findings, naïve HA mice were injected with 3mg/kg of CD45-Sap255 retro-orbitally. Mice were then transplanted at 6, 9, or 12 days after infusion with 20 million non-modified whole bone marrow cells. Contrary to Palchadhuri *et al.* 2016, no measurable levels of engraftment could be measured in transplanted mice.

**Figure 4.8**

**Figure 4.8 Clearance of anti-CD45 clone 104 in naïve HA mice.** In order to simulate clearance of CD45-Sap255 immunotoxin, biotinylated IgG (Clone 104) was injected into naïve HA mice. Clearance of CD45 Clone 104 was determined by flow cytometric analysis as a percentage of total CD45 available in peripheral blood.



## 4.5 Discussion

Using a heterobifunctional linker system, we generated a cell-specific immunotoxin by conjugating a recombinant saporin molecule to a commercially available anti-CD45 antibody. **(Figure 4.2)** Previously published data had determined this monoclonal antibody was capable of cellular internalization and therefore could provide saporin entry into the cell. Once inside the cell, saporin can initiate cell death through a variety of pathways, most notable protein synthesis inhibition.[205] Immunotoxin mediated killing is dependent on the cell type, the density of the target antigen, the binding affinity of the IgG portion of the immunotoxin, and the cellular routing of the toxin. CD45 is an ideal target for initial immunotoxin based conditioning studies, as it is present in high concentration on the cell surface of all cells of hematopoietic origin.

Our CD45-Sap255 immunotoxin demonstrated dose-dependent depletion on stem and progenitor cells in the bone marrow compartment. **(Figure 4.3)** While these results were not considered to be statistically significant, it is important to note that the initial experimental set up was not powered to detect differences within sub-compartments of the bone marrow. Given the expression pattern of CD45, our initial hypothesis was that excess infusion of immunotoxin would result in near complete depletion of the bone marrow compartment and the experiments presented in this dissertation were powered to detect the presence or absence of engrafted cells. In addition to stem and progenitor cells, CD45-Sap255 provided substantial depletion of bone marrow mononuclear cells at both doses of immunotoxin. **(Figure 4.4)**

Given these results, we examined the use of CD45-Sap255 in a murine model of HSCT gene therapy for HA. **(Figure 4.5)** Mice were treated with either CD45-Sap255 and anti-thymocyte globulin (ATG) or CD45-Sap255 alone. Previously published studies have shown that T-cell depletion is necessary for successful engraftment of FVIII gene-modified cells and prevention of an anti-FVIII humoral immune response. However, despite measurable levels of depletion, transplantation studies did not result in efficacious engraftment of FVIII gene-modified cells. Two out of eight mice showed measurable levels of engraftment, however, the data suggested that a

threshold of 20% engraftment was necessary to detect circulating FVIII in the plasma of transplanted mice. Mice conditioned with 11Gy TBI demonstrated over 90% chimerism in peripheral blood confirming that transduced cells were both engraftable and could undergo hematopoiesis. Inhibitor analysis also showed no humoral responses to FVIII suggesting there was no immune-mediated clearance of transduced cells in the CD45-Sap255 + ATG experimental cohort. Bone marrow analysis from a single mouse of the experimental cohort still demonstrated depletion of the CD48<sup>-</sup> compartment relative to age-matched controls. **(Figure 4.6)** We then questioned whether a CD48<sup>-</sup> depleted compartment is engraftable by FVIII gene-modified cells. However, a sequential transplantation of the experimental cohort still resulted in poor engraftment of gene-modified cells. **(Figure 4.7)** Furthermore, transplantation of naïve whole bone marrow cells did not result in measurable levels of engraftment after conditioning with a higher dose of CD45-Sap255.

Our data differs from the results reported by Palchaudhuri et al. 2016.[201] In their transplantation studies, CD45-SAP conditioning resulted in 70-90% long-term donor cell engraftment with measurable levels of chimerism in peripheral blood by 4 weeks post-transplantation. While the focus of our studies was HSCT gene therapy for HA, CD45-Sap255 depletion could not facilitate engraftment of unmodified whole bone marrow cells as well. We achieved near complete ablation of short-term and quiescent stem cells at 3mg/kg, however, reasonable numbers of progenitor cells could still be detected by flow cytometry in both the CD48<sup>+</sup> and Lin<sup>-</sup> compartment. Given that it is now accepted that progenitor cells are the major contributors to hematopoiesis, deeper depletion of the progenitor compartment may be necessary for engraftment in an immune-competent mouse model.[206] This also coincides with the conclusions of Uchida et al. 2014 that determined deep depletion within the bone marrow compartment is necessary for engraftment of gene-modified cells.[196] Typical transplantation protocols utilize radiation or chemotherapeutics that cause significant stress to the bone marrow compartment including non-hematopoietic cell death, vascular damage, and the release of pro-inflammatory

cytokines, as well the pressure to promote hematopoiesis due to progenitor cell death.[207] It is possible that increased depletion of progenitors is not just necessary to provide adequate space within the bone marrow compartment, but also to promote engraftment and hematopoiesis of transplanted gene-modified stem cells.

It is also important to recognize that these studies utilized a different immunotoxin molecule than that described in the previous paper, a biotinylated CD45 antibody purchased from Biolegend and a streptavidin-saporin molecule purchased from Advanced Targeting Systems.[201] While the saporin conjugation was guided by the addition of a free cysteine engineered at the C-terminus, conjugation of the antibody to the heterobifunctional linker was random. Additionally, our conjugation process resulted in higher molecular weight products with a minimal estimated ratio of 3 saporins per molecule of IgG. Initially, we hypothesized that an increase in the number of saporins per molecule of immunotoxin would increase the potency of CD45-Sap255, however this may have increased the number of “dead” molecules unable to bind the CD45 receptor. In future experiments, a potency assay should be put in place to quality control the inhibitory potential of each conjugation reaction. Furthermore, a head to head analysis of bone marrow depletion will also be useful to determine if our immunotoxin can achieve the same efficacy. Lastly, there are several isoforms of saporin that can be extracted from the seeds of *Saponaria officinalis*, each with similar  $IC_{50}$  values in a translation inhibition assay using bacterial cells. Our immunotoxin conjugate incorporates the saporin-3 isoform and was previously shown to have comparable ribosomal inhibitory activity to that of commercially available saporin from Advanced Targeting Systems.[202] However, this analysis was based on protein inhibition within bacterial cells, and a differential in toxicity maybe exist between isoforms of saporin in mammalian cells.

It is also possible that there is a lag time between when progenitor cells begin depleting and when stem cells demonstrate substantial ablation at day 6. Without complete depletion of the progenitor compartment, these cells may be rapidly repopulating as stem cells are dying off. This is likely not true as significant CD45 antibody could still be detected bound to the surface of

peripheral blood cells at days 2 and 4. (**Figure 4.8**) Residual cells present after depletion may be resistant to CD45 mediated internalization of saporin, and an added immunotoxin targeting perhaps the c-kit receptor would aid in achieving 100% depletion in the progenitor compartment. If our conclusions are correct and deeper depletion within the progenitor compartment is necessary to facilitate engraftment, future experiments would benefit from the use of an immunotoxin cocktail to target multiple receptors present on the surface of progenitor cells.

#### **4.6 Acknowledgments**

We would like to thank the laboratory of Dr. Pete Lollar (Emory Children's Center, Aflac Cancer and Blood Disorders Center), specifically Ernie Parker and John Healey, for their expertise and guidance in obtaining and purifying the saporin molecule used in this chapter. This work was supported by funding from the National Institutes of Health/National Heart, Lung and Blood Institute (Doering and Spencer) and Hemophilia of Georgia (Doering and Spencer).

## Chapter 5

### **Developing Alternative Conditioning Regimens Using Lamprey-Antibody Based Immunotoxins**

Allison Lytle performed and designed the experiments, and wrote and edited the chapter

NaYoon Paik performed experiments

Trent Spencer and Chris Doering conceived the experiments and edited the paper

## 5.1 Abstract

Developing non-genotoxic conditioning agents is of considerable interest to both the field of gene therapy and stem cell transplantation. One approach has been to develop immunotoxins that target the immunophenotype of stem and progenitor cells. However, this method has been limited to known cell surface receptors and epitopes present on bone marrow cells. The lamprey adaptive immune system, which diverged from a common ancestor approximately 550 million years ago, has been shown to generate variable lymphocyte receptors that mediate immune responses through antigen-specific immune mechanisms. This chapter presents a methodology for developing VLR-fusion proteins that can be utilized in a variety of biochemical assays and demonstrate cell-specific binding using flow cytometric analysis.

## 5.2 Introduction

Sea lampreys are jawless vertebrates that diverged from a common ancestor 550 million years ago. Their life cycle begins in a larvae stage upon hatching from fertilized eggs in muddy streambeds. After 3-14 years, the wormlike larvae metamorphose into eel-like parasitic adults and migrate to the ocean. After a year to 18 months, adult lampreys return to the streams where they mate and die shortly after.

Lampreys are considered to have the most primitive adaptive immune system that survived to present day comprised of lymphocyte-like cells that are similar to their human counterpart. In addition to leukocytes, lampreys utilize VLRs to mediate antigen recognition similar to human immunoglobulin. The VLR structure can be divided into highly variable leucine rich repeat modules that can vary in number as well as size. However, despite this variability, lamprey VLRs are encoded by a single gene that remains incomplete in germ line cells. In mature immune cells, leucine rich repeat genomic cassettes assemble into the complete gene providing a diverse VLR repertoire similar to human immunoglobulin.[208]

The lamprey adaptive immune system has been shown to produce antigen-specific VLRs to a variety of immunogens.[209] In these studies, we attempted to generate VLR-fusion proteins

capable of binding the surface of murine HSCs. Given the primitive nature of their adaptive immune system, we hypothesized that VLRs may recognize novel antigens or epitopes present on the cell surface of mouse stem cells. Our studies demonstrate that lampreys can launch an antigen-specific VLR-based immune response against murine stem cells. Additionally, the sequence of these VLRs can be isolated and used to develop VLR-fusion proteins that can be purified directly from conditioned media. Lastly, these VLR-fusion proteins are amenable to multiple biochemical assays and demonstrate cell binding profiles consistent with the immunizing cell population used to generate the initial lamprey immune response.

## 5.2 Materials and Methods

*Animal Experiments.* Sea lampreys of approximately 12–15 cm in length were obtained from commercial fishermen (Lamprey Services, Ludington, MI). Lampreys were maintained in sand-lined and aerated aquariums at 16–20 °C. All lamprey experiments were approved by the Emory Institutional animal care and use committee (IACUC).

*Immunization of lampreys.* Lampreys were immunized three times at two week intervals with 1million Lin<sup>-</sup> Sca<sup>+</sup> cKit<sup>+</sup> stem cells purified from whole bone marrow cells. For each immunization, forty C57/Bl6 mice 6-8 weeks old were euthanized and whole bone marrow was flushed from the femurs and tibias using a syringe and PBS with 5% FBS. Following red cell lysis at room temperature, cells underwent lineage depletion following the manufacturer's protocol (Milltenyi Biotec, catalogue number: 130-090-858). The resultant cell suspension was sorted for double positive ckit<sup>+</sup> and Sca<sup>+</sup> cells using fluorescent activated cell sorting and 1 million cells were injected immediately into the coelomic cavity of lampreys.

*Identifying single clones.* Total leukocytes from immunized lampreys were isolated and screened using a streptavidin-fluorophore conjugate and an anti-VLR antibody compatible with flow cytometry to determine positive binding of biotinylated HSC lysate. Total RNA was collected and converted to cDNA using SuperScript<sup>®</sup> III reverse transcriptase (Invitrogen<sup>™</sup>) and oligo-dT primers. Nested PCR was then performed using primers that overlapped with 50bp of a yeast

display vector with an inducible promoter and anti-Myc tag epitope engineered at the end of the VLR insert as described in Velásquez et al. *Plant Methods* 2017. Transformation and yeast display were carried out as described previously.[210] Single colonies were then picked and screened using flow cytometric analysis to confirmed positive binding to HSC lysate. Positive binders were determined by double staining of yeast cells positive for the streptavidin-fluorophore conjugate and an anti-Myc tag antibody. Genomic DNA was then isolated from positive binding yeast cells and primers targeting the constant regions of the VLR cDNA sequence were used to PCR amplify the VLR cDNA for sequence analysis.

*Establishing stable producing VLR cell lines.* Sequence analysis identified 11 unique VLR sequences that were synthesized into double-stranded gene fragments using Integrated DNA Technologies gBlocks<sup>®</sup> services. The gene fragments were engineered to include a 6x-histidine tag at the N-terminus of the VLR along with compatible restriction sites at both the 5' and 3' end to be cloned into our lentiviral expression vector. Additionally, a murine IgG2a-Fc scaffold was included at the 3' of the gene fragment in order to generate VLR fusion proteins that will dimerize through a disulfide linkage in the Fc region. Viral accessory plasmids along with the bioengineered VLR gene fragment encoding expression plasmid incorporating a strong viral promoter were transiently transfected into 293T-17 cells using a calcium phosphate transfection method to generate LV vectors pseudotyped with the VSVG envelope similar to the method described previously except for the utilization of the calcium phosphate transfection reagent (Sigma Aldrich, St. Louis, MO). [169] Conditioned media was collected for three days beginning at 48 hours post transfection and passed through a 0.45µm filter. Virus was concentrated by overnight centrifugation at 10,000 x g overnight, followed by filtration using a .22 µm filter. Viral concentrate titers were determined using quantitative real time PCR analysis. LVs generated for each VLR were used to transduce HEK 293 cells in complete DMEM with 10% FBS and polybrene. Transduced cells were expanded and then plated into 96-well plates using a limiting dilution. In order to identify single-cell colonies secreting VLR-fusion proteins, a sandwich ELISA was performed on conditioned media using an



anti-6x-histidine antibody to capture VLR antigen and a biotinylated anti-IgG2a-Fc antibody for detection. An alkaline-phosphatase-based detection method was employed to determine relative expression and only the cells that produced the highest OD values for each VLR were expanded and banked for protein purification.

*Purification of VLR-fusion proteins.* Stable producing cells lines were expanded into triple flasks and switched to AIM-V media at approximately 80% confluency. Conditioned media was then collected every other day for one week and stored at 4 degrees celcius after centrifugation to eliminate any cell debris. Using the manufacturer's protocol, conditioned media was applied directly to an equilibrated Ni-sepharose excel column at a flow rate of approximately 1mL/min overnight (catalogue number: 17-3712-02). The VLR-fusion proteins were eluted off the column in fractions and analyzed by SDS-PAGE gel and coomassie staining. Pooled fractions were buffer exchanged using an Amicon filter into HBS with 0.01% sodium azide.

*Flow analysis of VLR-fusion fluorophore conjugates.* VLR-fusion proteins were conjugated to a FITC fluorophore using the Pierce FITC Antibody Labeling Kit according to the manufacturer's instructions (catalogue number: 53027). Whole bone marrow from C57/Bl6 mice were flushed from the femurs and tibias of mice and lysed with red cell lysis buffer at room temperature for 10 minutes. Cells were then washed in PBS with 1% FBS and stained with a lineage cocktail, anti-Sca antibody, anti-ckit antibody, and the VLR conjugated for flow cytometry analysis for 30 minutes at 4 degrees celcius. Leukocytes were gated off of FSC-A and SSC-A analysis followed by removal of doublets from the data set. Positive staining from the lineage cocktail was used to eliminate lineage committed cells. Finally, anti-Sca and anti-cKit analysis was used to identify Lin<sup>-</sup> Sca<sup>+</sup> cKit<sup>+</sup> stem cells present within the data set. Analysis of VLR-fusion protein FITC staining was performed using median fluorescence intensity comparing the lineage committed compartment to the Lin<sup>-</sup> Sca<sup>+</sup> cKit<sup>+</sup> stem cell compartment.

## 5.4 Results

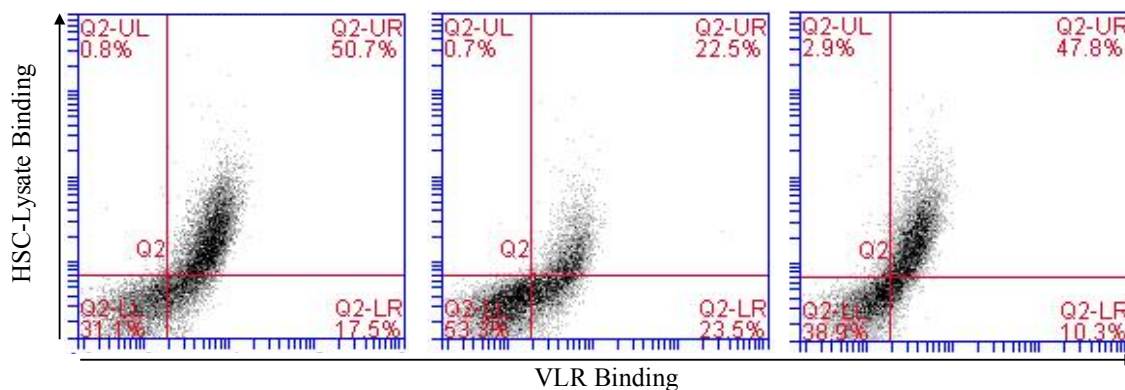
*Generating HSC specific VLR-fusion proteins.* In order to generate a murine HSC- specific VLR repertoire, Lin<sup>-</sup> Sca<sup>+</sup> cKit<sup>+</sup> stem cells were purified from whole bone marrow and injected into the coelomic cavity of lampreys. The Lin<sup>-</sup> Sca<sup>+</sup> cKit<sup>+</sup> compartment of bone marrow cells enrich for murine stem cells capable of undergoing self-renewal and repopulating the hematopoietic niche.[211] Following immunizations, the lampreys were exsanguinated and peripheral lamprey leukocytes were incubated with biotinylated HSC lysate and screened for positive binding using flow cytometric analysis. Total RNA was collected from the leukocytes of lampreys that demonstrated a positive immune response and was used to generate a yeast library. Using yeast surface display and flow cytometric techniques, single clones of yeast cells expressing a single VLR were identified based on binding to biotinylated HSC lysate. **(Figure 5.1)** Additionally, primers targeting sequences within the yeast display expression vector were used to PCR amplify VLR cDNA for sequence analysis.

Flow cytometric and sequence analysis identified eleven unique VLR sequences capable of binding murine HSCs. These sequences were then synthesized into double-stranded gene fragments using Integrated DNA Technologies gBlocks<sup>®</sup> services that were cloned into our LV expression vector used to produce recombinant lentivirus. The VLR expression cassette was designed to produce VLR fusion proteins with a 6x-histidine tag at the N-terminus of the protein and an IgG2a-Fc scaffold at the C-terminus in order to facilitate dimerization. Before vector production, VLR plasmids were transiently transfected into HEK293 cells and conditioned media was analyzed by Western blot to confirm protein production. **(Figure 5.2)** As expected, a single band could be observed at approximately 98kDa for each VLR sequence. However, one VLR-fusion protein was observed to have a higher molecular weight due to the two LRRV repeats observed within the cDNA coding sequence. LVs generated for each VLR were used to repeatedly transduce HEK 293 cells in order to create stable producing cell lines. Using limiting dilutions, cells were plated onto 96-well plates and an ELISA assay was used to identify single clones

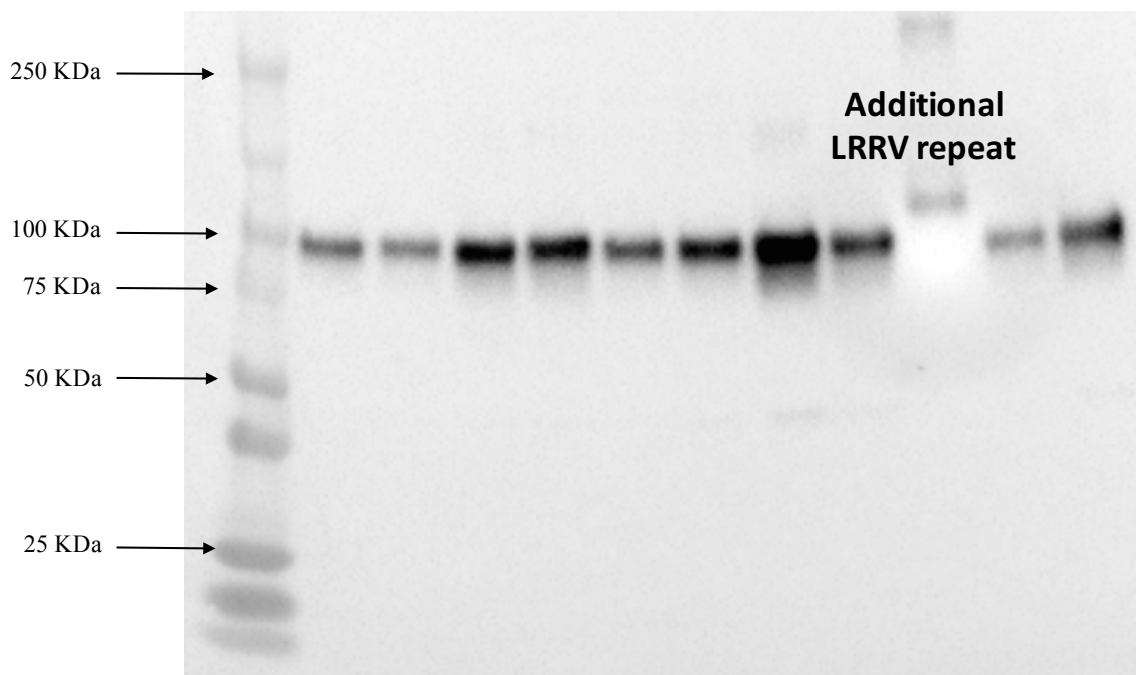
secreting recombinant VLR-fusion proteins. Cells that generated the highest OD values were expanded and banked for protein purification.

VLR fusion proteins were purified using a Tricorn column packed with Ni Sepharose excel resin purchased from GE healthcare. Eluted fractions were analyzed by SDS-page gel electrophoresis and pooled fractions were buffered exchanged into HBS with 0.01% sodium azide using Amicon filtration. **(Figure 5.3)** Analysis showed purified VLR-fusion protein dimers with minimal contaminants in each fraction. Purification resulted in approximately 1mg of VLR-fusion protein per liter of conditioned media, although variability was observed between each unique VLR.

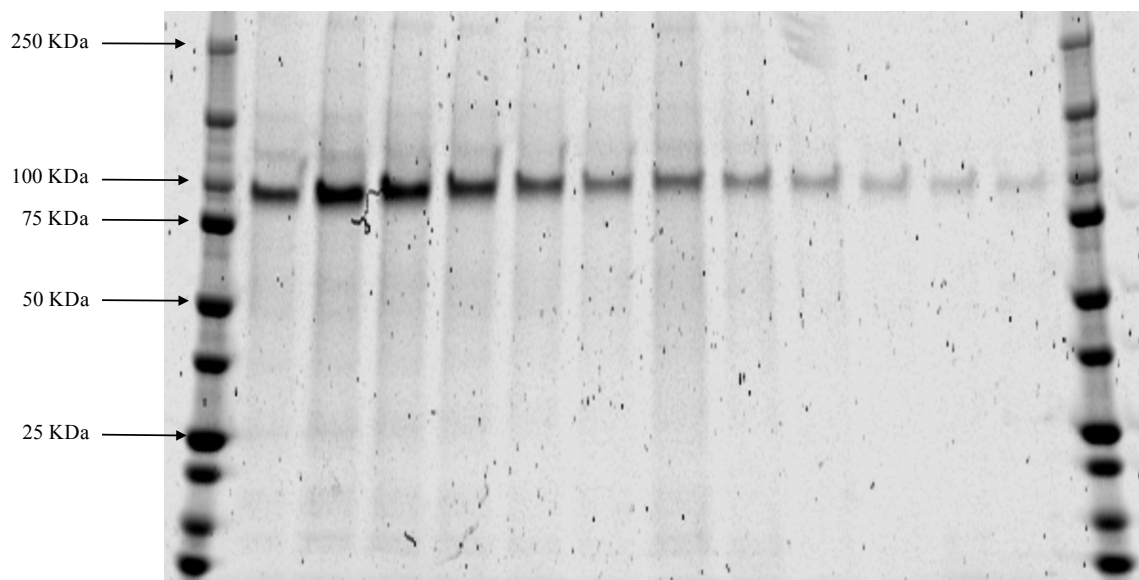
*Identifying the cellular targets of VLR-fusion proteins within the hematopoietic compartment.* In order to develop a VLR-based immunotoxin that can be used as a conditioning agent for stem cell transplantation, VLR-fusion proteins must bind hematopoietic stem cells within the bone marrow compartment. To identify binding events, flow cytometry was performed on whole bone marrow cells from C57/Bl6 mice incorporating a VLR-fusion protein conjugated to the FITC fluorophore. The three top VLR-fusion protein candidates were chosen for initial screening based on the available binding data to biotinylated HSC lysate during yeast display screening. Given that VLRs were generated from the immunization of lampreys using  $\text{Lin}^- \text{Sca}^+ \text{cKit}^+$  stem cells, this population of cells was identified within the data set to examine VLR-fusion protein binding. Analysis showed enriched staining by the VLR-fusion protein FITC conjugate within the  $\text{Lin}^- \text{Sca}^+ \text{cKit}^+$  stem cell compartment. Interestingly, the VLR-fusion protein with two LRRV repeats demonstrated stronger staining within the  $\text{Lin}^- \text{Sca}^+ \text{cKit}^+$  stem cell compartment compared to other candidates. **(Figure 5.4)** Furthermore, a 1.6-fold increase in median fluorescence intensity was observed in the  $\text{Lin}^- \text{Sca}^+ \text{cKit}^+$  stem cell compartment compared to lineage committed cells when staining with each VLR-fusion protein FITC conjugate (data not shown). These observations suggest that the VLR-fusions proteins preferentially bind HSCs within the bone marrow.

**Figure 5.1**

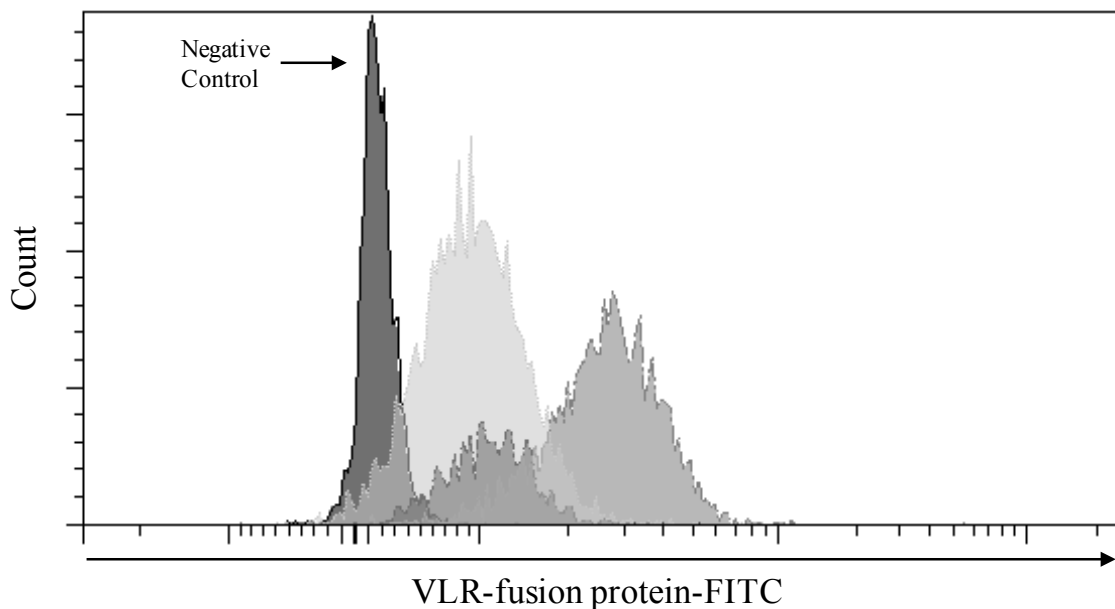
**Figure 5.1 Flow cytometric analysis of yeast surface display.** Transfected yeast cells were induced to express individual VLR proteins on the cell surface. Induced cells were incubated with biotinylated by HSC lysate and analyzed by flow cytometry. Positive binders were identified by demonstrating positive staining for the streptavidin-conjugate indicated on the x-axis and positive anti-VLR antibody staining indicated on the y-axis.

**Figure 5.2**

**Figure 5.2 Transient transfection of VLR sequences in HEK 293 cells.** Western blot analysis of VLR-fusion proteins sequences detected in the conditioned media of transiently transfected HEK 293 cells. VLR fusion proteins were detected using an anti-mouse IgG2a Fc antibody. The western blot results show VLR-fusion proteins to approximate the predicted size of 98kDa with the exception of a single VLR expected to have a higher molecule weight due the addition of another LRRV repeat sequence.

**Figure 5.3**

**Figure 5.3 SDS-PAGE analysis of elution fractions from the purification of VLR-fusion proteins.** Ni sepharose excel resin packed into a Tricorn column was used to purify VLR-fusion proteins from the conditioned media of HEK 293 stable producing cell lines. Analysis showed purified VLR-fusion protein dimers with minimal contaminants in each fraction.

**Figure 5.4**

**Figure 5.4 Flow cytometry analysis of VLR-fusion protein FITC conjugates to  $\text{Lin}^- \text{Sca}^+ \text{cKit}^+$  stem cells within the bone marrow compartment.** Using flow cytometry, whole bone marrow cells were analyzed for positive staining with the VLR-fusion protein FITC conjugate. Given that lampreys were immunized with  $\text{Lin}^- \text{Sca}^+ \text{cKit}^+$  stem cells, we compared the staining of this population with a negative control (bone marrow cells incubated with all staining antibodies minus the VLR-fusion protein FITC conjugate).

## 5.5 Discussion

Using the lamprey immune system, we generated a VLR-based antibody capable of recognizing murine HSCs in vitro. Repeated immunizations of lampreys with primary Lin<sup>-</sup> Sca<sup>+</sup> cKit<sup>+</sup> stem cells resulted in an antigen specific immune response producing murine HSC specific lamprey leukocytes. Yeast surface display and sequence analysis resulted in the identification of 11 unique VLR sequences that could be synthesized into double-stranded gene fragments. These gene fragments were then subcloned into an LV expression vector to generate stable producing cell lines that can secrete VLR-fusion proteins. These proteins are designed to have a 6x-histidine tag on the N-terminus for ease of purification. Furthermore, the C-terminus includes a mouse IgG-2a Fc scaffold to facilitate dimerization as VLR's require dimerization in order to facilitate binding. Additionally, the Fc scaffold allows the VLR-fusion protein to be more adaptable to qualitative and quantitative analysis including conjugation of fluorophores for flow cytometry techniques, and ELISA. Using a VLR-fusion protein conjugated to FITC, flow cytometry analysis confirmed that lamprey immunizations resulted in variable lymphocyte receptors capable of binding murine HSCs. Analysis of the median fluorescence intensity demonstrated a 1.6-fold shift within the Lin<sup>-</sup> Sca<sup>+</sup> cKit<sup>+</sup> compartment compared to lineage committed cells, suggesting flow cytometry offers a means to screen VLR-fusion proteins for stem cell binding.

In the future, we hope to develop methods to identify the cognate binding partners of our VLR-fusion proteins in order to identify novel receptors or epitopes present on the surface of stem cells. These binding sites could serve as potential therapeutic targets for the development of non-genotoxic conditioning agents, as one of the primary goals of this dissertation is to improve the safety of conditioning regimens for HSCT. Currently, our knowledge of stem cell biology and physiology is limited to murine animal models, however, this technology offers a means to one day explore the surface expression profile of human stem cells. The lamprey immune system may provide a high-throughput platform for developing VLR-fusion proteins that could bind cell surface receptors on any given cell population, both mouse or human. Furthermore, the ease of expression,



purification and long-term stability make VLRs an excellent candidate for therapeutic development.

### **5.6 Acknowledgements**

We would like to thank the laboratory of Dr. Max Cooper (Emory Vaccine Center), specifically Brant Herrin, for their expertise and guidance in developing the methods used in this chapter. This work was supported by funding from the National Institutes of Health/National Heart, Lung and Blood Institute (Doering and Spencer) and Hemophilia of Georgia (Doering and Spencer).

## **Chapter 6**

### **General Discussion**

## 6.1 Summary of Results

Since the first clinical trial in 1990, the field of gene therapy has experienced rapid expansion and development. The *Journal of Gene Medicine* has reported gene therapy trials covering at least 8 different indications, with over 100 clinical studies initiated in 2016 alone. Substantial progress has been made improving both safety and efficacy of *in vivo* and *ex vivo* gene transfer methods, including vector design and delivery. However, with decades of research behind us, the field has recognized that designing a therapeutic gene therapy platform is not one size fits all. The size of the gene, the cell and tissue source of gene expression, and the method of delivery all play a role in vector design and differ significantly given the context of disease. Furthermore, the immunological consequences of ectopic protein expression can vary with method of gene transfer as well as the gene product.[212] Both *in vivo* (AAV vector infusion) and *ex vivo* (LV-HSCT) gene transfer methods have shown efficacy in preclinical mouse models of HA, offering the unique opportunity to examine the strengths and weaknesses of each approach in the context of the same disease.

Chapter 2 explores the *in vivo* expression of an oversized high-expressing FVIII transgene (ET3) 125% the size of the packaging capacity of an AAV vector. This transgene incorporates the porcine sequences of the A1 and A3 domains of FVIII resulting in 10-100 fold increased expression in a heterologous cell culture system.[90] These studies addressed whether or not the superior biological activity of ET3 would be maintained in a liver-directed AAV gene therapy platform. Given the possibility that the AAV packaging capacity may convey limitations to FVIII expression, initial studies used hydrodynamic injections of the vector packaging plasmid to compare *in vivo* expression levels. As predicted, liver-directed expression of ET3 maintained a 20-fold higher plasma concentration to that of human FVIII. Additionally, AAV-ET3 provided phenotypic correction in a mouse model of HA determined by both plasma FVIII levels as well as hemostatic challenge. However, these studies also confirmed that the molecular assembly of ET3 packaged virus resulted in the truncation of ssDNA genomes within the viral capsid. Furthermore, specific

activity analysis confirmed that very little if any truncated FVIII protein was being transcribed *in vivo*. These studies confirmed that incorporation of a high-expressing human/porcine FVIII transgene could overcome the inferior biosynthetic properties of non-modified human FVIII in an AAV gene therapy setting. Lastly, the ET3 vector system demonstrated increased potency by achieving therapeutic levels of FVIII expression at reduced vector doses compared to previously published data.[213, 214]

In chapter 3, we began examining the immunological implications of FVIII expression in a gene therapy setting. The liver is considered to be an immune-privileged organ capable of inducing antigen-specific regulatory T cells (Tregs) that provide immune surveillance and maintain peripheral tolerance. These Tregs are suggested to facilitate immunological tolerance to the transgene product in an AAV gene transfer platform, as was demonstrated in AAV-FIX studies.[215] AAV was also shown to modulate FIX immunity by eradicating pre-existing anti-FIX inhibitory antibodies.[157] However, we were unable to replicate these results using an AAV-FVIII vector. Furthermore, peripheral infusions of AAV-FVIII were unable to facilitate robust peripheral tolerance capable of preventing immune responses to intravenous injections of recombinant FVIII protein.

Contrary to AAV, HSCT is hypothesized to result in transplantation tolerance of gene-modified cells, an immunological state in which the transplant recipient can launch pathogen specific immune responses while preventing immune responses against the transgene product. This state of tolerance is thought to be achieved through central and peripheral mechanisms in which both T cell education takes place as well as induction of antigen-specific Tregs. LV-HSCT gene transfer of FVIII prevented the development of anti-FVIII IgG after several infusions of recombinant FVIII. These results taken together suggest that bone marrow chimerism of FVIII gene-modified cells may offer superior protection against immune responses to exogenous FVIII.

Lastly, chapter 4 explores the development of non-genotoxic conditioning agents that could be used in a preparative regimen for HSCT. Conditioning agents are an integral part of LV-HSCT

gene therapy as they provide space in the bone marrow compartment for newly transplanted cells to engraft and undergo hematopoiesis. As was described in chapter 4, the acute and chronic toxicities associated with conditioning diminish the risk-to-benefit ratio of LV-HSCT gene therapy for the treatment of HA. Therefore, we investigated the use of an immunotoxin, specifically targeting the CD45 cell surface receptor, to facilitate engraftment of FVIII-gene modified cells in a mouse model of LV-HSCT gene therapy for HA. Anti-CD45 IgG was conjugated to recombinant saporin engineered to have a free cysteine at position 255 of the amino acid sequence. Saporin is a type 1 ribosomal inhibitor protein that requires cellular internalization mediated through the binding of a secondary ligand to the cell surface to elicit its cytotoxic effects. The resulting immunotoxin showed dose-responsive depletion of progenitor and stem cells, as well as depletion of mononuclear cells within the bone marrow compartment. However, conditioning with CD45-Sap255 was unable to provide consistent engraftment of genetically modified cells. Our results demonstrated only 2 out of 8 conditioned mice to have measurable levels of donor chimerism. Clearance analysis indicated the immunotoxin was no longer present in peripheral blood and bone marrow by day 6 after infusion. To substantiate these findings, we performed a series of experiments varying the time between immunotoxin conditioning and time of transplantation. However, our results again demonstrated levels of engraftment inconsistent with the levels of depletion achieved by CD45-Sap255 conditioning. These studies concluded that therapeutic levels of engraftment may require deeper depletion of the progenitor cell compartment.

## **6.2 Implications of Findings**

The focus of this dissertation was to evaluate the immunological implications of *in vivo* and *ex vivo* FVIII gene transfer in a preclinical mouse model of HA. While both approaches of gene therapy have their unique strengths and challenges, AAV and LV-HSCT mediated gene transfer will facilitate therapeutic levels of circulating FVIII in plasma. However, the primary focus of HA gene therapy development has been therapeutic efficacy and vector safety, very little effort has

been made understanding the mechanisms of immunotolerance and how they may apply to previously untreated patients.

Given that long-term therapeutic FVIII expression is repeatedly observed, we know that both liver or HSC-directed FVIII gene transfer results in immune-nonresponsiveness. These observations are particularly significant as current clinical trials for AAV-FVIII vectors exclude patients that have not received factor replacement therapy or have a history of immune mediated adverse events. While these current exclusion criteria are necessary to ensure the safety of patients during initial clinical studies, preclinical data suggests that HA gene therapy may induce anti-FVIII immune responses in previously untreated patients. AAV gene transfer studies in nonhuman primates resulted in anti-FVIII immune responses and loss of transgene expression.[102] Furthermore, as what was observed in the clinical studies for hemophilia B, it is possible that patients may require some level of factor replacement therapy in spite of gene therapy.[37] An analysis in the durability of immune-nonresponsiveness, particularly in the context of recombinant FVIII infusions, will be significant to the design of clinical protocols involving previously untreated patients.

The results of the experiments presented in chapter 3 confirm our hypothesis that chimerism within the bone marrow compartment offers a more robust induction of immune-nonresponsiveness to FVIII antigen. These findings suggest that LV-HSCT gene therapy for HA offers minimal risks in terms of inhibitor development, particularly in the population of previously untreated patients. Furthermore, these findings suggest that subtherapeutic outcomes from LV-HSCT gene transfer, such as low plasma levels of circulating FVIII or breakthrough bleeding episodes, can be easily managed using protein replacement therapy without the development of anti-FVIII IgG. Animal experiments presented in chapter 3 suggest that chimerism within the bone marrow compartment is a stronger driver of immune tolerance induction than peripheral FVIII expression. Therefore, in addition to phenotypic correction, LV-HSCT gene therapy offers the added benefit of robust tolerance induction to FVIII, a therapeutic outcome previously left

unmeasured in clinical gene therapy development. Despite the superior immune modulation offered by LV-HSCT gene transfer, the risks of acute and chronic toxicities attributed to conditioning regimens diminishes the benefits of treatment in the context of HA. While chapter 4 explores the development of non-genotoxic agents to circumvent these risks, current options for preparative regimens could hinder the use of LV-HSCT gene therapy for treating HA.

Liver-directed AAV gene transfer has been described to induce antigen-specific regulatory T cells that facilitate induction of peripheral tolerance. However, contrary to FIX, our studies show that this mechanism of immunotolerance is unable to prevent an immune response to exogenous FVIII in our mouse model of HA. Nonetheless, the results in chapter 2 establish that liver-directed AAV gene transfer results in sustained therapeutic plasma levels of circulating FVIII, and the results of chapter 3 do not discredit AAV-FVIII as a viable clinically efficacious treatment for HA. While it is unclear whether AAV-FVIII may be less immunogenic than recombinant protein replacement therapy in previously untreated patients, current available clinical data has not reported any anti-FVIII IgG related adverse events in patients considered to be tolerized to FVIII.

The observations and results of this dissertation suggest that the future landscape of treatment for HA is highly diversified and that improvement in clinical care is possible for the global community of persons with HA. The current standard of care in the United States includes prophylactic use of recombinant FVIII, a standard that is not available in countries with emerging markets that house close to 75% of the worlds hemophilia population. At a cost of \$300,000 annually for factor replacement therapy, gene therapy offers a means to reduce the financial burden of treatment as well as costs associated with comorbidities like hemarthropathy due to inadequate care. Furthermore, 20-30% of patients treated with recombinant FVIII develop inhibitory antibodies and require therapeutic interventions that may cost close to \$1,000,000 per patient. The immunomodulatory effects of both stem cell and liver-directed gene transfer may reduce the incidence of inhibitor formation as observed with conventional protein replacement therapy, but this information will not be known until clinical trials begin including previously untreated patients.

Despite the potential to improve quality of care, the nature of vector development and gene transfer creates a substantial price tag for gene therapy. Glybera, the first AAV product approved by the European commission, became the world's most expensive medicine at a price point of a million dollars. Unfortunately, the product had very little demand and as of 2017 the company, Uniqure, has plans to discontinue Glybera from their portfolio. These results are concerning and may leave people questioning whether there is a place for gene therapy in the global market, particularly in countries with underprivileged economies. However, it is important to note that there are several defining factors that separate gene therapy for HA from Glybera. The field can very clearly define clinical efficacy due to decades of evidence demonstrating how even moderate changes in FVIII plasma levels can cause major improvements in clinical outcomes. Evidence of immunomodulation and a potential to reduce the risks of a highly expensive complication, anti-FVIII inhibitor formation, offers a unique advantage to gene therapy for HA. Lastly, tracking clinical improvement post treatment is both feasible and easily interpretable, making gene therapy for HA attractive to investment companies and insurance companies interested in amortizing treatment costs based on patient improvement. Despite the substantial price tag, this dissertation suggests that combination gene therapy is the future of clinical care for HA. LV-HSCT gene transfer FVIII may provide robust immunotolerance induction to FVIII antigen, while AAV-FVIII gene transfer can be used to boost FVIII expression levels to compensate for potential subtherapeutic outcomes post-transplantation.

### **6.3 Limitations and Future Directions**

A major limitation of this study, as with all studies of this kind, is the use of a murine HA model to predict human immune responses to vector gene transfer. While mouse models have shown evidence of innate and humoral immune responses against the AAV capsid, these models were unable to predict the CD8<sup>+</sup> T cells responses observed in clinical trials that caused acute liver toxicity in liver-directed AAV gene transfer.[40] Along these same lines, repeated infusions of recombinant FVIII protein result in uniform production of inhibitory anti-FVIII IgG in immunized



mice, an incidence of inhibitor formation not observed in people with severe HA. Therefore, the lack of immunomodulation observed in these AAV-FVIII studies may not accurately predict the outcomes observed in humans. It is plausible that replacement protein therapy due to inadequate FVIII plasma levels after AAV gene transfer may not result in the formation of anti-FVIII inhibitory antibodies observed in chapter 3. However, currently available preclinical animal models are unable to simulate or predict human anti-FVIII immune responses, specifically in persons with HA who not been exposed to recombinant FVIII protein. Nonetheless, efforts to improve tolerance induction in the context of FVIII gene transfer will be useful.

Clinical observations have determined that AAV gene transfer is dose-limiting due to a CD8<sup>+</sup> T cell anti-capsid immune response that causes acute liver inflammation and injury marked by increased plasma levels of alanine transaminase and aspartate transaminase. However, attenuation of anti-FVIII humoral immunity was observed in mice treated with vector doses above this threshold, suggesting that vector optimization could result in more robust tolerance induction. Ongoing studies in the lab have produced more potent vector systems capable of achieving supraphysiological levels of plasma FVIII at vector doses an order of magnitude lower than previously published (publication currently under review). It is our hope that future studies using these novel vector systems will demonstrate more robust tolerance induction and the prevention of anti-FVIII IgG development after immunological challenge with recombinant FVIII. These vectors may also prove to be more efficacious in eradicating pre-existing inhibitory anti-FVIII antibodies. However, questions remain concerning the mechanistic relationship between circulating antigen secreted from AAV transduced hepatocytes and tolerance induction to FVIII. Our data suggests that higher levels of FVIII activity are required to induce tolerance. Contrary to our results, Merlin et al. 2017 reported robust tolerance induction using an LV liver directed vector that restricted FVIII expression to liver sinusoidal endothelial cells or resident liver macrophages, both of which are believed to be responsible for inducing antigen-specific Tregs. In these studies, HA mice expressing below normal levels (1 unit/mL) of FVIII were tolerized to exogenous FVIII.

Furthermore, their vector system achieved successful eradication of pre-existing inhibitors followed by phenotypic correction and measurable levels of circulating plasma FVIII. [216] These results suggest that future studies using *in vivo* gene transfer methods for HA may benefit from targeting liver antigen presenting cells rather than hepatocytes to modulate pre-existing immunity or prevent immune responses from exogenous FVIII.

Contrary to AAV, there are decades of clinical data demonstrating robust tolerance induction facilitated by chimerism within the bone marrow compartment. While these mechanisms are not completely understood, important cellular “players” have been identified and studies are ongoing to determine their contribution to transplantation tolerance.[217] We hypothesize that similar mechanisms associated with transplantation tolerance apply to LV-HSCT facilitated unimolecular microchimerism, low-level engraftment of genetically-modified transplanted cells (microchimerism with <10% genetically-modified peripheral blood mononuclear cells) expressing a single transgene product (unimolecular neoantigen) that is absent from the host proteome. The results of chapter 3 support this hypothesis as LV HSCT treated mice did not develop an immune response to exogenous FVIII. However, harnessing the immunomodulatory capabilities of LV HSCT gene therapy requires the development of safer preparative regimens prior to transplantation.

Current conditioning protocols typically use the alkylating agent busulfan because of its ability to kill non-cycling stem cells and lymphocytes, however, it can cause serious acute toxicities including veno-occlusive disease and lung injury. Thus far, clinical trials involving LV-HSCT and non-immune compromised patients have used myeloablative conditioning regimens to achieve therapeutic engraftment.[27, 28] These doses of busulfan can put patients at a significant risk of conditioning-related complications. Even reduced-intensity conditioning can be associated with risks of regimen-related toxicity.[218] To circumvent these problems, groups have attempted to develop antibody-based conditioning regimens that target the immunophenotype of cells, specifically the c-kit receptor, to try and reduce off-target side effects. However, these studies used naked antibodies that proved ineffective in immune-competent mice.[191] These results were

puzzling as Fc-mediated effector functions were hypothesized to be the mechanism of action utilized to deplete stem cells, a mechanism that should be more efficient in a functional immune system.[219]

Our approach, first reported by Palchaudhuri et al. 2016, has been to develop a saporin-based immunotoxin that targets the cell surface receptor CD45 present on all HSCs.[201] Rather than depend on the effector of functions of the host immune system, this mechanism of action requires internalization of the toxin through binding of the CD45 receptor. However, while an excellent proof of concept target, our studies suggest more substantial depletion of the progenitor compartment is necessary for engraftment. The CD45 receptor has 8 different human isoforms and their expression patterns are dictated by cell type as well as differentiation/maturation status.[197] Additionally, previous clinical studies using lytic anti-CD45 antibodies were unsuccessful in achieving substantial depletion within the bone marrow.[198] Using only an anti-CD45 antibody-based conditioning regimen is likely not clinically translatable and future studies should focus on the development of an immunotoxin cocktail targeting cell surface receptors that will facilitate deeper depletion of both progenitor and stem cell compartments to increase the efficacy of transplantation.

Using the conjugation process described in chapter 4, we have revisited the c-kit narrative using our saporin-based approach. By incorporating a monoclonal antibody shown to initiate receptor internalization, initial studies have demonstrated depletion in the progenitor bone marrow compartment of mice. In addition to c-kit, we have performed similar experiments targeting other receptors present on the cell surface of primitive quiescent stem cells and platelets. We hypothesize that an immunotoxin cocktail targeting multiple receptors will behave synergistically to provide deep depletion of both progenitor and stem cells that were otherwise resistant to CD45-Sap255 cytotoxicity. Experiments are ongoing to determine if the combinatorial approach to conditioning will increase the efficacy of LV-HSCT gene therapy.

Future approaches should also include efforts to identify novel epitopes or cell surface receptors present on progenitor and stem cells. We have developed a novel technology with the help of the laboratory of Dr. Max Cooper to engineer lamprey VLR fusion proteins capable of binding murine HSCs. Lampreys are jawless vertebrates that diverged from a common ancestor 550 million years ago and VLRs are the functional unit of the lamprey adaptive immune system. Similar to mammalian IgG, VLRs are generated from a recombinatorial process that results in an extensive repertoire to mediate antigen-specific immune responses.[220] We have created VLRs that target murine stem cells through a multi-step immunization and screening process. Using primers directed to conserved regions of the VLR, sequences were amplified from mature lymphocytes of immunized lampreys. These sequences were then cloned in a yeast display expression system to create a library of VLRs. Yeast cells expressing VLR peptides on the surface were then screened using flow cytometry to detect positive binding of stem cell lysate. This screening process resulted in the identification of 11 unique VLR sequences that were cloned into an expression plasmid to generate VLR fusion proteins containing an IgG2a Fc scaffold.

Using flow cytometric analysis, initial studies have demonstrated VLR-fusion proteins enrich for HSCs. Their binding profile appears to be specific for the Lin<sup>-</sup>ckit<sup>+</sup>Sca<sup>+</sup> compartment within mouse bone marrow, however non-specific VLR fusion proteins should be generated to confirm binding. In the future, we hope to identify the cognate binding partners to our VLR sequences. Further analysis will also include cellular internalization assays to determine if a VLR-Sap255 immunotoxin could be used to deplete stem and progenitor cells within the bone marrow compartment. If proven successful, the lamprey immune system offers us an opportunity to interrogate any cell population and generate variable lymphocyte receptors with the potential to bind novel epitopes or surface proteins previously unknown to be expressed on the cell surface.

#### **6.4 Conclusion**

This dissertation investigated the immunomodulatory potential of *in vivo* and *ex vivo* gene transfer methods used in gene therapy platforms for the treatment of HA. While the data in this

dissertation demonstrate both AAV-FVIII and LV-HSCT to be curative in a murine model of HA, bone marrow chimerism from LV HSCT results in more durable tolerance induction to the transgene product. However, safer conditioning regimens are necessary to maintain an acceptable risk to benefit ratio for LV-HSCT gene therapy for HA. This dissertation explores the use of an immunotoxin based conditioning regimen to facilitate engraftment of FVIII gene-modified cells. While transplantation efficacy still remains a challenge, these studies suggest that optimization and the use of an immunotoxin cocktail targeting multiple cell surface receptors will result in phenotypically corrective levels of engraftment.

Clinical gene therapy for the treatment of HA is still in its infancy. In the late 1990's and early 2000's, several clinical trials were initiated using oncoretroviruses but were terminated due to poor FVIII expression. For the next decade, clinical gene therapy slowed as the field focused on developing recombinant vector systems with increased transduction efficiencies and improved safety profiles. Finally, in 2016, the field of gene therapy initiated an AAV-based clinical study directing FVIII expression to liver hepatocytes. While the most recent reported results indicate therapeutic levels of FVIII expression, patient enrollment is restricted to individuals who have undergone protein replacement therapy with no immunological adverse events.

Determining immunological responses to transgene products, particularly FVIII, remain a challenge for clinical gene therapy. Current animal models are unable to adequately predict immune responses to both vector proteins as well as the transgene product. Given these limitations, it is difficult to see a clear path for bringing clinical gene therapy to patients who have not established immunological tolerance to FVIII or have not undergone protein replacement therapy. The studies summarized in this dissertation help to provide insight to this problem. HSCT gene therapy demonstrated robust tolerance induction to intravenous infusions of recombinant FVIII in an inhibitor prone murine model of HA. Additionally, there have been decades of stem cell transplantation research providing evidence of immunological tolerance to allotransplants in people. Taken together, these observations suggest that HSCT gene therapy may provide a path to

broadening clinical gene therapy for both HA and other hematological nonmalignant disease in patients that have not been exposed to the transgene product.

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