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Pharmacologic evaluation of fVIII expression in retroviral mediated gene therapy for hemophilia A

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B.S., University of Maryland, 2001

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An Abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences Program in Molecular and Systems Pharmacology

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ABSTRACT

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By Kerry Titus Dooriss

Hemophilia A is an excellent candidate for gene therapy because it can be cured by the transfer of a single gene into any cell with access to the bloodstream. Unfortunately, the success of gene therapy has been hampered by low and transient transgene expression, the risk of insertional mutagenesis, and the frequent induction of an immune response. We hypothesized that the development of a safer viral vector incorporating a high-expressing fVIII transgene for the transduction and transplantation of hematopoietic stem cells can overcome the barriers of low and transient expression with a decreased risk of insertional mutagenesis. With respect to the development of a high-expressing transgene, a number of human, porcine, and human/porcine chimeric transgenes were developed for the purpose of determining which transgene exhibits the highest fVIII expression following lentiviral-mediated hematopoietic stem cell transduction and transplantation in a mouse model of hemophilia A. We discovered that porcine fVIII and one chimeric fVIII transgene express at significantly higher levels than any of the human constructs. We also demonstrated sustained fVIII expression in hemophilic mice following lentiviral-mediated transduction and transplantation of gene-modified hematopoietic stem cells. To evaluate the ability of a lentivirus to mediate lineage-specific fVIII expression in gene-modified bone marrow cells, self-inactivating lentiviral vectors

were developed to express porcine fVIII under the control of the β-globin promoter and locus control region. We demonstrated high-level fVIII expression following lentiviral mediated gene-transfer into a myelogenous leukemic cell line, as well as from gene-modified hematopoietic cells in transplanted mice. Although we confirmed this lentiviral-mediated gene transfer platform is capable of GFP and fVIII expression in mice, hematopoietic stem cell transduction efficiency was limiting. Transduction efficiency was lower with the porcine fVIII-containing lentivirus compared to the GFP-containing lentivirus. Together, these studies demonstrate that a porcine or human/porcine chimeric transgene is able to mediate high level fVIII expression, that erythroid-specific fVIII expression is possible, and that hematopoietic stem cell transduction is the limiting factor in the future success of this erythroid-specific lentiviral-mediated gene transfer system.

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouricil
AAV	adeno-associated virus
ALD	adrenoleukodystrophy
APC	allophycocyanin
APPT	activated partial thromboplastin
BDD	B domain deleted
β-globin	beta globin
bGHpA	bovine growth hormone polyA
BHK-M	baby hamster kidney
BiP	Immunoglobin binding protein
bp	base pair
BU	Bethesda unit
CD	cluster of differentiation
CGD	chronic granulomatous disease
CMV	cytomegalovirus
CPPT	central polypurine tract
CTS	central termination sequence
DIG	digoxigenin
DM	double mutation (L303E/F309S)
DMEM	Dulbecco's modified eagle media
$E16^{-/-}$	exon 16 null

EF1α	elongation factor 1 alpha
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FACT	factor assay control plasma
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
fIX	coagulation factor IX
FRT	*flp* recognition target
fVIII	coagulation factor VIII
fvp	function viral particle
fX	coagulation factor X
γ-retrovirus	gamma-retrovirus
HAMSTeRS	Haemophilia A Mutation, Structure, Test, and Resource Site
HEK-293T	human embryonic kidney 293T
hFlt3	human flt3 ligand
hfVIII	human factor VIII
hIL-11	human interleukin 11
HS 2,3,4	hypersensitive sites 2, 3, 4
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplant
IDUA	alpha-L-iduronidase

ITR	inverted terminal repeat
kb	kilobase
KSL	c-kit^{+}/sca-1^{+}/lin^{-}
LacZeo	beta galatasidase/zeocin fusion gene
LCR	locus control region
LTR	long terminal repeat
LV	lentiviral particle
MFI	mean fluorescence intensity
μg	microgram
μL	microliter
mIL-3	murine interleukin 3
mL	milliliter
MMLV	Moloney murine leukemia virus
MOI	multiplicity of infection
mSCF	murine stem cell factor
MSCV	murine stem cell virus
NK	natural killer
PACE	paired basic amino acid cleaving enzyme
PCR	polymerase chain reaction
PE-Cy-7	Phycoerythrin-Cy7
PEI	polyethyleneimine hydrochloride salt
PGK	phosphoglycerate kinase
pfVIII	porcine factor VIII

PBS	phosphate buffered saline
Ψ	psi packaging signal
RBC	red blood cells
RRE	rev reverse element
SCID-X1	X-linked severe combined immunodeficiency
SD	standard deviation
SIN	self-inactivating
SIV	simian immunodeficiency virus
TNFα	tumor necrosis factor alpha
U	units
VSVG	vesicular stomatitus virus
vWF	von Willibrand factor
WBC	white blood cell
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element

Chapter 1:

Introduction

1.1 – Hemostasis and the blood coagulation cascade

Hemostasis is a complicated and highly regulated process involving many coagulation factors and cofactors present in blood plasma. Normal hemostasis is achieved by tight regulation of the levels of these factors and even the slightest change may tilt the balance toward either thrombosis or anti-coagulation. The role of each coagulation factor is best depicted by the classical coagulation cascade **(Figure 1.1A),** which consists of the intrinsic and extrinsic pathways**.** In the extrinsic pathway, circulating factor VII (fVII) is activated to become fVIIa, which then activates factor X (fX) to fXa. FXa works with its cofactor, activated factor V (fV), to activate a small amount of prothrombin to thrombin. This small burst of thrombin, formed by initial vessel injury, participates in a positive feedback loop. Thrombin then activates factor VIII (fVIII) and fV, to allow activation of more thrombin. With sufficient levels of thrombin in circulation, fibrinogen is activated to become fibrin, which in the presence of activated factor XIII, polymerizes to form a cross-linked fibrin clot at the site of injury. Although the initial burst of thrombin can be achieved in the absence fVIII and other components of the intrinsic clotting cascade, the formation of a fibrin clot cannot be achieved. FVIII is a large glycoprotein of ~170 kDa with domain structure A1-A2-B-A3-C1-C2, based on internal sequence homology (**Figure 1.1B**). Endogenous fVIII is present in circulation at ~100 ng/mL, and is mainly bound to von Willebrand factor (vWF) to form a stable complex. Upon activation by thrombin, fVIII is released from vWF and is proteolytically cleaved at arginine sites 372, 740, and 1689 to generate the activated A1/A2/A3-C1-C2 heterotrimer.

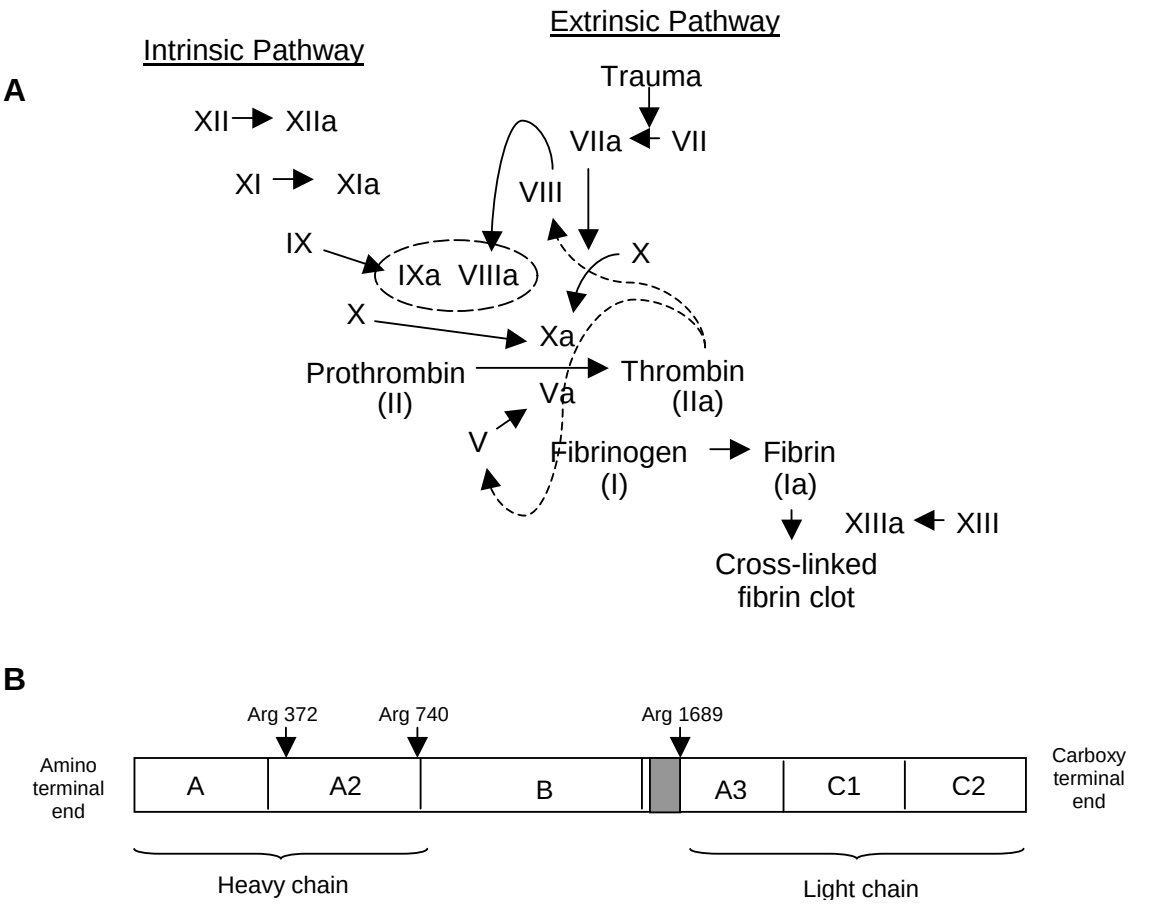

Figure 1.1 – Blood coagulation cascade and the role of fVIII. A) Activated fVIII (VIIIa) works with its cofactor IXa in the intrinsic pathway of the coagulation cascade to activate factor X. Factor Xa works with its cofactor Va to activate thrombin, which activates fibrinogen to form fibrin. Fibrin polymerizes and cross-links to form a stable blood clot at the site of vessel injury. B) FVIII is comprised of domain structure A1-A2-B-A3-C1-C2. Upon activation by thrombin, fVIII is cleaved at arginine sites 372, 740, and 1689, whereby the B domain is released leaving the A1-A2 heavy chain and A3-C1-C2 light chain. In circulation, fVIII is stabilized and bound to von Willebrand factor (vWF) at the shaded acidic region of the A3 domain.

1.2 – Genetic and clinical implications hemophilia A

Hemophilia A is an inherited coagulation disorder resulting from a deficiency in fVIII, due to structural defects in the fVIII gene. The frequency of occurrence of hemophilia A is 1 in every 5,000 to 10,000 live male births, with approximately 50% of patients diagnosed with the severe form of the disease. People with severe hemophilia A have less than 1% of the normal level of fVIII in the blood and suffer from recurrent spontaneous bleeding and minor-injury induced joint bleeding. Many different mutations are responsible for the inactivation of fVIII and disease severity is dependent on the type of mutation. The 2 most common fVIII mutations are inversions of intron 1 and intron 22, which occur in 2% and ~50% of severe hemophilia A patients, respectively (Bagnall et al 2002; Lakich et al 1993). A variety of nonsense, missense, and splicing mutations, as well as large and small deletions and insertions are responsible for the inactivation of the fVIII gene in the remaining severe hemophilia A patients **(Table 1.1).** Cases of moderate and mild hemophilia are generally due to missense mutations (Jacquemin et al 2003). As of January 2007, 1209 unique mutations had been discovered and are cataloged in the hemophilia A mutation database on the Haemophilia A Mutation, Structure, Test, and Resource site (HAMSTeRS) on the World Wide Web at http://europium.mrc.rpms.ac.uk.

Depending on the disease phenotype, hemophilia A can lead to a severely increased risk of prolonged bleeding from superficial injuries. The repeated presence of blood in the joints leads to synovial hypertrophy and eventually

Table 1.1 – Summary of reported unique fVIII mutations

	Point Mutations			Deletions		Insertions
Exon	Missense	Nonsense (stop)	Splicing	Small	Large	
1	15	2	4	2	n/a	1
2	10	1	5	8	n/a	4
3	25	0	6	5	n/a	0
4	35	7	4	1	n/a	1
5	13	1	10	4	n/a	1
6	11	2	6	4	n/a	2
7	38	5	4	8	n/a	1
8	27	6	1	8	n/a	1
9	27	3	5	7	n/a	3
10	10	3	3	5	n/a	0
11	36	1	4	2	n/a	1
12	20	5	4	2	n/a	1
13	32	3	2	7	n/a	3
14	43	52	6	77	n/a	39
15	19	2	3	4	n/a	0
16	26	7	2	7	n/a	0
17	28	3	2	6	n/a	5
18	34	5	1	5	n/a	4
19	16	1	8	4	n/a	4
20	8	1	0	2	n/a	2
21	9	5	1	0	n/a	1
22	19	5	4	3	n/a	1
23	30	1	4	7	n/a	0
24	13	5	4	3	n/a	2
25	13	2	2	7	n/a	3
26	26	3	0	9	n/a	0
TOTAL	**583**	**131**	**95**	**197**	**135**	**80**

destruction of the synovium, cartilage, and bone, causing chronic pain and limitation of movement. Intracranial bleeding was the major cause of death in hemophiliacs until the 1970s – 1990s, when AIDS became the major cause of death in this population. At that time, treatment of hemophilia was limited to intravenous infusion of plasma-derived fVIII, and it was not known that much of the blood and plasma contained in blood banks harbored the lethal human immunodeficiency virus (HIV). Today, much safer treatment options exist. Though plasma-derived fVIII replacement therapy is still used, considerable improvements in blood-screening techniques have been made to ensure the safety of blood before use. An even safer option for replacement therapy is the use recombinant fVIII. Since the cloning and characterization of the fVIII gene in 1984 (Gitschier et al 1984), recombinant fVIII has become a major source of this protein. However, this therapy is extremely costly due to the expense of production and purification. The high cost and limited availability of recombinant fVIII prevents most patients from prophylactic use, limiting them to treatment only after acute bleeding episodes.

1.3 – Gene therapy as a cure for hemophilia A

Gene therapy is an attractive alternative to intravenous protein replacement therapy. FVIII gene therapy would prevent the need for commercial manufacture of recombinant fVIII by providing the patient with the machinery to produce his own functional fVIII. It could also eliminate the need for frequent venous injections, which can lead to infection of the infusion port. Hemophilia A is an

excellent candidate for gene therapy because it is monogenic and its characteristic bleeding episodes can be prevented by only minor increases in circulating fVIII. FVIII levels as low as 1-2% of normal are enough to prevent the most severe bleeding episodes experienced by patients with severe hemophilia, or those endogenously producing less than 1% of normal circulating fVIII. As well, any cell with access to the bloodstream is a potential target cell for transduction, which can then become a dispensary for therapeutic protein. Unfortunately, hemophilia A gene therapy studies have typically been hampered by low expression of fVIII within heterologous systems and to date, no clinical trials have been able to achieve sustained, high levels of fVIII. Three hemophilia A gene therapy clinical trials have been initiated, each using a different gene delivery system (reviewed in Chuah et al 2004, White 2001). In the first trial in 1998, dermal fibroblasts were transfected *ex vivo* with the human B-domain-deleted fVIII transgene (BDD-hfVIII). Transduced cells were isolated, expanded, and injected back into the patient's omentum, resulting in transient increases in circulating fVIII for at least 18 months. Initial results indicated that *ex vivo*, non-viral gene transfer is safe, however, only low levels of circulating fVIII were observed throughout the trial. The second study used a replication-deficient Moloney murine leukemia virus (MMLV)-derived vector containing BDD-hfVIII administered directly into the bloodstream of 13 patients, aged 18 – 55 years old. This resulted in transient measurable levels of fVIII and decreased bleeding frequency for at least 6 months. Although no subject demonstrated sustained fVIII levels over 1% of normal (whereby 100% normal is ~100 ng/mL), 6 subjects

exhibited fVIII levels over 1% on at least 2 occasions and isolated levels of 2.3 – 6.2% of normal were reported for 3 subjects. This trial was shut down due to the detection of vector sequence in a single semen sample from one subject 9 weeks into the study. The third trial involved only a single patient who received an infusion of an adenovirus expressing full-length fVIII under the control of a liver-specific promoter. Although measurable levels of fVIII were detected, this trial was shut down due to the development of hepatotoxicity. Taken together, these clinical studies demonstrate the ability to increase circulating fVIII to levels associated with improved hemostasis in hemophilia A patients, albeit only temporarily. However, to many patients, even temporary improvement of the hemophilia A phenotype would be considered a success.

The low levels of fVIII expressed from mammalian cell systems are due to a number of factors, including mRNA instability and inefficient fVIII secretion. Understanding fVIII on a molecular level has proved critical in the development of higher expressing fVIII transgenes. It has been shown that specific modifications made to the human fVIII cDNA sequence can increase fVIII protein secretion by optimizing intracellular protein interactions. Miao et al demonstrated that providing 6 N-linked glycosylation sites from the deleted B domain increased fVIII interaction with mannose-binding lectin protein (LMAN1), resulting in more efficient carbohydrate-facilitated transport from the endoplasmic reticulum to the Golgi apparatus (Miao et al 2004; Swaroop et al 1997). Swaroop et al demonstrated that mutating amino acids L303 and F309 to their homologous

amino acids in coagulation factor V decreased fVIII binding to immunoglobin binding protein (BiP) and increased fVIII secretion (Swaroop et al 1997). Additionally, we have demonstrated that B domain-deleted (BDD) porcine fVIII is expressed at 10-100 fold greater levels than BDD-hfVIII *in vitro* (Doering et al 2002b; Dooriss et al 2009) and have identified sequences within porcine fVIII (pfVIII) that are necessary and sufficient for high-level expression (Doering et al 2004). Using these higher-expressing fVIII transgenes incorporated into various viral vectors, a number of groups have been able to achieve high-level sustained fVIII expression in hemophilia A mice (Cerullo et al 2007; Dooriss et al 2009; Ide et al 2007; Moayeri et al 2005; Ohmori et al 2006; Shi et al 2007; Tiede et al 2003). With the creation of these higher expressing transgenes, future clinical trials may no longer be hindered by low levels of fVIII. However, lack of long-term expression and the development of a safe and effective viral delivery system remain as limitations to hemophilia A and other gene therapy trials.

1.4 – Successes and setbacks in gene therapy

Several different types of viral and non-viral delivery systems are currently being explored in gene therapy applications, each with its own advantages and disadvantages. **Table 1.2 (top)** lists some of the most common viral-mediated gene delivery platforms and their major characteristics. Non-viral approaches typically require large amounts of DNA for direct injection and offer predominantly transient expression in a limited number of tissues. However, viral-mediated gene transfer can be achieved by a number of different viral vectors.

Table 1.2 – The Basic Characteristics of Viral Vectors Commonly Used in Hematopoietic Stem Cell (HSC) Gene Therapy

Virus Name	Class	Genome size	HSC integration
MLV	γ-retrovirus	10 kb	Yes
HIV-1	Lentivirus	11 kb	Yes
Foamy Virus	Spumavirus	11 kb	Yes
AAV	Parvovirus	4.7 kb	Yes/No
Adenovirus	Adenovirus	36 kb	Yes/No

Relative sizes of promoters and transgenes

	Promoters		
Transgene	EF1α (1140 bp)	β-globin (3.4 kb)	MSCV-LTR (520 bp)
Wild type hfVIII (7.1 kb)	8.2 kb	10.5 kb	7.6 kb
BDD-fVIII (4.4 kb)	5.5 kb	7.8 kb	4.9 kb
BDD-N6-fVIII (5.1 kb)	6.2 kb	8.5 kb	5.3 kb
eGFP (750 bp)	1.9 kb	4.2 kb	1.3 kb

Clinical trials with recombinant adeno- and adeno-associated virus

Adenovirus and adeno-associated virus (AAV) are beneficial in gene therapy due to their efficiency in transduction and ease of production. However, an adenovirus carrying the gene for ornithine transcarbamylase (OTC) was responsible for the death of Jesse Gelsinger in 1999 due to a massive immune response to the vector. This was the first documented death in a gene therapy clinical trial. An investigation by the U.S. Food and Drug Administration concluded that the scientists involved in the trial, including the lead researcher, Dr. James M. Wilson from the University of Pennsylvania, broke several rules of conduct, resulting in Jesse Gelsinger's untimely death. First, Jesse should have been excluded from the trial due to his having circulating ammonia levels higher than the maximum allowed for the protocol. Increased ammonia levels are a symptom of OTC deficiency, however, Jesse's levels at the time of the study put him at high risk of encephalopathy or even death, and should have excluded him from the trial. Second, the study failed to report that 2 patients experienced severe side effects during the trial. Lastly, the informed consent documentation did not mention that a number of monkeys died after being given similar treatment.

AAV is a small, replication incompetent, non-enveloped virus. The AAV genome is built of single-stranded DNA comprised of inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames: *rep* and *cap.* The small genome size and resulting packaging capacity (4.7 kb) limits their use for

gene therapy applications utilizing large transgenes and/or large promoters **(Table 1.2, bottom)**. AAV has been used to mediate fVIII gene transfer, however the fVIII sequence must be divided into 2 parts, which are packaged and delivered separately as the heavy and light chain. Upon co-transduction of heavy chain and light chain AAV particles, the heavy chain and light chain are translated as separate proteins and using this 2-vector system, transduced cells have been shown to secrete functional fVIII at levels equivalent to wild type (Burton et al 1999; Yonemura et al 1993). AAV can infect both dividing and non-dividing cells and has the ability to stably integrate into the host cell genome at a specific site in the human chromosome 19 (Surosky et al 1997). However, this feature is prevented in AAV gene therapy vectors by removal of the *rep* and *cap* cDNA. At least 11 different AAV serotypes exist, each with different cellular infectivity properties. Tissue specificity is determined by the capsid serotype , however pseudotyping of AAV vectors to alter their tropism may be necessary to achieve optimal transduction for successful AAV-mediated gene therapy. AAV2 is the most commonly researched serotype and has been shown to induce robust humoral immunity in animal models and humans, whereby up to 80% of humans are thought to be seropositive for AAV2. Since AAV manifests episomally in gene therapy applications, most transgene expression is transient. Prior to the death of a patient treated with an AAV-derived vector in July 2007, these vectors were thought to be the safest of all viral delivery vectors. In an experimental gene therapy trial for rheumatoid arthritis, 127 patients received an injection of AAV expressing a tumor necrosis factor-alpha (TNF-α)-blocker directly into an

inflamed joint. After a single dose, no subjects showed serious side effects. Seventy-four subjects received a second dose, one of whom developed a serious adverse event and died of massive organ failure 4 days later (Kaiser 2007). Following this death, the National Institute of Health's Recombinant DNA Advisory Committee launched an investigation to determine if gene therapy killed Jolee Mohr. Doctors at the University of Chicago Hospital where she was treated determined her cause of death was due to the presence of *Histoplamsa capsulatum*, a fungus endemic in the Midwest. Although it was unclear why her immune system was unable to fight the infection, doctors suspect that her taking the TNF-α-blocker adalumumab (Humira), possibly in combination with the TNF-α-blocker gene therapy, could have caused severe immune suppression resulting in the spread of infection throughout her body. In other cases of AAV-mediated gene therapy, immune response to a specific AAV serotype has been the cause of adverse events. Currently, much research is focused on finding a specific AAV serotype that is not subject to immune clearance *in vivo.*

Recombinant retroviruses for use in gene therapy

Retroviruses are a class of enveloped virus capable of inserting their proviral sequence into the genome of infected cells. When cells are transduced *ex vivo*, all retroviral structural proteins are removed prior to transplantation of gene-modified cells, making the chance for immune response to the virus itself very low. Retroviral particles are made up of two identical copies of single-stranded RNA, which are contained within a capsid shell. The capsid is surrounded by a

lipid membrane containing envelope glycoproteins on its outer surface which confer retroviral specificity **(Figure 1.2**). The tropism of a retrovirus depends on the specific envelope proteins present on the surface of the virus **(Table 1.3)**. Upon cellular infection, the retroviral RNA genome is reverse transcribed into linear double-stranded DNA. The DNA remains associated with a nucleoprotein complex known as the preintegration complex (PIC), made up of viral and host cellular proteins. The PIC is translocated to the nucleus and viral DNA becomes integrated into the host genome (**Figure 1.3**). The integrated DNA is then transcribed by the host machinery for the life of that cell and its progeny. Each gene-modified cell is then capable of making therapeutic protein.

Recombinant retroviruses used for gene therapy have been extensively modified to be replication-incompetent once inside an infected cell. While wild type retroviruses produce new retroviral particles upon transcription of integrated viral DNA, replication incompetent retroviruses no longer contain the sequences for the core viral proteins necessary to produce and package new viral particles. Included in the wild type retroviral genome are three regions: gag, pol and env encoding for capsid proteins, viral enzymes and envelope proteins, respectively, flanked on either end by a 5' and 3' long terminal repeat (LTR) (**Figure 1.4A**). The viral LTRs are responsible for the initiation of viral DNA synthesis, integration of proviral DNA, and the regulation of viral gene expression. Retroviruses are engineered to be replication incompetent and suitable for gene transfer applications by the removal of the gag, pol and env genes. These genes are

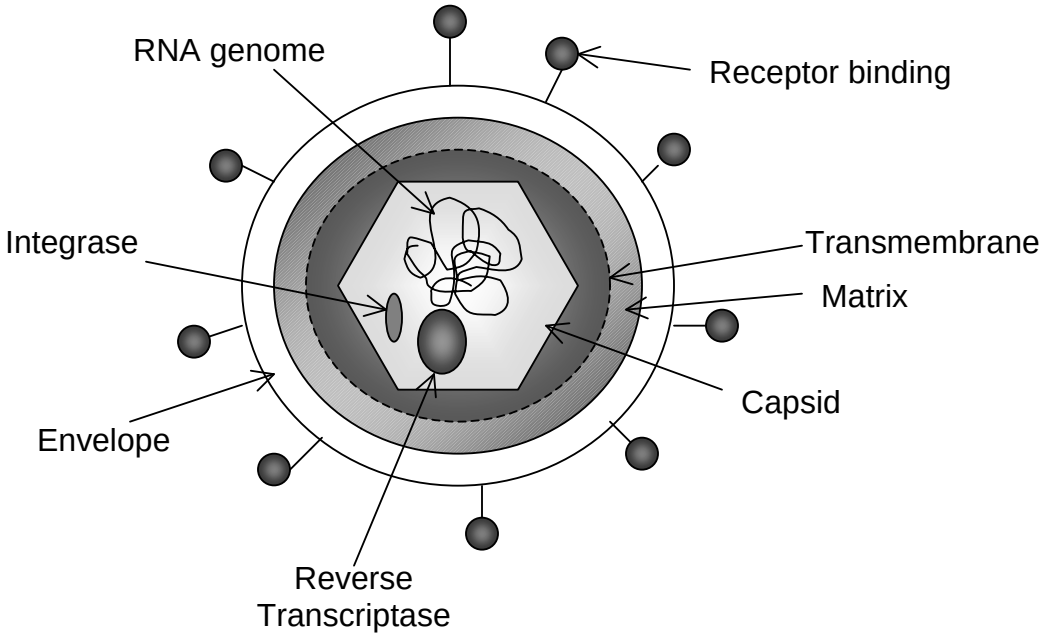

Figure 1.2 – Schematic of retrovirus virion structure. The main components of a retroviral virion include two identical strands of RNA genome, viral capsid proteins, reverse transcriptase for synthesis of viral DNA, integrase for integration into host DNA, and envelope proteins to confer tropism for receptor binding.

Table 1.3 – Envelope Glycoproteins Commonly Used for Retroviral Pseudotyping

Envelope	Receptor	Tropism	Stable for concentration
Ecotropic	Cationic amino acid transporter	Rodent	Yes
Amphotropic	Sodium-dependent phosphate transporter	Human and murine	No
VSV-G	Ubiquitous phospholipids	Any cell type	Yes

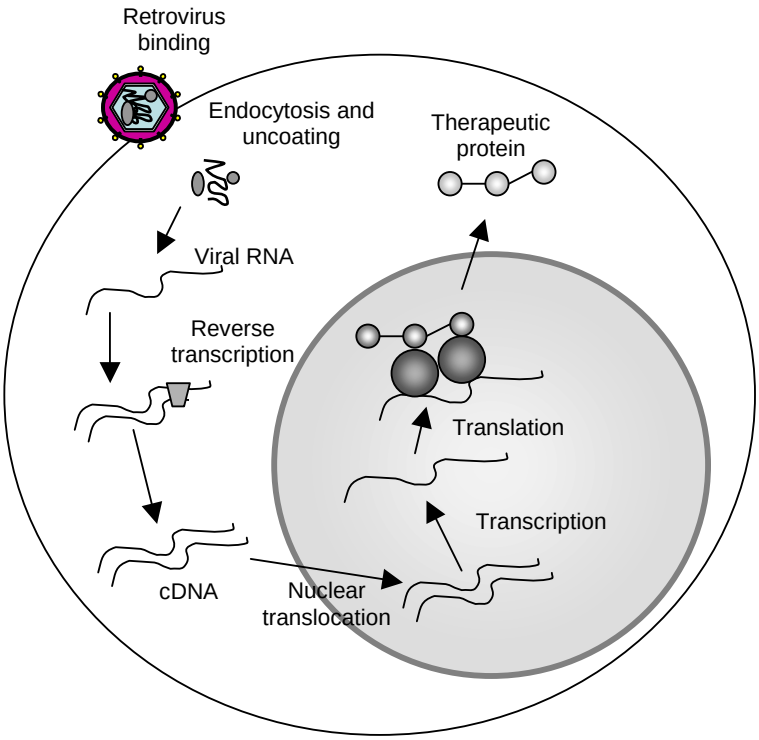

Figure 1.3 – Overview of the retroviral transduction process. Upon binding to a host cell, a retrovirus enters the cell via endocytosis where the viral coat breaks down to release viral RNA into the cytoplasm. Cellular reverse transcriptase converts RNA to cDNA, which is imported into the nucleus and integrated into the host genome using cellular integrase. The proviral sequence is then transcribed to make mRNA, which is translated into proteins encoded in the viral genome. In retroviral vectors used for gene therapy, the viral genome has been replaced with genes that code for a therapeutic protein. Upon integration, host cellular machinery will transcribe and translate the protein of interest instead of the viral proteins originally designed in the retroviral genome. These recombinant retroviruses contain none of the code necessary to make new virus, indicating a replication incompetent virus.

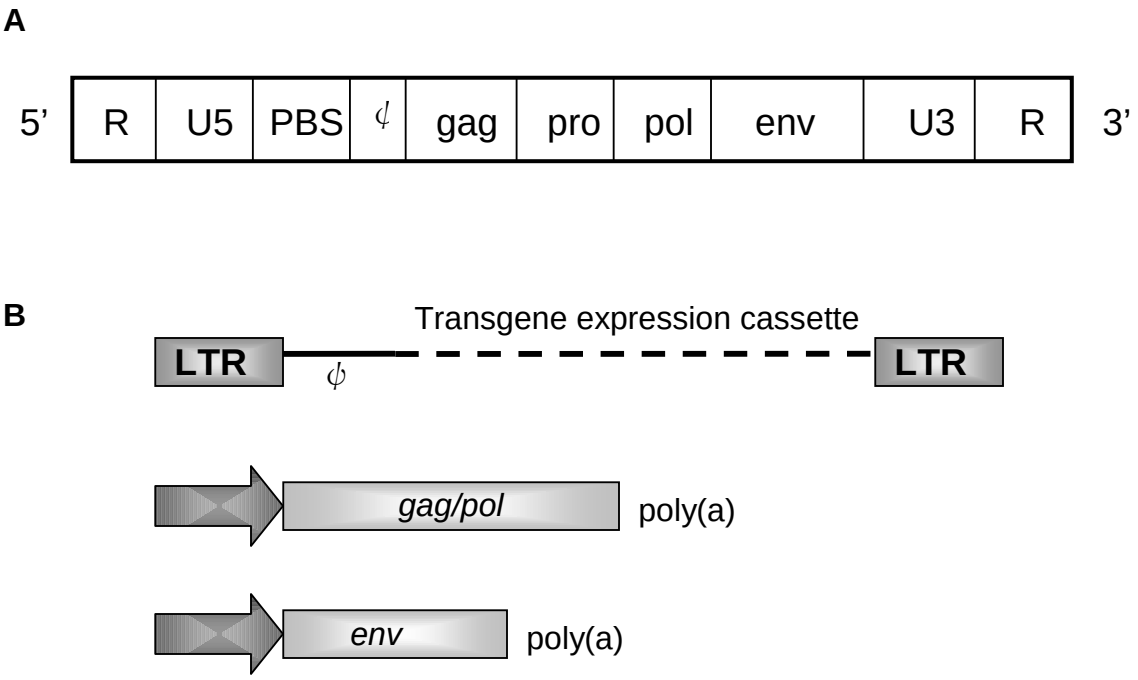

Figure 1.4 – Schematic of a retroviral genome. A) The wild type retroviral genome consists of a 5` and 3` long terminal repeat necessary for initiation of viral DNA synthesis, tRNA primer binding site (PBS), psi packaging region (ψ), *gag* genes which encode structural proteins to make up the capsid, *pol* genes which code for the viral enzymes reverse transcriptase and integrase, *env* genes which code for envelope proteins, and a *pro* gene to make viral protease. Upstream of the gag sequence is the psi region, necessary for viral packaging and virus production. B) Viruses used in gene therapy are engineered replication-incompetent by removing the viral genes from the viral genome. These genes are provided on 2 or 3 separate plasmids and are provided transiently in viral producer cells during viral production. In place of the viral genes, an expression cassette containing a transgene of interest is inserted. Viral LTRs and psi region are retained in order to package and produce recombinant virus.

provided transiently, on separate plasmids, in the packaging cell used to make virus **(Figure 1.4B)**. In place of these genes within the reengineered viral genome is an expression cassette containing the transgene of interest. The essential LTR and psi elements are retained in order to allow transgene expression and packaging into viral capsids during virus production. As a result, retroviral vectors are capable of transducing cells and expressing the foreign gene, but further viral particles can no longer be produced due to the lack of structural and enzymatic genes.

Potential effects of retroviral integration

The most efficient retroviral vectors are based on the Moloney murine leukemia virus (MMLV) due to the simplicity of its genome and its high transduction efficiency. However, MMLV and other γ-retroviruses can only infect dividing cells, which limits their application in gene therapy. Lentiviruses such as HIV and simian immunodeficiency virus (SIV) are a subclass of retrovirus able to infect both dividing and quiescent cells. Due to their efficiency in transduction and ability to integrate into the host genome, retroviral vectors are most beneficial in the development of long-term expression. It was previously thought that retroviral integration was random, making the chance for oncogenic transformation extremely low. However, this risk was realized during a clinical trial for severe combined immunodeficiency disease (SCID-X1) initiated in Paris in 1999 (Cavazzana-Calvo et al 2000; Hacein-Bey-Abina et al 2003). Originally, the trial was credited as the first successful gene therapy in humans, as 9 of 11 patients

treated showed full phenotypic correction of the disease after genetic modification and transplantation of $CD34^+$ cells, using a MMLV-derived γ-retroviral vector. However, within 2 years of the trial's initiation, 4 of the 11 patients developed leukemia. In 2 patients, vector integration occurred within the LMO2 locus, which codes for a known T cell oncogene (Hacein-Bey-Abina et al 2003). To date, three patients have been treated successfully with chemotherapy and one has died (Associate Editor 2007). Though it was not known if the deregulation of LMO2 was solely responsible for the oncogenic transformation, the U.S. Food and Drug Administration put a hold on all U.S. clinical trials using retroviruses in gene therapy of HSCs in 2003, pending further studies surrounding retroviral integration events (Yi et al 2005). It was soon revealed that integration is not random as was once thought, but that retroviruses favor sites of active gene transcription (Schroder et al 2002). More specifically, murine leukemia virus (MLV) and MLV-derivatives favor integration near promoters while HIV and other lentiviruses prefer integration downstream of the transcriptional start site (Schroder et al 2002; Wu et al 2003).

The majority of mutations from retroviral insertion are caused by the presence of viral enhancers and their wide target area (Uren et al 2005). Enhancer elements present in viral LTRs are capable of activating genes up to 100 kb away (Metais & Dunbar 2008) **(Figure 1.5A)**. Transcriptional read-through due to weak viral polyadenylation signals also contributes to unregulated increases in gene transcript levels (Schambach et al 2007) **(Figure 1.5B)**. Conversely, decreased

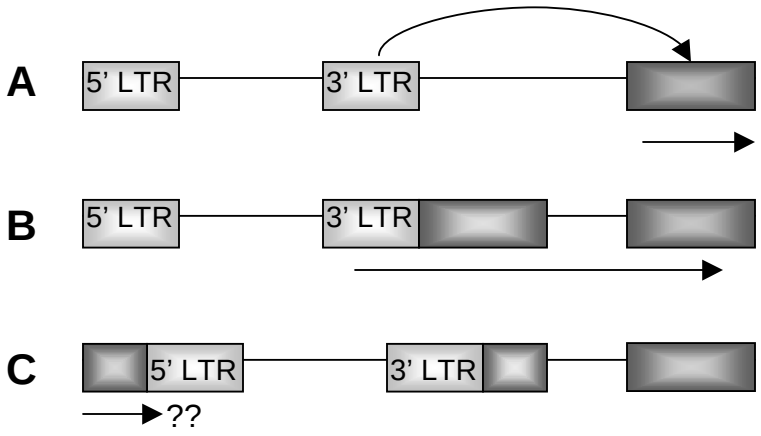

Figure 1.5 – Schematic of the pathways to retroviral-mediated insertional mutagenesis. A) Retroviral insertion upstream of genes allows for LTR-mediated activation of *cis* genes up to 100 kb away. B) 3' LTR may fuse to endogenous promoter regions which can cause abnormal gene regulation. Transcriptional read-through due to a weak polyadenylation signal is also possible. C) Insertion within a gene could generate inactive transcripts by nonsense or frameshift mutations.

protein levels can be seen as a result of proviral insertion downstream of the promoter producing a truncated gene transcript **(Figure 1.5C)**, though inactivating mutations represent only a minority of oncogenic mutations because both gene copies must be inactivated in order to completely block protein expression. For this reason it is thought that lentiviruses, whose proviral DNA integrates well within the boundaries of the transcriptional unit, are the safest retroviral vectors for use in gene therapy. However, the strong viral LTRs must be inactivated in order to prevent deleterious enhancer effects resulting in potential proto-oncogene activation or inactivation.

1.5 – Improving the safety of gene therapy

The development of a safe viral delivery vector with a minimized risk for insertional mutagenesis is crucial in moving forward in the field of gene therapy. A number of vector modifications have already been shown to decrease the risk of insertional mutagenesis and have created a new generation of viral vectors. First was the choice of viral platform for gene transfer. Lentiviruses are inherently safer than γ-retroviruses because of their positional preference in integration. Second was to provide the sequences for essential viral proteins on separate plasmids in order to prevent viral replication in transduced cells and to decrease the risk of recombination in the packaging cell line resulting in a replication-competent virus. Another safety feature is preventing localized and long-distance activation of endogenous genes from the integrated proviral sequence by inactivation of the viral LTRs. Lentiviruses possess a 3’ and 5’ LTR, each

containing a strong promoter and enhancer in its U3 region that drives viral transcription. Although these elements are efficacious to produce high transgene expression, they are also capable of long distance transcriptional activation (West & Fraser 2005). In order to decrease this risk of enhancer mutations, self-inactivating (SIN) vectors have been developed in which the U3 regions of the LTRs have been deleted. Modlich and colleagues showed a 12-fold decrease in the rate of vector-mediated transformation by using a SIN vector coupled with a single internal U3 promoter (Modlich et al 2006). Another method used in the development of a potentially safer vector is to limit transcription by using a cell- or tissue-specific promoter. By decreasing the number of cells actively transcribing the inserted proviral sequence, the risk of insertional mutagenesis also decreases. Although viral promoters typically induce much higher rates of transcription than eukaryotic promoters, their ability to cause enhancer mutations, as well as their tendency for silencing by methylation limit their effectiveness in gene therapy studies (Gonzalez-Murillo et al 2008; Ott et al 2006; Papadakis et al 2004). Recent data have demonstrated successful erythroid- and platelet-specific expression of various transgenes following *ex vivo* genetic modification of bone marrow using lentiviral vectors (Sadelain 2002; Shi et al 2007) as well as liver-specific expression of fVIII and fIX using AAV vectors (Chao et al 2000; Ishiwata et al 2009; Snyder et al 1999). Since transcription of the integrated proviral sequence may be greatly reduced using a cell-specific promoter compared to using viral LTRs, it will be essential to optimize cell-specific protein production by using the highest expressing transgene and

choosing a cell population with enough cells actively manufacturing and secreting protein in order to reach therapeutic levels. Lastly, limiting the number of vector copies per cell would be desirable to limit the risk of induction of cooperating mutations by 2 or more integrated proviral sequences.

1.6 – Hematopoietic stem cell-mediated gene therapy

The use of hematopoietic stem cells (HSCs) as the target cell population in lentiviral-mediated gene therapy applications is one way to overcome the barrier of transient expression. First, lentiviruses have the ability to insert their proviral sequence into the genome of transduced cells. Once the sequence is integrated into the genome, host cellular machinery will begin to transcribe the proviral sequence containing the gene of interest. Upon translation and secretion of protein, the transduced cell will become a source of therapeutic protein for the life of that cell. Second, since that cell is a HSC, it can both self renew into another gene-modified HSC, or differentiate into all of the blood cell types in the hematopoietic compartment (**Figure 1.6**). In theory, transduction and transplantation of a single gene-modified HSC can result in the complete repopulation of the hematopoietic compartment, whereby all cells are gene-modified and secreting therapeutic protein. However, the need to limit the risk of insertional mutagenesis is especially important when the target cell population includes hematopoietic stem cells. By definition, stem cells are capable of both self-replication and differentiation. While these features make them ideal for use in gene therapy, they also cause an exponential increase in the risk of insertional

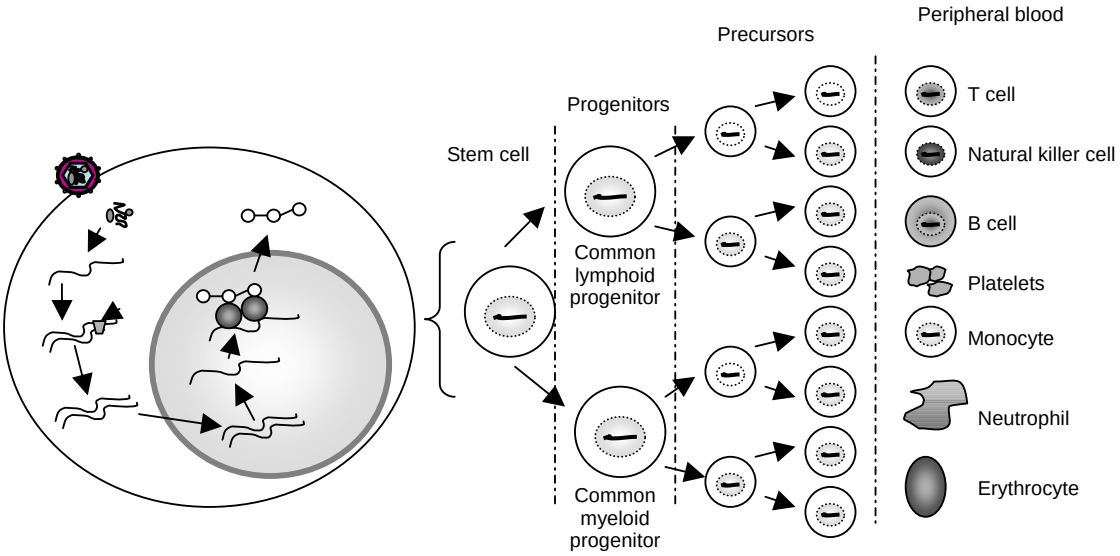

Figure 1.6 – Lentiviral-mediated hematopoietic stem cell gene therapy. Upon transduction of a hematopoietic stem cell with a recombinant lentivirus, the proviral sequence containing the gene of interest is integrated into the host cell genome. The gene-modified HSC can then self renew to form another gene modified HSC or it can differentiate into a gene-modified myeloid or lymphoid progenitor cell. Each progenitor cell will then mature into various precursor cells and finally into a terminally-differentiated peripheral blood cell, each one containing the gene of interest in its cellular DNA. Each gene-modified cell has the machinery to produce the therapeutic protein of interest for the life of that cell.

mutagenesis. For example, if a single HSC receives a mutagenic integration event, each progeny formed by either self-renewal or differentiation will also contain the mutagenic integration.

Diseases that have seen success in animal models using HSC gene therapy include hemophilias A and B (Barrette et al 2000; Doering et al 2007; Dooriss et al 2009; Ide et al 2007; Moayeri et al 2005; Shi et al 2007), X-linked adrenoleukodystrophy (ALD) (Cartier et al 2009), X-linked chronic granulomatous disease (CGD) (Ott et al 2006), severe combined immunodeficiency disease (SCID) (Hacein-Bey-Abina 2008; Hacein-Bey-Abina et al 2003; Ott et al 2006), sickle cell disease (Pawliuk et al 2001), metachromatic leukodystrophy (Biffi et al 2004), as well as a number of cancers and immune disorders (Alderuccio et al 2009; Alderuccio et al 2006; Li et al 2009; McIvor 1999). A number of successful clinical trials using hematopoietic stem cell gene therapy have been also been reported. ALD, CGD, SCID, HIV, and sickle cell anemia have all benefitted recently from gene therapy in clinical trials, without the occurrence of adverse events. In 1999, SCID-X1 became the first genetic disease in which a gene therapy clinical trial was initiated. In this hallmark study, Cavazzana-Calvo et al report the successful correction of SCID-X1 disease by oncoretroviral-mediated gene transfer of the γ_c cytokine receptor gene into HSCs (Cavazzana-Calvo et al 2000). Unfortunately, 3 of nine patients cured of SCID-X1 developed leukemia due to disregulation of LMO2 within 3 years of treatment. To date, 5 cases of T-cell carcinogenesis have been reported from SCID-X1 clinical trials. Because the

transferred proviral sequence controlled transgene expression from a ubiquitous viral promoter, every cell derived from the transduced HSC was susceptible to oncogenic disregulation. In this case, the sequence integrated into a T cell oncogene, causing specific T cell expansion. For this reason, limiting active transcription to only a fraction of gene-modified cells by using a cell or tissue-specific promoter prevents the chance of endogenous gene activation in the majority of gene-modified cells. Of note, HIV proviral integration is not thought to play a direct role in cellular transformation. Of all patients infected with the HIV virus, only one documented case of lymphoma due to HIV integration has been reported (Herndier et al 1992).

Chapter 2:

Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A

2.1 – Abstract

Successful gene therapy of hemophilia A depends on the sustained expression of therapeutic levels of factor VIII (fVIII). Because of mRNA instability, interactions with resident endoplasmic reticulum (ER) chaperones, and the requirement for carbohydrate-facilitated transport from the ER to the Golgi apparatus, fVIII is expressed at much lower levels from mammalian cells than other proteins of similar size and complexity. A number of bioengineered forms of B domain-deleted (BDD) human fVIII have been generated and shown to exhibit enhanced expression. Previously, we demonstrated that recombinant BDD porcine fVIII exhibits high-level expression due to specific sequence elements that increase biosynthesis via enhanced posttranslational transit through the secretory pathway. For the studies described in Chapter 2, high-expression recombinant human, porcine, and human/porcine chimeric fVIII constructs were compared directly in order to determine the relative expression of the various bioengineered fVIII transgenes. The data demonstrate that BDD porcine fVIII expression is superior to that of any of the human fVIII variant constructs tested. Mean fVIII expression of 18 units/10^6 cells/24 hr was observed from HEK-293 cells expressing a single copy of the porcine fVIII transgene, which was 36- to 225-fold greater than that of any human fVIII transgene tested. The human/porcine chimeric transgene HP-fVIII also expressed at significantly higher levels than any human transgene tested, whereby fVIII expression of 10 units/10^6 cells/24 hr was observed from cells containing a single HP-fVIII transgene. Furthermore, greater than 10-fold higher expression was observed in

human cells transduced with BDD porcine fVIII versus BDD human fVIII-encoding lentiviral vectors, even at low proviral copy numbers, supporting its use over other human fVIII variants in future hemophilia A gene therapy clinical trials.

2.2 – Introduction

Hemophilia A is an X-linked recessive disorder characterized by insufficient production of functional fVIII. Intravenous infusion of either plasma-derived or recombinant human fVIII (hfVIII) is generally effective at alleviating spontaneous bleeding episodes characteristic of severe hemophilia A, though treatment is expensive due to the high cost of fVIII products. Hemophilia A is an excellent candidate for gene therapy since 1) only a modest increase (~ 1 – 5% of normal) in fVIII levels is necessary to alleviate spontaneous bleeding, 2) murine and canine models of hemophilia A already exist, and 3) endogenous tissue-specific fVIII expression is not necessary. Although success has been achieved in animal models, previous clinical trials for gene therapy of hemophilia A using hfVIII transgenes have failed to achieve sustained, therapeutic fVIII expression, for review see (Chuah et al 2004). Low fVIII expression primarily is due to inefficient transport of the primary translation product from the ER to the Golgi apparatus resulting in expression levels approximately 2-3 orders of magnitude lower than other secreted proteins of similar size (Dorner et al 1987; Marquette et al 1995). The incorporation of bioengineered hfVIII constructs into gene-transfer systems has provided some improvements in the duration and level of expression following gene transfer *in vitro* and *in vivo* (Cerullo et al 2007; Kang et al 2005;

Moayeri et al 2005; Moayeri et al 2004; Saenko et al 2003). The initial breakthrough in the development of higher-expression recombinant fVIII molecules came with the discovery that removal of the entire B domain resulted in up to a 20-fold increase in mRNA and primary translation product (Meulien et al 1988; Pittman et al 1993). However in these studies, only a 2 – 3-fold increase in secreted fVIII was observed. Further insight into the translation and secretory pathway of fVIII revealed a 19 amino acid hydrophobic cluster in the A1 domain that was predicted to bind the endoplasmic reticulum (ER) resident protein chaperone, immunoglobin-binding protein (BiP), and be responsible for poor fVIII expression (Marquette et al 1995). Coagulation factor V (fV), a cofactor that works with coagulation factor X to activate prothrombin to thrombin, does not have this hydrophobic cluster and is not subject to poor expression. Substitution of single amino acids within the hydrophobic cluster of fVIII with the homologous amino acids present in fV resulted in profound changes in fVIII expression. Specifically, the amino acid substitution F309S was shown to decrease fVIII binding to BiP and increase expression by 2 - 3-fold over full length fVIII, while the double mutation L303E/F309S increased expression slightly further (Swaroop et al 1997) **(Table 2.1)**. Previous work also demonstrated that efficient secretion of fVIII requires carbohydrate-facilitated transport by LMAN1 mediated by mannose residues of N-linked oligosaccharides post-translationally attached to the B domain (Moussalli et al 1999). By incorporating the first 226 amino acids, or approximately 34% of the deleted B domain, Pipe and colleagues generated a

Table 2.1 – Recombinant fVIII transgenes and reported increases in expression

Modification to fVIII cDNA	Change in expression	Change compared to which fVIII
BDD-hfVIII	2-3 fold	Wt hfVIII
6 glycosylation sites (N6)	4-9 fold	BDD-hfVIII
F303S	2-3 fold	Wt hfVIII
F303S/N6	4-7 fold	BDD-hfVIII
L303E/F309S	3-4-fold	BDD-hfVIII
pfVIII	10-14 fold	BDD-SQ-hfVIII

fVIII molecule that retains 6 N-linked glycosylation sites and demonstrated improved expression 4 – 9-fold over BDD-hfVIII (Miao et al 2004).

Several bioengineered hfVIII constructs currently are being evaluated in various *in vivo* gene-transfer systems. FVIII expression has been observed in mice following administration of transduced bone marrow cells expressing BDD-hfVIII (Dwarki et al 1995; Moayeri et al 2005), BDD-hfVIII containing L303E/F309S (Kang et al 2005; Moayeri et al 2004), partial BDD-hfVIII (Cerullo et al 2007), and partial BDD-hfVIII containing F309S (Cerullo et al 2007; Sinn et al 2007). We previously demonstrated that BDD-pfVIII is expressed at 10 – 14-fold greater levels than BDD-SQ-hfVIII *in vitro,* whereby BDD-SQ-hfVIII is a B domain deleted hfVIII transgene with a 14 amino acid SQ linker containing the PACE/furin recognition sequence to enhance protein processing (Doering et al 2002b). Doering et al then showed that the reason for the increase in fVIII expression is because BDD-pfVIII is secreted more efficiently than BDD-SQ-hfVIII (Doering et al 2004). We also demonstrated high-level expression of BDD-pfVIII following *ex vivo* genetic modification and transplantation of hematopoietic stem and progenitor cells into genetically immunocompetent mice. In these studies, moderate donor-cell chimerism and sustained high-level expression of BDD-pfVIII was achieved in mice after transplantation under myeloablative and reduced intensity conditioning (Gangadharan et al 2006; Ide et al 2007). Furthermore, by employing a similar hematopoietic stem cell transplantation

(HSCT) protocol, we demonstrated sustained BDD-pfVIII expression even in the presence of pre-existing fVIII immunity (Doering et al 2007; Ide et al 2007).

Although a number of fVIII transgenes have been published claiming to be high expression constructs, never have they all been compared using the same expression systems. The previous experiments varied in cell types used, transfection protocols, expression vectors, and comparators, and were performed by different researchers. The goal of the current experiments was to determine which of the above mentioned fVIII transgenes is truly the highest expresser *in vitro* and if that those properties translated into high level sustained fVIII expression in a mouse model of hemophilia A following lentiviral-mediated HSC transduction and transplant. We compared various high-expression fVIII constructs using transient transfection, analysis of stable clones, isogenic stable cell lines, and recombinant lentiviral gene transfer in an effort to determine the optimal fVIII transgene and to overcome the transient, low-level fVIII expression that hindered previous clinical gene therapy trials for hemophilia A. As shown in this chapter, direct comparison of bioengineered human and porcine fVIII transgenes demonstrated that BDD-pfVIII is expressed at significantly greater levels than any other bioengineered human fVIII variant supporting its future success in hemophilia A gene therapy applications.

2.3 – Materials and Methods

Materials. Dulbecco's Modified Eagle medium (DMEM), Advanced D-MEM/F-12 medium, Aim V medium, Opti-MEM medium, heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin solution, GlutaMax™, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Invitrogen™ life technologies (Carlsbad, CA). Zeocin™ and hygromycin B antibiotics for maintenance and selection of Flp-In™ 293T cells were purchased from Invitrogen™ life technologies. Cell transfections were performed using Lipofectamine™ 2000 from Invitrogen™ life technologies. Human fVIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KS). Automated activated partial thromboplastin reagent was purchased from BioMérieux, Inc. (Durham, NC). Clotting times were measured using a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France). Genomic DNA and total RNA from cultured cells was isolated using QIAGEN DNeasy® Blood & Tissue Kit and RNeasy® Mini Kit, respectively (Germantown, MD). *Taq* PCR Master Mix Kit was purchased from QIAGEN (Germantown, MD). FVIII RNA quantitation was performed using SYBR® GREEN PCR Master Mix, TaqMan® Reverse Transcription Reagents, and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) unless otherwise specified. Southern and Northern blot analysis was performed using the DIG Nonradioactive Nucleic Acid Labeling and Detection System by Roche (Indianapolis, IN).

Construction of fVIII Expression Vectors. ReNeo constructs – BDD-SQ-hfVIII and BDD-OL-pfVIII transgenes in the ReNeo mammalian expression vector were generated as described previously (Doering et al 2002b). Both constructs contain the RHQR PACE/furin recognition sequence responsible for increased protein processing and it was previously published that expression data was similar for a BDD porcine fVIII transgene containing either the 14 amino acid SQ linker or the 24 amino acid OL linker (Doering et al 2004). Human B-domain-deleted fVIII (BDD-hfVIII) in the ReNeo vector was a gift from Dr. Pete Lollar (Emory University, Atlanta, GA). L303E/F309S BDD-SQ-hfVIII (BDD-DM-SQ-hfVIII) and L303E/F309S BDD-hfVIII (BDD-DM-hfVIII) were created by polymerase chain reaction-mediated site-directed mutagenesis of BDD-SQ-hfVIII and BDD-hfVIII template DNA, respectively, using Stratagene's Quick Change™ Site Directed Mutagenesis kit (La Jolla, CA) with forward primer (5` - GAAGAGATATGACAAGACAGTAGAAA – 3`) and reverse primer (5`- CTCTTGATGGACGAAGGACAGTTTCT – 3`). BDD-N6-SQ-hfVIII was created by XhoI/AvrII digest of full length, wild-type fVIII and ReNeo/BDD-SQ-hfVIII plasmid DNA followed by gel purification and ligation of the resulting 2,282 bp and 10,163 bp fragments, respectively. BDD-N6-hfVIII was similarly created by subcloning of the 2,282 bp XhoI/AvrII fragment into the BDD-hfVIII construct. BDD-DM-N6-hfVIII was created by subcloning the 2,282 bp XhoI/AvrII fragment into the BDD-DM-hfVIII construct. All ReNeo constructs drive transgene

expression from an adenovirus type 2 major late promoter and a G-418 resistance gene for selection of transfected cells.

pcDNA5/FRT constructs – BDD-SQ-hfVIII and BDD-OL-pfVIII in the pcDNA5/FRT vector (pcDNA5/FRT/BDD-OL-pfVIII and pcDNA5/FRT/BDD-SQ-hfVIII) were gifts from Dr. Pete Lollar. pcDNA5/FRT/BDD-hfVIII was created by SpeI/XbaI digest of ReNeo/BDD-hfVIII and pcDNA5/FRT/BDD-SQ-hfVIII plasmid DNA followed by gel purification and ligation of the resulting 3,636 bp and 5,718 bp fragments, respectively. pcDNA5/FRT/BDD-DM-SQ-hfVIII was created by SpeI/MluI digest of ReNeo/BDD-DM-SQ-hfVIII and pcDNA5/FRT/BDD-SQ-hfVIII plasmid DNA followed by gel purification and ligation of the resulting 784 bp and 8,612 bp fragments, respectively. pcDNA5/FRT/BDD-DM-hfVIII was similarly created by subcloning of the 784 bp SpeI/MluI fragment into the pcDNA5/FRT/BDDhfVIII construct. pcDNA5/FRT/BDD-N6-SQ-hfVIII was created by SpeI/XbaI digest of ReNeo/BDD-N6-SQ-hfVIII and pcDNA5/FRT/BDD-SQ-hfVIII plasmid DNA followed by gel purification and ligation of the resulting 4,371 bp and 5,718 bp fragments, respectively. pcDNA5/FRT/BDD-N6-hfVIII was created by SpeI/XbaI digest of ReNeo/BDD-N6-hfVIII and pcDNA5/FRT/BDD-hfVIII plasmid DNA followed by gel purification and ligation of the resulting 4,329 bp and 5,718 bp fragments, respectively. pcDNA5/FRT/BDD-DM-N6-hfVIII was created by MluI/XbaI digest of ReNeo/BDD-N6-hfVIII and pcDNA5/FRT/BDD-hfVIII plasmid DNA followed by gel purification and ligation of the resulting 3,545 bp and 6,502 bp fragments, respectively.

Lentiviral Vector Production. Lentiviral vectors containing fVIII transgenes were manufactured by Lentigen™ (Gaithersburg, MD). Briefly, BDD-SQ-hfVIII and BDD-OL-pfVIII were sub-cloned into the LentiMax™ expression vector, a highly optimized HIV-based lentiviral vector. Transgene expression was controlled by the human elongation factor 1 alpha (EF-1 α) promoter and research grade lentiviral vector (LV) particles were manufactured using the LentiMax™ Production System. Titers were obtained from the manufacturer by quantitative PCR (qPCR) for the *gag* region of the LV vector and/or p24 ELISA on extracted genomic DNA from transduced 293FT cells. LV particles were subsequently titered by performing competitive PCR analysis for fVIII transgene (as described below) on extracted genomic DNA from transduced 293T cells. Lenti-GFP™, a LentiMax™ LV expressing the enhanced green fluorescent reporter protein (eGFP), was used in transduction experiments as a negative control.

Cell Culture, Transfection, Transduction, and Clonal Selection. *Transient transfection of fVIII ReNeo vectors* – Baby hamster kidney-derived (BHK-M) cells were grown to 90% confluency in 24-well plates containing 1 mL Advanced DMEM/F12 supplemented with 10% FBS. Cells were transfected with 0.8 µg plasmid DNA from each ReNeo construct and 2 µL Lipofectamine™ 2000 per well in 0.5 mL media. Media was changed to fresh Advanced DMEM/F12 with 10% FBS the following morning. Twenty-four hours later, media was replaced

with 0.5 mL serum-free AIM V media. Cells were cultured another 24 hours before fVIII activity was measured by one-stage coagulation assay.

Stable transfection of fVIII ReNeo vectors – BHK-M cells were grown to 90% confluency in 6-well plates containing 2 mL Advanced DMEM/F12 media supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were transfected with 4 µg plasmid DNA from each ReNeo construct and 2 µL Lipofectamine™ 2000 per well in 2 mL Opti-MEM media. Twenty-four hours after transfection, cells were split into 100 mm cell culture plates and cultured in Advanced DMEM/F12 supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.5 mg/mL G-418 for 10 – 14 days. Visible colonies were picked with a pipette tip and transferred to a 96-well plate. Cells were then expanded onto a 24-well plate and grown to 90% confluency, before changing media to serum-free AIM V media. FVIII activity was measured by one-stage coagulation assay 24 hours later.

Stable transfection of fVIII pcDNA5/FRT vectors – Three million Flp-In™ 293 cells were plated in 10 mL DMEM containing 4.5 g/L D-glucose, 25mM HEPES, and L-glutamine, supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL Zeocin in a 100 mm cell culture plate. Flp-In™ 293 cells were engineered by Invitrogen to contain a *lacZ*-Zeocin fusion gene and an integrated FLP recombination target (FRT) site just downstream from the *lacZ*-Zeocin ATG initiation codon. Upon co-transfection at a 9:1 ratio of

pOG44 expressing FLP recombinase and pcDNA5/FRT expressing the fVIII transgene homologous recombination occurs between the FRT site in the cell and the FRT site in the expression plasmid. Transfected cells can then be selected by hygromycin B resistance, Zeocin sensitivity, and/or lack of β-galactosidase expression. Cells were transfected using Lipofectamine™ 2000 in DMEM containing 10% FBS. Twenty four hours after transfection, media was changed to DMEM containing 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL hygromycin B. Ten to fourteen days later, cells were split in limiting dilution to allow for the propagation of individual colonies from a single cell per well of a 96-well plate. Cells were expanded into 6-well plates and when 90% confluent, media was changed to AIM V and fVIII activity was measured by one-stage coagulation assay 24 hours later.

Lentiviral Transduction - 293T were maintained in complete Advanced DMEM/F-12 supplemented with 10% FBS, 1% penicillin-streptomycin solution (100 units/mL each), and 2 mM GlutaMax™ at 37 ^{o}C with 5% CO_2. Transductions of LentiMax™ LV particles at various multiplicity of infections (MOI) were performed by incubating approximately 100,000 cells/well plated onto collagen-1 coated 24-well plates (BIOCOAT®, Becton Dickinson) with virus in a final volume of 500 µL of complete Advanced D-MEM/F-12 supplemented with 8 µg/mL of polybrene (Specialty Media). Twenty-four hours post-transduction, virus-containing media was replaced with fresh complete Advanced D-MEM/F-12 and transduced cells were cultured until reaching approximately 70-90% confluence, upon which each

sample was transferred equally onto three wells of a 6-well plate for measurement of fVIII activity, integration events, and transcript expression as described below. Clonal isolation was performed by single cell cloning by limiting dilution onto 96-well plates and visual examination of wells for single cell. After several weeks in culture, single cell clones were expanded in sequence from a 96-well plate onto a 48-well, 24-well, 12-well, 6-well, and 100 mm dish for further testing.

Measurement of FVIII Activity. For all *in vitro* studies, fVIII activity was measured using the activated partial thromboplastin reagent-based one-stage coagulation assay in a STart Coagulation Instrument (Diagnostica Stago, Asnieres, France) using human fVIII-deficient plasma as the substrate as previously described (Doering et al 2002b). All coagulation assays were carried out on cells cultured in low serum-containing Advanced D-MEM/F-12 media (2% FBS) or serum-free (AIM V) media for 24 hours prior to assaying fVIII activity in conditioned medium. Cells were counted at the time of assay in order to express fVIII levels as units/10^6 cells/24 hr. For *in vivo* fVIII measurement, blood was collected from mice through the retro-orbital plexus and fVIII measured using a chromogenic substrate assay (COATEST SP FVIII) as described previously (Doering et al 2007).

Measurement of FVIII Transgene Copy Number. Total DNA from transduced cells was isolated using the DNeasy® Blood & Tissue Kit following the

manufacture's protocol for cells. DNA was quantitated spectrophotometrically by A_{260} in dH_2O. To determine transgene copy number, a competitive PCR protocol was developed in which a known amount of fVIII copies amplified from the pUA2 plasmid is added to the PCR reaction containing unknown total DNA **(Figure 2.1)**. The derived product from the pUA2 plasmid comprises a fVIII DNA fragment containing a 70 bp deletion that can be distinguished from the integrated fVIII transgene. During the PCR reaction, the pUA2 template competes with the integrated fVIII transgene in the genomic DNA and the point of equivalent amplification is used to determine the number of fVIII transgenes in the unknown sample. The PCR reactions were carried out in 25 µL of total volume containing 1X TAQ PCR Master Mix, 2 µM forward and reverse primers, and 100 ng of total DNA. PCR cycling was carried out in a Mastercycler gradient (Eppendorf, Westbury, New York, USA) by performing one cycle at 95^{o}C for 3 minutes followed by 30 cycles at 95 ^{o}C for 1 minute, 58 ^{o}C for 1 minute, and 72 ^{o}C for 1 minute, and a final extension at 72 ^{o}C for 10 minutes. The oligonucleotide primers used were located within the A2 domain of the fVIII sequence at positions 1,402 – 1,420 for the forward primer (5'-TGGGACCTTTACTTTATGG-3') and 1,923 – 1,946 for the reverse primer (5'-AAAAACATAGCCATTGATGCTGTG-3') of the human fVIII cDNA sequence. The PCR products (520 bp for fVIII transgene and 450 bp for competitor) were separated in a 1.5 % agarose gel and an ethidium bromide image was obtained using a UV transilluminator (BioDoc-It™System by UVP). Densitometry of PCR products was obtained using the ImageJ 1.37v Software (developed by Wayne

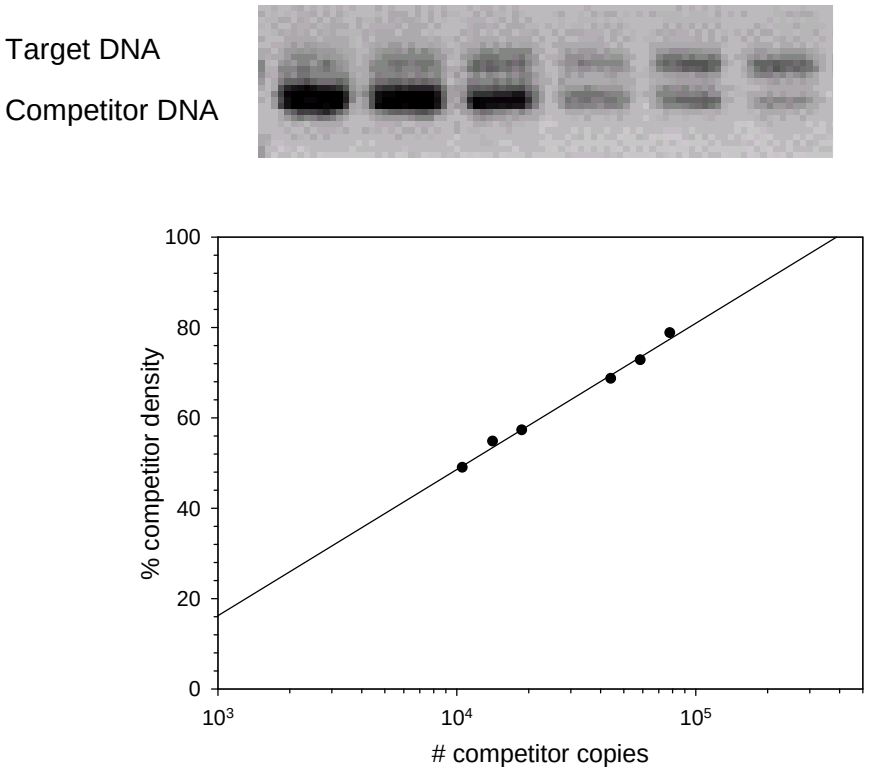

Figure 2.1 – Lentiviral titering by competitive PCR. A competitive PCR protocol was developed in which a known amount of fVIII copies amplified from the pUA2 plasmid is added to the PCR reaction containing unknown total DNA. The derived product from the pUA2 plasmid comprises a fVIII DNA fragment containing a 70 bp deletion that can be distinguished from the integrated fVIII transgene. The PCR products (520 bp for fVIII transgene and 450 bp for competitor) were separated in a 1.5 % agarose gel and an ethidium bromide image was obtained using a UV transilluminator. Densitometry of PCR products was obtained and plots were performed to determine the % competitor density as a function of the number of copies per reaction at the point of equivalence determined by linear regression analysis.

Rasband, National Institutes of Health, USA). Plots were performed to determine the % competitor density as a function of the copies per reaction and the point of equivalence determined by linear regression analysis.

Measurement of FVIII Transcript Expression. Total RNA was extracted from cell lines using the RNeasy® Mini Kit following manufacturer's instructions using QIAshredder homogenizers. RNA concentrations were determined by A_{260} in 10 mM Tris-Cl pH 7.0. *In vitro* transcribed fVIII RNA standards were generated using the mMessage mMachine Kit by Ambion (Austin, TX) as described previously (Doering *et al.*, 2002a). PCR reactions were carried out in 25 µl total volume containing 1X SYBR Green PCR master mix, 300 µM forward and reverse primers, 12.5 units of MultiScribe™, 10 units of RNase Inhibitor, and 5 ng of sample RNA. PCR reactions using the porcine fVIII RNA standard also included 5 ng of yeast tRNA to mimic the RNA environment of sample RNA using serial dilutions (10^2 - 10^6 transcripts/reaction) as template RNA. The oligonucleotide primers used were located in the fVIII sequence encoding the A2 domain located at positions 2,047 – 2,067 for forward primer (5'-ATGCACAGCATCAATGGCTAT-3') and 2,194 – 2,213 for reverse primer (5'-GTGAGTGTGTCTTCATAGAC-3') of the human fVIII cDNA sequence. Primers recognize both human and porcine fVIII as sequences in this region are identical between both templates. One-step real time RT-PCR was performed by incubation at 48 ^{o}C for 30 minutes for reverse transcription followed by one cycle at 95 ^{o}C for 10 minutes, and 40 amplification cycles of 90 ^{o}C for 15 sec then 60

^{o}C for 1 minute. Post-reaction dissociation analysis was performed to confirm single-product amplification. Absolute quantitation was performed as described previously (Doering et al 2002b)

Northern Blot Analysis. Total RNA was extracted from cell lines using the RNeasy® Mini Kit following manufacturer's instructions using QIAshredder homogenizers. RNA concentrations were determined described above. A total of 2.5 μg or 5 μg of RNA sample were separated, transferred, hybridized, and detected using a protocol involving denaturing conditions in a 1% agarose/formaldehyde/MOPS gel following the technique outlined in the DIG Application Manual for Filter Hybridization (Roche). Digoxigenin (DIG)-labeled RNA molecular weight marker I (Roche) was used as a marker. Hybridization was performed using a PCR DIG-labeled probe specific to either porcine A1 domain (A1 probe) or human A2 domain (A2 probe) generated using the PCR DIG Probe Synthesis Kit (Roche). The oligonucleotide primers used to generate for A1 probe were forward 5`-CGGCAAAGTGAACTCCTCCGTG–3` and reverse 3`-CTGACGTGAGCCTCCATGCCACCA-5` from the porcine fVIII cDNA sequence. The oligonucleotide primers used to generate for A2 probe were forward 5`-ACATTGCTGCTGAAGAGGAGGACT–3` and reverse 3`-ATGTTGGAGGCTTGGAACTCTGGA– 5` from the human fVIII cDNA sequence. Post-hybridization wash and block for immunological detection of DIG-labeled probes was performed using the DIG Wash and Block Buffer Set (Roche) at a dilution of anti-DIG-AP of 1:25,000. CDP-*Star* (Roche) was used for

chemiluminescent substrate detection. Imaging was performed using a Luminescent image analyzer (LAS)-3000 (FUJIFILM) followed by X-ray film exposure (Kodak).

Southern Blot Analysis. To confirm that pcDNA/FRT clones contain a single integration event, total genomic DNA was isolated from transfected cells using the DNeasy® blood and tissue kit (Qiagen) and analyzed by Southern blot. Eight µg of genomic DNA from each stable clone was digested with ScaI and fragments were separated on a 1% agarose gel. DNA was transferred by capillary action using 20x SSC transfer buffer to a positively charged nylon membrane and hybridized overnight with a probe complimentary to either the porcine A1 domain or the human A2 domain. Probes were created by PCR-mediated digoxigenin (DIG)-labeling using the DIG Nonradioactive Nucleic Acid Labeling and Detection System (Roche). In order to determine the limit of detection of the DIG-labeled probe, pcDNA/FRT/pfVIII plasmid DNA dilutions from 20 µg – 20 fg were spiked into 10 µg genomic DNA and run on gel. Following transfer to a positively charged nylon membrane, the membrane was probed with the DIG-labeled probe and found to be sensitive to ~200 pg **(Figure 2.2)**. Stable transfectants with the correct single integration were confirmed by a single band at 12,460 bp for BDD-OL-pfVIII clones, 12,430 bp for BDD-SQ-hfVIII and BDD-DMSQ-hfVIII clones, 13,120 bp for BDD-N6-SQ-hfVIII clones, 12,390 bp for BDD-hfVIII and BDDDM-hfVIII clones, and 13,080 bp for BDD-N6-hfVIII and BDD-DM-N6-hfVIII clones. All human clones also produced a band at 8.6 kb

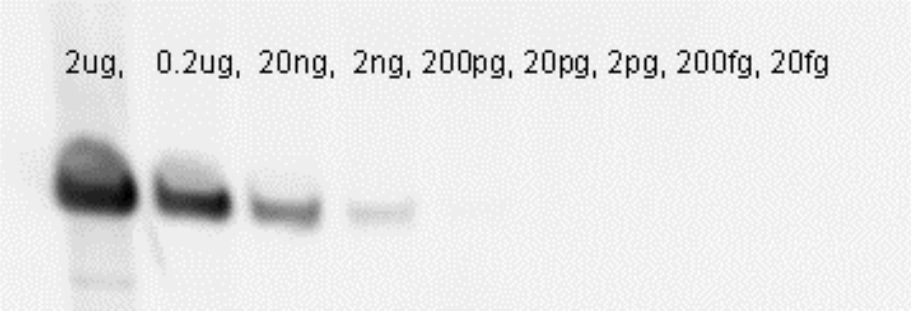

Figure 2.2 – Determination of DIG-labeled probe sensitivity. 293T genomic DNA was spiked with pcDNA/pfVIII plasmid DNA (2 μg – 20 fg) then digested with NotI and NheI restriction enzymes. Digested DNA was separated on an agarose gel then transferred to a nylon membrane by capillary action. The membrane was probed using a DIG-labeled pfVIII-specific probe to determine the sensitivity of the DIG labeling and detection system. Using this system, approximately 0.2 – 2 ng transgene DNA could be detected in 50 ng genomic DNA.

and 5.5 kb when probed with the human A2 probe due to homology with two portions of the endogenous fVIII sequence when digested with ScaI. Porcine clones were probed with a porcine A1 probe and exhibited 3 endogenous bands when digested with ScaI. Transduced 293T clones were also analyzed by Southern Blot in order to determine number of integration events using 5 –10 µg of genomic DNA digested with AvrII.

Hematopoietic stem cell isolation, transduction, and transplant. Bone marrow was harvested from femurs and tibias of C57BL/6 and enriched for Sca-1^+ cells using positive immunomagnetic bead selection, resulting in 80-95% Sca-1^+ cells. Cells were cultured in serum-free media and stimulated for 2 days with murine stem cell factor (100 ng/mL), murine interleukin-3 (20 ng/mL), human interleukin-11 (100 ng/mL) and human Flt3 ligand (100 ng/mL) in order to induce cell cycling. On days 3 and 4, cells were transduced consecutively with 2.5 functional viral particles (fvp)/cell using either SIV-BDD-SQ-hfVIII or SIV-BDD-OL-pfVIII. Plasmids necessary for generating SIV viruses were a kind gift from Dr. Arthur Nienhuis (Hanawa et al 2004a). Transduced cells were transplanted into $E16^{-/-}$ hemophilia A mice by tail vein injection at a dose of 7.5×10^5 cells/mouse. Mice were 8-12 weeks old at time of transplant and were lethally irradiated prior to transplantation using a Gammacell 40 Exactor (Nordion, Ottawa, ON).

2.4 – Results

Expression of bioengineered fVIII constructs in heterologous mammalian cells

In order to determine if one high expression fVIII transgene is significantly higher than the others, so as to overcome the barrier of low transgene expression associated with hemophilia A gene therapy applications, we compared the expression of several previously described bioengineered fVIII transgenes. The following fVIII variants were generated: BDD human fVIII (Toole et al 1986) (herein referred to as BDD-hfVIII); BDD-hfVIII containing the L303E/F309S double mutation (Swaroop et al 1997) (herein referred to as BDD-DM-hfVIII); BDD-hfVIII containing 6 N-linked glycosylation sites (Miao et al 2004) (herein referred to as BDD-N6-hfVIII); BDD-hfVIII containing the double mutation and 6 N-linked glycosylation sites (herein referred to as BDD-DM-N6-hfVIII); BDD-hfVIII containing a 14 amino acid SQ linker between the A2 domain and the activation peptide which includes the RHQR recognition sequence to increase secretion via PACE/furin processing (Doering et al 2002b; Seidah & Chretien 1997) (herein referred to as BDD-SQ-hfVIII); BDD-SQ-hfVIII containing L303E/F309S (herein referred to as BDD-DM-SQ-hfVIII); BDD-SQ-hfVIII containing 6-N-linked glycosylation sites (herein referred to as BDD-N6-SQ-hfVIII), and BDD porcine fVIII containing a 24 amino acid linker (designated OL) between the A2 domain and the activation peptide, described previously (Doering et al 2002b) (herein referred to as BDD-OL-pfVIII) **(Figure 2.3)**. All fVIII cDNA were cloned into the

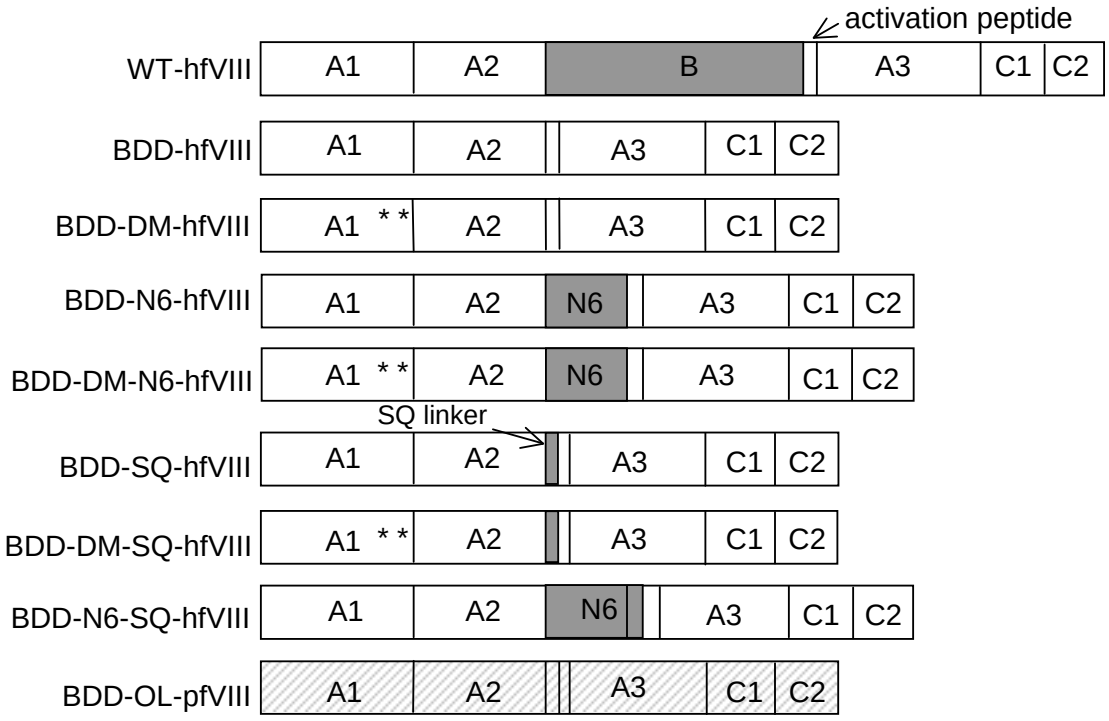

Figure 2.3 - Schematic of recombinant fVIII constructs. Eight fVIII transgenes were generated for comparison of fVIII activity. Full-length wild type fVIII was not used in these comparisons, but is shown here to illustrate the complete fVIII domain structure (A1-A2-B-ap-A3-C1-C2). Human fVIII domains are shown in white with the exception of the B domain, which is shown in gray. Porcine fVIII sequence is identified by hatching. The location of L303E/F309S in double mutant (DM) constructs is depicted by asterisks.

ReNeo mammalian expression vector. Transgene sequences were confirmed by automated DNA sequencing and found to be correct **(Figure 2.4)**

To compare fVIII expression, BHK-M cells were transfected with plasmids encoding each transgene and one-stage coagulation assays were performed after incubation for 24 hours in serum-free medium. No significant differences were observed among the human fVIII transgenes. However, transient expression of BDD-OL-pfVIII was approximately 15-fold higher than any of the human fVIII variants (p<0.001, ANOVA) **(Figure 2.5A)**. Stable clones (n = 17 – 42) were selected by antibiotic resistance, and expanded in 6-well plates. After expansion, individual clones were incubated for 24 hours in serum-free medium prior to assaying for fVIII activity by one-stage coagulation assay. Again, no significant differences were observed among the human fVIII transgenes. However, mean expression of BDD-OL-pfVIII was 10 units/10^6 cells/24hr, which was 5-16-fold higher than any of the human fVIII variants (p<0.001, ANOVA) **(Figure 2.5B).**

Comparison of fVIII expression using targeted single transgene integration

Due to the large variances in fVIII expression observed for the human fVIII transgenes from stable BHK-M clones, we performed an additional, potentially more direct comparison of transgene expression. The Flp-In™ System is designed to allow expression of a single transgene from a specific location in the genome, thus eliminating the effects of random integration and position effect

(Signalpeptide)ATGCAAATAGAGCTCTCCACCTGCTTCTTTCTGTGCCTTTTGCGATTCTG
CTTTAGT

(A1domain)GCCACCAGAAGATACTACCTGGGTGCAGTGGAACTGTCATGGGACTATATGC
AAAGTGATCTCGGTGAGCTGCCTGTGGACGCAAGATTTCCTCCTAGAGTGCCAAAATCTTTT
CCATTCAACACCTCAGTCGTGTACAAAAAGACTCTGTTTGTAGAATTCACGGTTCACCTTTTC
AACATCGCTAAGCCAAGGCCACCCTGGATGGGTCTGCTAGGTCCTACCATCCAGGCTGAGG
TTTATGATACAGTGGTCATTACACTTAAGAACATGGCTTCCCATCCTGTCAGTCTTCATGCTG
TTGGTGTATCCTACTGGAAAGCTTCTGAGGGAGCTGAATATGATGATCAGACCAGTCAAAGG
GAGAAAGAAGATGATAAAGTCTTCCCTGGTGGAAGCCATACATATGTCTGGCAGGTCCTGAA
AGAGAATGGTCCAATGGCCTCTGACCCACTGTGCCTTACCTACTCATATCTTTCTCATGTGG
ACCTGGTAAAAGACTTGAATTCAGGCCTCATTGGAGCCCTACTAGTATG**TAGAGAAGGG**
AGTCTGGCCAAGGAAAAGACACAGACCTTGCACAAATTTATACTACTTTTTGCTGTATT
TGATGAAGGGAAAAGTTGGCACTCAGAAACAAAGAACTCCTTGATGCAGGATAGGGATGCT
GCATCTGCTCGGGCCTGGCCTAAAATGCACACAGTCAATGGTTATGTAAACAGGTCTCTGCC
AGGTCTGATTGGATGCCACAGGAAATCAGTCTATTGGCATGTGATTGGAATGGGCACCACTC
CTGAAGTGCACTCAATATTCCTCGAAGGTCACACATTTCTTGTGAGGAACCATCGCCAGGCG
TCCTTGGAAATCTCGCCAATAACTTTCCTTACTGCTCAAACACTCTTGATGGAC**CTT**GGACA
GTTTCTACTG**TTT**TGTCATATCTCTTCCCACCAACATGATGGCATGGAAGCTTATGTCAAAGT
AGACAGCTGTCCAGAGGAACCCCAACTACGAATGAAAAATAATGAAGAAGCGGAAGACTAT
GATGATGATCTTACTGATTCTGAAATGGATGTGGTCAGGTTTGATGATGACAACTCTCCTTCC
TTTATCCAAATTCGC

(A2domain)TCAGTTGCCAAGAAGCATCCTAAAACTTGGGTACATTACATTGCTGCTGAAGA
GGAGGACTGGGACTATGCTCCCTTAGTCCTCGCCCCCGATGACAGAAGTTATAAAAGTCAA
TATTTGAACAATGGCCCTCAGCGGATTGGTAGGAAGTACAAAAAAGTCCGATTTATG
GCATACACAGATGAAACCTTTAAGACGCGTGAAGCTATTCAGCATGAATCAGGAATCTTGGG
ACCTTTACTTTATGGGGAAGTTGGAGACACACTGTTGATTATATTTAAGAATCAAGCAAGCAG
ACCATATAACATCTACCCTCACGGAATCACTGATGTCCGTCCTTTGTATTCAAGGAGATTACC
AAAAGGTGTAAAACATTTGAAGGATTTTCCAATTCTGCCAGGAGAAATATTCAAATATAAATG
GACAGTGACTGTAGAAGATGGGCCAACTAAATCAGATCCTCGGTGCCTGACCCGCTATTACT
CTAGTTTCGTTAATATGGAGAGAGATCTAGCTTCAGGACTCATTGGCCCTCTCCTCATCTGCT
ACAAAGAATCTGTAGATCAAAGAGGAAACCAGATAATGTCAGACAAGAGGAATGTCATCCTG
TTTTCTGTATTTGATGAGAACCGAAGCTGGTACCTCACAGAGAATATACAACGCTTTCTCCCC
AATCCAGCTGGAGTGCAGCTTGAGGATCCAGAGTTCCAAGCCTCCAACATCATGCACAGCA
TCAATGGCTATGTTTTTGATAGTTTGCAGTTGTCAGTTTGTT**TGCATGAGGTGGCATAC**
TGGTACATTCTAAGCATTGGAGCACAGACTGACTTCCTTTCTGTCTTCTTCTCTGGATATA
CCTTCAAACACAAAATGGTCTATGAAGACACACTCACCCTATTCCCATTCTCAGGAGAAACTG
TCTTCATGTCGATGGAAAACCCAGGTCTATGGATTCTGGGGTGCCACAACTCAGACTTTCGG
AACAGAGGCATGACCGCCTTACTGAAGGTTTCTAGTTGTGACAAGAACACTGGTGATTATTA
CGAGGACAGTTATGAAGATATTTCAGCATACTTGCTGAGTAAAAACAATGCCATTGAACCAA
GA

(Bdomain(N6)):AGCTTCTCCCAGAATTCAAGACACCCTAGCACTAGGCAAAAGCAATTTAA
TGCCACCACAATTCCAGAAAATGACATAGAGAAGACTGACCCTTGGTTTGCACACAGAACAC
CTATGCCTAAAATACAAAATGTCTCCTCTAGTGATTTGTTGATGCTCTTGCGACAGAGTCCTA
CTCCACATGGGCTATCCTTATCTGATCTCCAAGAAGCCAAATATGAGACTTTTTCTGATGATC
CATCACCTGGAGCAATAGACAGTAATAACAGCCTGTCTGAAATGACACACTTCAGGCCACAG
CTCCATCACAGTGGGGACATGGTATTTACCCCTGAGTCAGGCCTCCAATTAAGATTAAATGA
GAAACTGG**GGACAACTGCAGCAACAGAGTTGA**AGAAACTTGATTTCAAAGTTTCTA
GTACATCAAATAATCTGATTTCAACAATTCCATCAGACAATTTGGCAGCAGGTACTGATAATA
CAAGTTCCTTAGGACCCCCAAGTATGCCAGTTCATTATGATAGTCAATTAGATACCACTCTAT
TTGGCAAAAAGTCATCTCCCCTTACTGAGTCTGGTGGACCTCTGAGCTTGAGTGAAGAAAAT

AATGATTCAAAGTTGTTAGAATCAGGTTTAATGAATAGCCAAGAAAGTTCATGGGGAAAAAAT
GTATCGTCAACAGAGAGTCCTAGGAG

(SQlinker)CTTCTCTCAGAATCCACCAGTCTTGAAACGCCATCAACGG(activationpeptide)
GAAATAACTCGTACGACTCTTCAGTCAGATCAAGAGGAAATTGACTATGATGATACCATATCA
GTTGAAATGAAGAAGGAAGATTTTGACATTTATGATGAGGATGAAAATCAGAGCCCCCGC

(A3domain)AGCTTTCAAAAGAAAACACGACACTATTTTATTGCTGCAGTGGAGAGGCTCTG
GGATTATGGGATGAGTAGCTCCCCACATGTTCTAAGAAACAGGGCTCAGAGTGGCAGTGTC
CCTCAGTTCAAGAAAGTTGTTTTCCAGGAATTTACTGATGGCTCCTTTACTCAGCCCTTATAC
CGTGGAGAACTAAATGAACATTTGGGACTCCTGGGGCCATATATAAGAGCAGAAGTTGAAGA
TAATATCATGGTAACTTTC**AGAAATCAGGCCTCTCGTCCCTAT**TCCTTCTATTCTAGC
CTTATTTCTTATGAGGAAGATCAGAGGCAAGGAGCAGAACCTAGAAAAAACTTTGTCAAGCC
TAATGAAACCAAAACTTACTTTTGGAAAGTGCAACATCATATGGCACCCACTAAAGATGAGTT
TGACTGCAAAGCCTGGGCTTATTTCTCTGATGTTGACCTGGAAAAAGATGTGCACTCAGGCC
TGATTGGACCCCTTCTGGTCTGCCACACTAACACACTGAACCCTGCTCATGGGAGACAAGTG
ACAGTACAGGAATTTGCTCTGTTTTTCACCATCTTTGATGAGACCAAAAGCTGGTACTTCACT
GAAAATATGGAAAGAAACTGCAGGGCTCCCTGCAATATCCAGATGGAAGATCCCACTTTTAA
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GGCTCAGGATCAAAGGATTCGATGGTATCTGCTCAGCATGGGCAGCAATGAAAACATCCATT
CTATTCATTTCAGTGGACATGTGTTCACTGTACGAAAAAAAGAGGAGTATAAAATGGCACTGT
ACAATCTCTATCCAGGTGTTTTTGAGACAGTGGAAATGTTACCATCCAAAGCTGGAA**TTTGG
CGGGTGGAATGCCTTATTG**GCGAGCATCTACATGCTGGGATGAGCACACTTTTTCTG
GTGTACAGCAATAAGTGTCAGACTCCCC

(C1domain)TGGGAATGGCTTCTGGACACATTAGAGATTTTCAGATTACAGCTTCAGGACAA
TATGGACAGTGGGCCCCAAAGCTGGCCAGACTTCATTATTCCGGATCAATCAATGCCTGGA
GCACCAAGGAGCCCTTTTCTTGGATCAAGGTGGATCTGTTGGCACCAATGATTATTCACGGC
ATCAAGACCCAGGGTGCCCGTCAGAAGTTCTCCAGCCTCTACATCTCTCAGTTTATCATCAT
GTATAGTCTTGATGGGAAGAAGTGGCAGACTTATCGAGGAAATTCCACTGGAACCTTAATGG
TCTTCTTTGGCAATGTGGATTCATCTGGGATAAAACACAATATTTTTAACCCTCCAATTATTGC
TCGATACATCCGTTTGCACCCAACTCATTATAGCATTCGCAGCACTCTTCGCATGGAGTTGAT
GGGCTGTGATTTAAATAGTTGC

(C2domain)AGCATGCCATTGGGAATGGAGAGTAAAGCAATATCAGATGCACAGATTACTG
CTTCATCCTACTTTACCAATATGTTTGCCACCTGGTCTCCTTCAAAAGCTCGACTTCACCTCC
AAGGGAGGAGT**AATGCCTGGAGACCTCAGGTGAAT**AATCCAAAAGAGTGGCTGCAA
GTGGACTTCCAGAAGACAATGAAAGTCACAGGAGTAACTACTCAGGGAGTAAAATCTCTGCT
TACCAGCATGTATGTGAAGGAGTTCCTCATCTCCAGCAGTCAAGATGGCCATCAGTGGACTC
TCTTTTTTCAGAATGGCAAAGTAAAGGTTTTTCAGGGAAATCAAGACTCCTTCACACCTGTGG
TGAACTCTCTAGACCCACCGTTACTGACTCGCTACCTTCGAATTCACCCCCAGAGTTGGGTG
CACCAGATTGCCCTGAGGATGGAGGTTCTGGGCTGCGAGGCACAGGACCTCTACTGA**(end
fVIII)**

Figure 2.4 – FVIII cDNA with sequencing primers. Nine primers were used to span the entire fVIII cDNA sequence. Primer sequences are depicted in the cDNA sequence as underlined and bolded. FVIII domains are labeled in

parenthesis in black type throughout the cDNA sequence. The sites of the L303E/F309S mutations are marked in underlined black type within the A1 domain. In the wild type, L303 (leucine) is encoded by CTT and F (phenylalanine) is encoded by TTT. In the double mutation (DM) constructs, these sequences become glutamic acid encoded as GAA and serine encoded as TCT.

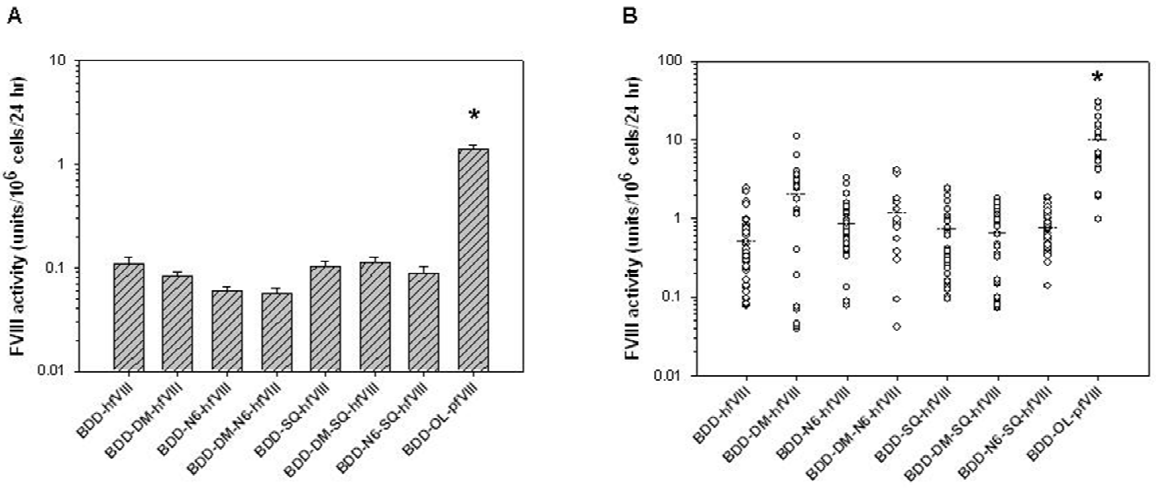

Figure 2.5 - Heterologous expression of fVIII transgenes. BHK-M cells were transfected with fVIII transgenes in the ReNeo mammalian expression vector. **(A)** Transient fVIII expression was measured from individual wells of a 24-well plate by one-stage coagulation assay. Vertical bars represent fVIII activity (mean ± sample standard deviation) of four independent replicates. Statistical analysis revealed that BDD-OL-pfVIII activity was significantly higher than any human construct (*p<0.001, ANOVA). **(B)** Transfected cells were plated in selective antibiotic reagent for the generation of stable fVIII-expressing clones. G-418-resistant clones were cultured in 6- well plates and fVIII expression was measured by one-stage coagulation assay. Horizontal bars represent the mean of each set of clones. Statistical analysis revealed BDD-OL-pfVIII activity was significantly higher than any human construct (*p<0.001, ANOVA)

variegation that may have lead to the high variability in fVIII expression observed among the BHK-M clones. We used Flp-In™ human embryonic kidney 293 cells (hereby referred to as Flp-In 293) that were engineered to contain a single FLP recombination target (FRT) site. The FRT site serves as the binding and cleavage site for the recombinase and is located in the 5′-end of the open reading frame of a β-galactosidase/Zeocin fusion gene (*lacZeo*). This cell line is Zeocin-resistant, *lacZ*-positive, and hygromycin-sensitive prior to transfection with the pcDNA5/FRT expression vector. As shown in **Figure 2.6**, FLP recombinase mediates site-specific recombination between the FRT site in the host cell line and the FRT site in the pcDNA5/FRT/fVIII expression vector allowing transgene integration into the same transcriptionally-active genomic location in every cell. After recombination, the fVIII transgene is expressed under the control of a cytomegalovirus (CMV) promoter and resistance to hygromycin B is driven by a SV40 promoter to allow selection of genetically-modified cells. The efficiency of FLP-mediated, targeted recombination and ease in generating isogenic cell lines makes this an effective and potentially more predictive *in vitro* system for the comparison of relative transgene expression properties (Liu et al 2006; Wirth & Hauser 2004).

Following selection of 20 – 40 clones per fVIII transgene, the clonal cell lines were expanded and cultured for 24 hours in serum-free medium before fVIII activity was measured by one-stage coagulation assay. Cell viability and growth rates were also determined and found to be similar among all fVIII-expressing

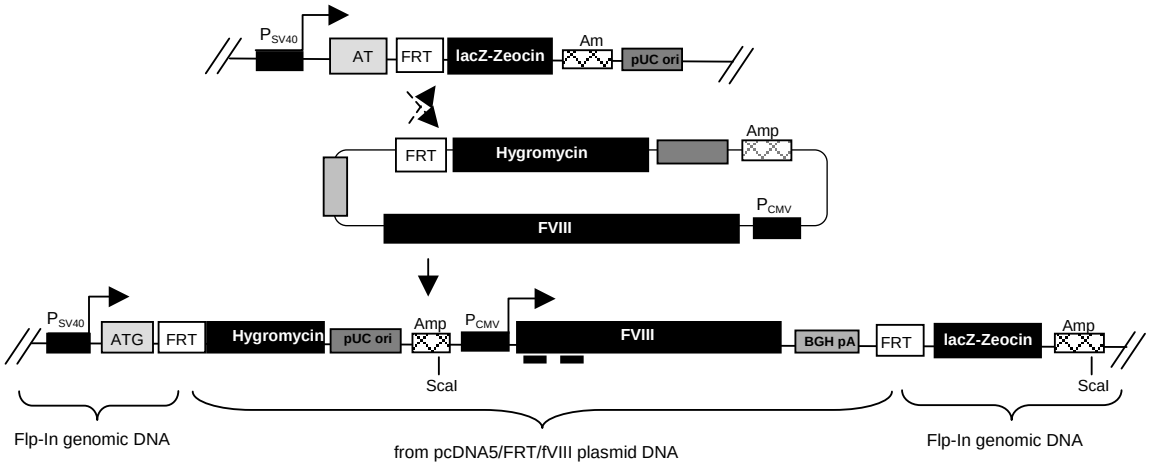

Figure 2.6 - The Flp-In™ System incorporating pfVIII. (A) The Flp-In™ system utilizes a host cell line with an integrated FRT site to direct site-specific integration of a gene of interest upon co-transfection of the pcDNA5/FRTexpression plasmid and a FLP recombinase plasmid (pOG44). Resulting isogenic cell lines were selected in hygromycin B. Horizontal bars under the fVIII transgene represent the binding sites for the human (A2) or porcine (A1) probes used in Southern blot analyses.

clones **(Figure 2.7).** Genomic DNA was harvested from Flp-In 293 clones and screened by Southern blot to identify clones with multiple and/or incorrect integrations. A representative Southern blot analysis of genomic DNA from clonal cell lines expressing various correctly and incorrectly integrated human fVIII transgenes is shown in **Figure 2.8**. All clones exhibited bands at 8.6 kb and 5.5 kb when probed with the human A2 probe. These bands represent endogenous fVIII intron-containing sequences present in Flp-In 293 cells. Transgene integration into the correct genomic location only is shown in lanes 3, 4, 6, and 7. Lanes 1 and 8 depict transgene integration into the correct location as well as an additional random integration potentially resulting in expression of 2 copies of the fVIII transgene. Lane 2 shows an incorrect integration event and lane 5 represents a clone with no probe-specific fVIII transgene sequence in the genome. **Figure 2.9** shows the relative fVIII activity from Flp-In 293 clones expressing a single correctly targeted fVIII transgene. Mean BDD-OL-pfVIII activity was 18 units/10^6 cells/24 hr, which was 36 – 225-fold higher than any of the human transgenes (p<0.001, ANOVA). Although no significant differences were found among human transgenes, mean BDD-SQ-hfVIII expression was the greatest at 0.5 units/10^6 cells/24 hr, and only slightly higher than BDD-DM-N6-hfVIII with a mean fVIII expression of 0.4 units/10^6 cells/24 hr. A 3-fold increase in fVIII expression was observed with the addition of the N6 segment to BDD-hfVIII (0.25 units/10^6 cells/24 hr and 0.08 units/10^6 cells/24 hr, respectively), which is similar to a previously published report (Miao *et al.*, 2004). These data further demonstrate that BDD-OL-pfVIII is expressed at significantly higher levels than

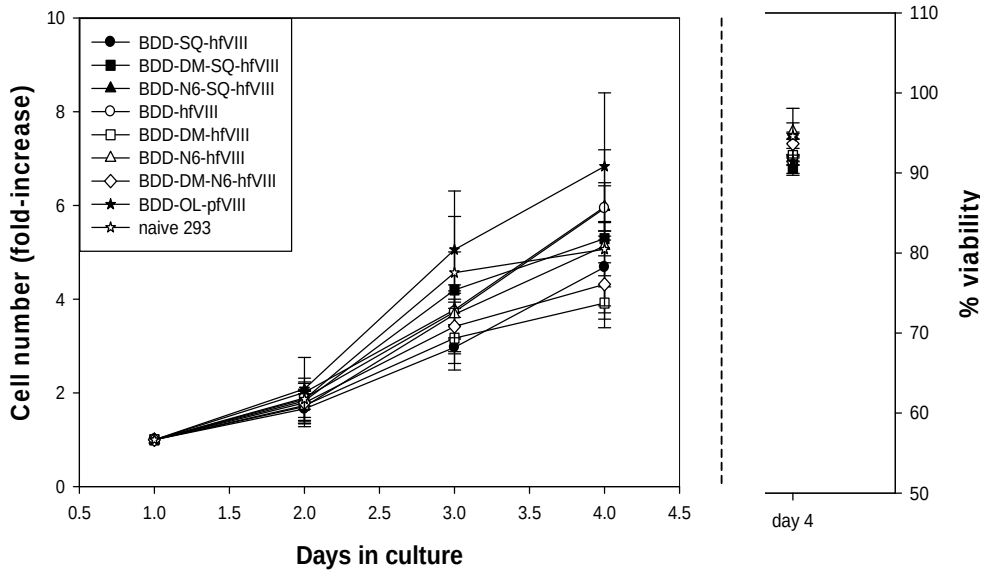

Figure 2.7 – Flp-In 293T clone growth rates and viability. One clone expressing each different transgene was plated in triplicate wells of a 6-well plate. On days 1, 2, 3, and 4 after plating, cells were counted in trypan blue to determine cell count (left panel) and viability (right panel). Naïve 293T cells were also included as an experimental control.

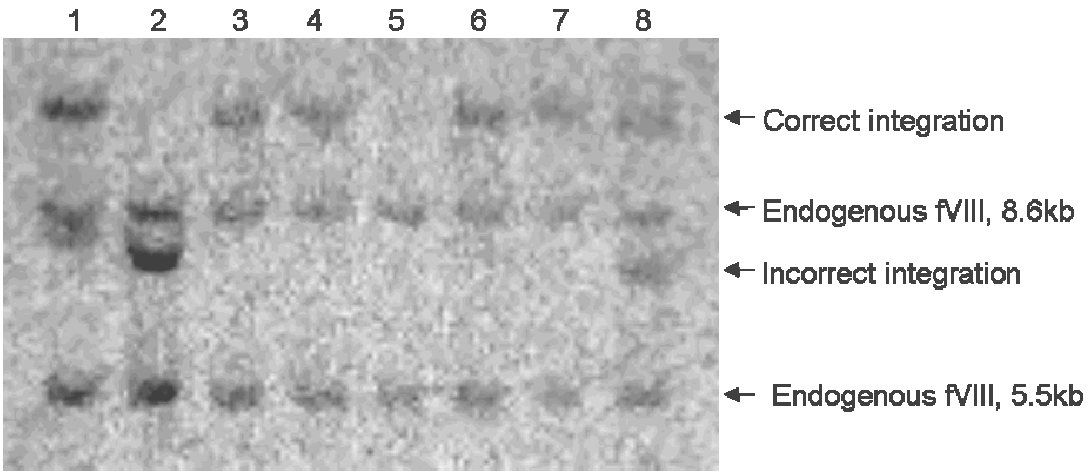

Figure 2.8 – Representative Southern blot of Flp-In clones. A representative Southern blot analysis of clones expressing various fVIII transgenes is shown. Endogenous human fVIII sequences in 293T cells generate bands at 8.6 kb and 5.5 kb when digested with ScaI. Correct single integrants are predicted to display a band at 12.5 kb. Lanes containing bands other than 12.5 kb represent clones with randomly integrated plasmid DNA

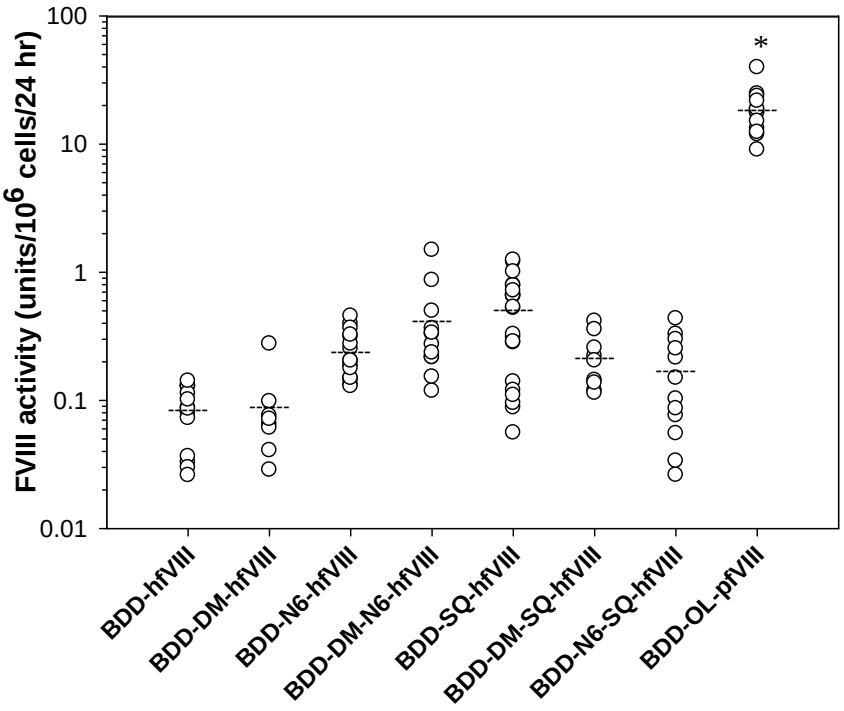

Figure 2.9 – FVIII expression using the Flp-In System. Flp-In™ 293 cells were transfected with each pcDNA5/FRT/fVIII expression vector and stable integrants were selected for hygromycin resistance and plated at limiting dilution. Individual clones were assayed for fVIII activity by one-stage coagulation assay. Only clones with single correct transgene integration were used in the comparative fVIII expression analysis. Statistical analysis confirmed that BDD-OL-pfVIII expression was significantly greater than observed for any human construct (*$p<0.001$, ANOVA).

previously characterized human fVIII transgenes. In order to further characterize fVIII expression from Flp-In 293 clones, four BDD-SQ-hfVIII clones were plated in limiting dilution for the creation of subclones. Single cells were expanded and fVIII measured from conditioned medium. FVIII activity from subclones was variable and suggests the importance of standardization in cell number, cell density, and time in culture for comparable fVIII analyses **(Figure 2.10).**

High level fVIII expression from human/porcine chimeric fVIII transgenes

Although BDD-OL-pfVIII is expressed at very high levels following gene-modification of heterologous cells, its clinical implications for us are limited, as a commercial BDD-OL-pfVIII product is already in Phase 3 clinical trial for treatment of hemophilia A patients harboring high titer anti-hfVIII antibodies. In order to take advantage of the high expression properties of BDD-OL-pfVIII and to create a novel transgene to take into clinical trial, a number of human/porcine chimeric fVIII transgenes were created. It was previously shown that the increased expression from BDD-OL-pfVIII was due to the presence of porcine-specific A1 and A3 domains. Metabolic labeling experiments comparing BDD-SQ-hfVIII and a human/porcine chimeric fVIII transgene containing human-specific A2, C1, and C2 domains and porcine-specific A1 and A3 domains, named BDD-HP47, revealed that the difference in fVIII expression from human to porcine was due to increased protein secretion (Doering et al 2004). Based on this data, 2 new chimeric transgenes were created: BDD-HP69 which is BDD-SQ-hfVIII containing a porcine A1 domain, and BDD-HP70 which is BDD-SQ-

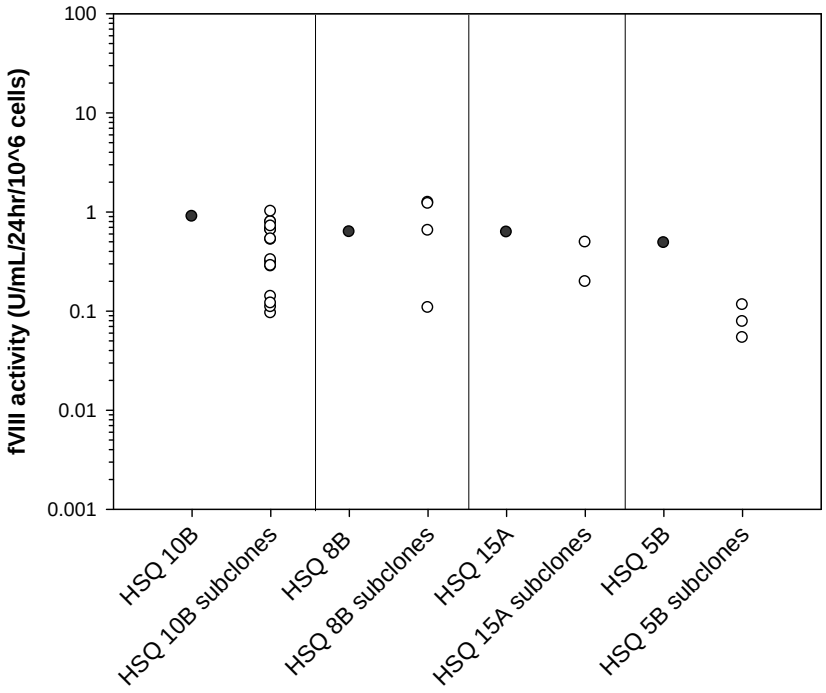

Figure 2.10 – FVIII activity from BDD-SQ-hfVIII Flp-in clones and subclones. Four BDD-SQ-hfVIII Flp-In clones (HSQ 10B, HSQ 8B, HSQ15A, and HSQ 5B) were plated in limiting dilution in a 96 well plate in order to create subclones from each original clone. Subclones were expanded into 6-well plates and cultured in serum-free media for 24 hours prior to measurement of fVIII activity by one stage coagulation assay. Parental clones are shown as a black circle and subclones shown as white circles.

hfVIII containing half of a porcine A1 domain **(Figure 2.11).** BDD-DM-HP47, BDD-DM-HP70, and BDD-DM-pfVIII were also created by introducing the L303E/F309S double mutation to decrease BiP binding and increase intracellular protein processing and secretion. Each transgene was cloned into the ReNeo mammalian expression vector and transfected into BHK-M cells. Stable clones were propagated in selection medium and fVIII measured by one stage coagulation assay to determine if any human/porcine chimeric transgenes could be expressed at levels comparable to BDD-OL-pfVIII. Of the three chimeric transgenes, BDD-HP47 was the highest expresser, showing no significant difference in expression as compared to BDD-OL-pfVIII **(Figure 2.12)**. The L303E/F309S double mutation did not increase expression in any of the chimeric transgenes, however, it completely abolished activity when incorporated into BDD-OL-pfVIII.

From this data, it was determined that BDD-HP47 was the highest expressing human/porcine chimeric transgene and warranted further characterization. Herein referred to as HP-fVIII **(Figure 2.13A)**, the sequence of this transgene containing porcine-specific A1 and A3 domains was compared to both human and porcine fVIII. A blastp alignment of amino acid sequence showed that HP-fVIII has 89% homology to BDD-SQ-hfVIII and 93% homology to BDD-OL-pfVIII. As described above, no significant difference in expression was observed between BDD-OL-pfVIII and HP-fVIII in transfected BHK-M cells. However, HP-fVIII expression was ~13-fold higher than BDD-SQ-hfVIII expression ($P < 0.0001$,

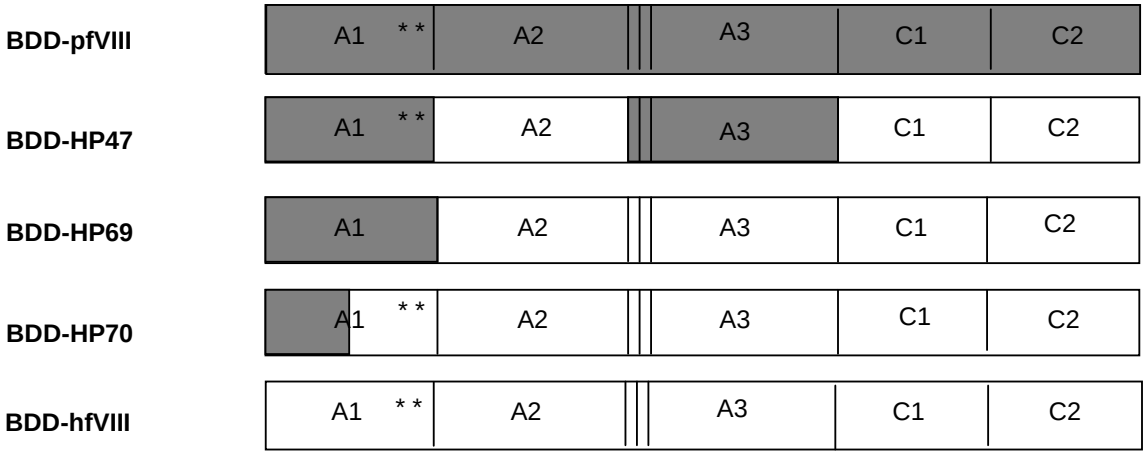

Figure 2.11 – Schematic of human/porcine chimeric fVIII transgenes. Three human/porcine chimeric fVIII transgenes were generated for comparison of fVIII activity. Human fVIII domains are shown in white. Porcine fVIII sequence is shown in gray. The location of L303E/F309S in double mutant (DM) constructs is depicted by asterisks.

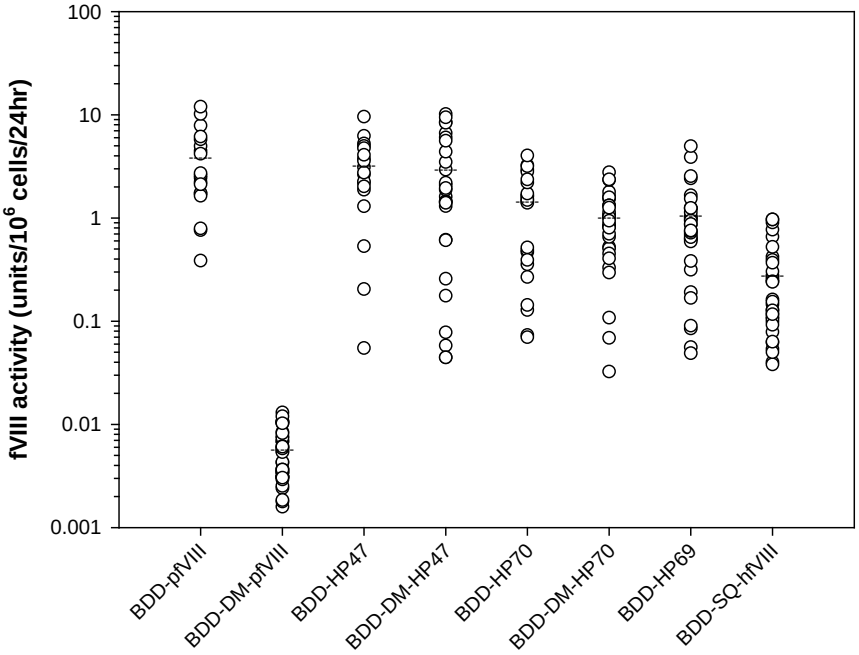

Figure 2.12 – Comparison of human/porcine chimeric fVIII transgene expression. BHK-M cells were transfected with each ReNeo/fVIII expression vector and stable integrants were selected by G-418 resistance and plated at limiting dilution. Individual clones were assayed for fVIII activity by one-stage coagulation assay. Mean fVIII activity is depicted by a horizontal dotted line.

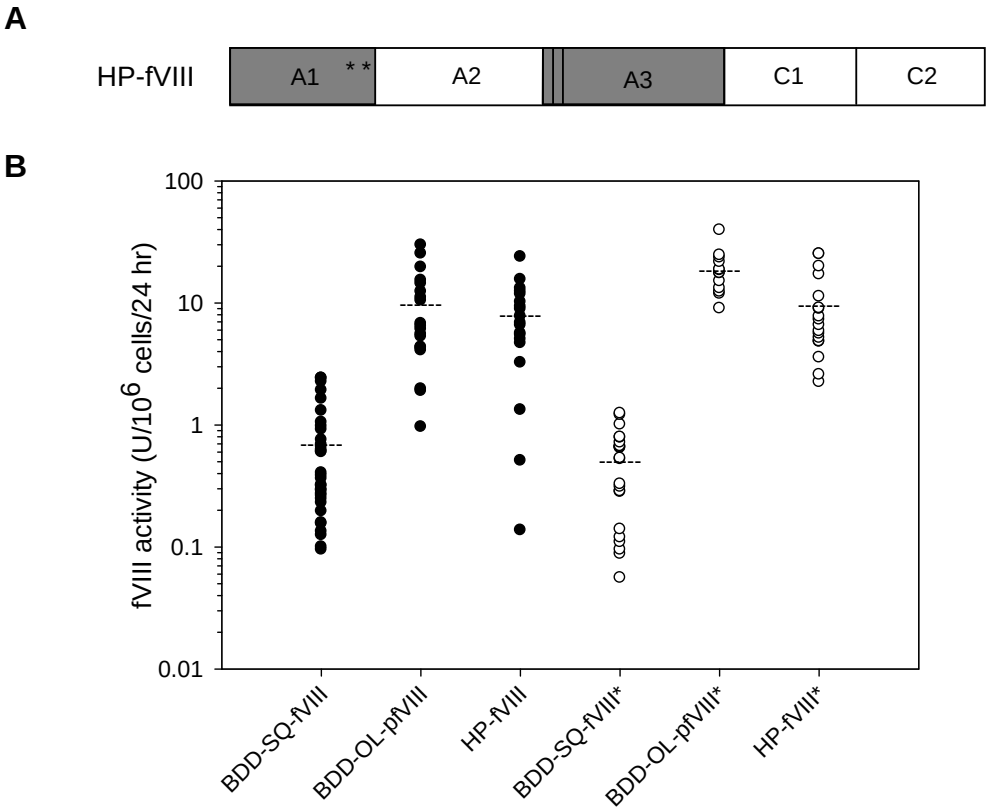

Figure 2.13 - Heterologous expression of human, porcine, and human/porcine chimeric transgenes. A) Schematic of human/porcine chimeric transgene (HP-fVIII). White domains are human-specific sequences and gray domains are porcine-specific sequences. B) Human transgene BDD-SQ-hfVII, porcine transgene BDD-OL-pfVIII, and the HP-fVIII transgene were transfected into both BHK-M and Flp-In 293 cells. BHK-M cells were modified using Lipofectamine 2000 and ReNeo plasmid constructs. Flp-In 293 cells were modified by FLP recombinase-mediated site-specific recombination. FVIII activity was measured from 19 to 33 BHK-M clones (black circles) and 13–22 singly integrated Flp-In clones (white circles) by one-stage coagulation assay. Mean fVIII activity is depicted by a horizontal dotted line.

Mann– Whitney *U* Test) **(Figure 2.13B)**. In an attempt to reduce interclonal variation due to position effect variegation, fVIII expression was compared using the Flp-In™ System. Clones were isolated by hygromycin resistance and those expressing fVIII were screened by Southern blot to eliminate any clones with multiple and/or incorrect integrations **(Figure 2.14)**. Only clones with a single correct integration event were used in the comparative analyses. As shown in **Figure 2.13B**, fVIII expression was greatest from BDD-OL-pfVIII at 19 units/10^6 cells/24 hour, which was 37-fold higher than BDD-SQ-hfVIII. HP-fVIII transgene expression was significantly lower at 10 units/10^6 cells/24 hour (P = 0.0002, Mann– Whitney *U* test), which was 19-fold higher than BDD-SQ-hfVIII. This is the first time a significant difference has been observed between BDD-OL-pfVIII and HP-fVIII. But for both BDD-OL-pfVIII and HP-fVIII, expression was significantly greater than BDD-SQ-hfVIII (P < 0.0001, Mann–Whitney *U* test).

Characterization of fVIII production and decay rates

It was shown previously that BDD-OL-pfVIII and BDD-SQ-hfVIII have similar specific activities and similar steady-state levels of fVIII mRNA in transfected cell lines suggesting that the increase in fVIII expression from BDD-OL-pfVIII is due to increased secretion resulting from more efficient translational and/or posttranslational modifications (Doering et al 2002b). To better understand the kinetics of fVIII in a cell-culture system, we followed the relative production and decay of BDD-OL-pfVIII and BDD-SQ-hfVIII *in vitro*. FVIII decay rates were assessed by adding 10 units of purified BDD-OL-pfVIII or BDD-SQ-hfVIII to 1 mL

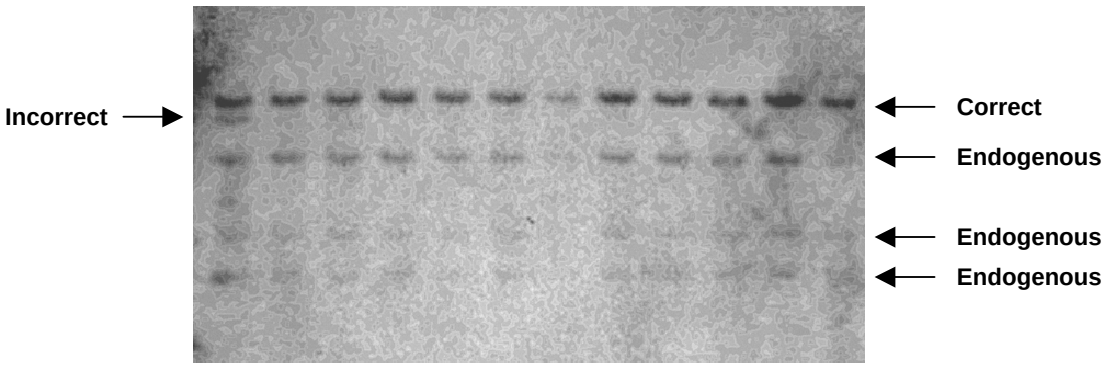

Figure 2.14 – Southern blot analysis of HP-fVIII Flp-In 293 clones. A representative Southern blot analysis of clones expressing HP-fVIII is shown. When probed with a porcine-specific A1 probe, endogenous human fVIII sequences in 293 cells generate 3 bands when digested with ScaI. Correct single integrants are predicted to display a band at 12.5 kb. Lanes containing bands other than 12.5 kb represent clones with randomly integrated plasmid DNA.

of serum-free medium a 6-well plate containing a nearly confluent layer of naive Flp-In 293 cells to simulate fVIII expression from gene-modified cells. FVIII activity was measured by one-stage coagulation assay immediately after the addition of fVIII then at various time points for up to 48 hours. The resulting time-course analysis confirmed that BDD-OL-pfVIII and BDD-SQ-hfVIII exhibit similar decay rates *in vitro* **(Figure 2.15)** suggesting that the higher fVIII activity levels observed for BDD-OL-pfVIII were attributable to increased biosynthesis and not increased stability in conditioned medium. In order to confirm the increased rate of protein production for BDD-OL-pfVIII, cells were plated from BDD-OL-pfVIII and BDD-SQ-hfVIII Flp-In 293 clones expressing a correctly targeted single transgene and fVIII activity was measured over time in serum-free medium by one-stage coagulation assay. Specific clones were selected whose 24 hr fVIII activity measurement was within one standard deviation of the mean expression level for that transgene. The rate of BDD-OL-pfVIII production was 18-fold greater than the rate of BDD-SQ-hfVIII production with a BDD-OL-pfVIII production rate of 0.92 units/10^6 cells/hr and a BDD-SQ-hfVIII production rate at 0.05 units/10^6 cells/hr **(Figure 2.16)**. The BDD-OL-pfVIII clone produced 75 fVIII molecules/cell/sec, while and the BDD-SQ-hfVIII clone produced 5 fVIII molecules/cell/sec. Quantitative RT-PCR performed on RNA isolated from each clone confirmed that the BDD-OL-pfVIII and BDD-SQ-hfVIII clones expressed a similar number of transcripts, 1216 ± 214 and 1610 ± 123 transcripts/cell, respectively **(Figure 2.17)**. Cells with similar transcripts/cell were chosen in order to confirm that the increase in fVIII expression from BDD-OL-pfVIII is due to more

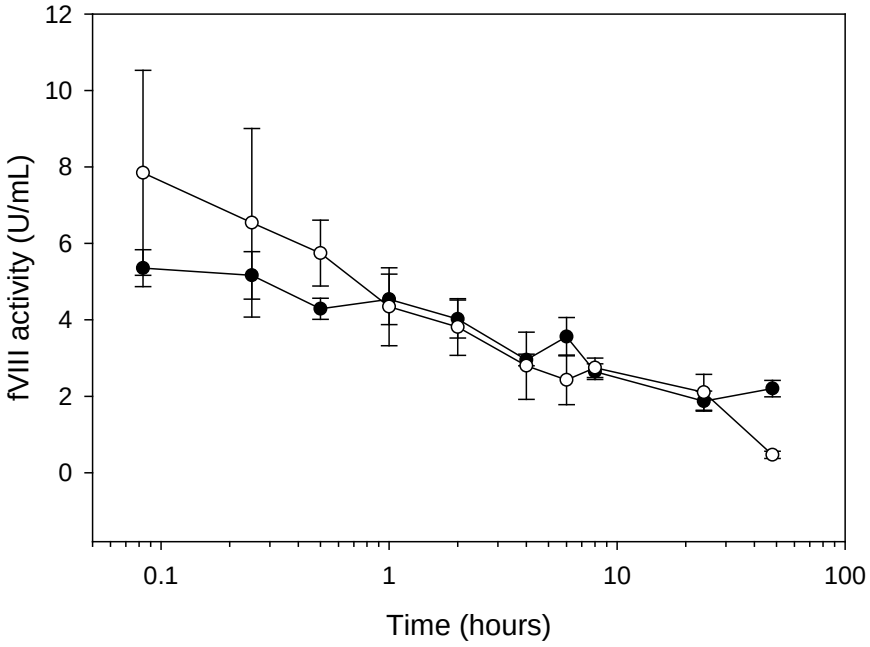

Figure 2.15 - Rates of decay of BDD-SQ-hfVIII and BDD-OL-pfVIII. Purified BDD-OL-pfVIII and BDD-SQ-hfVIII were spiked into serum-free AIM-V medium at 10 U/mL in 6-well plates containing a confluent layer of naïve 293T cells. FVIII activity was measured by one stage coagulation assay immediately after addition of purified fVIII to medium and then at various time points for up to 48 hours. BDD-SQ-hfVIII activity is shown as black circles and BDD-OL-pfVIII activity is shown as white circles. Each circle represents 2 measurements from 2 separate experiments.

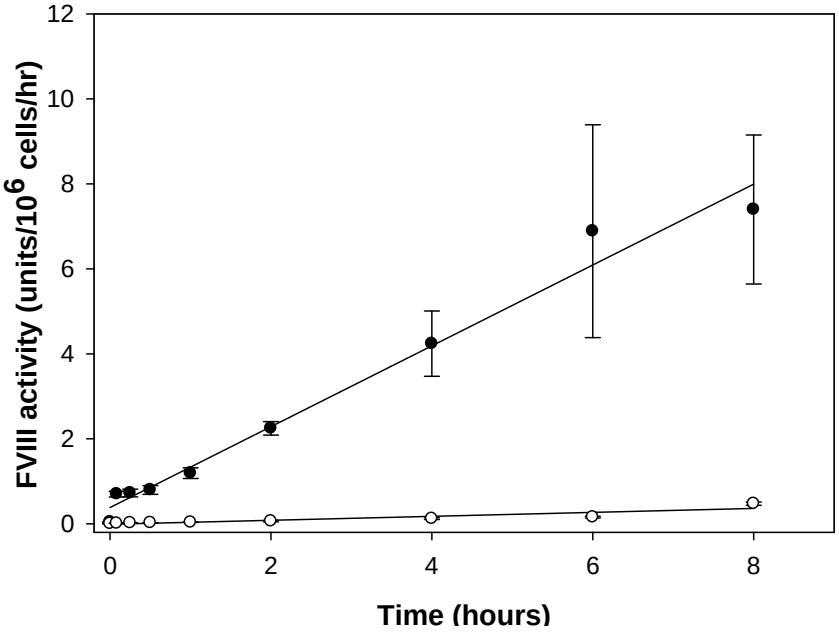

Figure 2.16 – Rates of fVIII production. Two million cells were plated in 6-well plates and cultured for 48 hours before washing with PBS and replacing conditioned medium with serum-free medium. FVIII activity was measured by one-stage coagulation assay at time = 0, 5, 15, and 30 minutes, then at 1, 2, 4, 6, and 8 hours in order to determine the rates of fVIII production from each clone. The BDD-OL-pfVIII clone is represented by the black circles and the BDD-SQ-hfVIII clone represented by the white circles. Each circle is the mean value of 3 independent experiments ± sample standard deviation at each time-point.

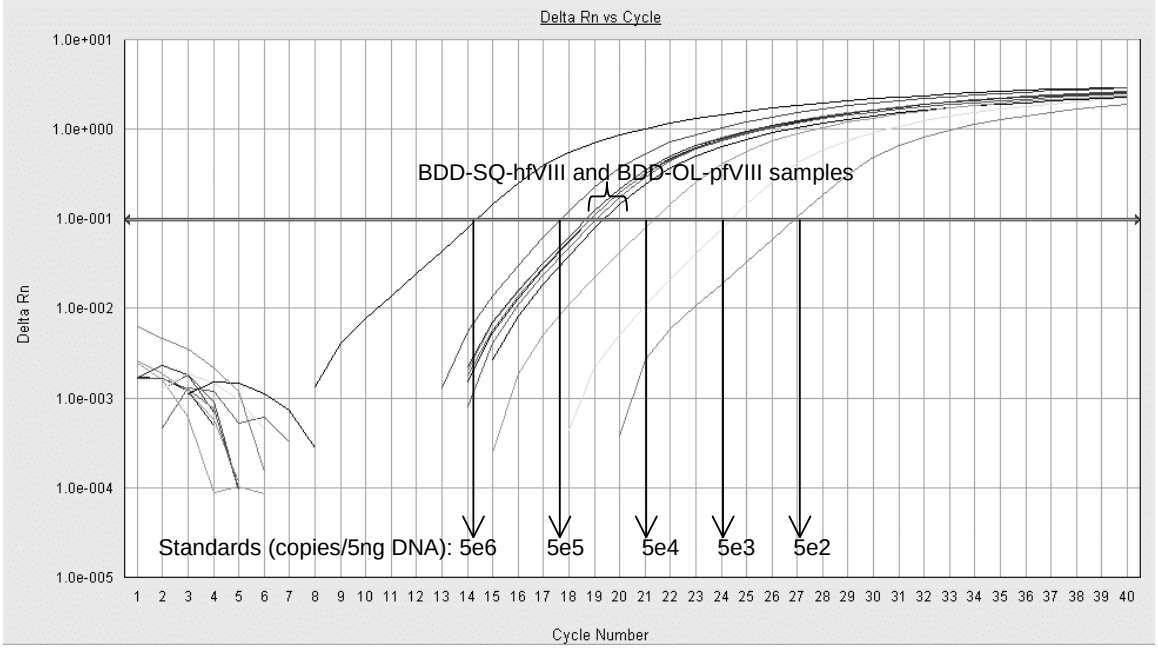

Figure 2.17 – RT-PCR analysis of clonal Flp-In 293 transcripts/cell. RNA was isolated from 4 BDD-SQ-hfVIII and 4 BDD-OL-pfVIII Flp-In 293 clones. A standard curve was generated using RNA standards from 5 x 10^6 – 5 x 10^2 copies in 5 ng genomic RNA. Graph shows experimental data from one BDD-SQ-hfVIII and one BDD-OL-pfVIII clone expressing similar transcript numbers. These 2 clones were used in the determination of fVIII production rates.

High-level expression of BDD-OL-pfVIII using lentiviral gene transfer

Lentiviral vectors are an attractive tool for transgene delivery as they have the potential to transfer proviral DNA sequences into the genome of dividing and non-dividing cells thereby generating a source of long-term transgene expression. In order to compare human and porcine fVIII expression in cells following lentiviral-mediated gene transfer, self-inactivating HIV-1-based lentiviral vectors encoding either BDD-OL-pfVIII or BDD-SQ-hfVIII were generated **(Figure 2.18A)**. 293T cells were transduced at low multiplicities of infection (MOI) equal to 0.3, 0.9, and 2.7, in order to achieve low-level integration of proviral DNA. These MOIs were chosen because low-level integration is beneficial in a clinical setting in order to limit the risk of insertional mutagenesis in gene-modified cells. At 7 days post-transduction, BDD-OL-pfVIII expression levels were on average 7–8-fold higher than BDD-SQ-hfVIII. At the highest MOI, BDD-OL-pfVIII expression was 38 units/10^6 cells/24 hr compared to 4.5 units/10^6 cells/24 hr for BDD-SQ-hfVIII **(Figure 2.18B)**. Cell viability and growth rates were determined and found to be similar among GFP control and hfVIII-expressing cells **(Figure 2.19)**. Quantitative RT-PCR analysis performed on total RNA isolated from transduced 293T cells indicated that the increased BDD-OL-pfVIII expression was not the result of disproportionately high steady-state mRNA levels. In the populations tested, BDD-SQ-hfVIII-expressing cells had higher steady-state mRNA levels than BDD-OL-pfVIII at each MOI examined **(Figure 2.18C)**. When fVIII activity was normalized to transcript expression, BDD-OL-pfVIII expression was 21-fold higher than that of BDD-SQ-hfVIII **(Figure 2.20)**. Northern blot

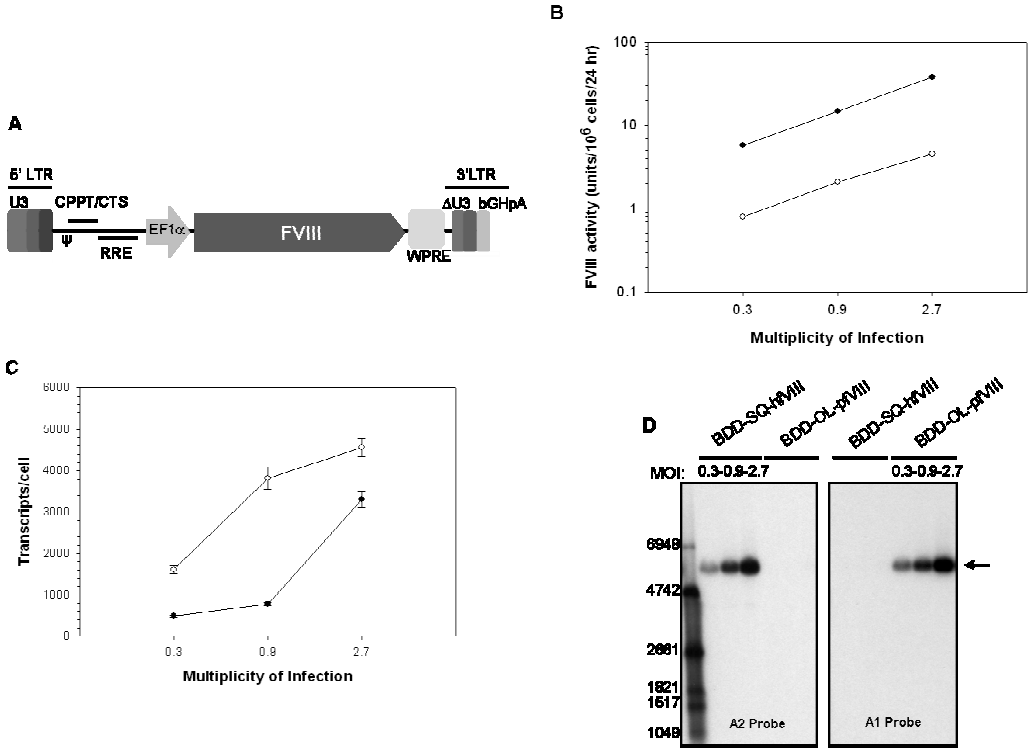

Figure 2.18 - Expression of BDD-SQ-hfVIII and BDD-OL-pfVIII following lentiviral transduction of 293T cells. A) A schematic diagram of the HIV-1 based, SIN lentiviral vectors encoding either the BDD-SQ-hfVIII or BDD-OL-pfVIII transgene is shown. Vectors include a 5` HIV-1 long-terminal repeat (LTR) and a 3' HIV-1 LTR with self-inactivating (SIN) U3 deletion, bovine growth hormone polyA signal (bGHpA), packaging signal (ψ), central polypurine tract and central termination sequence (CPPT/CTS), Rev-responsive element (RRE), elongation factor 1-alpha promoter (EF-1α) and Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). **(B)** 293T cells were transduced at increasing MOIs of 0.3, 0.9, and 2.7 with either BDD-SQ-hfVIII or BDD-OL-pfVIII-encoding lentivirus. At day 7 post-transduction, fVIII activity was measured by one-stage coagulation assay and normalized to cell number. Data points

represent the mean of two measurements of the sample. BDD-OL-pfVIII-expressing cells are represented by the black circles and BDD-SQ-hfVIII-expressing cells represented by the white circles. **(C)** Steady-state fVIII RNA levels from BDD-SQ-hfVIII or BDD-OL-pfVIII transduced 293T cells were determined at 7 days post-transduction. Steady-state fVIII RNA levels were measured from harvested total RNA using quantitative RT-PCR. Data represent the mean ± sample standard deviation from three independent replicates. BDD-OL-pfVIII-expressing cells are depicted by black circles and BDD-SQ-hfVIII-expressing cells by white circles. **(D)** Northern blot analysis of BDD-SQ-hfVIII and BDD-OL-pfVIII transduced 293T cells at day 7 post-transduction. The arrow depicts fVIII-specific products of the correct molecular weight. RNA molecular weight markers positions are shown on the left.

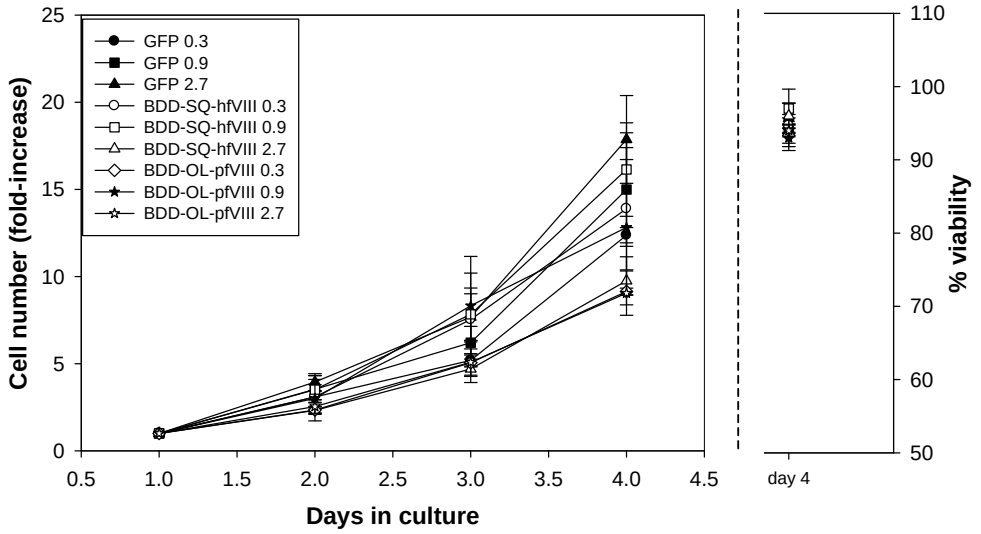

Figure 2.19 – Lentivirus-transduced 293T clone cell growth and viability.

Individual clones expressing GFP, BDD-SQ-hfVIII, or BDD-OL-pfVIII at 0.3, 0.9, or 2.7 copies per cell were plated in triplicate wells of a 6-well plate. On days 1, 2, 3, and 4 after plating, cells were counted in trypan blue to determine cell count (left panel) and viability (right panel). Naïve 293T cells were also included as an experimental control.

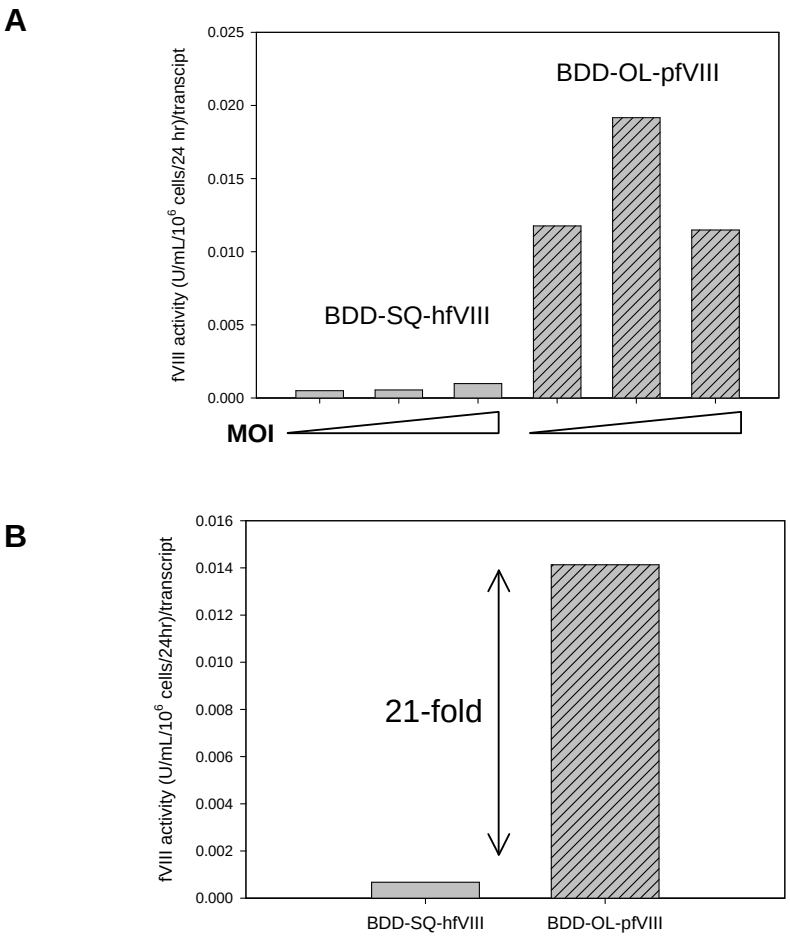

Figure 2.20 – FVIII expression per transcript in lentiviral-transduced clones. 293T cells were transduced at an MOI of 0.3, 0.9, and 2.7 with a HIV-1 based lentiviral vector expressing either BDD-SQ-hfVIII or BDD-OL-pfVIII. FVIII activity was measured by one-stage coagulation assay and RNA transcript number was measured by RT-PCR. A) Gray bars show BDD-SQ-hfVIII activity/transcript and hatched bars show BDD-OL-pfVIII activity/transcript at 3 different MOIs. The triangle below the graph signifies increasing MOIs equal to 0.3, 0.9, and 2.7. B) Gray bar shows the combined BDD-SQ-hfVIII activity/transcript and hatched bar shows combined BDD-OL-pfVIII activity/transcript for all MOIs tested.

analysis performed on the same samples, using either a human- or porcine-specific probe, showed expression of a single transcript of approximately 6,300 nucleotides **(Figure 2.18D)**. In order to further characterize expression of BDD-SQ-hfVIII and BDD-OL-pfVIII from transduced 293T cells, clones were isolated by limiting dilution 6 weeks post-transduction. Clonal BDD-OL-pfVIII expression was greater than that of BDD-SQ-hfVIII ranging from 0.05 – 57 units/10^6 cells/24 hr and 0.02 - 4.6 for BDD-OL-pfVIII and BDD-SQ-hfVIII, respectively **(Figure 2.21A)**. Significant differences in the median expression level of BDD-OL-pfVIII or BDD-SQ-hfVIII was observed from clones containing a single integration event ($p < 0.001$, Mann Whitney *U* Test) as well as from clones containing multiple integrants ($p = 0.008$, Mann Whitney *U* Test). Of note, BDD-OL-pfVIII clones derived from the population transduced at MOI of 2.7 exhibited a lower number of integrations than BDD-SQ-hfVIII clones at the same MOI. Although the viral titer of BDD-OL-pfVIII is slightly lower than that of BDD-SQ-hfVIII, cells were transduced at equivalent MOIs in order to prevent differences in the amount of virus introduced to the cells. As such, this difference in number of integrations cannot be due to differences in viral titer. Southern blot analysis was used to quantify integration of proviral transgene DNA within all clones and demonstrated mean proviral copy number/clone for BDD-SQ-hfVIII of 0.9 and 2.2, and for BDD-OL-pfVIII of 0.9 and 1.5 at MOIs of 0.9 and 2.7, respectively. Band sizes were above the minimum expected following genomic DNA digestion with AvrII, a restriction enzyme that recognizes a single site within the transgene cassette, indicating successful integration **(Figure 2.21B)**. Steady-state fVIII mRNA levels

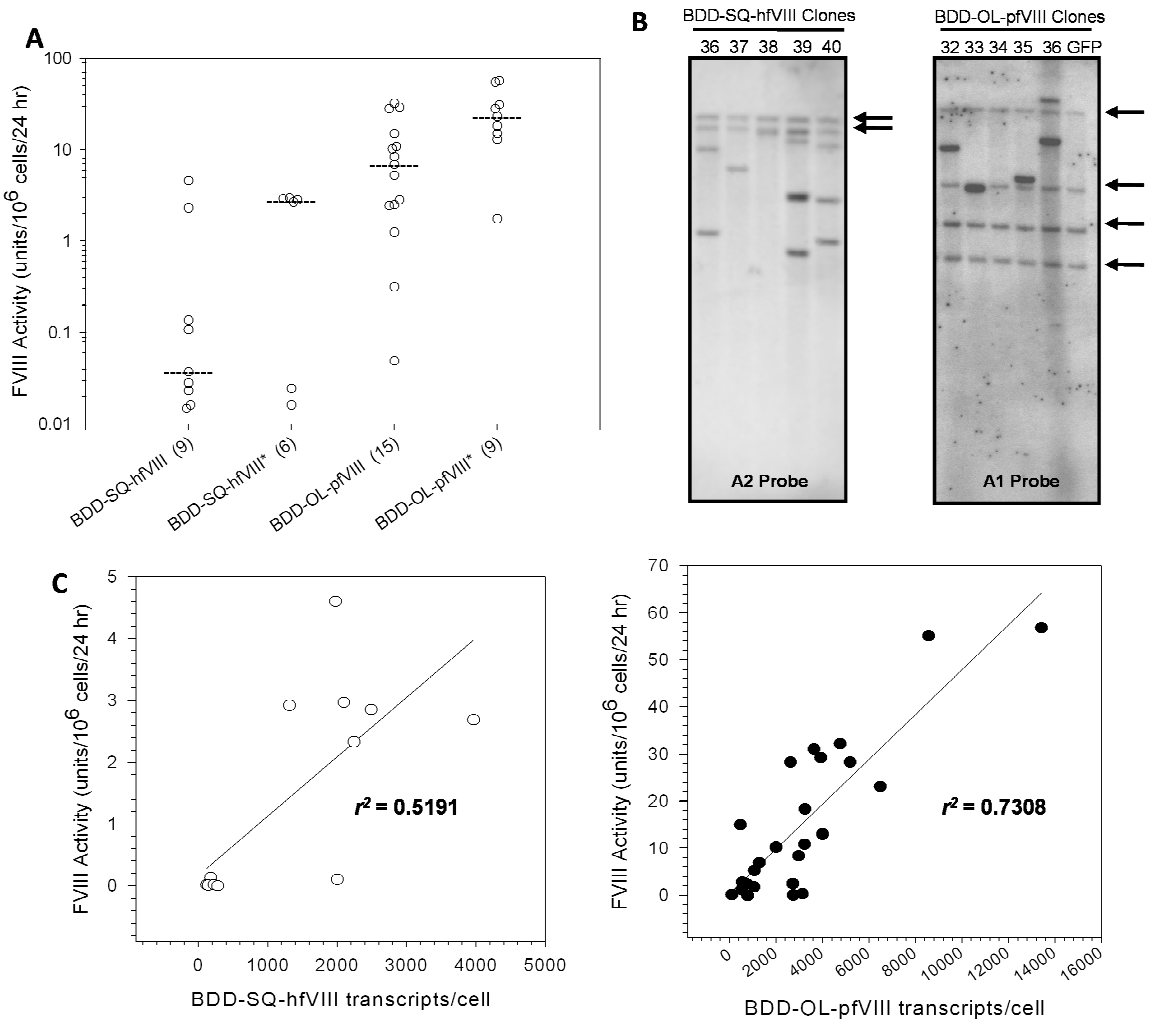

Figure 2.21 - Expression of BDD-SQ-hfVIII and BDD-OL-pfVIII from individual 293T transduced clones. (A) Individual clones were isolated by single cell limiting dilution. FVIII expression from each clone was measured by one-stage coagulation assay and normalized to cell number. The median value for all clones in each group is represented by a horizontal line and the value in parenthesis represents the number of clones in each group. Median fVIII activity of BDD-OL-pfVIII and BDD-SQ-hfVIII clones containing a single integration event was significantly different ($p < 0.001$, Mann Whitney U Test). Median fVIII activity

of clones with greater than a single integration event (BDD-SQ-hfVIII* and BDD-OL-pfVIII*) also was significantly different (p = 0.008, Mann Whitney U Test). **(B)** Representative Southern blot analysis of five clones transduced with BDD-SQ-hfVIII- or BDD-OL-pfVIII-containing lentivirus at MOI 2.7 is shown. Clone numbers are indicated above the blot and the arrows represent the positions of the bands expected for endogenous fVIII in 293T cells using each probe. **(C)** FVIII activity versus steady-state RNA levels for BDD-SQ-hfVIII and BDD-OL-pfVIII transduced 293T clones. Each clone was plated in duplicate 35 mm wells and grown to ~70 % confluence. FVIII activity was measured on conditioned medium by one-stage coagulation assay, and the cells were harvested for total RNA isolation. Steady-state fVIII RNA levels were measured by quantitative RT-PCR. Data represent mean ± sample standard deviation from three independent replicates. BDD-OL-pfVIII clones are represented by black circles and BDD-SQ-hfVIII clones are represented by the white circles. Clones demonstrating transcript expression below the limit of detection of the assay were not included in the analysis. BDD-SQ-hfVIII and BDD-OL-pfVIII expressing clones transduced at MOIs of 0.9 and 2.7 were combined in the analysis. Linear regression analysis revealed a significant correlation between fVIII activity and fVIII transcripts for both BDD-SQ-hfVIII and BDD-OL-pfVIII clones (p = 0.005 and $p < 0.0001$, respectively, two-tailed Student's t-test).

for all stable clones were measured by quantitative RT-PCR **(Figure 2.21C).** Linear regression analysis revealed a significant correlation between fVIII activity and fVIII transcripts for both BDD-SQ-hfVIII and BDD-OL-pfVIII (p = 0.005 and p < 0.0001, respectively, two-tailed Student's t-test). Several clones that exhibited transcript levels below the detection limit and fVIII activity close to or below the baseline (≤ 0.01units/10^6 cells/24 hr) were excluded from the analysis. These data demonstrate that the high-level BDD-OL-pfVIII expression achieved following transfection of heterologous mammalian cells is reproducible in cells modified by lentiviral-mediated gene transfer.

***In vivo* comparison of hfVIII and pfVIII expression**

To compare lentiviral vector expression of pfVIII and hfVIII *in vivo*, Sca-1^+ cells were isolated from donor mice and transduced with a self-inactivating lentivirus expressing either BDD-SQ-hfVIII or BDD-OL-pfVIII **(Figure 2.22).** Prior to transduction, cells were incubated 2 days in a standard cytokine cocktail to induce cell cycling. Although mitosis is not required for lentiviral transduction, it has been shown that cells are most efficiently transduced while cells are actively cycling (Groschel & Bushman 2005; Sutton et al 1999). Cells were transduced *ex vivo* with an SIV vector expressing either BDD-SQ-hfVIII or BDD-OL-pfVIII and transduced cells were transplanted into hemophilia A mice via tail vein injection. Plasma from mice transplanted with SIV-BDD-SQ-hfVIII-modified cells did not contain detectable fVIII activity at any time point, while mice transplanted with SIV-BDD-OL-pfVIII-modified cells showed sustained fVIII activity for at least 1

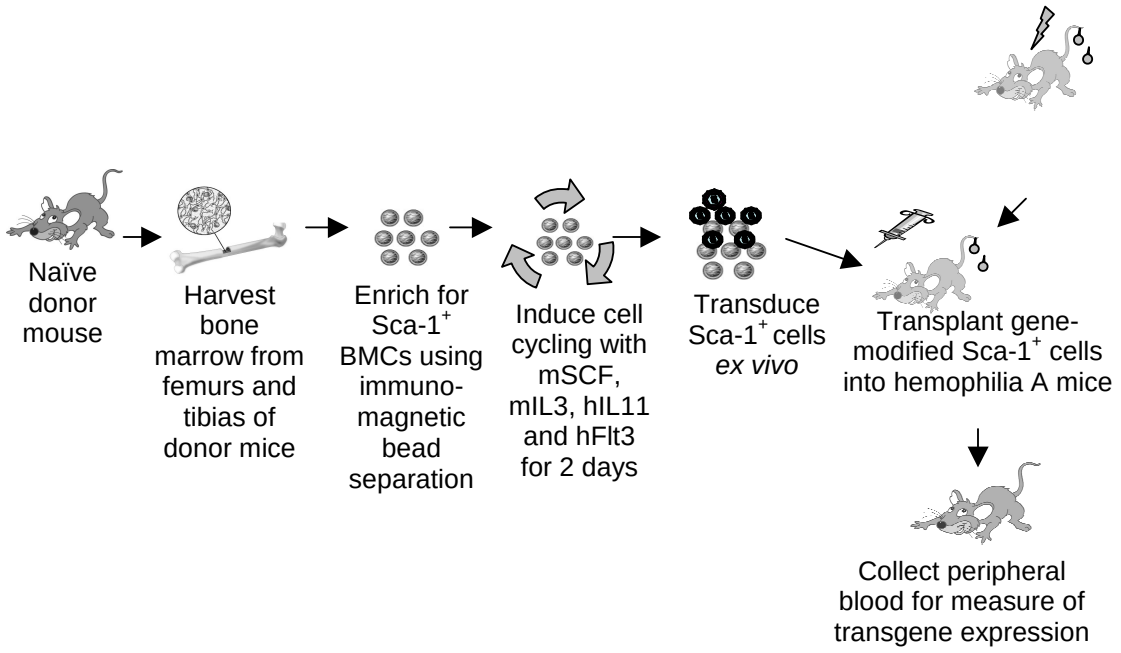

Figure 2.22– Schematic of HSC isolation, transduction, and transplant. Cells are flushed from femurs and tibias of donor mice and enriched for Sca-1$^+$ bone marrow cells using positive selection immunomagnetic bead separation. Sca-1$^+$ cells are plated in 6-well plates and incubated with 100 ng/mL murine stem cell factor, 20 ng/mL murine IL-3, 100 ng/mL human IL-11, and 100 ng/mL human Flt-3 to induce cell cycling for 2 days. On days 3 and 4, cells are transduced with virus at half the total MOI each time. Transduced cells are transplanted into recipient mice in 100μL PBS via tail vein or retro-orbital injection. Transgene detection begins 1-2 weeks post-transplantation using peripheral blood collected from the retro-obital plexis.

year **(Figure 2.23)**. For all transplanted mice, the fVIII transgene copy number was below the limit of detection of our RT-PCR assay (~0.6 copies/cell), suggesting that sustained fVIII expression is achievable from less than 6% gene-modified peripheral blood cells.

2.5 – Discussion

Low-level fVIII expression has been a limiting factor for both the commercial production of recombinant fVIII as well as for the success of hemophilia A gene therapy. With respect to gene therapy, a number of preclinical studies have demonstrated therapeutic levels of human fVIII and phenotypic correction of bleeding in mice using γ-retroviral vectors (Moayeri et al 2005; Moayeri et al 2004; VandenDriessche et al 1999), adeno-associated vectors (Chao et al 2000), and adenoviral vectors in mice (Balague et al 2000; Cerullo et al 2007; Reddy et al 2002) and dogs (Chuah et al 2003; McCormack et al 2006). In a clinical setting however, hemophilia A gene therapy has been unsuccessful. To date, 3 clinical trials for hemophlilia A have been initiated. The first two trials used BDD-hfVIII, while the third used full-length hfVIII though none were able to achieve sustained fVIII levels greater than 1% without treatment-related adverse effects (Chuah et al 2004). Based on these clinical and preclinical studies, it is clear that improving fVIII expression is critical for future success.

Recombinant DNA technology has led to the development of fVIII molecules with increased half-life (Pipe & Kaufman 1997), decreased immunogenicity

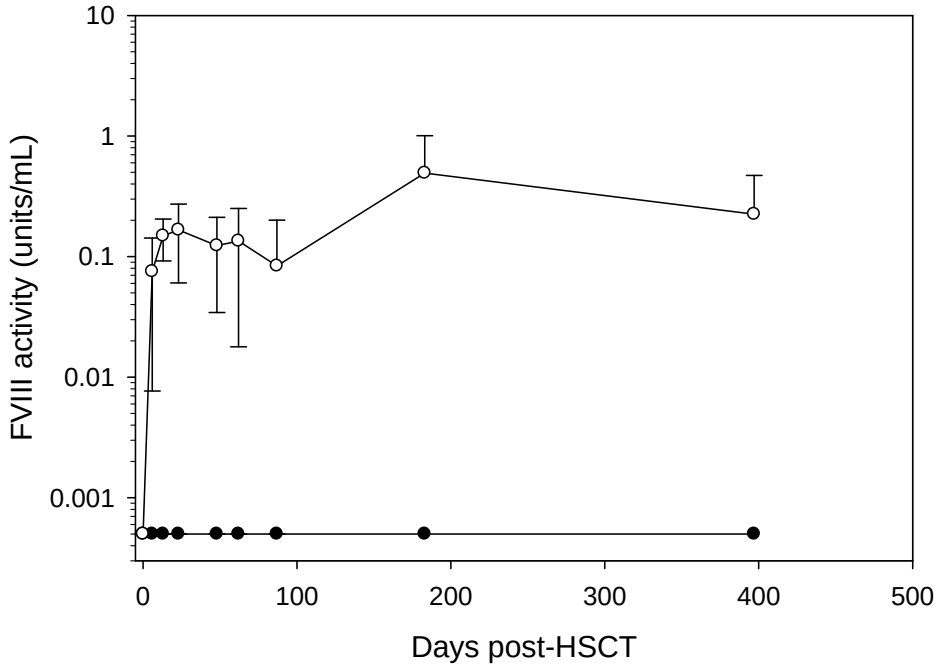

Figure 2.23 - *In vivo* expression of fVIII from genetically-modified hematopoietic stem cells. Plasma fVIII levels were determined in hemophilia A mice following transplantation of Sca-1^+ cells modified *ex vivo* with a lentivirus expressing either BDD-SQ-hfVIII (black circles) or BDD-OL-pfVIII (white circles). Mice were transplanted via tail vein injection with $7.5x10^5$ cells transduced at an MOI of 5 (n=6). By week 26, 3 mice from each group had died (n=3).

(Barrow et al 2000; Lubin et al 1997; Saenko et al 2003), and increased rates of processing and secretion (Doering et al 2002b; 2004; Miao et al 2004; Swaroop et al 1997). Significant advancements also are being made in vector development including the use of stronger promoters or enhancer elements to drive expression of transgenes, though this may lead to increased risk of insertional mutagenesis when employed in retroviral vectors (Papadakis et al 2004; West & Fraser 2005). The focus of the current study was on the comparison of the various bioengineered fVIII transgenes' expression level in order to determine an optimal construct for use in clinical gene transfer. A number of bioengineered high-expression human, porcine, and chimeric fVIII constructs compared in order to determine which is expressed at the greatest level. Among the human fVIII transgenes, we evaluated differences in expression due to specific modifications previously shown to decrease interaction with the resident ER protein chaperone BiP and increase carbohydrate-mediated transport from the ER to the Golgi (Dorner et al 1987; Marquette et al 1995). Our results show that BDD-OL-pfVIII is the optimal fVIII transgene with respect to expression levels as it is expressed at significantly greater levels than any human fVIII variant when tested both *in vitro* and *in vivo*. Upon transfection of each transgene into BHK-M cells, no significant differences in expression were observed among the human fVIII constructs. Although in each case BDD-OL-pfVIII expression was significantly greater, no conclusions could be made regarding differences in expression among the various human transgenes.

In an attempt to decrease the inter-clonal expression variability, we expressed each transgene in the Flp-In™ System and compared expression from resulting isogenic cell lines. The advantage of this system is that the integration of the gene of interest occurs in the exact same genomic location in each cell. Southern blot analysis can be used to screen for multiple and/or random integrations. Expected transgene integration has been shown to occur with high frequency and phenotypic characterization of expression to remain consistent as long as the transfected cells remain under selective pressure (Liu et al 2006). Using the Flp-In™ System, BDD-OL-pfVIII was expressed at significantly greater levels than any human transgene while again no significant differences were detected among the human transgenes. BDD-OL-pfVIII was expressed at 36-fold greater levels than BDD-SQ-hfVIII and 225-fold greater than BDD-hfVIII. Increased expression is not due to disproportionately higher number of transcripts/cell in BDD-OL-pfVIII clones compared to BDD-SQ-hfVIII clones. This is consistent with our previous finding of enhanced secretion resulting in a faster protein production rate (Doering et al 2002b; 2004). Currently, recombinant BDD-OL-pfVIII is being evaluated in a Phase 3 clinical trial on patients with congenital hemophilia A possessing antihuman fVIII inhibitory antibody titers (Mahlangu *et al.*, 2007). In a phase 2 trial, BDD-OL-pfVIII was successful in controlling all bleeds, even in the presence of fVIII antibodies.

Although BDD-OL-pfVIII provides an obvious advantage over human fVIII transgenes with respect to protein expression, we speculated that a humanized

fVIII transgene would provide additional advantages. For example, patients with hemophilia A are often treated with recombinant human fVIII products, and approximately 30% of hemophilia A patients develop complications due to anti-fVIII inhibitory antibody formation. However, the majority of patients are tolerant to the infused protein. Therefore, a high-expression fVIII protein engineered to closely resemble the clinically administered products could be more useful to patients previously tolerized to human fVIII. The availability of a less immunogenic product would be greatly advantageous for the treatment of both naïve and tolerized patients, as well as those patients already harboring anti-fVIII inhibitory antibodies. To test the utility of human/porcine chimeric transgenes, a number of human transgenes incorporating various high expression porcine-specific sequences were expressed in the ReNeo mammalian expression vector in transfected BHK-M cells. No statistically significant difference was observed between HP-fVIII and BDD-OL-pfVIII expression in BHK cells, as both were ~13-fold higher than BDD-SQ-hfVIII. Similar to data from the comparison of recombinant human transgenes, the addition of the L303E/F309S double mutation did not significantly increase expression of any chimeric transgene. However, the double mutation completely eliminated fVIII expression from BDD-OL-pfVIII. These results were surprising because the human and porcine sequences are identical from L300 through H315, and have only one different amino acid from P281 through H315. Sequencing of the A1 domain of BDD-DM-OL-pfVIII confirmed that no other mutations had been introduced, suggesting that the cause of the decrease in expression was solely due to the incorporation of

the L303E/F309S double mutation. Of all the chimeric transgene tested, HP-fVIII, which includes a porcine-specific A1 and A3 domain, was the highest expresser. BDD-SQ-hfVIII, BDD-OL-pfVIII, and HP-fVIII were then further characterized using the Flp-In™ System. Single-integrant Flp-In 293 clones demonstrated that HP-fVIII had a maximal clotting activity ~19-fold higher than human fVIII, which was slightly lower than that observed for BDD-OL-pfVIII. This is the first system in which this observation has been made and it is possible that this outcome is specific to the Flp-In™ System. Interestingly, we did not observe a dramatic decrease in the variability of fVIII expression from our clones using the Flp-In™ System. The basis for the variability observed is not understood, but is most likely due to variations in the fVIII activity assay including cell number at the time of assay, cell density, length of time the cells were in culture, and variability among reagents used on different days.

In a mouse model of hemophilia A, we previously showed that BDD-OL-pfVIII is expressed at significantly greater levels than BDD-SQ-hfVIII in HSCs and bone marrow-derived mesenchymal stem cells (Gangadharan et al 2006). Importantly, BDD-OL-pfVIII expression was sustained at levels greater than 1 unit/mL following HSCT without subsequent immune responses even when reduced intensity, radiation or chemotherapeutic-based conditioning was utilized (Ide et al 2007). Pre-immunized hemophilia A mice, harboring clinically significant anti-fVIII titers, treated with the same HSCT and reduced intensity conditioning protocol also exhibited sustained BDD-OL-pfVIII expression demonstrating the potential

for using a porcine fVIII transgene to treat patients harboring inhibitory antibodies to human fVIII (Doering et al 2007). These previous studies were performed using murine stem cell virus (MSCV) to drive genetic modification and expression of BDD-OL-pfVIII. In the current study, we aimed to compare human and porcine transgene expression in a more clinically-relevant lentivirus-mediated gene transfer system. Lentiviruses are emerging as the vector of choice for stable gene delivery due to several attractive features that include 1) efficient transduction of dividing and non-dividing cells, 2) the capacity to accommodate large genetic payloads, and 3) stable long-term transgene expression (Cockrell & Kafri 2007). An increasing body of evidence suggests that lentiviruses are inherently safer than γ-retroviruses possibly due to differences in integration site preference (Schroder et al 2002; Wu et al 2003). To date, no evidence exists to link HIV to cancer. However, lentiviruses possess a 3' and 5' LTR, each containing a strong promoter and enhancer, which are capable inducing long distance gene expression effects. To decrease this risk, SIN vectors have been developed in which the U3 regions of the LTRs have been deleted. Previous studies have demonstrated expression of therapeutic levels of fVIII expression in hemophilia A mice following systemic lentiviral gene delivery (Kang et al 2005; Kootstra et al 2003; Park 2003), transplantation of transduced bone marrow cells (Kootstra et al 2003), and transplantation of bone marrow cells with targeted platelet-specific expression (Ohmori et al 2006; Shi et al 2007). In the majority of these studies, only transient levels of fVIII expression were achieved due to the formation of neutralizing antibodies to fVIII, but platelet-specific fVIII expression

from either the GPIIb or GPIbα promoter has resulted in correction of bleeding in transplanted hemophilia A mice without inhibitor development (Ohmori et al 2006; Shi et al 2007). Using an alternative approach, Matsui et al. (Matsui et al 2007) demonstrated *in vivo* fVIII expression from implanted lentiviral-transduced blood outgrowth endothelial cells (BOECs) in hemophilia A mice, but anti-fVIII humoral immune response developed unless tolerization or immunosuppression was employed.

Using a HIV-1-based, SIN lentiviral vector, we tested transduction efficiency and expression levels of BDD-OL-pfVIII compared to BDD-SQ-hfVIII in human cells. 293T cells were efficiently transduced by the lentiviral vectors and expressed either BDD-SQ-hfVIII or BDD-OL-pfVIII, demonstrating a positive correlation between fVIII expression and MOI. Similar to our previous observations, BDD-OL-pfVIII exhibited enhanced expression compared to BDD-SQ-hfVIII with a 7 – 8 fold differential in expression at each MOI tested. Analysis of steady-state RNA levels indicated that higher BDD-OL-pfVIII expression was not due to increased transcript levels, which is consistent with our previous findings that the high-expression elements observed in pfVIII act through post-transcriptional differences that lead to enhanced secretion (Doering et al 2004). FVIII biosynthesis from individual BDD-SQ-hfVIII- or BDD-OL-pfVIII-expressing stable clones correlated with steady-state RNA levels and integration events. BDD-OL-pfVIII expression from individual clones was significantly greater than BDD-SQ-hfVIII when normalized to DNA copy number. Southern blot analysis revealed a

number of clones with integration events but no detectable levels of fVIII expression, possibly due to transcriptional silencing or insertion-site-dependent positional effects. Transgene expression driven by lentiviral vectors can be subject to epigenetic silencing by a number of pathways including DNA methylation and chromatin modification (Hamaguchi et al 2000; Hong et al 2007; Yao et al 2004). Genomic silencing mechanisms described to date for lentiviral vectors are not well established and may be dependent on cell type, differentiation state, and the specific promoter used. A recent study of SIN lentiviral-mediated fIX expression in bone marrow chimeras showed decreasing transgene expression within weeks after transplantation using the EF-1α promoter resulting in barely detectable fIX expression by week 30. However, stable transgene expression was maintained over time when driven by the β-globin locus control region (Chang et al 2006). Southern blot analysis of BDD-SQ-hfVIII and BDD-OL-pfVIII clones also indicated efficient transduction at the MOIs tested resulting in the expected range of 1-3 proviral integrants per cell. High-level BDD-OL-pfVIII expression was observed at low DNA copy numbers suggesting a decreased risk of insertional mutagenesis due to the requirement for fewer insertional events to achieve therapeutic levels of fVIII expression. Interestingly, although efficient transduction was achieved in human cells, it was observed that our HIV-based lentiviral vectors did not efficiently transduce murine Sca-1^+ hematopoietic stem and progenitor cells, resulting in undetectable plasma fVIII activity *in vivo* (personal communication with other lab members). However, when the fVIII transgenes were expressed in a SIV vector, efficient Sca-1^+ cell

transduction was achieved, resulting in sustained fVIII expression in mice transplanted with SIV-BDD-OL-pfVIII-transduced Sca-1^{+} cells. In summary, we have demonstrated significantly enhanced expression of BDD-OL-pfVIII and chimeric HP-fVIII compared to bioengineered human fVIII constructs using various gene transfer techniques. We also demonstrated the ability of a lentivirus to express BDD-OL-pfVIII in transduced cell lines and in HSCs following *ex vivo* transduction and transplantation. This data supports the use of lentiviral vector-mediated gene transfer and expression of BDD-OL-pfVIII in clinical gene therapy for hemophilia A.

Chapter 3:

Erythroid-restricted lentiviral gene therapy of hemophilia A incorporating high expression porcine factor VIII

3.1 – Abstract

Recombinant retroviruses are efficient vehicles for nucleic acid delivery and are capable of providing long-term transgene expression. The development of safe and efficient retroviral vectors is crucial for providing clinically relevant therapeutics. In the studies described in Chapter 3, self-inactivating lentiviral vectors were used to transfer the porcine fVIII (pfVIII) cDNA sequence, which we previously showed can be used to cure hemophilia A mice. SIV-based vectors were created containing an internal ankyrin-1 or β-globin promoter, designated SIV-Ank or SIV-βg, to drive either pfVIII or eGFP expression. The erythroid-specificity of each promoter was evaluated in vitro by measurement of fVIII expression following transduction of K562 and 293T cells with SIV-Ank-pfVIII and SIV-βg-pfVIII and revealed that myelogenous leukemic K562 cells expressed 4-5-fold greater levels of pfVIII transcripts per proviral copy compared to 293T cells. Hemophilia A mice were transplanted with hematopoietic stem cells transduced with each vector and the mean fluorescence intensity of GFP expression in SIV-βg-GFP gene-modified red blood cells was 4-fold greater than in white blood cells. Less than 10% of the SIV-βg-pfVIII-transplanted mice exhibited sustained fVIII expression, and the lack of long-term expression was due to inefficient stem cell transduction. However, the majority of transplanted mice were immunologically nonresponsive to administration of recombinant fVIII. Overall these data demonstrate the complexity of developing fVIII-encoding nucleic acid therapeutics, but demonstrate the potential for expressing fVIII using lineage-specific promoters.

3.2 – Introduction

Hemophilia A is an X-linked recessive disorder caused by mutations that prevent the synthesis of functional coagulation fVIII. Although it is generally accepted that the liver is the site of endogenous fVIII production (Bontempo et al 1987; Hollestelle et al 2001), HSCs are a potential target cell population for gene therapy of hemophilia A. HSCs are readily accessible for *ex vivo* modification, easily transplanted, undergo self-renewal and will repopulate the bone marrow following transplantation. We previously showed that HSCs transduced with γ-retroviral or lentiviral vectors containing a B-domain-deleted porcine fVIII (pfVIII) transgene can result in curative fVIII levels in hemophilia A mice (Dooriss et al 2009; Gangadharan et al 2006; Ide et al 2007). Recently it was shown that γ-retroviruses and lentiviruses have a differential preference for the site of provirus integration into host genomic DNA (Crise et al 2005; Liu et al; Mitchell et al 2004). Gamma-retroviruses prefer integration sites near the promoter of transcriptionally active genes, which can cause transactivation of crucial cell cycle regulators and lead to oncogenic transformation of cells. Evidence of this was observed in a clinical trial for severe combined immunodeficiency (SCID-X1) where several patients developed leukemia after being treated with a γ-retrovirus expressing the IL2 receptor γ_c gene (Hacein-Bey-Abina 2008; Hacein-Bey-Abina et al 2003). These cases have been the hallmark in understanding the causes of insertional mutagenesis and have led to the discovery of differential integration site preferences of retroviruses, including regional hotspots (Schroder et al 2002).

Following the discovery of leukemic transformation, tremendous effort has been directed toward creating a replication-incompetent retroviral vector with the ability to transduce target cells and efficiently drive transgene expression, but maintains a low risk of insertional mutagenesis (reviewed in Sinn (Sinn et al 2005)). Lentiviruses such as HIV and SIV generally do not integrate within promoter regions, and therefore are thought to have a decreased risk of insertional mutagenesis as compared to γ-retroviruses (Mitchell et al 2004; Schroder et al 2002). One modification made for the purpose of increasing the safety of recombinant lentiviruses was to create a self-inactivating (SIN) lentivirus. SIN lentiviruses contain a small deletion in the U3 domain of the 3' LTR which not only renders inactive the 3' LTR, but also the 5' LTR following reverse transcription within transduced cells. Inactivating the strong enhancer and promoter activity of the viral LTRs significantly reduces the ability of 3` LTR-directed expression and transactivation of downstream genes (Logan et al 2004). However, without a functional 5' LTR, proviral transgene expression must be controlled by an internal promoter, which also contributes to the safety profile of the virus. A ubiquitous promoter has the potential for transactivation in all transduced cells, while a non-ubiquitous promoter may only disrupt gene regulation in the cells in which the promoter is active. Therefore, limiting the number of cells with active transcription can further decrease the risk of viral-mediated transactivated disregulation (Modlich et al 2006; Ryu et al 2008). Others have already demonstrated phenotypic correction of hemophilia A in mice using lentiviral transduction of bone marrow cells with a platelet-specific

promoter, as well as in the presence of high titer inhibitory antibodies in a transgenic mouse model (Shi et al 2007; Shi et al 2003).

To determine if a lineage specific promoter is capable of driving high level fVIII expression, we constructed SIN SIV vectors containing the high expression pfVIII transgene or eGFP under control of either the human ankyrin-1 promoter or the human β-globin promoter flanked upstream by enhancing sequences, HS2, HS3, and HS4 from the locus control region. Erythroid-specific promoters were chosen because the amount of erythrocytes in the bloodstream is up to 100-fold higher than any other blood cell type **(Figure 3.1)**. As well, others have demonstrated the ability of erythroid promoters to drive expression of heterologous genes including GFP, coagulation factor IX, α-L-iduronidase (IDUA), and γ-globin following lentiviral gene transfer into hematopoietic stem and progenitor cells (Chang et al 2008; Chang et al 2006; Hanawa et al 2004a; Hanawa et al 2002). We previously showed that pfVIII is expressed at 10-100-fold higher levels than human fVIII *in vitro* and is capable of high level sustained expression in hemophilia mice transplanted with lentiviral transduced hematopoietic stem cells (Dooriss et al 2009). Based on this previous data, we hypothesize that using a high expression fVIII transgene and a promoter that is specifically active in the largest blood cell compartment, high level sustained fVIII expression is possible following lentiviral-mediated pfVIII gene transfer into HSCs. Here we show the potential for erythroid cells to mediate long-term delivery of pfVIII in a lentiviral

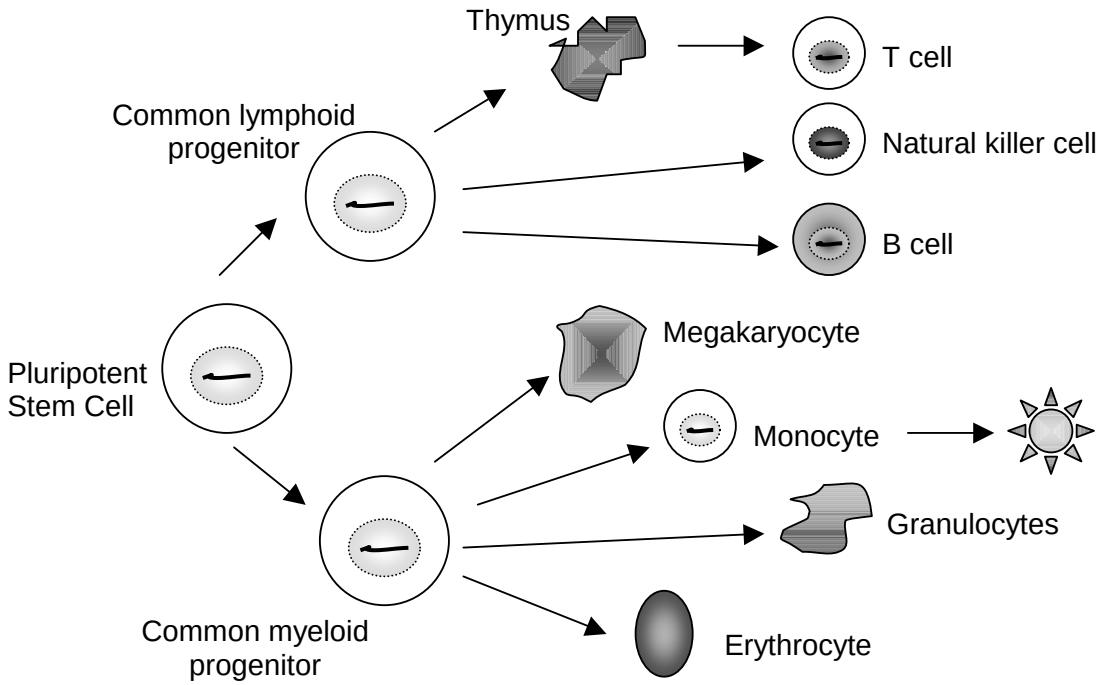

Figure 3.1 – Schematic of mammalian hematopoiesis. Use of a cell-specific promoter in lentiviral mediated HSCT will limit transgene expression to a fraction of gene-modified cells, depending in which cells the promoter is transcriptionally active. In a healthy mouse, erythrocytes are the most numerous blood cell type, at ~5 million cells/μL^3. WBCs are far less numerous, circulating at ~8,000 cells/μL. Five main types of WBCs exist in circulation and make up the following percentages of WBCs: neutrophils 60%, eosinophils 2%, basophils 1%, monocytes 4%, and lymphocytes 4%.

gene therapy hematopoietic stem cell transplantation (HSCT) model of hemophilia A mice using erythroid-specific promoters.

3.3 – Materials and Methods

SIV vector production. The pCL20, pSIV, and pCAG4 vectors used in this study was obtained from Arthur Nienhius (St. Jude Children's Hospital, Memphis, TN) and has been described previously (Doering et al 2009; Hanawa et al 2004b). To create SIV-Ank-GFP, human genomic DNA was amplified using primers that created a 314-bp sequence containing the ankyrin-1 promoter and 2 unique restriction sites, BstB1 and SacII. PCL20 (SIV-MSCV-GFP) was digested with BstB1 and SacII removing the MSCV promoter and the ankyrin-1 sequence was ligated into this site, creating a 10,716 bp product with ankyrin-1 located upstream of eGFP. To create SIV-βg-GFP, the β-globin promoter along with enhancer sequences HS2, 3, and 4 from the β-globin locus control region were amplified from the pCL20C-1.CM5 vector (Hanawa et al 2002) (obtained from Derek Persons, St Jude Children's Hospital, Memphis, TN) using primers that contained MluI and SacII restriction sites and cloned into the MluI and SacII sites of pCL20, creating a 10,127-bp product with the 180-bp β-globin promoter flanked upstream by enhancing sequences, HS2, HS3, and HS4 driving eGFP expression. To create SIV-Ank-pfVIII, a MluI site was engineered into SIV-Ank-GFP at the end of the GFP sequence using QuikChange II XL site-directed mutagenesis (Strategene, La Jolla, CA). The pfVIII sequence was removed from a previously created SIV vector containing pfVIII (SIV-MSCV-pfVIII(Doering et al

2009)) and subcloned into the SIV-Ank-GFP backbone following digestion of both with NotI/MluI. To make SIV-βg-pfVIII, an AgeI site was engineered into SIV-MSCV-pfVIII at the start of the pfVIII sequence using PCR amplification. SIV-MSCV-pfVIII and SIV-βg-GFP were digested with AgeI/NotI and the resulting pfVIII fragment was ligated to the SIV-βg-GFP backbone after removal of the eGFP sequence.

Production and titering of lentiviral particles. Lentiviral particles were produced by 4 plasmid co-transfection of 293T cells **(Figure 3.2)**. Briefly, 293T cells were plated in T75 flasks and each flask was transfected with 2μg pSIV, 1.25μg psPAX, 1.25μg pCAG, and 1.67μg PCL expression vector with 8μg polyethyleneimine hydrochloride salt (PEI) per 1μg total plasmid DNA. Transfection media was removed the next day and replaced with fresh media. Virus-containing conditioned media was collected twice daily for the next 3 days and stored at -80°C. Concentration was performed by overnight centrifugation at 9000 x g, and the viral pellet was resuspended in $1/100^{th}$ the original volume. Final virus stock concentrations typically range from 1×10^7 to 1×10^8. Virus was frozen in 1mL aliquots and stored at -80°C until ready to use. Viral titers were determined by RT-PCR on genomic DNA from 293T **(Figure 3.3).** Briefly, 0,5μL, 2μL, or 10μL of concentrated virus was added to ~200,000 cells in each well of a 6 well plate. After 6 days, transgene copy number was determined by real-time quantitative PCR of genomic DNA isolated from transduced cells using the following fVIII-specific primers: 5'-TGG GAC CTT TAC TTT ATG G-3' and

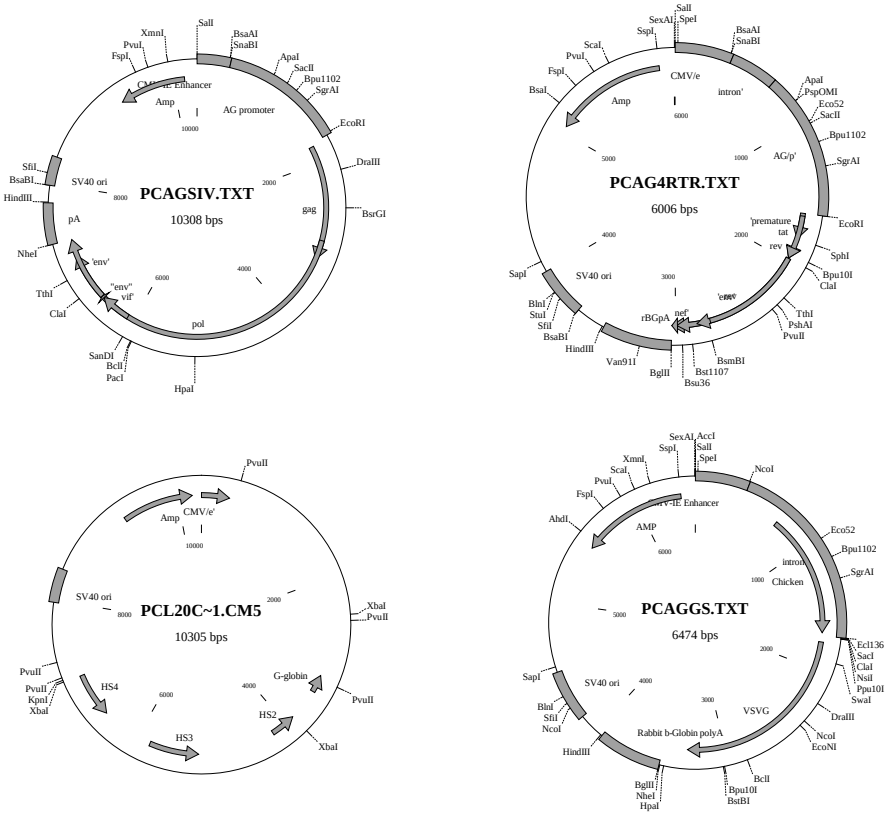

Figure 3.2 – Four-plasmid system for SIV production. Lentiviral production is carried out by 4-plasmid co-transfection of 293T cells. pCAGSIV contains the gag and pol genes which encode for capsid proteins, reverse transcriptase, and integrase. pCAG4RTR contains accessory protein genes (rev, tat, nef, env) for lentiviral function. pCL20 is the lentiviral expression vector containing the psi packaging signal, promoter, and transgene of interest (either pfVIII or GFP). pCAGGS contains the vesticular stomatitis virus-G (VSVG) envelope protein used to pseudotype the virus to infect a wide variety of cell types.

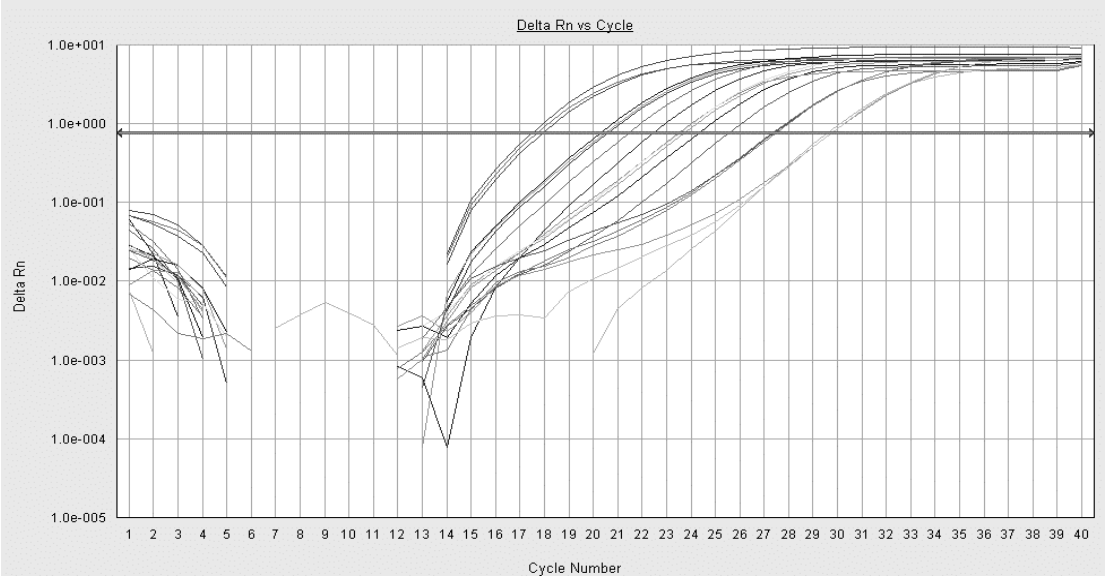

Figure 3.3 – Lentiviral titering by RT-PCR. 293T cells are seeded in 6-well plates at 100,000 cells/well. The next day, cells are counted and transduced with virus using 0.5, 2, 5, and 10uL per well. After 6 days, genomic DNA is isolated from each transduction well and gene copies are quantified by RT-PCR. A standard curve is created using plasmid DNA dilutions ranging from 5 x 10^6 to 5 x 10^2 copies per reaction well. These amplicons are depicted above in triplicate lines crossing the threshold at cycle numbers 17.5 – 29.5, respectively. The slope of the standard curve should be between 3.0 and 3.3. Sample genomic DNA amplicons are shown as a single line for each volume used for titer.

5'- AAA AAC ATA GCC ATT GAT GCT GTG-3'. Genomic DNA from cultured cells were isolated using Qiagen DNeasy Blood & Tissue Kit (Germantown, MD). PCR reactions were carried out in 25µL containing 1x SYBR Green PCR master mix, 250nM forward and reverse primers, and 50ng of genomic DNA per reaction. Cycle conditions are as follows: Incubation at 95°C for 10 minutes followed by 40 amplification cycles of 95°C for 15 seconds then 60°C for 1 minute. Post-reaction dissociation analysis was performed to confirm single-product amplification by performing a single cycle of 95°C for 15 seconds, 60° for 1 minute, and 95°C for 15 seconds. Viral titer is back calculated from the DNA copy number resulting from each volume of virus used on a known number of cells.

Cell culture and transduction. 293T and K562 cells were maintained in DMEM/F-12 media supplemented with 10% FBS and 1% penicillin-streptomycin solution (100 units/mL) at 37°C with 5% CO_2. Transductions with lentiviral particles were carried out in 6-well plates ~24 hours after seeding cells at 100,000/well in 1mL media containing 8 µg/mL polybrene.

Isolation, transduction, and transplantation of murine Sca-1$^+$ cells. Sca-1$^+$ cell isolation was performed as previously described using positive immunomagnetic bead selection (Gangadharan et al 2006; Ide et al 2007). Prior to transduction, cells were stimulated for 2 days in serum-free media containing murine stem cell factor (100 ng/mL), murine interleukin-3 (20 ng/mL), human

interleukin-11 (100 ng/mL), and human Flt-3 ligand (100 ng/mL). One million cells per well of a 6-well plate were transduced consecutively on days 3 and 4 with at the MOIs indicated in the text. Recipient mice were conditioned with total body irradiation (11*Gy*) on the day of transplant and $5x10^5$ - $1.5x10^6$ transduced cells were transplanted by intravenous tail injection. Exon 16-disrupted hemophilia A mice (Bi et al 1995) backcrossed to a C57BL/6 genetic background were obtained from Dr. Leon Hoyer (Holland Laboratories, American Red Cross) and a breeding colony was established. GFP expression was measured in peripheral blood cells using flow cytometry (BD LSRII). Secondary transplants were performed by harvesting whole bone marrow from previously transplanted hemophilia A mice and injecting $2x10^6$ – $4x10^6$ cells intravenously into 8-12 week old hemophilia A mice receiving total body irradiation.

Measurement of fVIII activity. For all *in vitro* experiments, fVIII activity was measured using the activated partial thromboplastin reagent-based one-stage coagulation assay in an STart Coagulation Instrument (Diagnostica Stago) using human fVIII-deficient plasma as a substrate as previously described (Doering et al 2002b). All coagulation assays were carried out on cells cultured in serum-free AimV media for 24 hours. For all *in vivo* experiments, fVIII activity was measured by COATEST assay as described previously (Doering et al 2002b). Human fVIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KS). Automated activated partial thromboplastin reagent was purchased from BioMérieux (Durham, NC).

Measurement of fVIII transcript expression. FVIII RNA quantification was performed using SYBR GREEN PCR Master Mix, TaqMan Reverse Transcription Reagents, and ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), as described previously (Doering et al 2009; Doering et al 2002b; Dooriss et al 2009). RNA was isolated using Qiagen's RNeasy kit (Germantown, MD). PCR reactions were carried out in 25µL containing 1x SYBR Green PCR master mix, 300 nmol forward and reverse primers, 12.5 units of MultiScribe reverse transcriptase, 10 units of RNase Inhibitor, and 5ng of sample RNA. The number of transcripts per cell was determined using an average value of 142 transcripts per 5ng of RNA.

Southern blot analysis. To confirm that transduced cells contain a single integration product, 10µg of genomic DNA was digested with KpnI and fragments separated on a 1% agarose gel. DNA was transferred by capillary action using 20x SSC transfer buffer to a positively charged nylon membrane and hybridized overnight with a probe complementary to the porcine A1 domain. Probe was created using the PCR DIG Probe Synthesis Kit (Roche) and primers: 5'-CGGCAAAGTGAACTCCTCCGTG-3' and 5'-CTGACGTGAGCCTCCATGC-CACCA-3'. Immunological detection of digoxigenin (DIG)-labeled probes was performed using the DIG Wash and Block Buffer Set (Roche). Disodium 2-chloro-5-(4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro) tricycle [3.3.1.1^{3,7}] decan) -4-yl) phenyl phosphate (CDP-Star, (Roche)) was used for chemiluminescent substrate

detection of alkaline phosphatase. Imaging was performed using a Luminescent image analyzer (LAS)-3000 (FUJIFILM) followed by X-ray film (Kodak).

Red blood cell lysis. Peripheral blood was collected from SIV-βg-pfVIII transplanted mice and centrifuged at 4°C and 3000 x g for 15 minutes. Plasma was removed and used for fVIII measure. Cells were washed 3 times in 40μL HBS Tween then lysed in 40μL HBS Tween containing 0.01% Triton X-100 and allowed to lyse on ice for 15 minutes. FVIII was measured in plasma, washes, and cell lysate by COATEST assay.

Sca-1$^+$/CD150$^+$ bone marrow cell isolation. Sca-1$^+$ cells were isolated as described above. Anti-biotin microbeads were removed from Sca-1$^+$ cells by adding 20μL/mL Multisort Release Reagent (Miltenyi) and incubated 10 minutes at 4°C. The microbead release reaction was stopped by the addition of Multisort Stop reagent (30μL/10^7 cells). PE-anti-CD150 antibody was added to cells (5μg/10^8 cells) and incubated 20 minutes. Cells were then incubated with anti-PE microbeads (20μL/10^7 cells) for 15 minutes at 4°C. Cells were washed in PBS then applied to an immunomagnetic bead column (Miltenyi) and collected as the positive fraction.

Anti-fVIII antibody assays. Bethesda assays were performed on murine plasma as described previously (Barrow et al 2000), whereby Bethesda titer is defined as the reciprocal of the plasma dilution that produces 50% inhibition of fVIII in the

one-stage fVIII coagulation assay. ELISAs were performed as described previously on plasma samples obtained by retro-orbital collection (Doering et al 2002a). Briefly, mice were challenged with 10 units of purified pfVIII (provided by Dr. Pete Lollar, Emory University) once weekly for 4-5 weeks. Plasma was collected weekly and assayed for presence of inhibitor antibodies by ELISA whereby 1.5μg/mL pfVIII in 20mM Bicine plus 2mM CaCl2 was adsorbed to microtiter plates (Dynex Immulon 2HB, Thermo Labsystems, Helsinki, Finland) followed by a 5 hour incubation in blocking buffer. Plates were then incubated in plasma sample dilutions overnight and presence of antibodies was detected using alkaline phosphatase-conjugated goat anti-mouse IgG and measure of absorbance at 405nm after 20-30 minute incubation. The ELISA titer is defined as the dilution of plasma that results in an absorbance value of 0.3 derived from a 4-parameter fitted curve.

3.4 – Results

Comparison of strength and specificity of two erythroid promoters

In this study we analyzed the expression of eGFP and pfVIII transgenes driven by the erythroid-specific promoters, human ankyrin-1 and β-globin, which were incorporated into a lentiviral system. The SIV-based lentiviral vectors contained a 3’ LTR with a self-inactivating U3 deletion, the bovine growth hormone polyA signal, a packaging signal (Ψ), and an internal ankyrin-1 or β-globin promoter, designated SIV-Ank and SIV-βg, respectively. The minimal 314-bp ankyrin-1

promoter and 180-bp β-globin promoter flanked upstream by enhancing sequences, HS2, HS3, and HS4 from the locus control region were cloned into the SIV vector backbone upstream from either eGFP or pfVIII **(Figure 3.4).** Also used for comparative analyses was a SIN SIV-based vector containing either pfVIII or eGFP controlled by an internal MSCV LTR.

To characterize the strength and erythroid specificity of the SIV-based vectors containing the human ankyrin-1 or β-globin promoters, K562 cells and 293T cells were transduced with either SIV-Ank-pfVIII or SIV-βg-pfVIII. K562 cells are a myelogenous leukemic cell line with erythroid properties, used as a surrogate for human erythroid cells. After 7 days in culture, DNA copy number and RNA transcript number were measured by RT-PCR and promoter strength and erythroid specificity were determined by the ratio of transcripts to copy number. As shown in **Figure 3.5A**, K562 cells transduced with SIV-βg-pfVIII contained 62.3 ± 25.8 transcripts/copy number, while similarly transduced 293T cells contained significantly fewer at 15.5 ± 5.3 transcripts/copy number ($p = 0.037$). K562 cells transduced with SIV-Ank-pfVIII contained 37.9 ± 6.8 transcripts/copy number, while similarly transduced 293T cells contained significantly fewer at 7.1 ± 1.1 transcripts/copy number ($p = 0.002$). Both SIV-Ank-pfVIII and SIV-βg-pfVIII were 4 – 5-fold more active in K562 cells than in 293T cells. Southern blot analysis was used to determine if the proviral sequence of each construct integrated into host genomic DNA without rearrangement. **Figure 3.5B** illustrates the integrated proviral sequence present in SIV-Ank-pfVIII and SIV-βg-pfVIII-

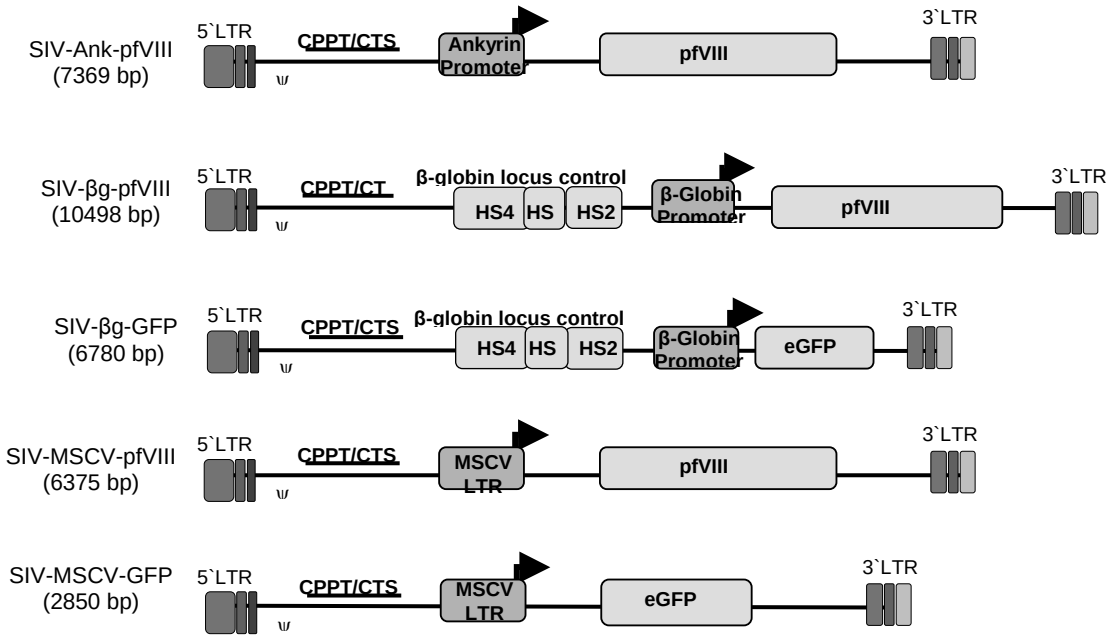

Figure 3.4 - Schematic of self inactivating SIV lentiviral vectors. Lentiviral constructs containing either eGFP or porcine fVIII transgenes driven by the erythroid-specific promoters ankyrin-1 or β-globin or an internal MSCV LTR. All constructs contain a 5` LTR, psi packaging sequence (ψ), central polypurine tract (cPPT) and the central termination sequence (CTS), rev response element (RRE), either eGFP or pfVIII transgene, and self inactivating 3`LTR containing a bovine growth hormone poly(A).

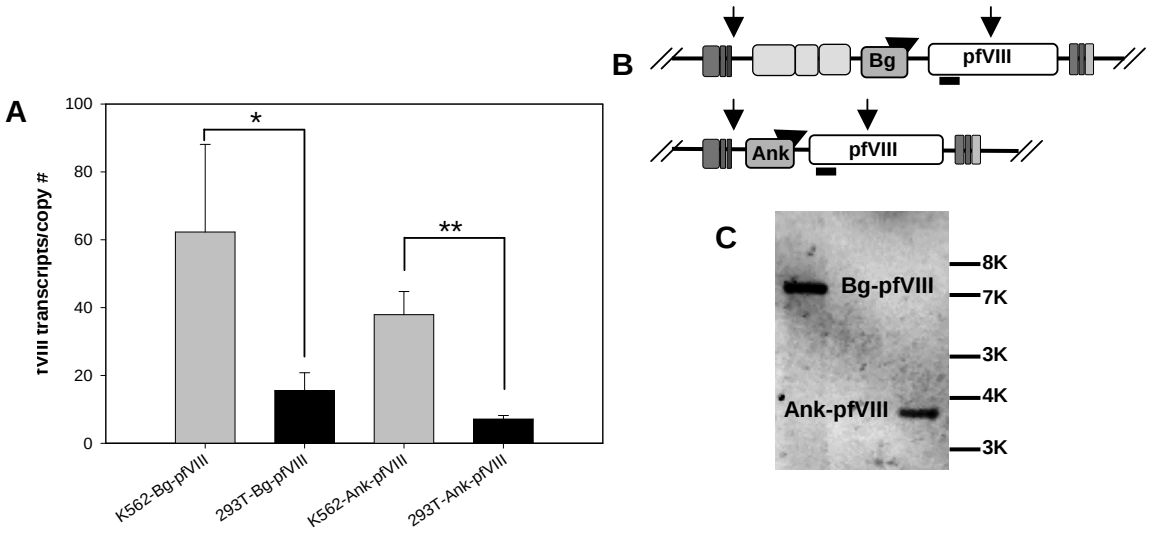

Figure 3.5 - Erythroid promoter specificity *in vitro*. K562 myelogenous leukemic cells and 293T human embryonic kidney cells were transduced with either SIV-Ank-pfVIII or SIV-βg-pfVIII to determine the erythroid specificity of each promoter. A) K562 cells (gray bars) and 293T cells (black bars) were transduced with either SIV-Ank-pfVIII or SIV-βg-pfVIII at an MOI of 10. DNA copy number and RNA transcript number were measured by RT-PCR and used to compare promoter strength and erythroid specificity. Each bar represents the mean ± S.D. of 3 wells, each measured in duplicate (*) p = 0.037, (**) p = 0.002. B) Schematic of genomic DNA from K562 and 293T cells transduced with either SIV-Bg-pfVIII or SIV-Ank-pfVIII. DNA was digested with KpnI (sites marked by black arrows) for Southern analysis. Probe binding position is denoted by a black line under pfVIII. C) Southern blot analysis of digested DNA gave rise to a single band of the expected band sizes of 3888 bp or 7017 bp for SIV-Ank-pfVIII and SIV-βg-pfVIII respectively in both 293T and K562 cells.

transduced cells. When digested with KpnI and analyzed by Southern blot, SIV-Ank-pfVIII and SIV-βg-pfVIII-containing genomic DNA gave rise to bands at the expected sizes of 3888 bp and 7017 bp respectively, confirming that the 5' end of the proviral sequence containing the promoter and approximately the first half of pfVIII integrated correctly into host cellular DNA **(Figure 3.5C).**

The relative strength of the β-globin promoter in driving fVIII expression compared to the strength of the strong ubiquitous MSCV LTR promoter was determined by transduction of 293T cells with SIV-MSCV-pfVIII at an MOI of 0.5, 1, 2.5, and 5 and K562 cells with SIV-βg-pfVIII at an MOI of 5, 10, 25, and 50. MOIs were chosen in order for transductions in K562 and 293T cells to result in similar copy numbers. Both populations of transduced cells exhibited a dose response for fVIII expression and proviral DNA copy number. K562 cells transduced with SIV-βg-pfVIII exhibited fVIII expression levels ranging from 0.13 – 1.2 U/10^6 cells/24hr and DNA copies ranging from 0.5 – 3.2 copies/cell. 293T cells transduced with SIV-MSCV-pfVIII exhibited fVIII expression levels ranging from 0.18 – 1.2 U/10^6 cells/24hr and DNA copies ranging from 0.07 – 1.6 copies/cell **(Figure 3.6A)**. No significant differences were seen in fVIII expression from the β-globin promoter compared to the MSCV promoter when similar copy numbers were achieved, suggesting that the β-globin promoter is as efficient at driving fVIII expression in its target cell type as is the strong MSCV-LTR. However, ~10x more SIV-βg-pfVIII was added to K562 cells in order to achieve similar copy numbers. No significant differences in fVIII expression were seen

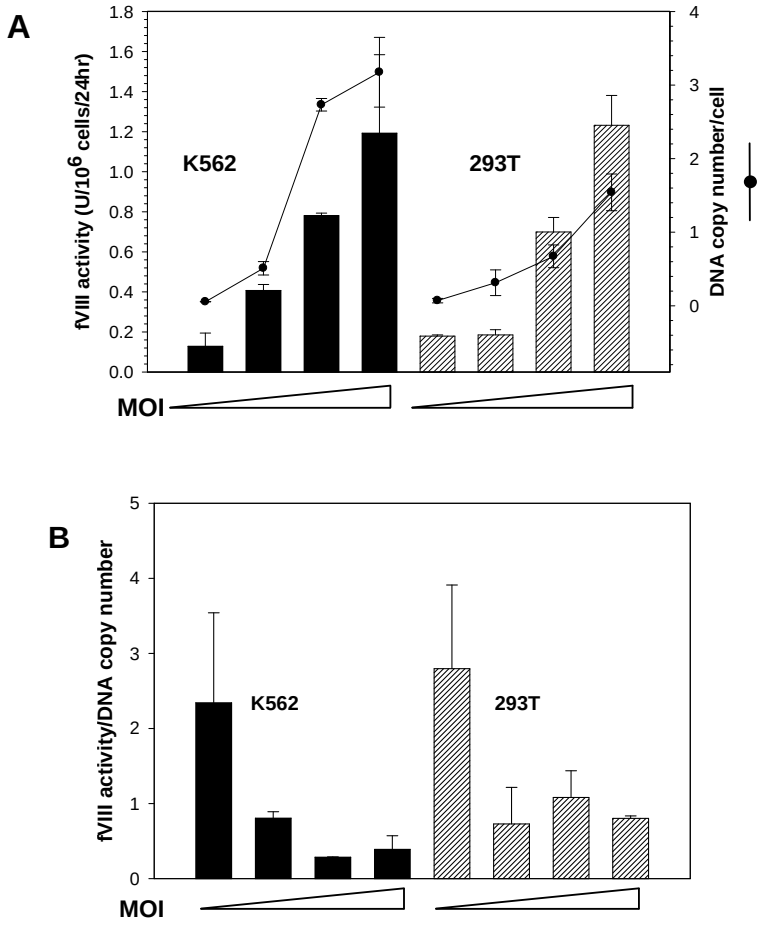

Figure 3.6 – *In vitro* erythroid promoter strength. To determine the relative strength of the beta globin promoter in driving pfVIII expression in K562 cells compared to the strength of the strong ubiquitous MSCV-LTR promoter, K562 cells were transduced at an MOI of 5, 10, 25, and 50 with either SIV-βg-pfVIII and 293T cells were transduced at an MOI of 0.5, 1, 2.5, and 5 with a SIV vector containing an internal MSCV-LTR promoter driving pfVIII expression. A) FVIII activity was measured using a one-stage coagulation assay 7 days after transduction. Black bars represent transduced K562 cells and hatched bars represent transduced 293T cells. Each bar represents the mean ± S.D. of 3

measurements from 2 separate experiments. DNA copy number per cell was measured using a real-time PCR assay. Each circle represents the mean ± S.D. of 2 separate experiments, n=4. B) FVIII activity/copy number was calculated, which showed no significant differences between SIV-βg-pfVIII and SIV-MSCV-pfVIII at any MOI tested. Black bars represent transduced K562 cells and hatched bars represent transduced 293T cells.

when fVIII activity was normalized to proviral DNA copy number **(Figure 3.6B).** In order to determine if fVIII activity is maintained in SIV-βg-pfVIII-transduced cells, K562 cells were transduced at an MOI of 10 and fVIII activity and DNA copy number were measured for up to 100 days after transduction. FVIII expression and DNA copy number correlated well over this time, but began to decrease after 25 days in culture **(Figure 3.7).** By 80 days post-transduction, DNA copy number had fallen below the limit of detection, however, cells continued to produce and secrete fVIII. By 100 days after transduction, fVIII activity was no longer detectable. The rise and fall of fVIII levels and DNA copy number up to 100 days can be explained by clonal dominance among the individual clones that make up the polyclonal population of transduced cells. At various points following transduction, different clones proliferate at different rates, resulting in increases or decreases in total copy number and resulting fVIII levels. Clonal analysis of cells derived from this population at 50 days after transduction showed high variability in fVIII expression and DNA copy number **(Figure 3.8).**

To characterize the strength and erythroid specificity of the SIV-based vectors *in vivo*, recipient mice were conditioned with total body irradiation and transplanted with Sca-1^{+} stem and progenitor cells transduced with either SIV-Ank-GFP, SIV-βg-GFP, or SIV-MSCV-GFP, a SIV vector driving eGFP expression from an internal MSCV LTR (Hanawa et al 2004b). Peripheral blood cells were then analyzed for GFP expression 16 weeks after transplant. SIV-Ank-GFP-transplanted mice exhibited low level GFP expression in WBCs and RBCs, 5.4%

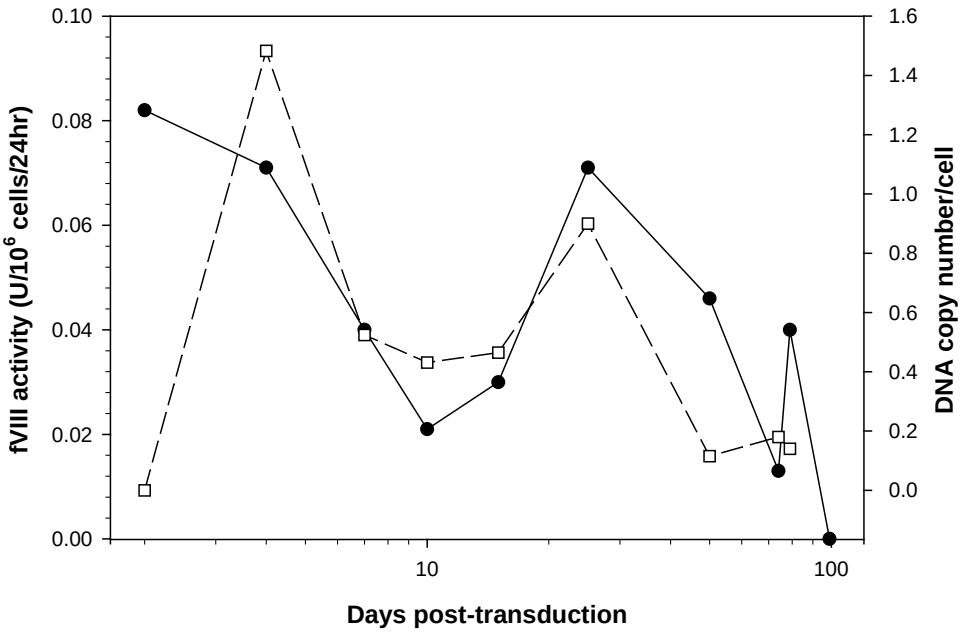

Figure 3.7 – FVIII activity and DNA copy number over time in SIV-βg-pfVIII-transduced K562 cells. K562 cells were transduced at an MOI of 10 and fVIII expression and DNA copy number/cell were measured for up to 100 days post-transduction. FVIII activity (black circles) was measured by one stage coagulation assay and DNA copy number (white squares) was measured by quantitative RT-PCR using primers specific to the A2 domain of fVIII.

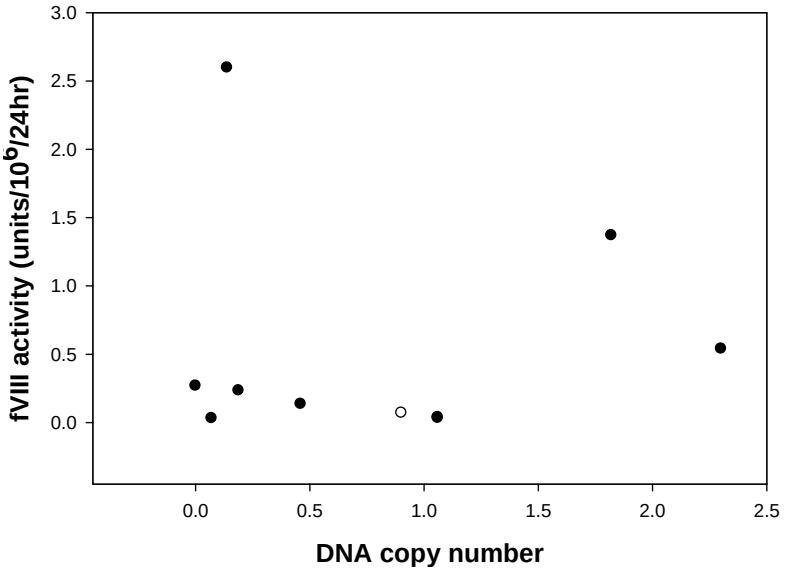

Figure 3.8 – Clonal analysis of SIV-βg-pfVIII transduced K562 cells. Clones were created by limiting dilution from the polyclonal population of K562 cells transduced at an MOI of 10. FVIII activity was measured from each of these clones by one stage coagulation assay 50 days after transduction and DNA copy number measured by RT-PCR. The original polyclonal cells are depicted above as an open white circle and each clone is depicted as a black circle.

and 4.9% respectively **(Figure 3.9)**. As shown in **Figure 3.10**, mice receiving cells transduced with SIV-βg-pfVIII possessed 8.1% ± 5.1% gene-modified RBCs and 10% ± 0.4% gene-modified WBCs. Mean fluorescence intensity (MFI) in these mice was 3118 ± 791 and 873 ± 251 for RBCs and WBCs respectively. The β-globin promoter exhibited red cell specificity, with a 4-fold higher MFI in RBCs than in WBCs ($p = 0.009$), despite a similar percentage of gene-marking in each cell type. Mice receiving cells transduced with SIV-MSCV-GFP possessed 37% ± 8% gene-modified RBCs and 56% ± 12% gene-modified WBCs. Mean fluorescence intensity in these mice was 850 ± 290 and 13200 ± 2200 for RBCs and WBCs respectively. However, the ratio of MFI in RBCs to MFI in WBCs was 66-fold higher for SIV-βg-GFP than SIV-MSCV-GFP ($p = 0.0361$) **(Figure 3.11)**, again demonstrating that the β-globin promoter is more active in its targeted cell type than the strong MSCV LTR ubiquitous promoter.

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High-level pfVIII expression in hemophilia A mice controlled by erythroid promoters

The efficiency of erythroid promoter-driven fVIII expression *in vivo* was evaluated by transplantation of Sca-1^+ hematopoietic stem and progenitor cells transduced with SIV-Ank-pfVIII or SIV-βg-pfVIII into hemophilia A mice. Six hemophilia A mice were transplanted with Sca-1^+ cells transduced with SIV-Ank-pfVIII at an MOI of 20. None of the SIV-Ank-pfVIII transplanted mice expressed sustained fVIII **(Figure 3.12).** For SIV-βg-pfVIII, an initial cohort (n=10) was injected with

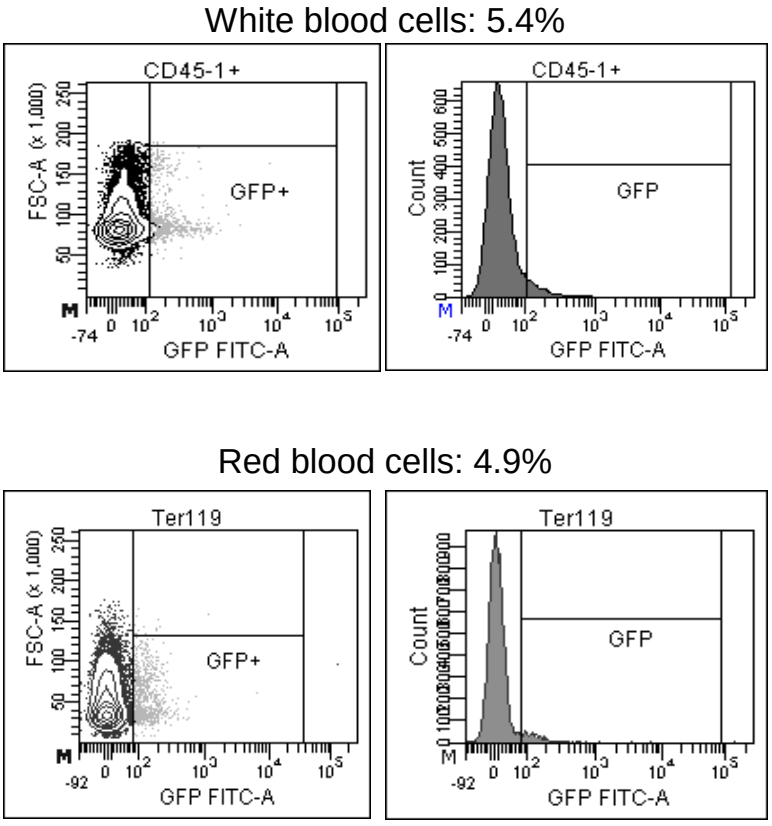


Figure 3.9 – GFP expression in SIV-Ank-GFP-transplanted mice. GFP expression was measured by flow cytometry in mice transplanted with Sca-1$^+$ cells transduced *ex vivo* with SIV-Ank-GFP. GFP expression in CD45.1$^+$ WBCs is shown in the top panel and GFP expression in Ter119$^+$ RBCs is shown in the bottom panel.

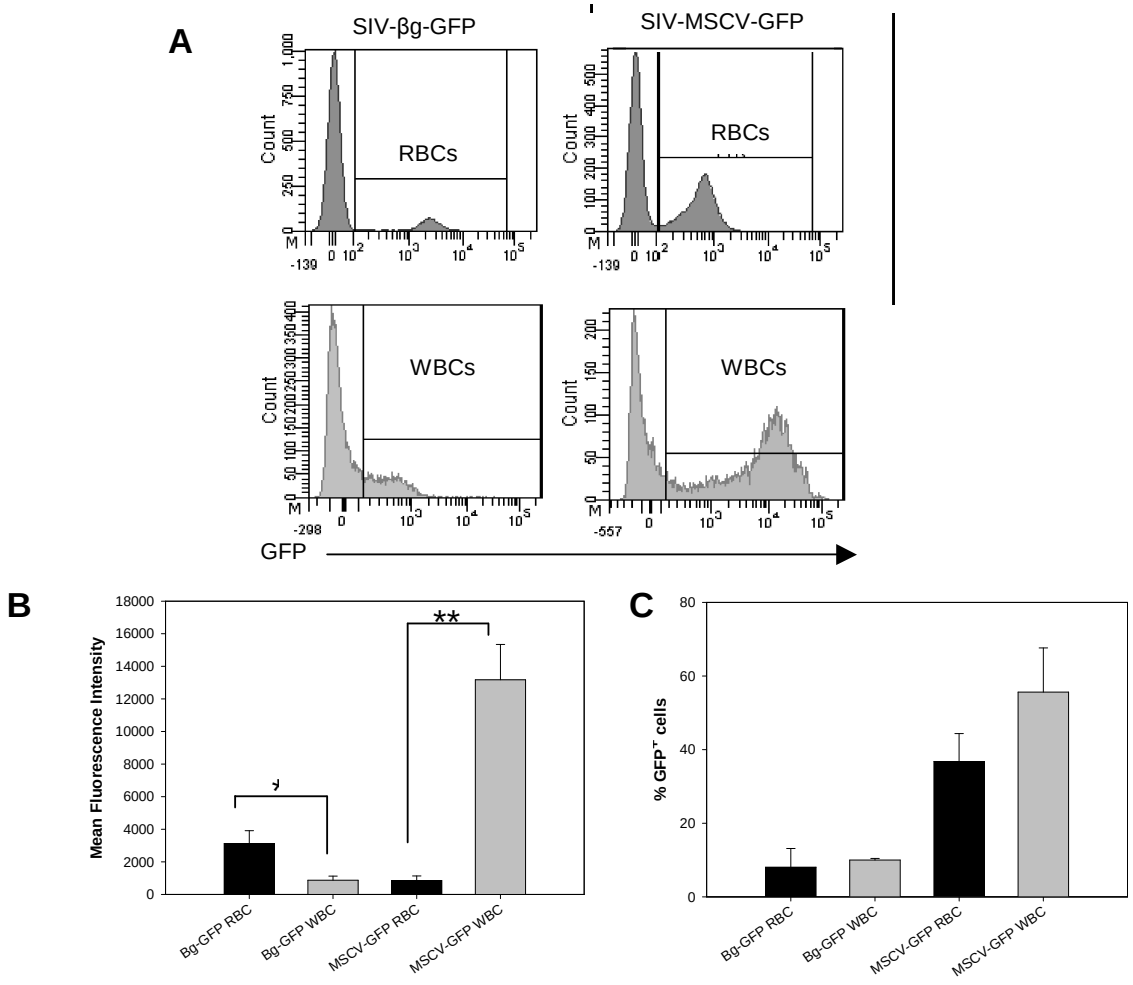


Figure 3.10 - Erythroid promoter strength and specificity *in vivo*. Recipient mice were transplanted with Sca-1$^+$ bone marrow cells transduced with either SIV-βg-GFP or SIV-MSCV-GFP. GFP expression was measured using flow cytometry 16 weeks post-transplant. A) GFP expression in donor white blood cells (WBCs) versus expression in Ter119 positive cells (marker of erythroid lineage, RBCs) in a representative SIV-Bg-GFP and SIV-MSCV-GFP mouse. B) Intensity of GFP expression *in vivo* as measured by MFI in WBCs versus RBCs

transduced with either SIV-βg-GFP or SIV-MSCV-GFP, n=3; (*) p = .009, (**) p = 0.0006. Bars represent the mean ± S.D. of 3 mice per virus. C) Percent gene-modified RBCs and WBCs in mice transplanted with cells transduced with either SIV-βg-GFP or SIV-MSCV-GFP (n=3).

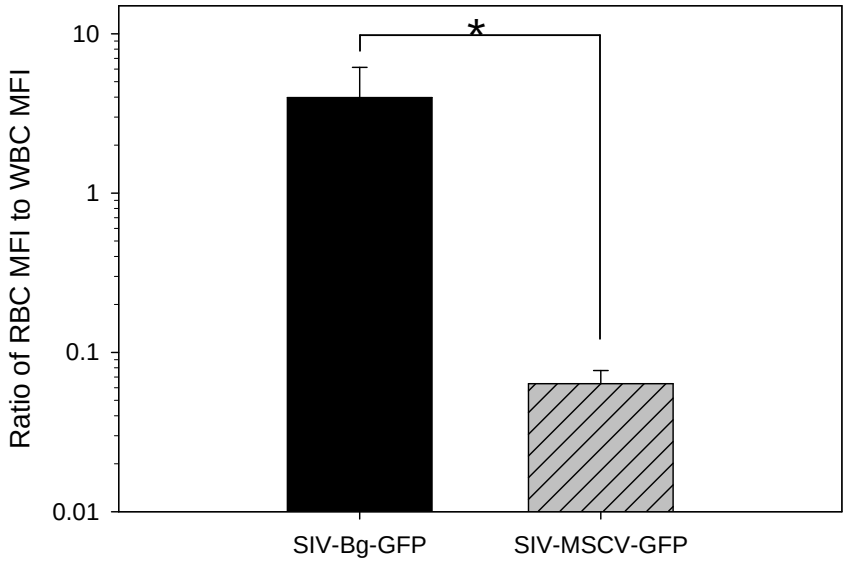


Figure 3.11 – Red blood cell-specific β-globin promoter strength. GFP expression was measured by flow cytometry in mice transplanted with Sca-1^+ cells transduced with either SIV-βg-GFP or SIV-MSCV-GFP. The difference in the ratio of MFI in RBCs to MFI in WBCs was calculated as a measure of promoter strength and specificity in RBCs, (*) p = 0.0361. Bars represent the mean ± S.D. of 3 mice per virus.

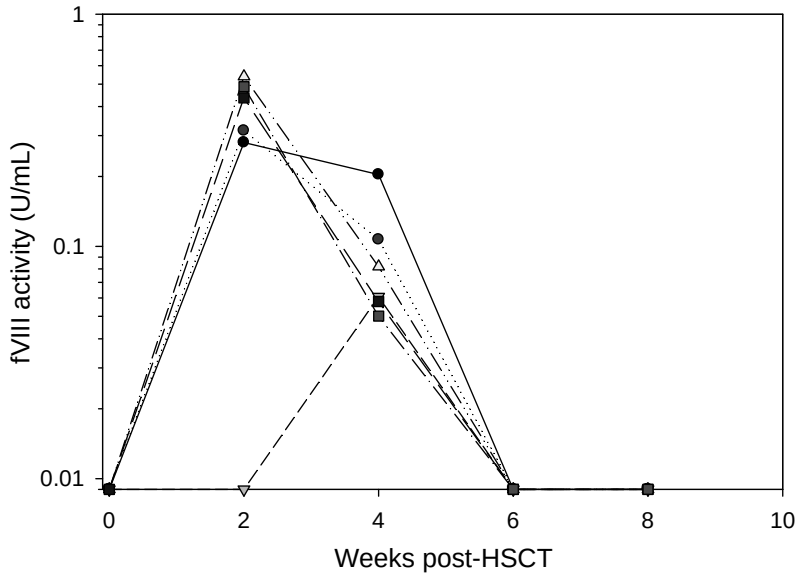


Figure 3.12 – Measure of fVIII expression in SIV-Ank-pfVIII transplanted mice. Six hemophilia A mice were lethally irradiated and transplanted with 500,000 Sca-1^{+} cells/mouse by tail vein injection. FVIII activity was measured in peripheral blood plasma by chromogenic assay starting at 2 weeks post-transplantation. All six mice exhibited a transient spike in fVIII expression lasting for 4-6 weeks.

cells transduced at an MOI of 35, giving rise to 2 mice with a transient spike in fVIII expression for approximately one month. A second cohort (n=13) was transplanted with cells transduced at an MOI of approximately 70, giving rise to two mice with sustained high level fVIII expression for at least 27 weeks **(Figure 3.13)** and 5 mice with a transient spike in fVIII expression between 2-4 weeks. A Bethesda assay was performed on blood plasma from mice with transient fVIII expression, which showed that no anti-fVIII inhibitors were present at 6 or 8 weeks post-transplantation. A third cohort (n=16) was transplanted with cells transduced at an MOI of ~350, giving rise to 7 mice with a transient spike in fVIII expression from 2-8 week, but no mouse achieved sustained long term expression. A final cohort of mice (n=5) was transplanted with cells transduced at a density of $1x10^7$/mL, compared to $1x10^6$/mL as in the previous transplants, with an MOI of ~3.5. Two mice from this cohort expressed a spike in fVIII from 2-10 weeks **(Figure 3.14)**. Of all the transplanted mice, 41% exhibited sustained or transient fVIII expression for approximately 4-10 weeks, but only 2 of 44 exhibited long-term expression.

To determine if those mice with sustained fVIII harbored gene-modified HSCs at the time of sacrifice, whole bone marrow was harvested and used for secondary transplants. None of the secondary transplant recipient mice expressed long-term fVIII (data not shown). In order to determine if the lack of fVIII expression in the majority of mice was due to sequestration of fVIII in enucleated peripheral red blood cells **(Figure 3.15),** blood was collected from one of the mice exhibiting

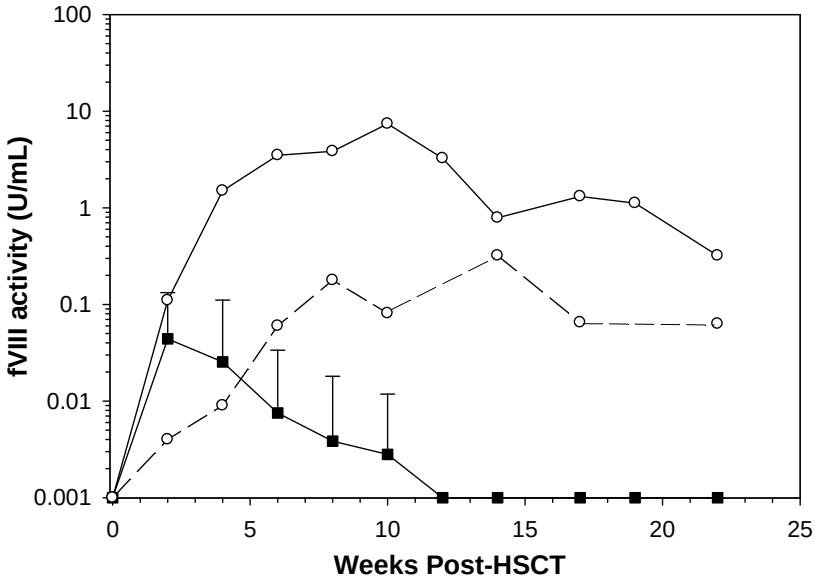


Figure 3.13 – SIV-βg-pfVIII expression *in vivo*. Hemophilia A mice were transplanted with Sca-1^{+} bone marrow cells transduced with SIV-βg-pfVIII (n = 44). FVIII expression was measured in peripheral blood plasma by chromogenic assay starting 2 weeks after transplantation. Only those mice exhibiting pfVIII expression are shown. 2 mice exhibited sustained fVIII expression (white circles) and 16 mice exhibited only a transient spike in fVIII expression for up to 10 weeks (black squares).

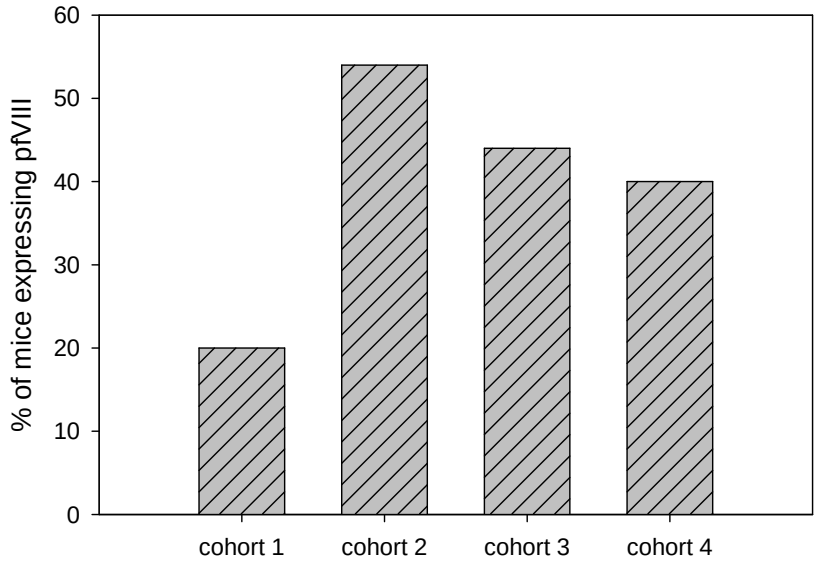


Figure 3.14– Measure of transient fVIII expression in all SIV-βg-pfVIII cohorts. FVIII expression was measured in all SIV-βg-pfVIII cohorts by chromogenic assay. The percentage of mice expressing fVIII was determined for each cohort. Cohort 1 (n=10) was transduced at an MOI of 35. Cohort 2 (n=13) was transduced at an MOI of 70. Cohort 3 (n=16) was transduced at an MOI of 350 and cohort 4 (n=5) was transduced at an MOI of 3.5.

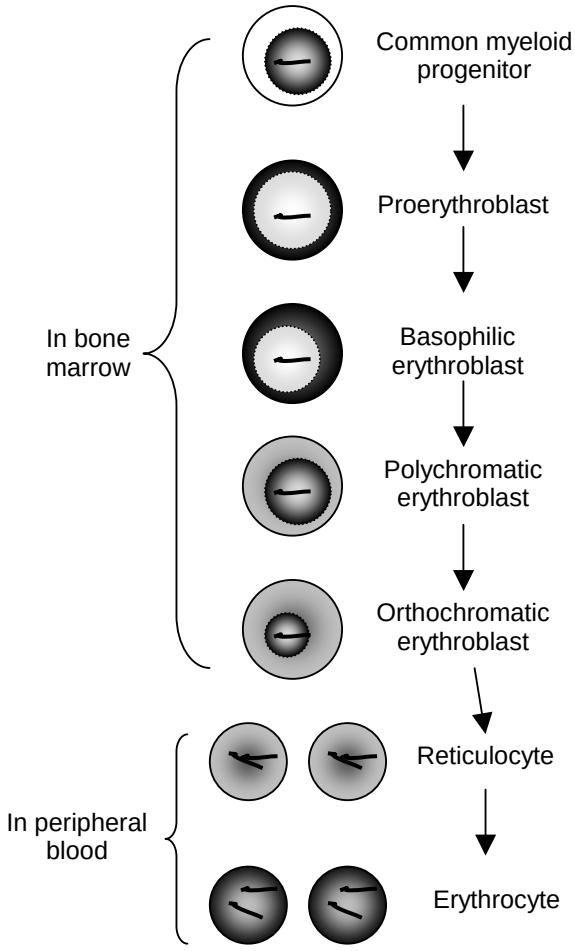


Figure 3.15 – Schematic of the stages of erythropoiesis. Erythroid cells begin as pluripotent stem cells, which differentiate into common progenitor cells. The first cell that is recognizable as specifically leading down the red cell pathway is the proerythroblast. As development progresses, the nucleus becomes smaller and the cytoplasm becomes more basophilic, due to the presence of ribosomes. This stage of differentation is called the basophilic erythroblast. The cell will continue to become smaller throughout development. As the cell begins to produce hemoglobin, the cytoplasm attracts both basic and

eosin stains, and is called a polychromatophilic erythroblast. The cytoplasm eventually becomes more eosinophilic, and the cell is called an orthochromatic erythroblast. The nucleus is then extruded and the cell enters circulation as a reticulocyte. As reticulocytes loose their polyribosomes they become mature red blood cells.

sustained fVIII expression, as well as a non-expressing mouse at 17 weeks post-transplantation. FVIII activity was measured in plasma as well as lysates of peripheral red blood cells. FVIII activity in the plasma of the expressing mouse was 0.8 U/mL **(Figure 3.16).** A small amount of fVIII (0.08 U/mL) was present in the first wash of the red blood cells, though 2 more washes were sufficient to remove any residual fVIII from the surface of the cells. Following these washes, RBCs were lysed and found to contain no detectable amount of fVIII. These data show that lack of long-term fVIII expression was not due to sequestration of fVIII in peripheral red blood cells. Red blood cell count, hemoglobin, and red blood cell morphology were normal despite the expression of fVIII **(Figure 3.16, inset)**.

Optimization of HSC purification and transplant from murine bone marrow

The lack of long term fVIII expression in the majority of primary transplant recipient mice and all secondary recipients suggests that within the Sca-1^+ donor cell population, SIV-βg-pfVIII preferentially targeted progenitor cells over HSCs, explaining why most transplanted mice exhibited only a 2-8 week transient spike in fVIII expression. We hypothesized that using a more highly HSC-enriched population of bone marrow cells as the targeted cell population may result in enhancement of stem cell transduction with greater engraftment of genetically modified cells. In order to test this hypothesis, a more highly HSC-enriched population of cells was isolated from murine bone marrow. HSCs are identified by their small size, lack of lineage markers, low staining side population with vital dyes such as rhodamine 123 or Hoechst 33342, and presence of various cluster

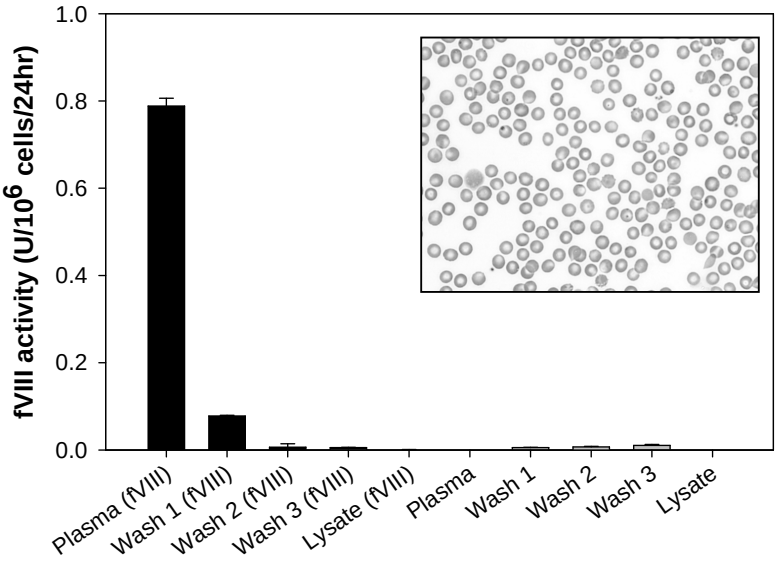


Figure 3.16 - Measure of fVIII expression in peripheral red blood cells. Blood was collected from a fVIII-expressing mouse (black bars, fVIII) and a non-expressing transplant recipient mouse (gray bars) at 17 weeks post-transplant. Peripheral red blood cells were lysed in HBS Tween containing 0.01% Triton-X100, then fVIII was measured by chromogenic assay. Inset shows normal RBC morphology from the fVIII-expressing mouse. Data is representative of 2 separate experiments. Bars are the mean ± S.D., measured in duplicate.

of differentiation (CD) antigenic markers on their surface. The receptor for stem cell factor, which is c-kit, is also a marker of HSCs. HSCs are negative for markers used for detection of lineage commitment, and for this reason are referred to as Lin^-. The most popular means of isolating HSCs is by fluorescence activated cell sorting (FACS) for $ckit^+/Sca\text{-}1^+/lin^-$ cells in whole bone marrow, also known as KSL cells. However, this method is time consuming, results in low yield, and often contaminates cells during the process, making bone marrow transplant impossible. For these reasons, a new method of HSC isolation was needed whereby a sterile population of cells could be isolated in a relatively short amount of time. We chose to isolate cells using immunomagnetic bead separation with 2 cell surface markers specific to HSCs, Sca-1 and CD150. CD150 is a cluster of differentiation cell surface marker present on HSCs and when co-expressed with Sca-1, is found in cells in the most primitive compartment of the hematopoietic developmental hierarchy, making Sca-1/CD150 a marker of multipotent HSCs (Bryder et al 2006; Weissman & Shizuru 2008) **(Figure 3.17)**. Because CD150 is also expressed on differentiated lymphoid cells, isolating cells by this marker alone will not result in primitive HSCs.

In order to achieve a highly purified $Sca\text{-}1^+/CD150^+$ cell population, a number of different protocols were tested to find the most optimal means of isolating this cell type. The first method tested was culturing whole bone marrow in a standard cytokine cocktail consisting of mSCF, mIL3, hIL-11, and hFlt3 for 3 days, then

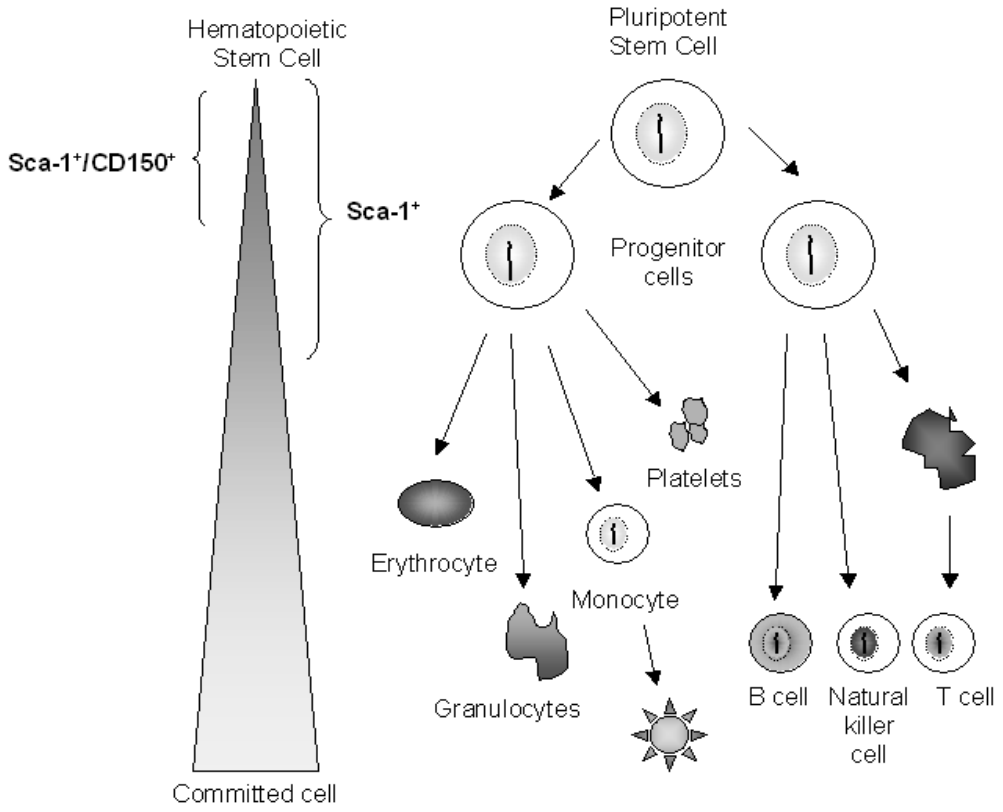


Figure 3.17 – Schematic of hematopoietic hierarchy. This schematic illustrates the various cell types expressing Sca-1 and CD150. Sca-1 is present on cells from HSC until they differentiate into common progenitor cells. CD150, is present on cells from HSC only until early multipotent progenitor cells.

$CD150^+$ cells were isolated using immunomagnetic bead separation. The hypothesis was that culturing in this cocktail would induce cell cycling and deplete non-HSC and non-progenitor cells, leaving only those cells with a stem cell phenotype. Flow cytometry analysis of the resulting cell population showed the percentage of $Sca\text{-}1^+$ cells after culturing in cytokines was increased compared to normal bone marrow, 38.5% compared to 1-3% normally, and the percentage of $CD150^+$ cells in whole bone marrow after cytokine conditioning was 8.5% **(Figure 3.18A).** After immunomagnetic bead separation of $CD150^+$ cells, the percentage of $Sca\text{-}1^+$ increased to 72.3% and the percent of $CD150^+$ increased to 78.7%. However, flow cytometry analysis of cells dually marked with both $Sca\text{-}1^+$ and $CD150^+$ showed only 10.3% $Sca\text{-}1^+/CD150^+$ cells **(Figure 3.18B)**. With such high percentages of $Sca\text{-}1^+$ cells in this population, it was unlikely that only 10.3% would be dually marked. We first hypothesized that the presence of anti-PE-conjugated microbeads used for the $CD150^+$ cell isolation was blocking binding of the PE-anti-CD150 antibody used in the detection of $CD150^+$ cells in the sample. However, analysis of the fluorescence of the microbeads with and without PE-stained $CD45^+$ cells revealed the PE signal was expressed fully in the presence of the microbeads **(Figure 3.19).** Rather, the reason for this discrepancy was that a new antibody was used for the detection of $Sca\text{-}1^+$ cells since 2 different fluorochrome conjugates were needed to distinguish Sca-1 and CD150 markers. The new FITC-anti-Sca-1 antibody did not efficiently bind to cells, resulting in a low percentage of $Sca\text{-}1^+$ cells in the $Sca\text{-}1^+/CD150^+$ population. After experimenting with a number of antibody

A

Marker	Whole bone marrow	$CD150^-$ fraction	CD150+ fraction
% Sca^+	38.5	36.7	72.3
% $CD150^+$	24.2	27.7	78.7
%$Sca^+/CD150^+$	8.5	9.1	10.3

B

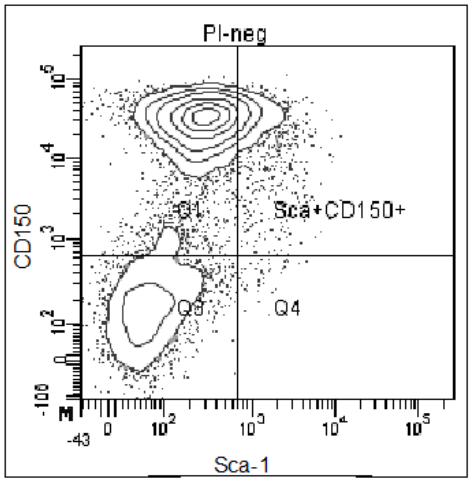


Figure 3.18 – Flow cytometry analysis of bone marrow cells before and after CD150 isolation. A) Whole bone marrow cells were cultured for 3 days in a cytokine cocktail to induce cycling while maintaining HSC phenotype. The percent of $Sca\text{-}1^+$ cells was measured using a PE-conjugated anti-Sca-1 antibody and the percentage of CD150 cells were measured using a PE-conjugated anti-CD150 antibody in whole bone marrow before CD150 immunomagnetic bead separation and after separation in both the positive and negative fractions. B) Flow cytometry analysis of the CD150 positive fraction stained with FITC-conjugated anti-Sca-1 and PE-conjugated anti-CD150.

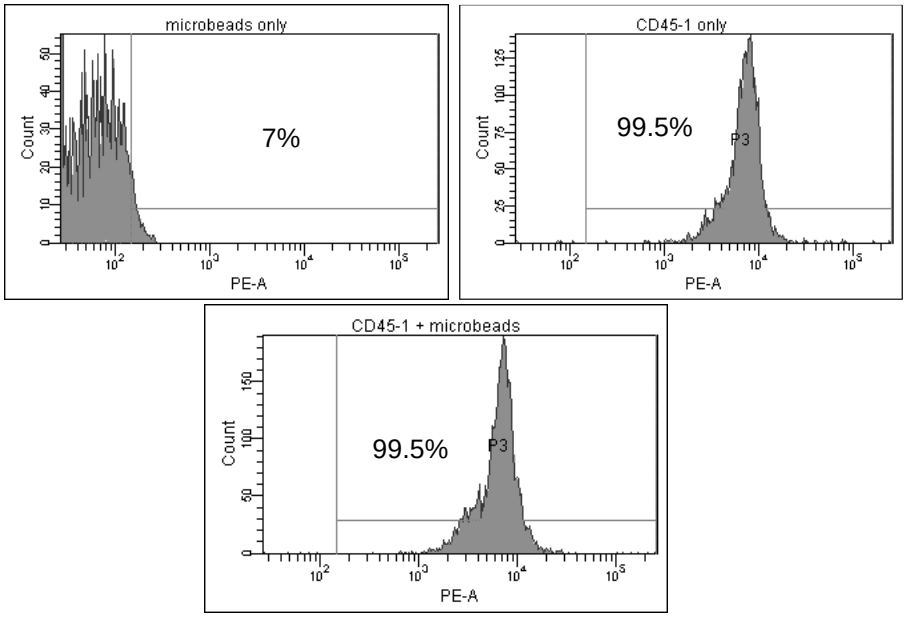


Figure 3.19 – Flow cytometry analysis to determine relative fluorescence of PE. Flow cytometry was performed on PE-stained CD45.1$^+$ peripheral blood cells, anti-PE-conjugated microbeads, and a population containing both PE-stained CD45.1$^+$ cells to determine if the presence of anti-PE-microbeads prevented fluorescence of the PE-anti-CD150 antibody used for detection.

combinations, we developed a new technique for Sca-1$^+$/CD150$^+$ cell isolation and detection of dually-marked cells. Bone marrow cells were labeled with a biotin-anti-Sca-1 (D7) antibody immediately after harvest, then purified through an anti-biotin-microbead column and separator (Miltenyi), identical to the current Sca-1$^+$ cell isolation protocol described in the Materials and Methods. Following Sca-1$^+$ cell isolation, the primary antibody was removed using a proprietary release reagent **(Figure 3.20)**. The biotinylated Sca-1$^+$ cells were stained with a PE-anti-CD150 (mSLAM) antibody then purified through an anti-PE-microbead column and separator. Resulting Sca-1$^+$/CD150$^+$ cells were cultured in a cytokine cocktail for 3 days before measurement of the percentages of each cell surface marker by flow cytometry. Cells were stained with either PE-anti-Sca-1 (E13-161.7) or PE-anti-CD150 (D7) and the percentages of each were determined separately. Direct measurement of the dually-labeled population was not possible due to antibody binding constraints. Following this protocol, the resulting cell population obtained from the final positive fraction was highly enriched for both Sca-1 and CD150 and used for transduction and transplantation. On the day of transplant, a sample of cells was analyzed to determine the percentages of Sca-1$^+$ and CD150$^+$ cells to be injected. Separate staining of each resulted in 87% CD150$^+$ cells and 91% Sca-1$^+$ cells **(Figure 3.21)**.

Gene modification of HSCs using Sca-1$^+$/CD150$^+$ bone marrow cells

Using this new highly stem cell-enriched Sca-1$^+$/CD150$^+$ population, cells were transduced with SIV-βg-pfVIII at an MOI of 20 and >20,000 cells were

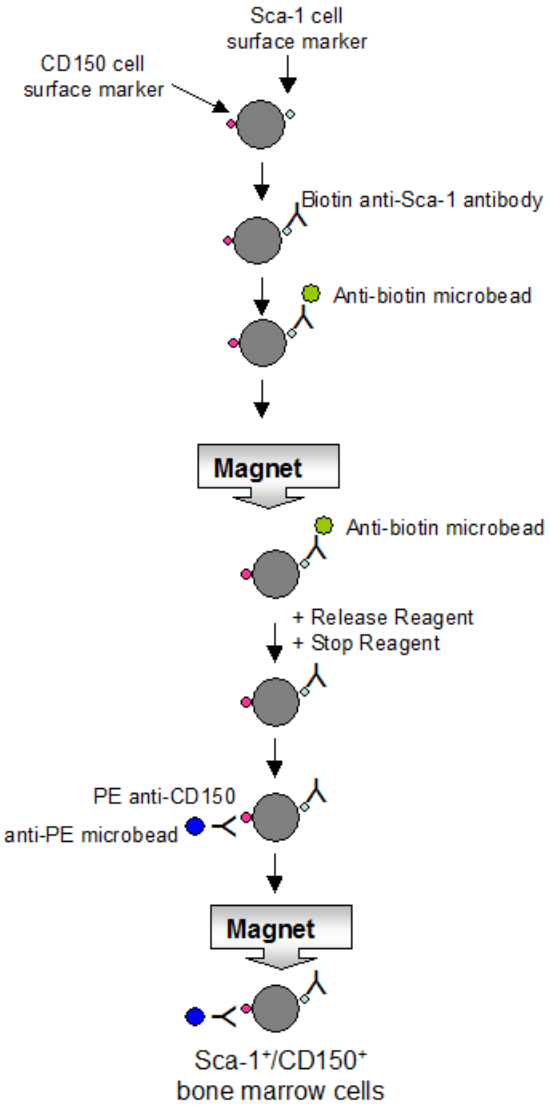


Figure 3.20 – Schematic of sequential Sca-1$^+$ and CD150$^+$ cell isolation. The steps of Sca-1$^+$/CD150$^+$ cell isolation are as follows: Stain whole bone marrow cells with biotin-anti-sca-1. Purify for Sca-1$^+$ cells by passing cells through an immunomagnetic biotin-microbead column. Remove anti-biotin microbeads from Sca-1$^+$ cells using release reagent. Stop release reaction with stop reagent. Stain Sca-1$^+$ cells with PE-anti-CD150. Purify for CD150$^+$ cells by passing cells through an immunomagnetic PE-microbead column.

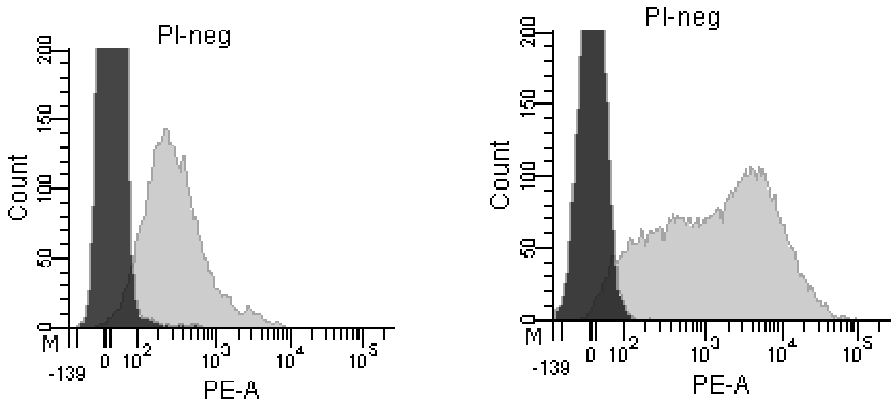


Figure 3.21 – Highly enriched Sca-1$^+$/CD150$^+$ cell population for HSCT. Representative flow cytometry analysis of transduced Sca-1$^+$/CD150$^+$ bone marrow cells before transplant. Cells were isolated using immunomagnetic bead separation to enrich for Sca-1$^+$ cells from whole bone marrow. CD150$^+$ cells were then enriched from the Sca-1$^+$ population after immunomagnetic bead removal and staining with a second antibody for separation through a second immunomagnetic bead column. Dark gray histogram shows mean fluorescent intensity of cells stained for the marker of interest in a population with no marker. Light gray histogram shows mean fluorescent intensity of cells stained for the marker of interest in Sca-1$^+$/CD150$^+$ cell population used for HSCT.

transplanted into lethally irradiated mice (n=4). All mice exhibited fVIII expression, but only one showed sustained expression for >5 months **(Figure 3.22A)**. At 22 weeks after the transplant, the mouse with sustained fVIII expression was sacrificed and whole bone marrow cells were used for secondary transplants. A complete blood count was performed on peripheral blood from the donor mouse, which showed that all blood cell types were present at normal levels. Of the 3 secondary recipients, 1 mouse expressed sustained fVIII for up to 15 weeks, suggesting that gene-modified long-term progenitor cells, but not HSCs, were included in the transplanted population **(Figure 3.22B).** A lineage analysis of whole bone marrow from the primary recipient mouse was also performed to determine the relative proportions of various blood cell types in this mouse at the time of sacrifice. Although most cell types appeared within the normal range, the percentage of granulocytes and macrophages was slightly increased in the transplanted mouse as compared to a naïve mouse**,** at 21% and 5% respectively**.** Although the reason for this increase is unknown, it may be worth investigating in future studies. Previous data from our lab suggests that granulocytes and macrophages may be the primary cell type making fVIII following HSCT using a γ-retrovirus with ubiquitous promoter driving pfVIII expression (Ide et al 2007). These results suggest that increasing the purity of HSCs in the transducing population does not greatly increase the percentage of gene-modified HSCs following transduction with SIV-βg-pfVIII.

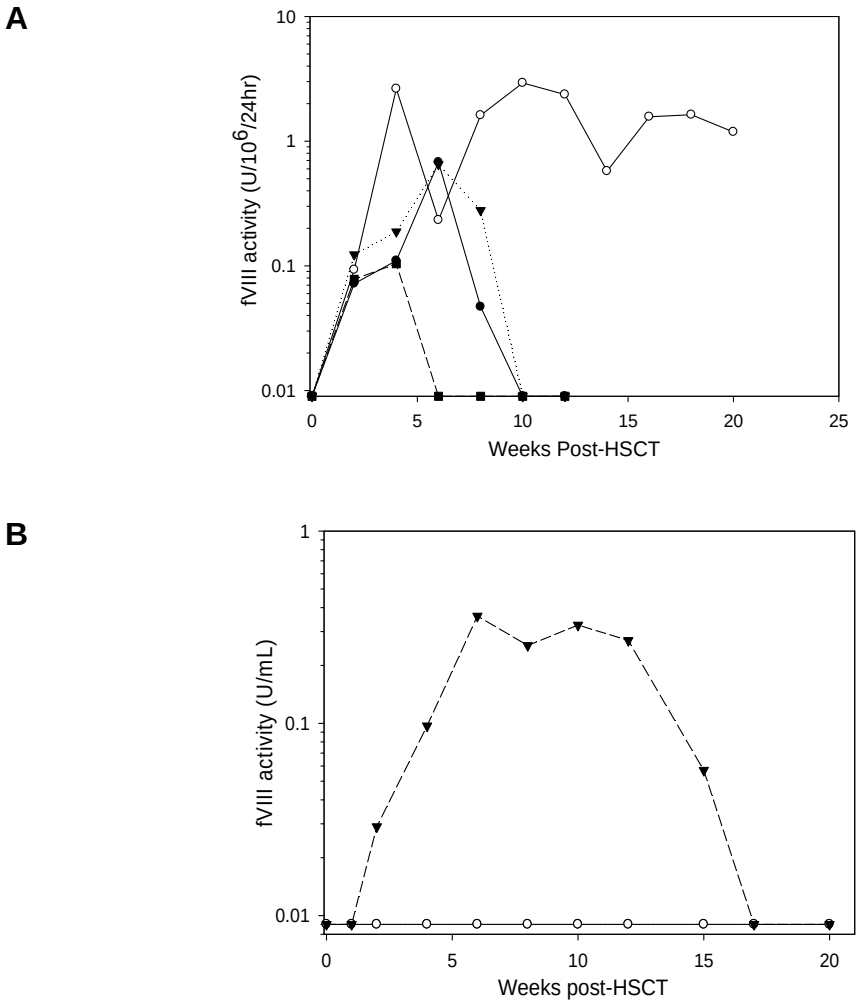


Figure 3.22 – *In vivo* fVIII expression in Sca-1$^+$/CD150$^+$ bone marrow cells. Hemophilia A mice were transplanted with Sca-1$^+$/CD150$^+$ bone marrow cells transduced *ex vivo* with SIV-βg-pfVIII (n = 4). A) FVIII activity was measured by chromogenic assay on blood plasma from peripheral blood of transplanted mice. Each line represents a single mouse. B) Secondary transplants were performed (n = 3) using whole bone marrow from the Sca-1$^+$/CD150$^+$ transplanted mouse with sustained expression at 22 weeks post-transplantation. Each line represents a single mouse.

Induction of immune tolerance by transient erythroid promoter driven fVIII expression

Following the disappearance of fVIII expression in Sca-1^{+}/CD150^{+} transplanted mice, these mice were challenged with 10 units of pfVIII once weekly for 4-5 weeks in order to determine if transient erythroid promoter driven fVIII expression could reduce or prevent the immune response against systemically delivered recombinant pfVIII. After challenges with pfVIII, as expected, naïve hemophilia A mice developed anti-pfVIII antibodies and three of four GFP-transplanted mice developed high titer inhibitor antibodies, from ~100-2000 Bethesda units (BU) **(Figure 3.23).** However, 2 of the Sca-1^{+}/CD150^{+} mice had no ELISA titer and one exhibited a very low-level inhibitor titer of 12 BU. These data suggest that transient erythroid promoter driven pfVIII expression can induce immunological non-responsiveness in transplanted mice.

3.5 – Discussion

The studies presented here focus on the use of erythroid-specific promoters to limit transgene expression to a specific lineage of genetically-modified cells following lentiviral transduction of HSCs. We first show that both the ankyrin-1 and β-globin promoters are more active in driving pfVIII expression in an erythroid-like cell line, but they do retain some ability to express low levels of fVIII in non-erythroid cells. Although 293T cells are inherently more susceptible to transduction than hematopoietic cell lines, promoter activity normalized to DNA copy number showed that both the ankyrin-1 and β-globin promoters exhibit

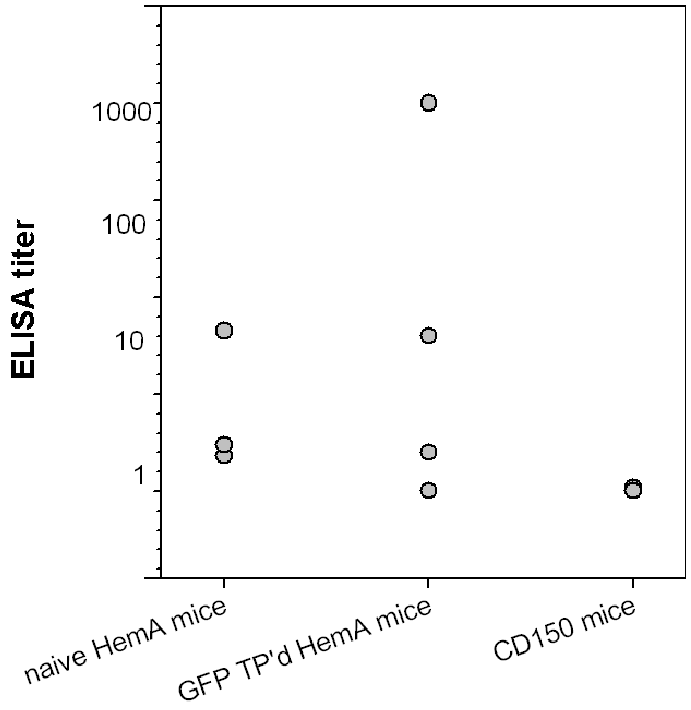


Figure 3.23 – Determination of fVIII immune responsiveness in transplanted mice. Porcine fVIII-transduced Sca-1^+/$CD150^+$ cell transplanted hemophilia A mice (n=3), GFP transduced Sca-1^+ cell transplanted mice (n=4), and naïve mice (n=3) were challenged for 4-5 weeks with 10 units pfVIII per injection once weekly. Mice were bled and plasma inhibitor levels were measured by ELISA. Each circle represents a single mouse.

some activity in these cells. Similar non-erythroid promoter activity was observed by others using the β-globin promoter and locus control region for transcriptional control of factor IX (Hanawa et al 2002). The non-erythroid activity observed in those experiments may be explained by specific vector design. Others have shown that the presence of globin regulatory elements in the 5’ to 3’ direction may result in non-erythroid-specific interactions with chromatin and transcriptional regulators, making the LCR act more like a classical ubiquitous enhancer than a cell-specific transcriptional regulator (Sadelain et al 1995).

In vivo data using GFP constructs confirmed that the β-globin promoter is more active in RBCs than in WBCs. However, the total percentage of gene-modified cells was much lower than expected based on previous studies using SIV vectors, suggesting transduction efficiency was dramatically impaired as compared to transduction with a SIV vector containing an internal MSCV LTR promoter. This decrease in transduction efficiency may be due to the increase in size or complexity of the SIV-βg-GFP vector as compared to SIV-MSCV-GFP. The additional size of the β-globin promoter and locus control region (~2000-bp) compared to SIV-MSCV-GFP may limit the ability of the viral RNA genome to be reverse transcribed, imported to the nucleus, and/or integrated into host cellular DNA, while the additional complexity may affect secondary structural interactions.

Despite the fact that a similar percentage of gene marking in RBCs and WBCs was observed, a greater overall number of gene-modified RBCs than WBCs are present due to the relative concentrations of each cell type in the bloodstream. In healthy humans, the normal amount of RBCs in circulation is ~$5x10^6$ /μL, created at a rate of 2-3 million RBCs per second. RBCs remain viable in circulation for up to 4 months before being recycled in the spleen. The normal amount of WBCs is ~$8x10^3$/μL, or approximately $1/1000^{th}$ the number of RBCs. Most WBCs remain in circulation for about 18-36 hours, though some have much longer half-lives. Although in the current experiment, it is not known at which stages of erythropoiesis fVIII is being produced and secreted, other studies have suggested the highest level of β-globin promoter-driven protein expression is in middle to late stage erythroblasts (Cowie & Myers 1988). The events of erythropoiesis would, therefore, suggest that middle to late stage erythroblasts are capable of protein production and secretion. For example, as erythrocytes differentiate from proerythroblasts to basophilic erythroblasts, the nucleus becomes smaller and the cytoplasm fills with ribosomes, preparing the cell to begin protein production. As the cell begins to produce hemoglobin, or in this case, pfVIII, it is termed a polychromatic erythroblast. The next stage of erythropoiesis is the orthochromatic erythroblast, which is the last stage of differentiation before nuclear loss. However, at this point, the cell maintains all the necessary machinery to produce and secrete pfVIII. The cell will then extrude its nucleus to become a reticulocyte and enter peripheral circulation. Reticulocytes are known to contain an endoplasmic reticulum (Grasso et al 1978)

and are filled with polyribosomes, but are lost upon terminal differentiation into mature RBCs. However, some pfVIII mRNA is likely present in the cytoplasm of reticulocytes whereby it will continue to be translated and secreted for approximately 3 days before terminal differentiation in to mature RBCs. At this point, further pfVIII production and/or secretion is not likely.

Given that others demonstrated the ability of erythroid cells to manufacture and secrete heterologous proteins, such as factor IX and IDUA (Chang et al 2006; Wang et al 2009), and based on our *in vivo* characterization of GFP-containing constructs, the lack of fVIII expression in mice transplanted with SIV-βg-pfVIII-transduced Sca-1^{+} cells was unexpected. Although some mice expressed sustained high levels of fVIII, most mice expressed only a brief spike in fVIII for 2-10 weeks after transplantation. One possible reason for a lack of circulating fVIII is that mature RBCs are not able to make and/or secrete functional fVIII. Since terminally differentiated RBCs are enucleated, it was unknown if sufficient RNA would be available for protein translation and secretion. However, lysis of peripheral red blood cells, which includes reticulocytes and terminally differentiated RBCs, revealed no fVIII was being sequestered in RBCs. These results suggest either 1) all functional fVIII was produced and secreted in the bone marrow, or 2) fVIII was produced and secreted from peripheral RBCs too quickly to be measured by this assay. Another potential reason for the lack of sustained fVIII expression in the majority of mice is the development of anti-fVIII antibodies. Bethesda and anti-fVIII antibody ELISA assays performed on blood

plasma at the time fVIII was lost showed that no inhibitory antibodies were present.

Secondary transplants using whole bone marrow from 2 mice with high-level sustained fVIII activity were performed in order to determine if the lack of fVIII expression was due to inefficient transduction and gene-modification of HSCs. None of the secondary transplant recipients expressed fVIII, suggesting that the primary recipient mice contained very few genetically-modified HSCs. Numerous studies have demonstrated the ability of lentiviruses to transduce cytokine-stimulated HSCs as well or better than similarly pseudotyped γ-retroviruses (Barrette et al 2000; Mostoslavsky et al 2005; Sutton et al 1999; Uchida et al 1998). When transducing Sca-1^+ cells with either a MSCV γ-retrovirus or a SIV lentivirus expressing GFP, we achieved upwards of 60% gene-modified cells, as shown in the bottom right panel of Figure 3.10A, confirming the efficiency of the lentiviral transduction protocol. However, as shown in the current study, incorporating the ~3 kb β-globin promoter and locus control region in place of an internal MSCV LTR decreases the efficiency of transduction, resulting in fewer genetically-modified cells. When the GFP transgene is replaced by the 4.4 kb pfVIII transgene, transduction of HSCs is almost completely prevented. We previously showed a similar decrease in the transduction efficiency of pfVIII-containing lentiviruses with a CMV promoter compared to GFP-containing lentiviruses (Doering et al 2009). However, the brief spike in fVIII expression observed in the transplanted mice suggests that hematopoietic cells are

transduced, however not long-term repopulating HSCs, and the elapsed time of fVIII expression in these mice suggests that progenitor cells in the Sca-1^+ cell population have been primarily transduced.

To determine if an increase in HSC transduction can be achieved with a pfVIII-containing lentivirus using a more highly purified target population, cells were isolated that express both Sca-1 and CD150, a cell surface marker associated with high expression in long term self renewing HSCs (Kiel et al 2005). This study was based on the hypothesis that if HSCs are able to be transduced, the majority of mice transplanted with genetically-modified Sca-1^+/$CD150^+$ cells will express sustained fVIII. If HSCs are inherently resistant to pfVIII-containing lentiviral transduction, no long-term fVIII expression would be observed. In fact, only 1 of 4 mice expressed sustained fVIII activity, suggesting that increasing HSC purity does not greatly increase HSC transduction. However, this data does show that HSC transduction with a lentivirus containing pfVIII is possible, but at a low efficiency. Although understanding the reason for this decrease in transduction efficiency requires additional studies, one possibility is that the size or complexity of the pfVIII transgene prevents optimal reverse transcription and/or integration into the host genome.

Even though the mice lost detectable fVIII expression, they were immunologically non-responsive to challenges with recombinant pfVIII. We and others showed previously that $CD4^+$ T cells from mice transplanted with bone marrow cells

modified to express fVIII were non-responsive to challenges with purified fVIII, and fVIII-specific T cells were either not present or had been inactivated in the transplanted mice (Hamel et al 1997; Ide et al; Madoiwa et al 2009). In mice transplanted with genetically modified bone marrow cells, tolerance can be achieved by thymic re-education and/or development of regulatory T cells. Thymic re-education would not necessarily be a primary mechanism in the current studies as pfVIII expression was limited to the erythroid lineage. However, because some expression was observed in a fraction WBCs, similar mechanisms may be involved. In contrast to the previous studies where no mice developed inhibitors after transplant of cells expressing long term sustained fVIII controlled by a strong ubiquitous promoter, one of 3 mice did develop low level anti-pfVIII inhibitors in the erythroid promoter driven pfVIII transplant. This suggests that the β-globin promoter system is not as robust as the ubiquitous expression system for the induction of tolerance to pfVIII. An interesting future study would be to determine if the reason for the lack of immune response in mice receiving platelet-specific fVIII (Gewirtz et al 2008; Shi et al 2007; Shi et al 2003) was due to induction of immune tolerance. However, the lack of inhibitor formation in these studies was most likely the result of the limited exposure of fVIII to the immune system due to the localized release of platelet-sequestered fVIII directly at the site of hemostatic injury.

In summary, we demonstrated the ability of erythroid cells to mediate systemic delivery of both transient and sustained pfVIII in a lentiviral gene therapy HSC

transplantation model of hemophilia A. The strength of the β-globin promoter at driving pfVIII expression in erythroid cells was comparable to that of the strong MSCV LTR promoter in 293T cells. The β-globin promoter was also efficient at driving sustained GFP expression in our HSCT murine model, however sustained pfVIII expression was only observed in a fraction of transplanted mice. Although the majority of mice that expressed pfVIII exhibited only transient expression, our studies indicate this is suitable to induce immune tolerance to pfVIII. Although the reasons for the lack of sustained fVIII expression in the majority of mice is unknown, it appears mostly due to a lack of HSC transduction by SIV-βg-pfVIII. These studies show that long-term erythroid promoter driven pfVIII expression is possible if HSC transduction levels are improved upon.

Chapter 4:

Characterization of lentiviral transduction efficiencies

4.1 – Abstract

Variability in retroviral transduction efficiencies is commonly observed, but not completely understood. Differences in γ-retroviral and lentiviral transduction can be explained by the different mechanisms of transduction inherent for each virus. However, variations in transduction efficiency among viruses containing similar viral backbones are not as easily explained. Previously, we demonstrated a decrease in the number of gene-modified HSCs following transduction with a MSCV or SIV vector containing the pfVIII transgene compared to GFP. In addition to potential transgene-specific differences, previous studies suggested the entire expression cassette may play a role in determining the efficiency of transduction. The studies described in Chapter 4 were designed to better understand the variations in lentiviral transduction efficiencies and to elucidate the reason for the observed low-level transduction of Sca-1^+ cells using a pfVIII-containing lentivirus. For this, a comprehensive characterization of the components involved in lentiviral transduction was performed. The transduction parameters examined included the type of cell transduced, the viral backbone used, kinetics of proviral reverse transcription in transduced cells, and various properties of the lentiviral stocks used including p24 titer, quantity of viral RNA, and functional viral titer. The resulting data suggest a number of differences may be present in the viral stocks of the GFP- or pfVIII-containing HIV-1-based lentiviruses used for these studies, including differences in viral packaging efficiency and functional viral titer for each virus. The pfVIII-containing lentiviral stock contained ~4-fold less viral RNA than the GFP-containing lentiviral stock

and required ~5-fold more total viral particles/cell to result in a similar number of integrated proviral copies. In addition, *in vitro* and *ex vivo* kinetic studies demonstrated the mechanism responsible for the decreased transduction of Sca-1^+ cells with a pfVIII-containing lentivirus occurs at a stage subsequent to proviral reverse transcription in transduced cells. Together these data indicate the complexity of retroviral transduction and suggest a better understanding of the kinetics of proviral nuclear transport and integration are necessary to improve HSC transduction efficiency.

4.2 – Introduction

To date, the success of HSC gene therapy has been limited by inefficient gene transfer. Gamma-retroviruses efficiently transduce many cell types, however, they are unable to transduce quiescent HSCs due to a requirement for cellular division at the time of transduction. Lentiviruses, however, contain a nuclear localization signal that allows entry of the pre-integration complex (PIC) into the nuclei of both dividing and non-dividing cells. The PIC forms after uncoating of a viral particle inside a cell and is composed initially of 2 strands of lentiviral RNA complexed with various viral and host cellular proteins. Several studies have demonstrated the superiority of lentiviral vectors compared to γ-retroviral vectors in the efficient transduction of HSCs (Akkina et al 1996; Barrette et al 2000; Case et al 1999; Hanawa et al 2004b; Mostoslavsky et al 2005; Uchida et al 1998), however, our data suggests the opposite is true. Using a γ-retroviral MSCV vector expressing eGFP to transduce Sca-1^+ hematopoietic stem and progenitor

cells, we have achieved upwards of 60-70% gene-modified cells. When the eGFP transgene was replaced with the pfVIII transgene, the percent of gene-modified cells decreased below our limit of detection (0.06 copies/cell), however high-level sustained pfVIII expression over 1 U/mL was achieved in all primary and secondary transplant recipients (Gangadharan et al 2006; Ide et al 2007). Converse to the high-level sustained fVIII expression in mice transplanted with MSCV-pfVIII-transduced Sca-1^+ cells, mice transplanted with Sca-1^+ cells transduced with a SIV vector expressing pfVIII exhibited sustained fVIII expression at least 10-fold lower than those observed using MSCV (Dooriss et al 2009). Using a SIV vector containing the pfVIII transgene under control of the beta globin promoter and locus control region for transduction and transplantation of Sca-1^+ cells, only 2 of 44 mice expressed sustained fVIII.

In addition to demonstrating variability in MSCV and SIV transduction efficiencies, our previous studies suggest that the size or complexity of the expression cassette may affect HSC transduction efficiency. In the erythroid promoter studies outlined in the previous chapter, decreased transduction was observed using a SIV vector containing the large β-globin promoter and locus control region (3.4 kb) as compared to a SIV vector containing the much smaller MSCV LTR (520 bp). Mice transplanted with Sca-1^+ cells transduced with SIV-MSCV-GFP exhibited up to 60% gene-modified cells, however SIV-βg-GFP exhibited up to only 10% gene-modified cells. As mentioned above, none of the mice transplanted with SIV-βg-pfVIII-transduced Sca-1^+ cells possessed a

detectable number of gene-modified cells. In addition, fVIII-expressing mice were used as donors for secondary bone marrow transplants, giving rise to no secondary recipients with sustained fVIII expression. Together, these data indicate that SIV-mediated HSC transduction is limiting and the specific transgene and/or promoter incorporated into a lentiviral vector may play a role in the efficiency of lentiviral transduction.

Several studies were initiated to determine 1) if HSC transduction efficiency could be increased, and 2) what is the mechanism for the poor SIV-mediated transduction of HSCs. Our attempts to increase HSC transduction efficiency included 5-FU-mediated enrichment of donor bone marrow cells, changing the vector backbone from SIV to HIV, and increasing viral production efficiency. Unfortunately, none of these methods resulted in increased transduction efficiency. In order to better understand the reasons for poor HSC transduction efficiency, 293T, K562, and Sca-1^{+} cells were transduced with HIV vectors ubiquitously expressing either pfVIII or GFP. The smaller size of the proviral sequence allowed for consistent generation of high titer virus, which was characterized by evaluation of functional titer, p24 titer, and kinetics of proviral reverse transcription in transduced cells. Overall, these studies showed 1) HIV-based vectors exhibit less efficient transduction than SIV in the cell lines tested, 2) pfVIII is produced less efficiently than GFP in SIV-transduced cells, and 3) similar kinetics of proviral reverse transcription are observed under conditions resulting in similar viral integration. These results suggest that the reason for the

observed difference in lentiviral-mediated HSC transduction efficiency was not due to a block in transduction at viral entry or reverse transcription, but rather, less pfVIII cDNA was integrating into the genome of transduced HSCs. Although the reasons for these differences are unknown, better characterization of the processes involved in lentiviral transduction of hematopoietic stem cells may lead to improvements in HSC transduction necessary to increase levels of integrated transgene and produce clinically therapeutic amounts of fVIII.

4.3 – Materials and Methods

HIV production and functional quantification. HIV-βg-pfVIII**,** HIV-pfVIII and HIV-GFP were created by 3-plasmid co-transfection into 293T cells **(Figure 4.1).** Briefly, a Nunc triple flask containing 90% confluent 293T cells was co-transfected with 80μg PCL expression vector (Addgene, Cambridge, MA) containing either eGFP or pfVIII, 40μg psPAX2, and 40μg pVSVG with 1.3 mg polyethyleneimine hydrochloric salt (PEI). Media was changed to fresh DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin the following day and virus-containing supernatant was collected once a day for the next 3 days. Note, this method of viral preparation may be scaled up or down as necessary. Virus was stored at -80°C then thawed in a 37°C water bath and filtered through a 0.2 μm vacuum filter before centrifuging overnight at 10,000 x g. Viral pellets were resuspended in 1/200th the original volume and concentrated virus was frozen at -80°C until ready to use. Viral titering was performed by adding 0.5, 2, 5, and 10 μL virus to ~150,000 293T cells in 1 mL complete medium containing

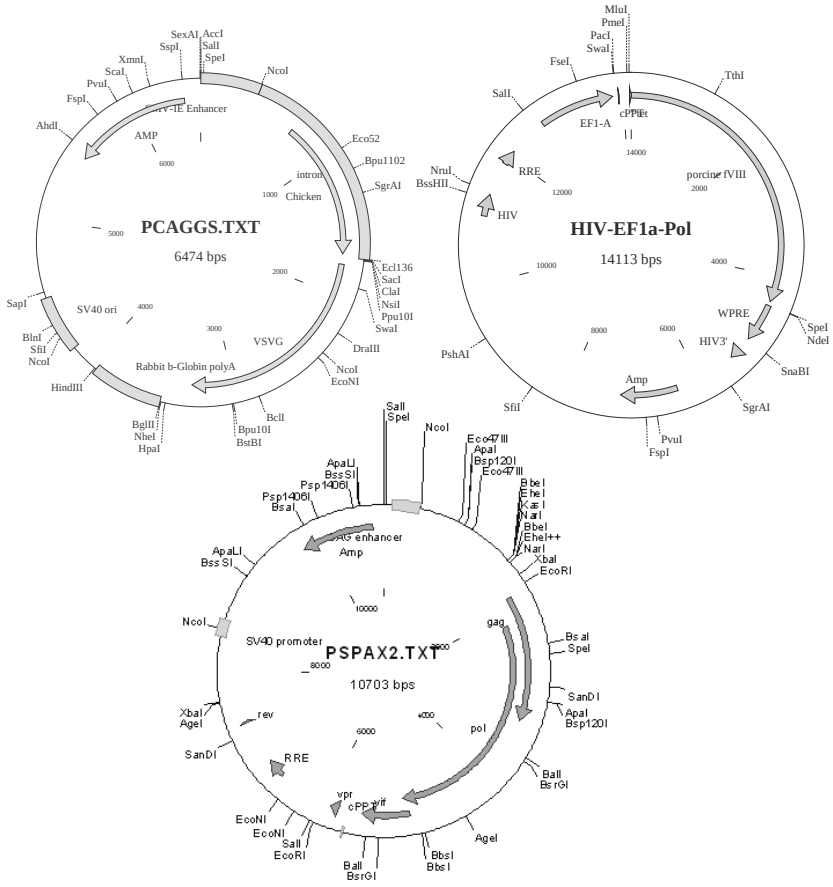


Figure 4.1 – 3-plasmid system for production of HIV. HIV virus is produced by 3-plasmid co-transfection into 293T cells. PCAGGS contains the gene for VSVG necessary for proper pseudotyping. PSPAX2 contains the gag, pro, pol genes encoding viral proteins necessary for viral particle production. HIV-EF1α-pfVIII is the HIV backbone containing the pfVIII transgene and the elongation factor 1α promoter.

8μg polybrene. Six days after transduction, genomic DNA was isolated from each well and the amount of provirus per cell was quantified using real-time PCR. HIV-βg-pfVIII was produced by the same method, however, the HIV-βg-pfVIII viral expression vector was first created as follows: a ~3.4 kb region containing the β-globin promoter and locus control region was amplified from SIV-βg-GFP plasmid DNA. The forward primer 5`-GGT TCG AAC GCG TCG ACA GGA TCA ATT CG-3` was designed to change an XbaI restriction enzyme recognition site to a SalI recognition site. Reverse primer 5`-CTT GCT CAC CAT GGT GGA TAT CGG TGG ATC-3` was designed to change an AgeI recognition site to a EcoRV recognition site. The PCR product was digested with SalI and EcoRV to give a 3429 bp product that was gel purified. A 1287 bp region containing the EF1α promoter was cut out of the HIV-pfVIII expression vector using SalI and SwaI and the 12.8 kb backbone was gel purified. The 3429 bp β-globin promoter region and the 12.8 kb HIV-pfVIII backbone were ligated together to create the HIV-βg-pfVIII plasmid. For comparative analyses, HIV-βg-pfVIII was also produced using Lipofectamine 2000, as described in previous chapters.

HIV antigen detection. HIV p24 antigen quantification was carried out using a HIV-1 p24 antigen capture assay kit (Advanced BioScience Laboratories, Inc; Kensington, MD). Briefly, 25 μL disruption buffer was added each well of a microtiter plate, which comes coated with 2 murine monoclonal antibodies against unique HIV-1 p24 epitopes. A standard curve was created by adding 100μL of p24 standard dilutions ranging from 100 pg/mL to 3.1 pg/mL, as this is

the linear range in which the assay is sensitive. Viral test samples were diluted 1:10^6 in order to fall within the range of the standard curve and samples were added to microtiter wells containing disruption buffer. Plate was covered and incubated for 60 minutes at 37°C. Wells were washed with 300 µL wash buffer then 100 µL conjugate solution was added to each well then incubated for 60 minutes at 37°C. Conjugate solution contains peroxidase-conjugated human anti-p24 polyclonal antibodies. Wells were washed again and 100 µL peroxidase substrate was added to each well. Plate was incubated at room temperature for 30 minutes, resulting in the substrate's blue color change. After this final incubation, 100 µL stop reagent was added to each well, which instantly changes the blue to yellow. Absorbance at 450 nm was read using a microelisa plate reader. Concentration of p24 in viral test samples was extrapolated from the standard curve.

Quantification of DNA copy number. Total DNA from transduced cells was isolated using the DNeasy® Blood & Tissue Kit following the manufacturer's protocol for genomic DNA isolation from tissue culture cells. DNA was quantified spectrophotometrically by A_{260} in dH_2O. DNA copy numbers were determined by real time PCR using forward primer 5`-ACTTGAAAGCGAAAGGGAAAC-3` and reverse primer 5`-CGCACCCATCTCTCTCCTTCT-3` which amplify a sequence within the psi packaging region of HIV vectors. DNA copy numbers were also quantified using forward primers 5` - CCGTTGTCAGGCAACGTG -3` and

reverse primer 5` - AGCTGACAGGTGGTGGCAAT -3` which amplify a sequence in the WPRE region.

Viral RNA isolation and quantification. Viral RNA was isolated from HIV-pfVIII and HIV-GFP viral stocks using the Qiagen's Viral RNA mini kit following the manufacturer's protocol. RNA was quantified by spectrophotometrically by $A_{260.}$ FVIII RNA quantification was performed using SYBR GREEN PCR Master Mix, TaqMan Reverse Transcription Reagents, and ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), as described previously (Doering et al 2009; Doering et al 2002b; Dooriss et al 2009). PCR reactions were carried out in 25µL containing 1x SYBR Green PCR master mix, 300 nmol forward and reverse primers, 12.5 units of MultiScribe reverse transcriptase, 10 units of RNase Inhibitor, and 5ng of sample RNA. The number of transcripts per cell was determined using an average value of 142 transcripts per 5 ng of RNA.

4.4 – Results

5-FU pretreatment for HSC enrichment

Attempting to increase fVIII expression in SIV-βg-pfVIII transplanted mice, the transducing cell population was changed from Sca-1^+ cells isolated from whole bone marrow by immunomagnetic bead separation, to 5-fluorouricil (5-FU)-pretreated whole bone marrow cells. 5-FU is part of a group of chemotherapy drugs known as the anti-metabolites that inhibit cell division by preventing DNA

synthesis. When administered systemically before bone marrow cell isolation, 5-FU induces apoptosis in dividing cells, leaving only quiescent cells in the bone marrow. In this study, 5-FU was administered at a dose of 150 mg/kg by intraperitoneal injection 2 days before bone marrow was harvested. 5-FU pretreatment increased the percent of Sca-1^+ cells in the bone marrow from 8.9% to 73.4% after 48 hours **(Table 4.1).** Following bone marrow cell harvest and *ex vivo* incubation in the presence of mSCF, mIL-3, hIL-11, and hFlt-3, cells were transduced with SIV-βg-pfVIII at an MOI of ~200 and transplanted into 5 lethally irradiated hemophilia A mice. Peripheral blood cells were measured by flow cytometry to ensure engraftment of donor cells **(Figure 4.2)**. Although all mice exhibited ~95% donor engraftment, none expressed sustained fVIII **(Figure 4.3).** These results suggest that pretreatment with 5-FU does not increase SIV-βg-pfVIII transduction efficiency of HSCs.

Comparison of viral production protocols

Due to the large amount of virus needed in Sca-1^+ cell transductions for the evaluation of *in vivo* transgene expression, a better method of viral production was needed. The current protocol for viral production used Lipofectamine 2000 to co-transfect 293T cells in a 10 cm cell culture dish. This method is extremely costly and resulted in only small volumes of concentrated virus. In order to evaluate the potential for HIV production using a different, and less expensive transfection reagent, HIV particles were created using PEI-mediated co-transfection instead of Lipofectamine 2000. Others in the lab optimized the PEI

Table 4.1 – 5-FU pretreatment for enrichment of HSCs

Mice	Total BMCs	Sca^+ cells	% Sca^+ in BM
No 5FU	9.8×10^7	8.7×10^6	8.9%
5FU pretreated (150 mg/kg)	7.9×10^6	5.8×10^6	73.4%

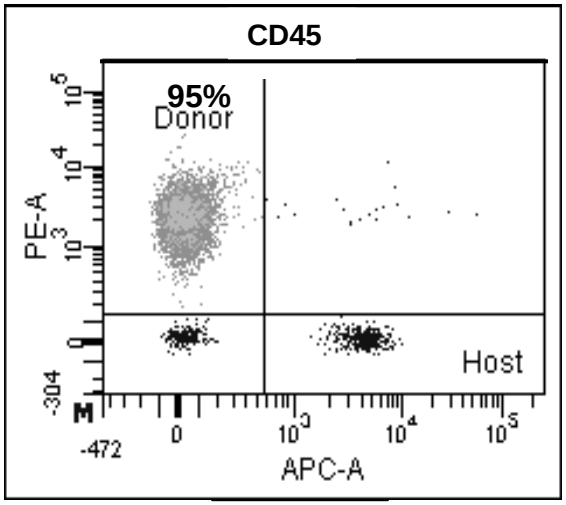


Figure 4.2 – Engraftment in a representative mouse receiving 5FU-pretreated whole bone marrow cells. Following transplantation of transduced cells into hemophilia A mice, engraftment of donor cells was measured by flow cytometry. Donor cells were stained with a PE-conjugated CD45.1 antibody and host cells stained with a APC-conjugated CD45.2 antibody. The percent engraftment was determined by the ratio of donor cells to host cells.

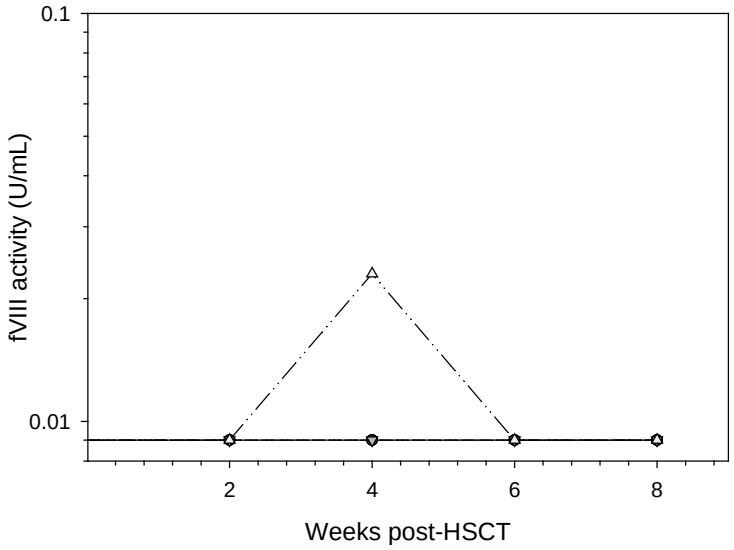


Figure 4.3 – Measure of fVIII activity in 5-FU pretreated bone marrow cells. Two mice were treated with 150 mg/mL 5-Fluorouricil 48 hours before harvest of bone marrow cells. After harvest, cells were stimulated with cytokines for 2 days then transduced with SIV-βg-pfVIII at an MOI of ~200. The percentage of Sca-1^+ cells was measured by flow cytometry and found to be ~74% Sca-1^+ prior to transplant of 500,000 cells per mouse, n = 5.

transfection protocol for transfection of a single plasmid into BHK-M cells and observed no difference in transfection efficiency compared to Lipofectamine 2000 (personal communication with Pete Lollar's lab). Using a new HIV-based lentiviral vector designed for the comparison of SIV and HIV transduction efficiencies, HIV-βg-pfVIII viral particles were produced using both Lipofectamine 2000-mediated co-transfection and PEI-mediated co-transfection of 293T cells. HIV-βg-pfVIII contains the pfVIII transgene under control of the β-globin promoter and locus control region **(Figure 4.4A)**. All other transfection parameters remained constant in this comparative analysis, including the amount of plasmids used, volume of medium in the transfection well, size of the transfection well, and number of cells to be transfected. Plasmid DNA was combined in serum-free, antibiotic-free medium as follows: 4 μg psPAX, 8 μg HIV-βg-pfVIII expression vector, and 4 μg pVSVG with either 40 μL Lipofectamine 2000 or 6 μg PEI. Plasmid DNA was incubated separately with each transfection reagent complex at room temperature for 20 minutes before adding to a T75 tissue culture flask containing 90% confluent 293T cells. The following day, transfection medium was replaced with fresh DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin. Virus-containing medium was collected twice daily for the next 3 days. After concentrating virus in 1/100th the original volume, viral titer was determined by adding 2, 10, and 25 μL of either Lipofectamine-produced HIV-βg-pfVIII or PEI-produced HIV-βg-pfVIII to each well of a 6-well plate containing ~150,000 cells. The amount of proviral copies present in cells of each transduction well was quantified by RT-PCR 6 days after transduction. The

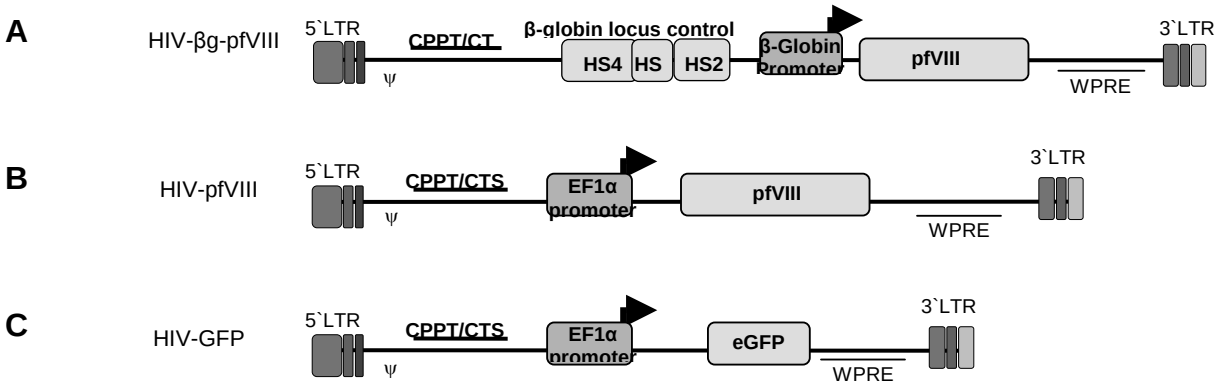


Figure 4.4 – Schematic of HIV vectors. All HIV vectors used in these studies contain a 5' LTR, psi packaging sequence (ψ), central polypurine tract (cPPT) and the central termination sequence (CTS), rev response element (RRE), either eGFP or pfVIII transgene, WPRE sequence, and self inactivating 3`LTR containing a bovine growth hormone poly(A). Transgene expression was controlled by either the β-globin promoter and locus control region (A) or the EF1α promoter (B and C).

resulting quantification showed the percent of gene-modified cells from each transduction sample volume was similar **(Figure 4.5)**, resulting in a Lipofectamine-mediated viral titer of 3.1 x 10^6 functional viral particles (fvp)/mL and a PEI-mediated viral titer of 2.0 x 10^6 fvp/mL. Neither viral production protocol resulted in a HIV-βg-pfVIII titer within an optimal range for high-level transduction, whereby viral titers of >10^7 fvp/mL are needed for transduction of Sca-1^+ cells for use in animal studies. Low viral titer was not a surprise as it is well documented that the size of the proviral sequence negatively correlates with viral titer (Kumar et al 2001). However, based on these results we chose to change our standard transfection protocol to the new PEI protocol due to cost effectiveness. A scaled up system for HIV production using Nunc triple flasks was devised by others in the lab whereby upwards of 8 mL of high-titer concentrated virus can be produced from 4 flasks in 4 days, as described in the Materials and Methods for this chapter.

Comparison of SIV- and HIV-based viruses for erythroid promoter driven pfVIII expression

Based on our previous studies demonstrating the poor efficiency of SIV-βg-pfVIII to transduce Sca-1^+ cells, we hypothesized that changing the lentiviral vector backbone may result in increased transduction efficiency. In order to determine if HIV-βg-pfVIII could effectively transduce cells and secrete protein more efficiently than SIV-βg-pfVIII, we constructed a HIV based vector containing pfVIII under control of the β-globin promoter, HIV-βg-pfVIII **(Figure 4.4A)**. The size of the

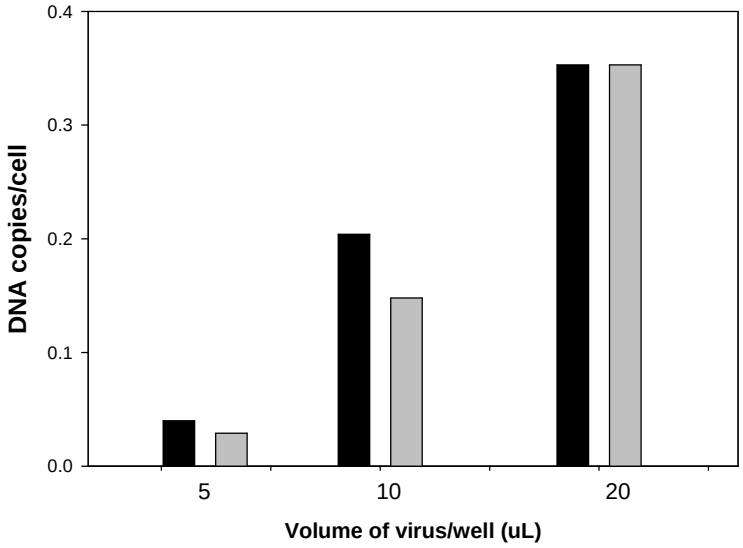


Figure 4.5 – Comparison of viral production protocols using Lipofectamine 2000 or PEI. 293T cells were co-transfected with a 3-plasmid system for the manufacture of 2 batches of SIV-βg-pfVIII using either Lipofectamine 2000 or PEI. Virus was collected and concentrated by overnight centrifugation at 10,000 x g. Cells were seeded at 100,000 cells/well in duplicate 6-well plates and 5, 10, or 20μL of each virus batch were added to the wells. DNA copy number was determined by RT-PCR 7 days after transduction. Black bars represent data from Lipofectamine-produced virus and gray bars represent data from PEI-produced virus.

HIV-βg-pfVIII proviral sequence was comparable to that of the SIV-βg-pfVIII sequence, at 10.6 kb and 10.4 kb respectively. K562 cells were transduced at an MOI of 1, 5, and 10 with either SIV-βg-pfVIII or HIV-βg-pfVIII. DNA copy number and fVIII expression were measured from each transduction well 6 days after transduction. SIV-βg-pfVIII-mediated fVIII expression in K562 cells ranged from 0.01 – 0.2 units/10^6 cells/24hr and DNA copies ranged from 2 – 17 copies/cell **(Figure 4.6A).** HIV-βg-pfVIII-mediated fVIII expression in K562 cells ranged from 0.01 – 0.03 units/10^6 cells/24hr and DNA copies ranged from 3 – 7 copies/cell**.** When fVIII activity was normalized to DNA copy number for all MOIs tested, a 3-fold difference in expression was observed for SIV-βg-pfVIII and SIV-βg-pfVIII, whereby SIV-βg-pfVIII-transduced cells produced 0.011 units/10^6 cells/24hr/copy and HIV-βg-pfVIII-transduced cells produced 0.003 units/10^6 cells/24hr/copy respectively **(Figure 4.6B)**. Based on these results, we determined that transduction with HIV-βg-pfVIII resulted in less fVIII activity per copy compared to transduction with SIV-βg-pfVIII and changing to HIV-βg-pfVIII would most likely not increase HSC transduction.

Characterization of HIV-pfVIII and HIV-GFP titers

Complete characterization of each viral batch used for transduction is essential in order to achieve reproducible and interpretable results. This was especially true for the transductions outlined above because the method of viral production was changed. Using the new PEI transfection protocol, HIV-pfVIII and HIV-GFP **(Figure 4.4B and C)** were produced at high titer for use in the analysis of

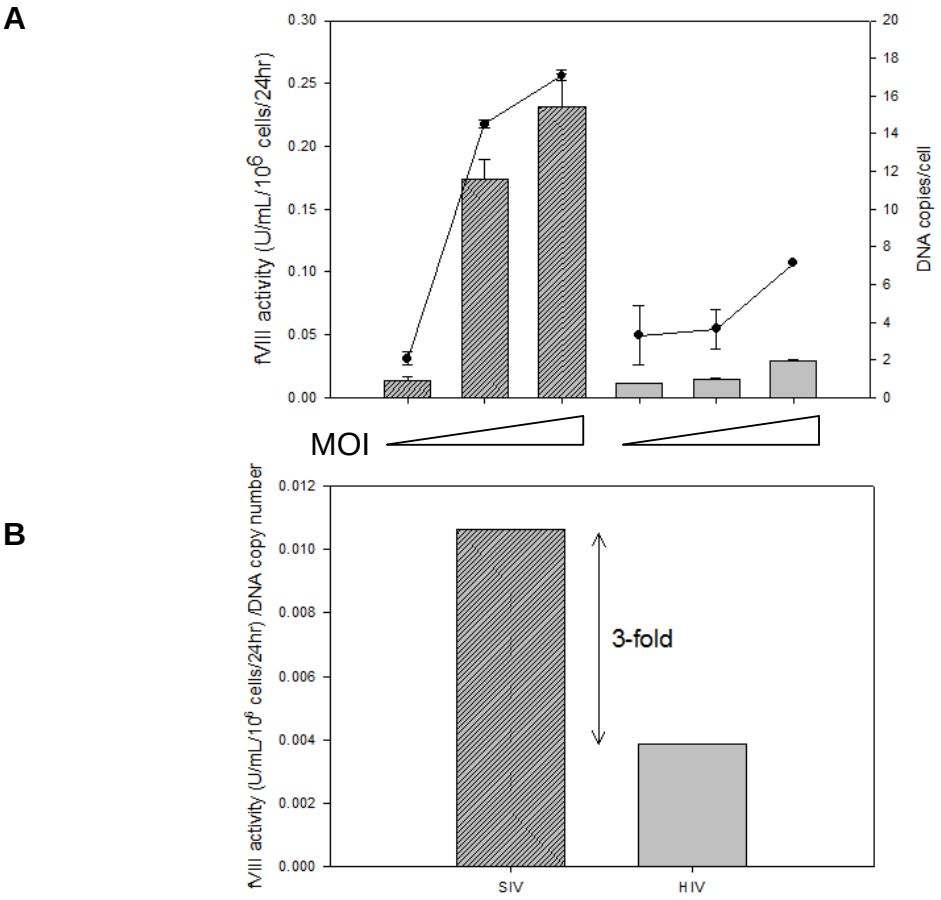


Figure 4.6 – Comparison of SIV-βg-pfVIII and HIV-βg-pfVIII. K562 cells were transduced in duplicate wells with either SIV-βg-pfVIII or HIV-βg-pfVIII at an MOI of 1, 5, and 10. Six days after transduction, fVIII activity was measured by one stage coagulation assay and DNA copy number was measured by real time PCR. A) SIV-βg-pfVIII activity is depicted as hatched bars and HIV-βg-pfVIII activity is depicted as gray bars. DNA copy number per cell is shown as black circles. Triangles below graph signify the increasing MOIs used for transduction, equal to 1, 5, and 10 fvp/cell. B) FVIII activity was normalized to DNA copy number. Each bar represents the average fVIII activity per DNA copy number of

all 3 MOIs tested for either SIV-βg-pfVIII- or SIV-βg-GFP-transduced K562 cells. SIV-βg-pfVIII fVIII/copy is shown as a hatched bar and HIV-βg-pfVIII fVIII/copy is shown as a gray bar.

potential transgene-specific and/or cell type-specific variances in transduction efficiency. Both viral vectors contain a self-inactivating U3 deletion and the EF1α promoter driving ubiquitous transgene expression. HIV-pfVIII titers typically ranged from 1 x 10^7 – 5 x 10^7 fvp/mL and HIV-GFP titers from 1 x 10^8 – 5 x 10^8 fvp/mL using PEI co-transfection for lentiviral production. Following viral production and concentration, the functional viral titer was determined by adding 0.5 – 10 µL of virus to each well of a 6-well plate containing ~150,000 cells in a total of 1 mL of complete medium. Six days after transduction, genomic DNA was isolated from each well and DNA copy number was quantified by real time PCR. Using primers that amplify a sequence within the WPRE region present in both HIV-pfVIII and HIV-GFP **(Figure 4.7A)**, the functional viral titer for HIV-pfVIII was 4.8 x 10^7 fvp/mL and 6.7 x 10^8 fvp/mL for HIV-GFP. Using primers that amplify a sequence within the psi packaging region, functional viral titer for HIV-pfVIII was 4.5 x 10^7 fvp/mL and 2.1 x 10^8 fvp/mL for HIV-GFP. HIV-GFP titer was 4-9-fold higher than the functional viral titer for HIV-pfVIII using an average of titers calculated using the psi primers and the WPRE primers **(Figure 4.7B)**. As a loading control, DNA was also amplified with a β-actin primer set. Analysis of the number of β-actin copies per cell confirmed that equal amounts of each DNA sample were loaded **(Figure 4.8).** In order to maintain consistency in DNA quantification throughout these experiments, only the psi primer set was used for viral titering and all other DNA quantification by RT-PCR. A single experiment was performed to ensure all transduction parameters were established and equal amounts of proviral integration were achieved by calculation of virus volume from

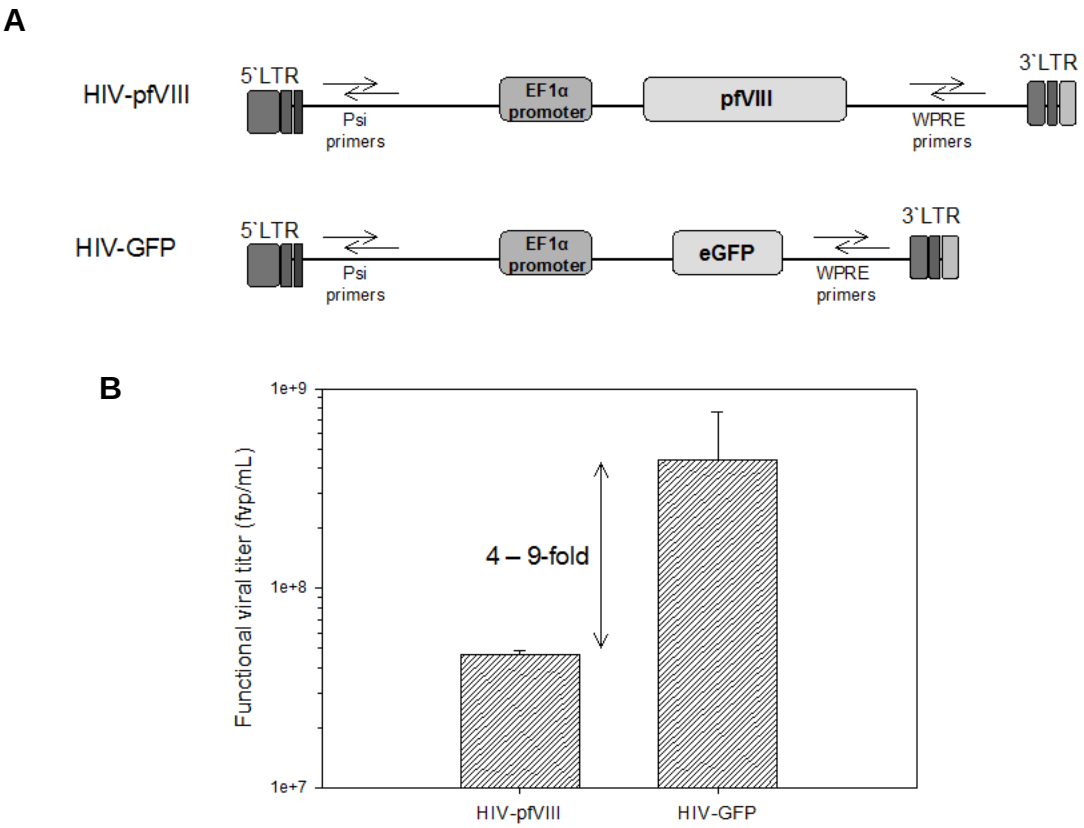


Figure 4.7 – Primers binding sites and calculation of functional viral titer. HIV-pfVIII and HIV-GFP were titered on 293T cells using 2 sets of primers. A) Schematic of HIV-pfVIII and HIV-GFP showing the regions where the psi and WPRE primer sets amplify. B) Average of functional viral titers for HIV-pfVIII and HIV-GFP calculated using both sets of primers is shown.

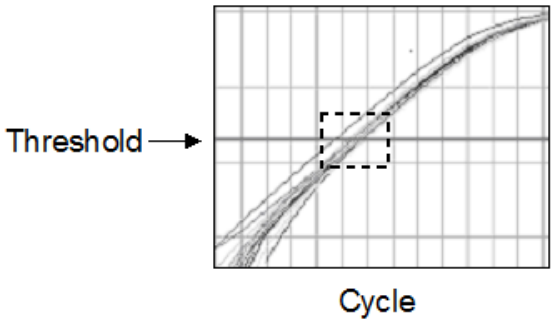


Figure 4.8 – β-actin amplification of HIV-GFP and HIV-pfVIII sample DNA. DNA samples used for titering HIV-GFP and HIV-pfVIII were quantified by RT-PCR using β-actin specific primers to ensure correct and consistent loading of DNA into each well. The dotted box shows β-actin amplication of all gDNA sample wells crossing the threshold at nearly equivalent cycle numbers.

the functional viral titer. 293T cells were transduced with either HIV-pfVIII or HIV-GFP at an MOI of 5 and reverse transcribed proviral cDNA copies were measured over time. The number of DNA copies/cell at each time point was similar for HIV-GFP and HIV-pfVIII, ranging from ~3 –13 copies/cell from 6 hours to 3 days after transduction **(Figure 4.9)**. Although functional viral titering is commonly used to describe the amount of active viral particles present in a viral stock, a number of other characteristics may be helpful in describing a batch of virus. Functional viral titering is beneficial in determining the amount of virus integrated into the host cellular genome, however, the amount of virus that does not integrate is also important in the characterization of viral stocks. In order to calculate the total amount of virus, including both functional and non-functional viral particles, an ELISA was performed to measure the amount of p24 antigen in each viral stock. P24 is a core protein present within each viral particle and the amount of p24 correlates with the number of viral particles. HIV-pfVIII and HIV-GFP viral stocks were serially diluted to $1:10^6$ in order to fall within the range of the assay and p24 titer was calculated from a standard curve of known p24 dilutions. The p24 titer for HIV-pfVIII was 1.4×10^4 ng/mL and 1.6×10^4 ng/mL for HIV-GFP. When comparing these titers to the functional titers determined by RT-PCR, 5.3-fold more total viral p24 of HIV-pfVIII than HIV-GFP were needed in order to achieve the same functional viral titer, assuming both HIV-pfVIII and HIV-GFP contain a similar ratio of p24 to total viral particles. Viral stocks were also characterized by

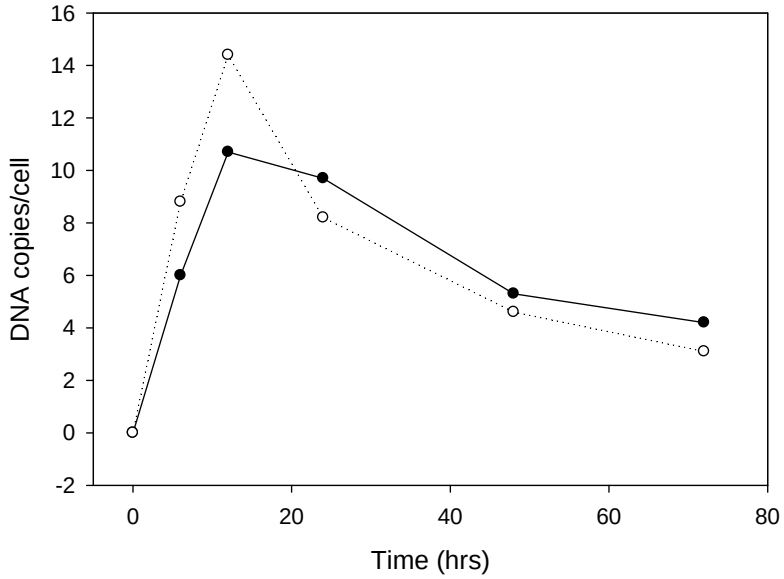


Figure 4.9 – HIV-GFP and HIV-pfVIII DNA cDNA copies after transduction. 200,000 cells/well in a 6-well plate were transduced with either HIV-GFP or HIV-pfVIII at an MOI of 5. The volume of virus needed to achieve this MOI was calculated based on the titer determined by RT-PCR using the psi primer set. HIV-GFP DNA copy number/cell is shown as black circles and HIV-pfVIII copy number is shown as white circles.

quantification of the amount of proviral RNA present. Analysis of viral RNA by RT-PCR revealed 3.7-fold more HIV-GFP transcripts than HIV-pfVIII transcripts were present in each respective viral stock. Viral RNA quantification was complicated by the use of carrier RNA for viral RNA isolation. As such, the total quantity of RNA isolated from each viral stock contained both viral and carrier RNA. However, since equal amounts of carrier RNA were used in the isolation of HIV-GFP and HIV-pfVIII viral RNA, the difference in calculated viral transcripts per nanogram of RNA is equal to the difference in the number of viral transcripts in each viral stock **(Table 4.2)**. Together, these data suggest that fewer HIV-pfVIII than HIV-GFP viral RNA genomes are efficiently packaged into viral particles, and fewer HIV-pfVIII than HIV-GFP viral particles per volume of virus are able to integrate into the genome of transduced cells.

Determination of transgene- or cell-specific differences in lentiviral transduction

Based on previous studies by myself and coworkers, a difference in the transduction abilities of a pfVIII-containing lentivirus and a GFP-containing lentivirus may exist in hematopoietic stem and progenitor cells. From p24 quantification in HIV-pfVIII and HIV-GFP viral stocks, we know a greater amount of total HIV-pfVIII particles are needed in order to achieve the same amount of integrated provirus as HIV-GFP in 293T cells. However, little is known about the kinetics of HIV-pfVIII proviral reverse transcription or nuclear import prior to integration. In order to determine if a transgene-specific difference in transduction

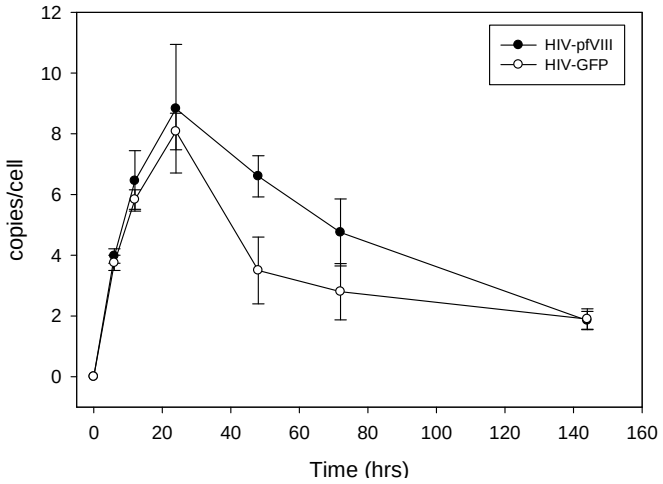
Table 4.2 – Comparison of viral quantification methods

Virus	Functional viral titer	p24 titer	RNA concentration
HIV-pfVIII	4.5×10^7 fvp/mL	1.6×10^4 ng/mL	4.6×10^5 transcripts
HIV-GFP	2.1×10^8 fvp/mL	1.4×10^4 ng/mL	1.7×10^6 transcripts

efficiency exists, 293T, K562 and Sca-1^+ cells were transduced at an MOI 5 and the number of proviral DNA copies/cell were calculated over time. Transduced 293T and K562 cells were measured for up to 6 days and Sca-1+ cells were measured for up to 3 days. In transduced 293T cells, pfVIII copies/cell ranged from 1.9 ± 0.3 – 7.4 ± 1.9 from 6 hours to 6 days post-transduction and GFP copies/cell ranged from 1.9 ± 0.3 – 6.8 ± 0.3 from 6 hours to 6 days post-transduction **(Figure 4.10A)**. No significant differences in copy number or percent of maximum copy number were seen during this time, whereby the maximum copy number/cell was reached at 24 hours after transduction for both HIV-pfVIII and HIV-GFP **(Figure 4.10B)**. In transduced K562 cells, pfVIII copies/cell ranged from 1.5 ± 0.1 – 4.9 ± 0.7 from 6 hours to 6 days post-transduction and GFP copies ranged from 1.0 ± 0.5 – 3.7 ± 0.7 from 6 hours to 6 days post-transduction **(Figure 4.11A).** Similar to the 293T transduction, no significant differences in copy number or percent of maximum copy number were seen during this time **(Figure 4.11B).** However, by 6 hours after transduction, 97% of maximum number of copies per K562 cell had been reached, whereas only 43% had been reached by 6 hours in 293T cells, suggesting that lentivirus entry and reverse transcription occur faster in K562 cells than in 293T cells. However, the total number of integrated transgenes/cell was lower in K562 than in 293T cells**.**

In the erythroid promoter studies described in the previous chapter, mice transplanted with Sca-1^+ cells transduced with SIV-βg-GFP exhibited ~10%

A



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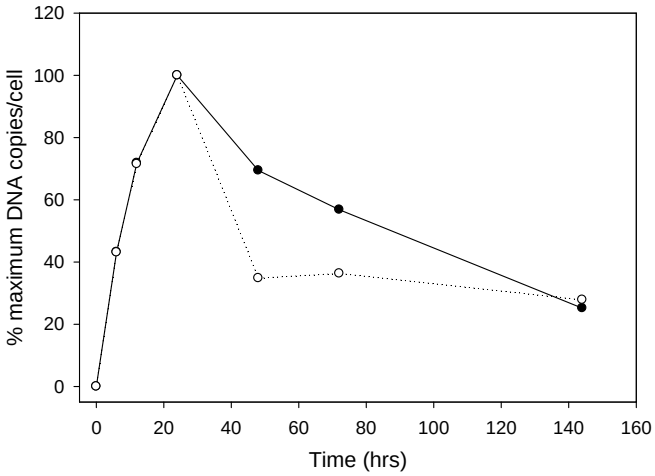
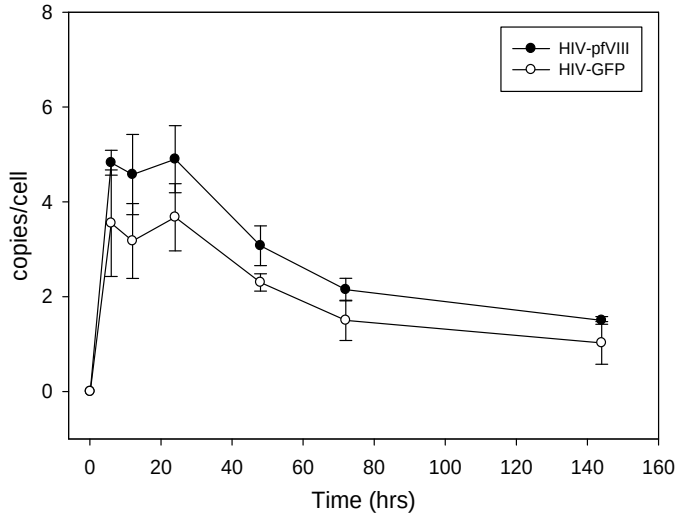


Figure 4.10 – Rates of HIV-pfVIII and HIV-GFP proviral production and degradation in 293T cells. ~300,000 293T cells were transduced in duplicate at an MOI of 5 in 1mL in a 6 well plate. DNA was isolated at 6, 12, 24, 48, 72, and 144 hours after transduction and A) the number of transgene copies/cell was determined by RT-PCR. B) Same data plotted as the % of maximum copy number. PfVIII copies are depicted as black circles and GFP copies are depicted as white circles. Each point represents 2 measurements of 2 identical transduction wells.

A



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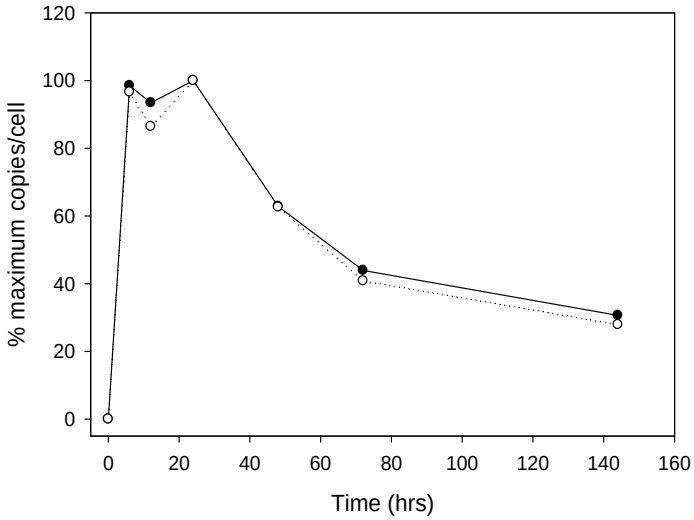

Figure 4.11 – Rates of HIV-pfVIII and HIV-GFP proviral production and degradation in K562 cells. ~300,000 K562 cells were transduced in duplicate at an MOI of 5 in 1mL in a 6 well plate. DNA was isolated at 6, 12, 24, 48, 72, and 144 hours after transduction and A) the number of transgene copies/cell was determined by RT-PCR. B) Same data plotted as % of maximum copy number. PfVIII copies are depicted as black circles and GFP copies are depicted as white circles. Each point represents 2 measurements of 2 identical transduction wells.

GFP$^+$ cells, but no mice transplanted with SIV-βg-pfVIII-transduced cells possessed detectable pfVIII-modified cells in either primary or secondary HSCT recipients. Using a SIV vector expressing GFP under control of the ubiquitous MSCV LTR, upwards of 60% of peripheral blood cells were GFP$^+$ after transduction and transplantation, however, no mice transplanted with SIV-MSCV-pfVIII-transduced cells possessed detectable gene-modified cells. In the current study, Sca-1$^+$ cells were transduced with HIV-GFP and HIV-pfVIII to determine if a transgene-specific difference in transduction efficiencies could be detected. Transduced Sca-1$^+$ cells exhibited similar kinetics of reverse transcription compared to 293T and K562 cells whereby both HIV-GFP and HIV-pfVIII viral RNA were efficiently reverse transcribed inside the cell. HIV-GFP-transduced cells possessed 7.5 ± 2.5 – 18.8 ± 2.1 copies/cell from 6 hours to 72 hours post-transduction and HIV-pfVIII-transduced cells possessed 15.5 ± 0.8 – 41.3 ± 8.0 copies/cell up to 72 hours after transduction **(Figure 4.12A).** HIV-GFP-transduced cells reached a maximum copy number of ~19 copies/cell 12 hours after transduction, which persisted up to 24 hours. HIV-pfVIII-transduced cells reached a maximum copy number of ~41 copies/cell 24 hours after transduction **(Figure 4.12B)**. These data show that similar kinetics of reverse transcription were exhibited by both HIV-pfVIII and HIV-GFP in 293T, K562, and Sca-1$^+$ cells, whereby all transduced cells reached a maximum number of proviral cDNA copies 12 – 24 hours after transduction **(Figure 4.13)**. By 72 hours after transduction, proviral copy number dropped 40% – 60% in all 3 cell types tested. These results suggest that the reason for the transgene-dependent divergence in

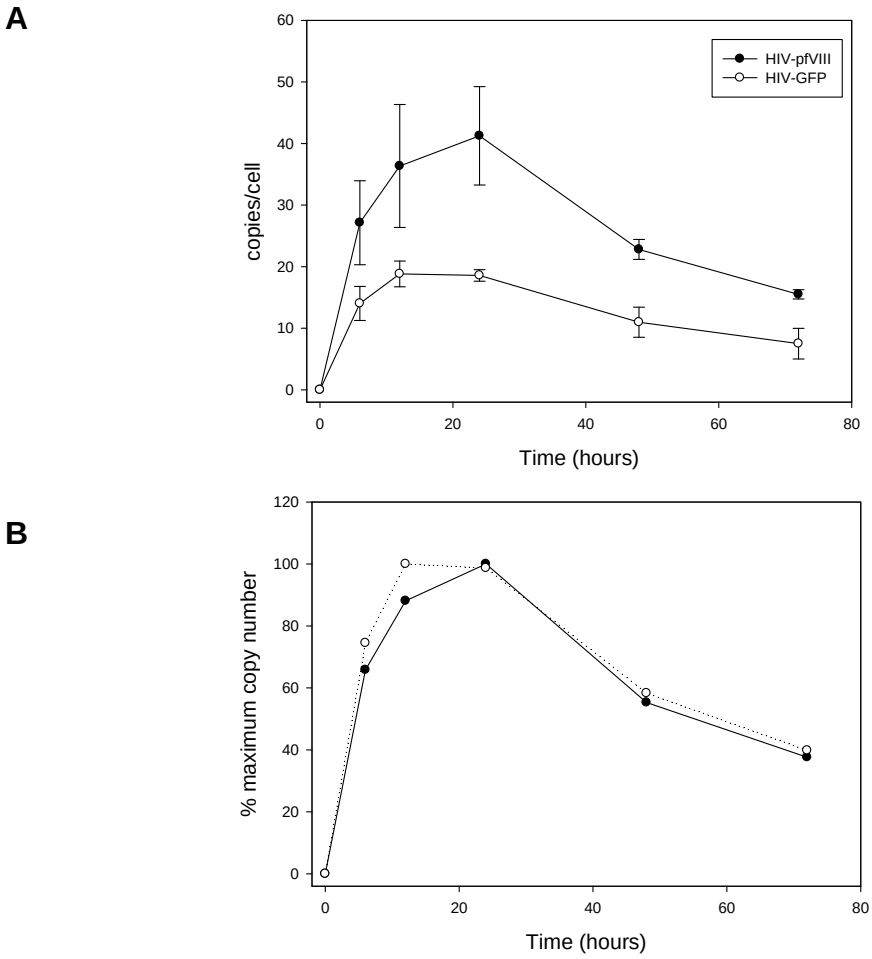

Figure 4.12 - Rates of HIV-pfVIII and HIV-GFP proviral production and degradation in Sca-1$^+$ cells. Sca-1$^+$ cells were isolated from mice and cultured in cytokine-containing media for 60 hours prior to transduction at an MOI of 5. Cells were transduced with either HIV-pfVIII or HIV-GFP at a density of 10^6 cells/mL in 500μL complete medium containing a standard cytokine cocktail in a 24-well plate. A) DNA was isolated at 6, 12, 24, 48, and 72 hours after transduction and the number of transgene copies/cell was determined by RT-

PCR. B) Same data plotted as % of maximum copy number. PfVIII copies are depicted as black circles and GFP copies are depicted as white circles. Each point represents 2 measurements of 2 identical transduction wells.

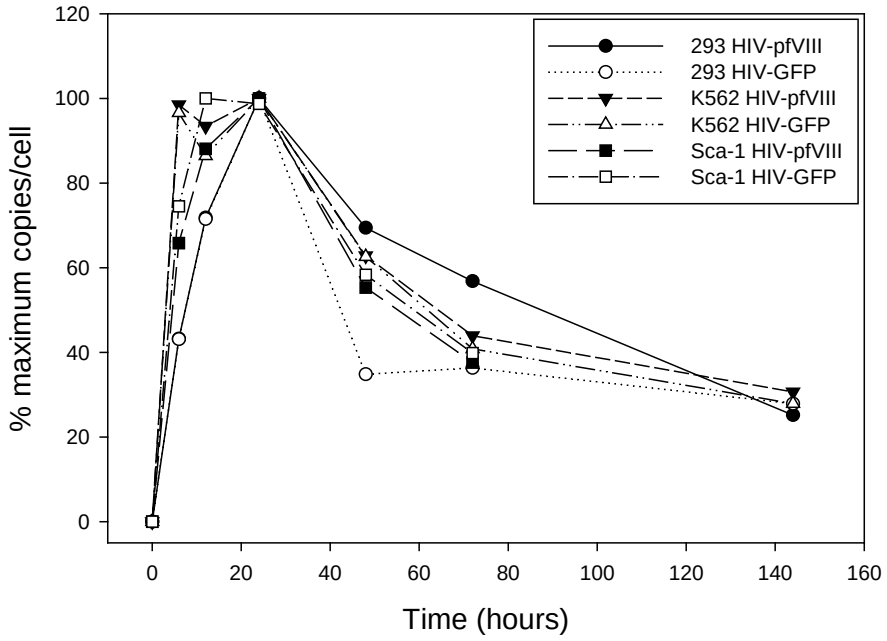

Figure 4.13 - Rates of HIV-pfVIII and HIV-GFP proviral production and degradation in various cell types. 293T, K562, and Sca-1$^+$ cells were transduced in duplicate under similar conditions and the number of DNA copies were measured from 6 hours to 6 days after transduction for 293T and K562 cells, and from 6 hours to 72 hours for Sca-1$^+$ cells. The number of proviral copies was calculated by RT-PCR using a primer specific to the psi region of HIV-pfVIII and HIV-GFP.

HSC transduction efficiencies is not inefficient viral entry or reverse transcription of proviral RNA, but most likely inefficient nuclear import or integration of proviral cDNA into the host genome.

4.5 – Discussion

The studies presented here focus on the complexity of lentiviral production, quantification, and transduction. Lentiviral transduction comprises a series of complicated and highly regulated steps from the production of viral particles to the integration of the proviral genome into transduced cells. We first showed that using 5-FU-pretreated bone marrow for transduction and transplantation did not give rise to any mice with fVIII expression, suggesting these cells were not transduced better than Sca-1^+ cells isolated by immunomagnetic bead selection. Next, we demonstrated that a HIV vector incorporating pfVIII under control of the β-globin promoter and locus control region did not transduce cells as well as a SIV vector with similar proviral sequence. Although others have demonstrated the successful transduction of human and murine HSCs using HIV-based vectors (Dupuy et al 2005; Sutton et al 1999; Sutton et al 1998), still others argue that SIV-based vectors are more efficient in HSC transduction (Hanawa et al 2004b; Kikuchi et al 2004; Ohmori et al 2006). Wild type HIV-1 displays less than 50% sequence homology with SIV, however both display common routes of transmission, long incubation periods, and kinetics of replication in the infected host. However, the pathogenicity and rate of HIV-1 transmission is greater than SIV (Gilbert & Wong-Staal 2001). We hypothesized that a HIV vector system

may also possess greater transduction efficiency than a SIV system. However, transduction of SIV-βg-pfVIII and HIV-βg-pfVIII into 293T and K562 cells resulted in fewer integrated proviral copies and less fVIII expression per copy in HIV-βg-pfVIII-transduced cells compared to SIV-βg-pfVIII-transduced cells. Viral titers achieved in the production of HIV-βg-pfVIII were consistently below levels necessary to transduce Sca-1^+ cells for *in vivo* studies. This was not surprising as it is generally accepted that lentiviral particles are capable of encapsidating vector RNA up to the length of the wild type proviral RNA. The 10.6 kb HIV-βg-pfVIII proviral sequence is near the limit of the packaging capacity of a HIV-1 virus, which is thought to accommodate an expression cassette of 8 – 11 kb (Srinivasakumar 2001). Proviral sequences larger than the wild type result in decreased titers (Kumar et al 2001)**.**

These studies also demonstrated the complexity of lentiviral quantification and the many parameters involved in the absolute quantification and characterization of lentiviruses. Although the most common means of viral quantification is by calculation of the number of integrated proviral genomes per volume of virus, many parameters must by optimized and standardized in order to achieve reproducible and interpretable data. After optimizing the transduction parameters and the primers used for DNA copy number analyses, we demonstrated that HIV-pfVIII and HIV-GFP have similar rates of proviral reverse transcription and degradation, suggesting they behave similarly in transduced 293T and K562 cells. However, quantification of the total amount of p24 antigen present in HIV-

pfVIII and HIV-GFP viral stocks revealed ~5-fold more HIV-pfVIII viral particles must be added to cells in order to result in the same number of integrated viral genomes as HIV-GFP in transduced cells. One reason for the difference in the percentage of functional viral particles in HIV-pfVIII and HIV-GFP viral stocks may be that the larger size of the expression cassette in HIV-pfVIII compared to HIV-GFP causes less efficient viral encapsidation, resulting in fewer properly packaged viral particles capable of cellular infection. However, others have shown that most viral particles present in the viral stock are initially active and viral integration is limited by variable rates of viral diffusion and decay (Andreadis et al 2000).

Previous studies with SIV vectors expressing pfVIII or GFP under the control of either the β-globin promoter or an internal MSCV LTR promoter demonstrated a transgene-specific difference in HSC transduction efficiency. Using HIV-pfVIII and HIV-GFP, Sca-1^+ cells were transduced and the rates of proviral reverse transcription were measured by quantification of the amount of reverse transcribed proviral cDNA present in transduced cells. We demonstrated that the kinetics of reverse transcription of HIV-pfVIII and HIV-GFP proviral RNA in Sca-1^+ cells were similar to that seen in 293T and K562 cells. Surprisingly, more pfVIII than GFP copies were reverse transcribed from 6 hours to 72 hours after transduction, suggesting that more HIV-pfVIII viral particles were entering Sca-1^+ cells than HIV-GFP particles. In all cell types tested, ~5-fold more HIV-pfVIII particles were added in order to achieve the same number of integrated proviral

copies as HIV-GFP, as determined by functional viral titering on 293T cells. However, Sca-1$^+$ cells were the only cells to exhibit a significant difference in the number of pfVIII and GFP copies/cell over time. Although this data demonstrates that both viruses efficiently enter cells and become reverse transcribed, the steps occurring after reverse transcription have not been investigated**.** Previous data from multiple experiments has shown that the final number of integrated pfVIII copies is lower than the final number of GFP copies in peripheral blood cells of mice transplanted with transduced Sca-1$^+$ cells. Taken together with the current data, these studies suggest that HIV-pfVIII and HIV-GFP transduction efficiencies diverge after proviral reverse transcription **(Figure 4.14)**. Although the reason for this divergence is unknown, it may be due to inefficient nuclear import of the lentiviral PIC or inefficient genomic integration.

In summary, these studies demonstrate the importance of viral characterization and standardization of viral production, transduction, and quantification protocols. We also presented data suggesting that the divergence in transduction efficiencies of a pfVIII-containing-lentivirus and a GFP-containing-lentivirus occurs at a step in transduction after the reverse transcription of lentiviral RNA within the cell. From these studies, we may now focus efforts to increase lentiviral-mediated transduction of Sca-1$^+$ cells on improving proviral cDNA nuclear translocation and/or genomic integration. With these future studies, it may be possible to increase HSC transduction efficiency and provide high-level sustained fVIII expression *in vivo* from gene-modified HSCs.

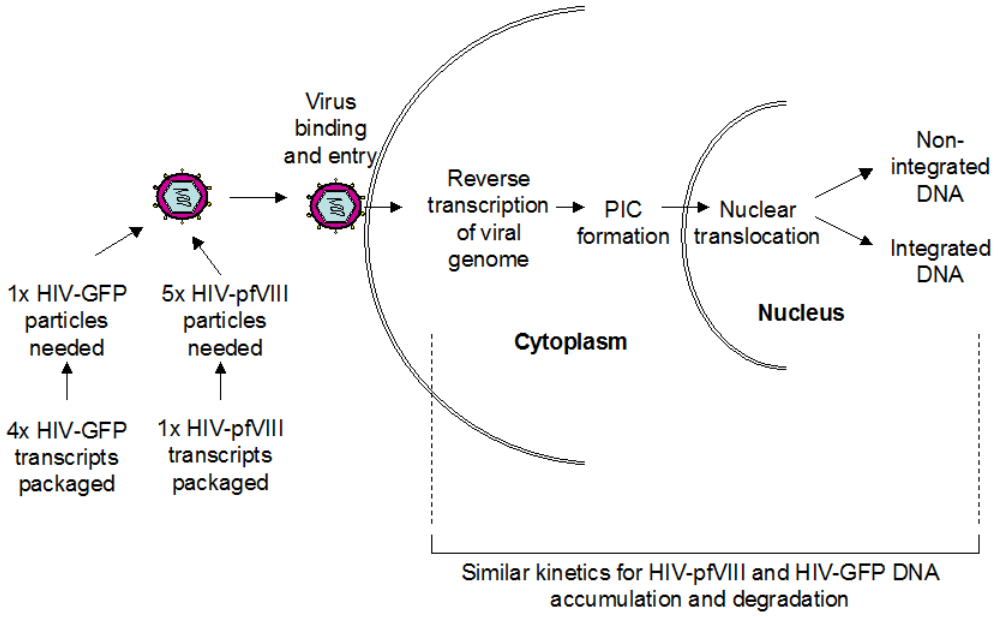

Figure 4.14 – Schematic of the similarities and differences in the kinetics of lentiviral transduction of Sca-1^{+} cells. HIV-pfVIII and HIV-GFP viral particles bind to cells equivalently by virtue of the VSV-G envelop proteins present on both. The viral RNA genome is reverse transcribed then complexed with viral and cellular proteins to form the pre-integration complex (PIC). The PIC is translocated into the nucleus via active transport where it either integrated into the cellular genome, or becomes circularized and initiates a pathway to degradation

Chapter 5:

Conclusions and Future Directions

5.1 – Conclusions

As outlined in the preceding chapters, the field of hemophilia A gene therapy has made significant advances in recent years. The reengineering of fVIII transgenes and viral vectors used for gene delivery has improved both the safety and efficacy of hemophilia A gene therapy. Although many diseases are considered candidates for treatment by gene therapy, hemophilias A and B are among the most prevalent monogenic diseases currently researched. Many factors contribute to the extensive research of hemophilia gene therapy including the availability of large and small animal models, disease prevalence, width of therapeutic window, availability of fVIII and fIX cDNA, and the presence of transducable target cells with access to the bloodstream. Currently, hemophilia A mouse, dog, and sheep colonies exist for preclinical research studies, which will be necessary to move any gene therapy product into clinical trials.

With proper treatment, hemophiliacs can live nearly normal lives, however life expectancy varies with disease severity and availability to adequate treatment. The disease phenotype for hemophilia A ranges from mild to severe, depending on the amount of functional fVIII in circulation, whereby 50% of patients are severe hemophiliacs and express less than 1% of normal fVIII activity. The characteristic symptoms include internal and external bleeding episodes, whereby pathogenesis varies with disease severity. Patients with the severe form of the disease are subject to spontaneous and frequent bleeding episodes while patients with mild hemophilia may only experience excessive bleeding after

surgery or serious trauma. However, the most serious sites of bleeding are joints, muscles, digestive tract, and the brain. Repeated joint bleeds can cause permanent joint damage and disfigurement that can lead to arthritis or immobility. Hemophilia A has a notoriously wide therapeutic window because only small increases in the amount of circulating fVIII are necessary to improve disease phenotype.

One of the most important aspects of gene therapy is a solid understanding of the viral platforms used for gene transfer. Since the discovery of oncogenic transformations in a number of patients involved in a clinical trial for SCID-X1 disease, the field of gene therapy has focused on the development of a safer gene transfer vector. The use of SIN lentiviral vectors in combination with an internal cell-specific promoter is gaining credence as a safe and effective gene transfer strategy, especially for use in HSC transduction. Although gene therapy as a cure for disease is still a number of years away from becoming a clinical treatment option, hemophilia A is positioned to be one of the first genetic diseases cured by gene therapy.

Development of recombinant fVIII transgenes and IP issues

One of the biggest obstacles hindering the success of hemophilia A gene therapy is low fVIII expression following gene transfer. We hypothesized that using a higher expressing recombinant fVIII transgene might overcome this barrier. Although we previously showed that pfVIII was expressed at 10-100-fold greater

levels than BDD-hfVIII and that the increased expression was due to enhanced protein secretion (Doering et al 2002b; Dooriss et al 2009), a number of novel recombinant human transgenes also claimed to exhibit increased expression compared to BDD-hfVIII. Extensive characterization of fVIII expression from each of these transgenes confirmed that pfVIII was expressed at greater levels than any of the recombinant human transgenes tested, even in lentiviral-mediated HSCT in a hemophilia A mouse model (Dooriss et al 2009). Although this was an important discovery in this aspect of hemophilia A gene therapy research whereby achieving the highest possible fVIII expression was the primary goal, the pfVIII transgene holds little translational ability for us. One reason for this is the intellectual property (IP) rights currently belong to Ipsen™, who are not interested in pursuing gene therapy research. Another reason is that this transgene is not human and patients have always been treated with human fVIII. Although a proprietary purified recombinant pfVIII protein product is currently in Phase III clinical trial initiated by Octogen™, it is unlikely that this transgene can be used in clinical gene therapy treatments. In order to take advantage of the high expression properties of pfVIII, Doering *et al* created a number of human/porcine chimeric transgenes to determine the pfVIII-specific domains necessary for high expression (Doering et al 2004). Using 2 different mammalian expression systems to compare fVIII expression from various human/porcine chimeric transgenes, we showed that only one exhibited fVIII expression levels comparable to pfVIII. This transgene, termed HP-fVIII, contained porcine-specific A1 and A3 domains, and human-specific A2, C1, and C2 domains. Currently, this

high expression humanized fVIII transgene is being researched at Expression Therapeutics (Tucker, GA) for gene therapy applications as well as for the creation of an infusible purified biotherapeutic product, both of which are nearing entry into clinical trials. Interestingly, the IP for the high expression properties of fVIII was owned by Emory originally, but Emory voided their gene therapy patents. This particular IP was returned to the National Institutes of Health (NIH) and discoveries were made with federal funding. Dr. Spencer and colleagues obtained the rights to the IP and started Expression Therapeutics™ to patent these rights and move forward with research projects using HP-fVIII.

Gene transfer platforms

The choice of vector for use in gene therapy depends on a number of factors. Although one vector may be more efficient at transduction and gene expression, it may not be the best-suited gene transfer platform for a specific disease study. Previously, γ-retroviral vectors were thought to offer the best chance of long-term heterologous gene expression by virtue of their stable chromosomal integration following transduction. We previously demonstrated the feasibility of using γ-retroviral vectors for long-term fVIII expression following HSC transduction and transplantation into hemophilia A mice (Gangadharan et al 2006; Ide et al 2007; Ide et al). However, following the development of leukemia in 5 of 20 patients involved in a SCID-X1 clinical trial initiated in Paris in 1999, the field of gene therapy has moved toward the development of vectors with a decreased risk of insertional mutagenesis. Lentiviruses are a subclass of retroviruses also capable

of chromosomal integration, however, lentiviral vectors have several advantages over traditional γ-retroviral vectors. First, lentiviruses are capable of transducing both dividing and non-dividing cells. This characteristic makes lentiviruses advantageous for transduction of quiescent cells such as HSCs. Second, lentiviral integration occurs randomly into open reading frames while γ-retroviral vectors prefer integration within transcriptional start sites (Mitchell et al 2004; Wu et al 2003). For this reason, lentiviruses are thought to have a decreased risk of full-length gene transcript activation due to proviral insertion (Baum & Fehse 2003; Hematti et al 2004; Modlich et al 2006; Zaiss et al 2002).

The lentiviral vectors used in the current studies were further engineered to increase safety by including a ~400 bp deletion in the U3 region of the 3' LTR. This deletion inactivates the strong promoter and enhancer elements in the 3' LTR, and also the 5' LTR following reverse transcription of the proviral RNA within a transduced cell. Inactivation of viral LTRs prevents activation of downstream genes due to LTR promoter insertion. Of note, all cases of leukemic transformation observed in clinical or preclinical gene therapy research involved the use of γ-retroviral vectors with intact LTRs. Using SIV-based SIN lentiviral vectors, we demonstrated efficient transfer of hfVIII, pfVIII, and eGFP into multiple cell types. We also demonstrated efficient gene transfer of eGFP into HSCs and less efficient lentiviral-mediated transfer of pfVIII into the genome of hematopoietic stem and progenitor cells. Efficient gene transfer is important in

gene therapy because in order to achieve permanent gene correction, transgene integration into a patient's cellular DNA is necessary.

Gene therapy transgene promoters

Retroviral LTRs are commonly used to drive transgene expression due to the robust and ubiquitous expression they provide. When using SIN vectors for gene therapy, it is necessary to provide an internal promoter to drive transgene expression due to inactivation of the native promoter. Although it is beneficial for safety in a gene therapy setting to eliminate the strong promoter and enhancer activity of the LTRs, transgene expression from an internal promoter is likely to decrease when compared to endogenous LTR-driven expression. In the current studies, a SIN SIV vector containing an internal MSCV LTR promoter to drive GFP expression was shown to transduce HSCs and express GFP in peripheral blood cells at levels comparable to a MSCV γ-retroviral vector. Although this SIN vector has a decreased risk of insertional mutagenesis compared to the wild type MSCV containing 2 active LTRs, the single internal LTR promoter retains the strong enhancer activity capable of long distance enhancer interactions. In fact, the ability of retroviral LTRs to transactivate distant genes suggests that even when incorporated internally into a SIN lentiviral vector, the oncogenic potential may be the same as if it were incorporated into a γ-retroviral vector. Many gene therapy vectors have incorporated a weaker ubiquitous promoter in order to decrease potential enhancer effects. The murine phosphoglycerate kinase (PGK) and cytomegalovirus immediate/early promoter were among the first non-γ-

retroviral LTR promoters used for gene therapy, however, as expected, both exhibited decreased levels of gene expression *in vivo* (Miyoshi et al 1999). Although lower expression was achieved, the correct thinking in the field of gene therapy is that decreased expression is better than insertional mutagenesis.

In the studies outlined in Chapter 3, we employed the human β-globin promoter to drive pfVIII or GFP expression. Use of the β-globin promoter in a gene therapy setting takes advantage of the strict regulation of globin gene expression by a number of DNase I hypersensitive (HS) sites located within the locus control region. It has been shown that when linked to a globin gene in *cis*, HS sites confer erythroid-specific and elevated transgene expression in cell lines and transgenic animals (Blom van Assendelft et al 1989; Grosveld et al 1987). Cell and tissue-specific vectors offer an additional element of safety in comparison to constitutively expressing vectors when transducing a mixed population of cells, especially one that includes HSCs. When transducing bone marrow cells, a constitutively expressing vector can be expressed in all blood lineages, including HSCs. However, the β-globin promoter used in these studies is thought to be silent in stem and progenitor cells, as well as in lymphocytes and early myeloid cells, although low-level activity has been seen in stem cells. Restricting promoter activity to a portion of erythroid cells, instead of all transduced cells, drastically reduces the probability of oncogenic gene activation in the majority of gene-modified cells. In these studies, we demonstrated the ability of the β-globin promoter and locus control region to drive high-level pfVIII expression in a

myelogenous leukemic cell line, K562. Expression of pfVIII in transduced K562 cells was comparable to MSCV LTR-mediated pfVIII expression in 293T cells with a similar gene copy number. The β-globin promoter was also efficient at driving GFP expression in $Ter119^+$ peripheral RBCs. Although some leaky promoter activity was observed in $CD45^+$ cells, GFP expression in $CD45^-$ /$Ter119^+$ cells was significantly higher than MSCV-driven GFP expression. β-globin driven pfVIII expression was also demonstrated in a murine model of hemophilia A, however low transduction efficiency limited expression to only 5% of transplanted mice. The “Future Studies” section below outlines a number of experiments designed to determine the reason for the observed low HSC transduction efficiency.

Hematopoietic stem cell transduction

Safe and effective gene transfer into HSCs is one of the main goals of gene therapy. HSCs have characteristic properties of self-renewal, differentiation, and proliferation, which make them ideal targets for the permanent correction of a variety of genetic diseases. However, the successful implementation of this method requires both the efficient transfer of the gene of interest into HSCs and the successful engraftment of gene-modified cells with a low risk of toxicity. Gamma-retroviral vectors were previously the gene transfer system of choice and have been approved for use in a number of clinical trials using *ex vivo* modification of human $CD34^+$ cells. Our previous studies using a pfVIII-containing retrovirus to transduce murine HSCs were successful in correcting

hemophilia A in transplanted mice (Gangadharan et al 2006; Ide et al 2007). However, gene transfer into HSCs using a pfVIII-containing lentivirus has been less successful. Lentiviruses, unlike γ-retroviruses, are capable of transducing non-dividing cells due to the presence of viral proteins that make up the PIC. These proteins include integrase, an accessory protein that is the product of the *vpr* gene, and the essential structural protein matrix encoded by the *gag* gene. The matrix protein contains a localization sequence that is recognized by import machinery in the nucleus of a cell and allows active transport of the PIC through the nucleopore. Although lentiviruses are capable of transducing quiescent cells, they display low permissivity to the vector and require cytokine stimulation to reach high-level transduction. It is well established that cells in the G_1 phase of the cell cycle are more readily transduced than non-dividing cells in G_0 (Korin & Zack 1999; Sutton et al 1999) **(Figure 5.1)**. It is also thought that reverse transcription of lentiviral RNA in a transduced cell cannot occur until the cell progresses into the G_{1b} phase, whereby the G_{1b} phase is defined as having RNA levels equivalent to those seen in early S phase before DNA synthesis (Hossle et al 2002). One possible reason for the lack of HSC transduction observed in our pfVIII-lentiviral HSCT is the cells were not transduced at an optimal time during cycling. For this reason, the cytokine cocktail used to induce cell cycling is very important. A delicate balance must be achieved in the *ex vivo* HSC population in order to produce effective cell cycling while maintaining the self-renewal, multipotentiality, and engraftment capacities of HSCs. While the combination of cytokines used varies depending on the researcher, the experiment, or the

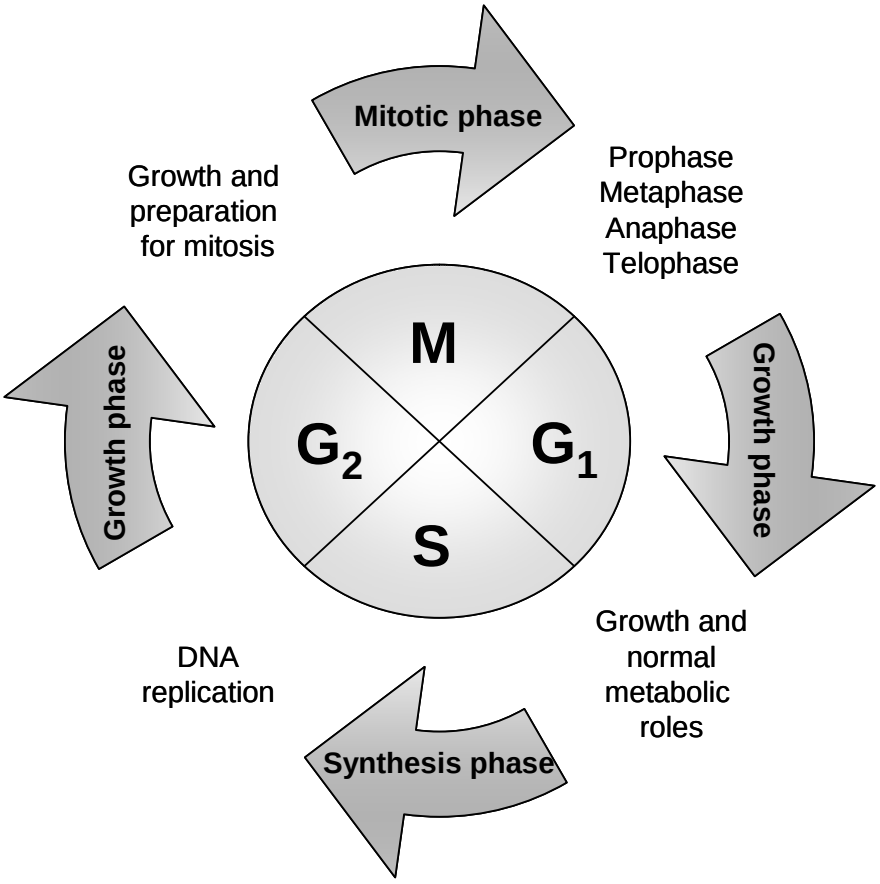

Figure 5.1 – Diagram of the phases of cell division. Once quiescent cells are induced to divide, they leave G_0 and enter the G_1 phase of cell division. This is the phase at which retroviral transduction is thought to be optimal. Cells enter interphase in G_1, S, and G_2 stages, whereby cells grow and DNA replicates in preparation for mitosis. Checkpoints are located at G_1 and G_2 to ensure the cell is ready to move on to the next stage of division. Cell growth stops in the M phase and the cell begins to divide into 2 daughter cells. A metaphase checkpoint also exists in the M phase to ensure the cell is ready for complete division.

species of target cell, it is generally thought that the use of cytokines for the stimulation of HSCs is beneficial to transduction and does not impair the ability of cells to engraft and repopulate following transplant (Zielske & Braun 2004). Although the mechanism by which cytokines affect cells is poorly understood, a recent study suggests that it is not their effect on cell cycling, but rather on proteosome-mediated protein degradation that enhances lentiviral-mediated gene transfer (Santoni de Sio et al 2006). Santoni de Sio *et al* showed that pharmacologic inhibition of proteosome activity during lentiviral transduction of HSCs dramatically increased transduction efficiency. In contrast to the lack of requirement for cell proliferation during lentiviral transduction, they showed that cytokine stimulation increased transduction efficiency due to down-regulation of proteosome activity in hematopoietic stem and progenitor cells.

We demonstrated the ability of a lentivirus containing pfVIII under the control of the β-globin promoter and locus control region to mediate long-term pfVIII expression in mice following transduction and transplantation of HSCs. The cytokine cocktail used in these studies was identical to that used in our previous studies whereby high level sustained fVIII was produced in mice following transplantation of Sca-1^+ cells transduced with a MSCV virus (Gangadharan et al 2006; Ide et al 2007). In the erythroid promoter studies however, low transduction efficiency in HSCs prevented long-term fVIII expression in the majority of transplanted mice. It may be interesting to determine if more efficient HSC transduction would be possible using a γ-retrovirus containing pfVIII under the

control of the β-globin promoter. However, it is unlikely that the large β-globin promoter and locus control region along with the 4.4 kb pfVIII transgene could be packaged into a γ-retrovirus system, as the limit for γ-retroviral packaging capacity is ~8 kb.

Lentiviral quantification

As the prevalence of gene therapy research increases, so does the importance of accurate titering of viral stocks. Currently the most widely used method for lentiviral quantification is by functional viral titer, or calculation of the number of gene transfer events per unit volume of virus stock. However, this method is not ideal for making accurate comparisons among viral stocks. Functional viral titer can be influenced by a number of factors, including the cell type used for transduction, cell number and density, volume of medium, viral particle half-life, and the parameters of the quantification assay. Further complicating this system for viral quantification is the fact that MOI is based on the functional viral titer. In the studies outlined above, the total amount of viral particles in each virus stock was calculated using a p24 ELISA assay and found that equal amounts of total viral particles were present in each HIV-pfVIII and HIV-GFP stock. However, using a qRT-PCR assay, we found the HIV-GFP functional viral titer to be 5x higher than the HIV-pfVIII functional viral titer. One alternate method to more accurately quantify viral titer is by measuring the amount of adsorbed active viral particles per cell (AVC) (Andreadis et al 2000). This method takes into account the diffusion and adsorption of viral particles on target cells, which is a critical

step in retroviral transduction. Using AVC as an alternative to MOI in the calculation of viral titer, Andreadis et al showed that most viral particles present in the viral stock are initially active, but variable rates of diffusion and virus decay limit the amount of viral particles able to infect a cell. Slower diffusion leading to increased virus decay may be the reason 5x less HIV-pfVIII than HIV-GFP is able to integrate into cellular DNA, however, the kinetic studies described in Chapter 4 suggest HIV-pfVIII and HIV-GFP enter the cell at equivalent rates.

Variability in viral transduction efficiencies

Previous studies by myself and co-workers have demonstrated a transgene-specific difference in Sca-1^+ cell transduction efficiency, whereby transduction efficiency is determined by the number of proviral copies in the genome of peripheral blood cells following HSC transduction and transplantation. We hypothesized that because 5x more HIV-pfVIII than HIV-GFP $p24^+$ viral particles were needed to achieve a similar number of integrated transgene copies/cell, the divergence in transduction efficiencies was most likely at the step of viral entry or reverse transcription. However, we demonstrated similar kinetics of viral entry and reverse transcription in 293T, K562, and Sca-1^+ cells transduced with each virus. These data suggest that the difference in transduction efficiencies occurred later in the lentiviral transduction pathway. One other possibility accounting for the lack of gene-modified cells in transplanted mice is that non-gene-modified cells have a competitive advantage over gene-modified cells in engraftment. However, previous data from our lab suggests that naïve and transduced cells

engraft equally in mice under similar pre-transplantation immunosuppression. Together, these data suggest that achieving therapeutic levels of pfVIII from lentiviral-mediated HSCT therapy is dependent on increasing genomic integration of therapeutic proviral DNA. With these improvements, lentiviral-mediated pfVIII- or HP-fVIII-modification of HSCs may soon become a clinical therapeutic option.

5.2 – Future directions

Although these studies demonstrated the potential for erythroid-restricted lentiviral-mediated gene therapy using a high expression pfVIII transgene, a number of future studies are necessary for this system to progress. First, pfVIII is not available to us for clinical use. However, we demonstrated high fVIII expression from a human/porcine chimeric transgene whose cDNA is available for future studies. Further characterization of this transgene in a lentiviral gene transfer setting is currently taking place at Emory University and Expression Therapeutics. Second, it may be necessary to elucidate the mechanisms for non-specific β-globin-controlled fVIII expression in non-erythroid cells before this system can be used clinically. While the non-specific protein expression in WBCs was initially a surprise, this may be beneficial for the induction of immune tolerance. Prevention of an immune response in a gene therapy setting would be hugely beneficial. Currently, 30% of patients receiving fVIII replacement develop inhibitors, thus preventing effective maintenance of the disease. However, before a better understanding of the functionality of the β-globin promoter at driving fVIII expression can be achieved, the matter of HSC transduction efficiency must be

addressed. If lentiviral transduction of HSCs cannot be increased, this study holds no clinical relevance and will never move forward. Understanding the transgene-specific and cell type-specific differences in lentiviral-mediated HSC transduction will lead to the development of a specific, yet standardized protocol for efficient and reproducible transduction.

Since the kinetic studies described in Chapter 4 demonstrated no difference in the rate or amount of total viral DNA incorporated in HIV-pfVIII or HIV-GFP-transduced cells, future studies must be designed to quantify viral DNA at each specific stage of transduction. We know from previous studies that less pfVIII than GFP integrates into similarly transduced cells, however measurement of total viral DNA following transduction could not distinguish how this occurs. Because the initial rates of viral cDNA formation were similar for HIV-pfVIII and HIV-GFP, this would suggest rates of reverse transcription were equivalent in similarly transduced cells. However, somewhere along the path of transduction from reverse transcription to integration of proviral DNA, HIV-pfVIII and HIV-GFP transduction efficiencies diverge. In order to determine which stage of transduction is responsible for the observed differences in transduction, qRT-PCR assays should be designed to measure 1) the amount of integrated proviral DNA, and 2) the amount of proviral DNA degraded prior to integration. Quantification of integrated proviral DNA can be carried out using primers specific to the viral sequence and the Alu genomic sequence, as described below. Quantification of degraded proviral DNA can be achieved using primers

specific to the pre-degradation products formed in the nucleus of transduced cells. These studies, in conjunction with the kinetic studies described in Chapter 4, will better characterize the stages of lentiviral transduction leading up to and including the integration of the proviral sequence.

In order to measure the rate of proviral integration and the amount of integrated provirus, specific qRT-PCR assays must be designed which can differentiate integrated DNA from total DNA. Quantification of integrated provirus can be accomplished using primers specific to the viral LTR and the Alu repeat sequence. Because proviral DNA integrates randomly throughout the genome of transduced cells, each integration event results in a differently sized amplicon, complicating quantification by RT-PCR. However, using a cellular DNA standard containing a known number of randomly integrated lentiviral DNA copies, a standard curve can be generated relating the number of proviral copies in a sample to the RT-PCR cycle number at which Alu amplification signal crosses the threshold. Using this, one can determine the amount of integrated DNA and its relation to the amount of total proviral DNA within a cell.

In relation to the observed decrease in Sca-1^{+} cell transduction with HIV-pfVIII compared to HIV-GFP, we know 1) fewer HIV-pfVIII than HIV-GFP copies become integrated, and 2) similar amounts of total HIV-pfVIII and HIV-GFP copies were present in cells following transduction, suggesting the amount of integrated DNA and the amount of degraded DNA are inversely related. To

confirm this inverse relationship, qRT-PCR assays can be designed to quantify the amount of degradative DNA products. These products form in the nucleus of transduced cells, and as such, may also be used as markers for nuclear translocation. 1-LTR circles form by homologous recombination between the 5' and 3' LTRs, while 2-LTR circles form by ligation of the 3' U5 region to the 5' U3 region of the LTRs **(Figure 5.2).** Measurement of 2-LTR circles can be carried out using qRT-PCR and primers designed to amplify a region spanning from the 3' U5 region to the 5' U3 region of the LTRs. The amount of 1-LTR circles can be calculated based on a known ratio of 9 1-LTR circles to 1 2-LTR circle, although quantification of 1-LTR circles may be estimated by Southern blot as well. Determining the amount of integrated versus non-integrated provirus in HIV-pfVIII- and HIV-GFP-transduced cells will better pinpoint the stage of transduction responsible for the divergence of transduction efficiencies. If a direct relationship between integrated DNA and circularized degradative DNA is not observed, a mechanism other than integration is most likely responsible for the difference in HIV-pfVIII and HIV-GFP transduction.

Another way to test for variable efficiencies of integration would be to transduce cells with a virus containing a defective integrase gene. With this, all viral transduction processes would be identical to previous transductions until the integration step. However, if integration is specifically blocked, an increase in 1-LTR and 2-LTR circle formation should occur because the DNA that would normally integrate is now forced down a pathway to degradation prior to

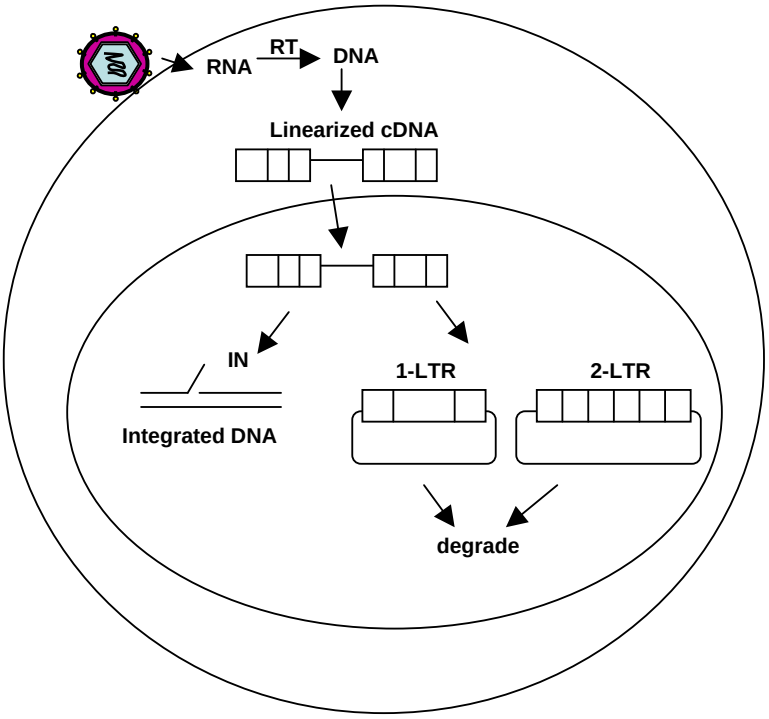

Figure 5.2 – Retroviral-mediated transduction leading to 1- and 2-LTR circle formation. Once the virus binds to the cell, it uncoats and injects its proviral load into the cytoplasm of the cell. Viral RNA is reverse transcribed by reverse transcriptase (RT) to form a linear strand of DNA. DNA is imported to the nucleus where it can become integrated into the host genome using cellular integrase (IN) or it can become circularized to form a 1-LTR or 2-LTR circle committed to a path of degradation.

integration. If the decreased transduction efficiency of HIV-pfVIII is due to less efficient integration, the difference in 1-LTR and 2-LTR circle formation using virus with or without a functional integrase gene will be smaller for HIV-pfVIII than HIV-GFP-transduced cells, since more HIV-pfVIII DNA is already blocked from integration. Inefficient integration of HIV-pfVIII DNA compared to HIV-GFP DNA would suggest the size or complexity of the transgene plays a role in overall transduction efficiency. Studies to increase integration might include incorporating different integrase genes in the HIV expression vector or increasing the ratio of integrase to proviral DNA. One possible reason for decreased integration of HIV-pfVIII compared to HIV-GFP could be that viral integrase is only capable of inserting a proviral sequence up to the size of the wild-type sequence. As such, a proviral sequence as large HIV-pfVIII may be less efficiently integrated into the cellular genome by the wild type SIV or HIV integrase. If inefficient integration is determined the reason for poor HIV-pfVIII transduction, future studies may want to test the utility of a spumavirus vector backbone for viral-mediated DNA transfer of large proviral sequences. Spumavirus is another subclass of retrovirus, whose ~13 kb genome is larger than any lentivirus. Although their utility in gene therapy has not been well characterized, spumaviruses such as the simian foamy virus may offer better viral encapsidation and integration of large proviral sequences due to their large genome size.

If the studies described above show no difference in the rate or amount of proviral integration, some other mechanism is most likely responsible for the divergence of HIV-pfVIII and HIV-GFP transduction efficiencies. Nuclear translocation of the PIC is another potential rate-limiting step in viral transduction. In order to measure the rate of proviral nuclear translocation and the total amount of nuclear proviral DNA, qRT-PCR assays can be designed to measure proviral DNA in the nucleus, following cell lysis and nuclear isolation. Kinetic measurement of proviral copies in the nucleus of cells following lentiviral transduction would demonstrate if differences exist in the rate of HIV-pfVIII or HIV-GFP PIC nuclear translocation. This can be accomplished by isolation of the nucleus via a sucrose gradient, nuclear DNA isolation, then qRT-PCR analysis of proviral copy number at various time-points following transduction. Measurement of HIV-pfVIII and HIV-GFP proviral copies in the nucleus versus the cytoplasm should deomonstrate if differences exist in the rate of nuclear translocation for each virus. If these studies demonstrate a difference in the rate of HIV-pfVIII nuclear translocation versus HIV-GFP, the next studies should be designed to increase nuclear transport efficiency of the HIV-pfVIII PIC. One way to increase nuclear transport might be to include an optimized nuclear localization signal in each component of the PIC so the entire complex may be transported efficiently across the nuclear membrane. One reason for the observed difference in HIV-pfVIII and HIV-GFP transduction could be that the HIV-pfVIII proviral complex is too large to be efficiently transported through the nuclear pore. Optimization of the nuclear localization signal may allow more efficient binding to importins

necessary for active transport. Similarly, codon optimization of the proviral sequence may lead to changes in DNA folding and protein interaction within the PIC, allowing for better nuclear translocation. Immunohistochemistry experiments may be helpful in tracking the movement of the PIC from the cytoplasm to the nucleus.

Converse to enhancing the late stages of lentiviral transduction, improvements made to the early stages of transduction may lead to an overall increase in the total amount of integrated provirus. Previously, others have demonstrated an increase in the early events of HIV transduction, including reverse transcription and development of circularized degradative products, using various reagents designed to arrest cell cycle progression (Groschel & Bushman 2005). For example, the anti-tumor agents etoposide and camptothecin arrest cell cycle in G_2/M via activation of a DNA damage checkpoint. Paclitaxel also arrests at G_2/M by modulation of microtubule polymerization. Groschel *et al* suggest the reason for the G_2/M arrest-dependent increase in transduction could be that the change in cellular physiology makes cells more conducive to lentiviral transduction, possibly due to increased cell surface area during the arrest. Others have demonstrated increased HSC transduction efficiency by knocking down the cyclin-dependent kinase p21 (Zhang et al 2007). They showed p21 plays an important role in intrinsic cellular defense against HIV-1 infection of HSCs and that its effect was not due to cell cycle modulation and was independent of other known mediators of HIV-1 resistance such as tripartite motif protein 5α (Trim 5α),

promyelocytic leukemia protein (PML), or IFN-α. Future studies should include siRNA-mediated p21 knockdown in HSCs prior to lentiviral transduction with the goal of increasing the amount of provirus entering the cell may contribute to an increase in the amount of integrated transgene.

It may also be necessary to evaluate the growth and engraftment properties of gene-modified HSCs in order to determine if the reason for low percentages of pfVIII gene-modified cells following transplantation is due to altered cellular proliferation properties. Recent studies of γ-retroviral-mediated gene transfer into HSCs demonstrated that proviral integration may confer a growth advantage and trigger clonal expansion in transplanted recipients (Schmidt et al 2005; Stein et al), however others have demonstrated lentiviral-mediated gene transfer into HSCs does not confer competitive repopulation advantages over non-gene-modified cells (Gonzalez-Murillo et al 2008). The viral vector, promoter, and incorporated transgene all play a role in the development or prevention of clonal expansion in these cases. In order to confirm that donor HSCs transduced with pfVIII or GFP possess similar properties of engraftment and proliferation following transplantation, competitive repopulation experiments should be designed as follows: 1) Isolate $CD45.1^+$ and $CD45.2^+$ HSCs and transduce with HIV-pfVIII or HIV-GFP, respectively. 2) Combine HIV-pfVIII- and HIV-GFP-transduced cells in various ratios such as 0, 10, 25, 50, 75, and 100% of HIV-pfVIII-transduced cells to 100, 75, 50, 25, and 10% of HIV-GFP-transduced cells. 3) Transplant an equal number of total transduced cells into lethally

irradiated recipient mice. 4) Measure the amount of $CD45.1^+$/HIV-$pfVIII^+$ and $CD45.2^+$/HIV-GFP^+ cells in the peripheral blood. If the rates of engraftment and proliferation are equal between HIV-pfVIII and HIV-GFP-transduced cells, the ratios of $CD45.1^+$ and $CD45.2^+$ donor cells will remain similar to the day of transplantation. One downfall of this study is that it may be difficult to determine if decreased engraftment of gene-modified cells is actually due to a change in cellular physiology resulting in decreased engraftment, or if it is due to increased cell death.

Another possible reason for the lack of gene-modified cells in mice transplanted with pfVIII-containing lentiviral-transduced Sca-1^+ cells in our studies is that pfVIII is toxic to cells and the production and expression of pfVIII from gene-modified cells decreases cell viability and proliferation. Although we demonstrated that stable 293T clones expressing hfVIII, pfVIII, or GFP display similar growth rates and viability compared to naïve cells, the effect of pfVIII production from gene-modified hematopoietic cells on growth rate and viability is unknown. In order to determine if pfVIII exposure is toxic to HSCs, murine HSCs should be isolated and cultured in the presence of purified fVIII. Cell count and viability can be measured over time using trypan blue exclusion to determine if HSCs in the presence of purified fVIII exhibit a difference in proliferation or viability compared to HSCs not exposed to fVIII. In addition to determining if fVIII exposure is toxic to HSCs, it will also be necessary to determine if fVIII or GFP production by HIV-pfVIII- or HIV-GFP-transduced cells has an effect on cellular viability or

proliferation. For this, murine HSCs can be isolated and divided into 3 groups. One group will be transduced with HIV-pfVIII, one group transduced with HIV-GFP, and one group will remain untransduced. Cell count and viability should be measured over time following transduction to determine if any differences exist among the three groups. If no differences are observed, it would suggest that production of pfVIII or GFP has no effect on hematopoietic cell proliferation or viability. One pitfall of this study is that because HSCs are transduced at such a low efficiency, it may be impossible to determine a difference in viability in the transduced cell population. To ensure the highest percentage of gene-modification, HSCs may be transduced with a pfVIII-containing MSCV instead of a lentivirus, however, previous studies suggest the percentage of MSCV-modified HSCs may also be too low to detect a difference in a background of mostly non-gene-modified cells.

In addition to determining the reason for the variability in lentiviral transduction efficiencies, future studies might also include the development of novel lineage-specific lentiviral expression systems designed for enhanced safety. Since previous studies from our lab suggested myeloid cells are the predominant manufacturers of fVIII following HSC transduction and transplantation, future studies should focus on the use of myeloid-specific promoters. The myeloperoxidase (MPO) promoter is an attractive choice for a safe lineage-specific promoter because it is turned on in the early stage of myeloid differentiation, but turned off once myeloid precursors are induced to

differentiate. This tight regulation would be beneficial in increasing the safety of gene therapy vectors. The chance of transformation into a clonally dominant cell would be very low, as active transcription would only occur in myeloid precursors. Three distinct MPO promoters have been shown to actively transcribe the myeloperoxidase gene, however only the P1 MPO promoter gives rise to full length transcripts and exhibits characteristic down-regulation following the induction of differentiation (Lin & Austin 2002). The CD68 promoter would also be a good choice based on our previous data showing Gr-1^+/Mac-1^+ cells contained the highest percentage of fVIII of all bone marrow and peripheral blood cells following HSCT. The human CD68 promoter has been shown to direct constitutive transgene expression *in vivo* specific to macrophages (Gough et al 2001).

Lentiviral transduction is a highly regulated and complicated series of events, each of which has a role in the final amount of integrated transgene and ultimately, the amount of protein produced. In a gene therapy setting, the main goal is to achieve curative levels of therapeutic protein without occurrence of adverse events. However, the biggest obstacle preventing the success of HSC gene therapy currently is transduction efficiency and engraftment of gene-modified cells. Once a method for increasing lentiviral integration in HSCs is developed, future studies may focus on determining the optimal number of gene-modified cells and proviral copies per cell, decreasing immune reactivity, and ultimately increasing the amount and duration of therapeutic protein delivered to

the patient. In my opinion, the future of gene therapy for hemophilia A is bright and it can be predicted that a successful clinical trial may take place within the next 5 years.

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