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Optimization of a Lentiviral Vector for use in an HSC Gene Therapy Protocol for Hemophilia A

By

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An Abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Graduate Division of Biological and Biomedical Sciences Program in Molecular and Systems Pharmacology
2013

ABSTRACT

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Gene therapy for hemophilia A has been hindered by limited expression of factor VIII (fVIII). In order to overcome the expression barrier, several high-expression fVIII transgene sequences were compared and identified enhanced expression of a B domain deleted porcine fVIII sequence. As a result, a high-expression fVIII transgene was engineered and used to genetically modify HSCs and effectively treat hemophilia A mice. Prior to clinical application, the components of the self-inactivating lentiviral vector system needed to be optimized in regards to viral production, transduction efficiency and transgene expression. Specifically, three parameters were evaluated: 1) the woodchuck hepatitis post-transcriptional regulatory element (WPRE), 2) HIV versus SIV viral vector systems, and 3) various internal promoters. Based on these studies, an optimized vector contains the HP-fVIII transgene driven by a CMV internal promoter within a SIV-based lentiviral backbone devoid of a WPRE. In addition, two obstacles faced by the field of gene therapy were confronted. Firstly, lentiviral vectors containing fVIII transgenes consistently have lower titers compared to similar vectors containing other transgenes. An alternative approach to lentiviral production was attempted in BHK-M cells. However, the transfection efficiency was too low to produce substantial amounts of virus. The second obstacle addressed is that of the inefficiency of lentiviral vectors to transduce HSCs. Limited transduction efficiency has been observed clinically with the use of lentiviral vectors and will need to be enhanced in scenarios in which a growth advantage is not expected as is the case for hemophilia A. Therefore, a high-throughput screen was performed to identify compounds that could enhance the transduction of HSCs by lentiviral vectors. A library of 1280 pharmacologically active compounds was screened using K562 cells in the presence of a SIN lentiviral vector encoding a GFP transgene. Among the positive hits were known enhancers of transduction, as well as phorbol 12-myristate 13-acetate (PMA). Transduction was enhanced in K562, EU1 and most notably $CD34^+$ cells following treatment with PMA prior to transduction. The results from this report were used to help develop a lentiviral vector that is currently being considered by the FDA for a clinical trial to treat hemophilia A.

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LIST OF ABBREVIATIONS

AAV	adeno-associated viral vector
ADA-SCID	adenosine deaminase deficient severe combined immunodeficiency
ADP	adenosine diphosphate
ANOVA	analysis of variance
APTT	activated partial thromboplastin
AT	antithrombin
BGpA	bovine growth hormone poly A sequence
BHK	baby hamster kidney
BiP	immunoglobulin binding protein
BOEC	blood outgrowth endothelial cells
bp	base pairs
Cβ	β-actin promoter
CD	cluster of differentiation
cDNA	complementary deoxyribose nucleic acid
CFU	colony forming unit
CMV	cytomegalovirus
CPPT	central polypurine tract
DAG	diacylglycerol
DNA	deoxyribose nucleic acid
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethyl sulfoxide
EFIα	human elongation factor-1 alpha

EIAV	equine infectious anemia virus
ELISA	enzyme linked immunosorbent assay
ENH	enhancer
ES	embryonic stem cells
FACT	normal human plasma
FDA	food and drug administration
FITC	fluorescein isothiocyanate
FU	fluorouracil
fV	factor V
fVa	active factor V
fVII	factor VII
fVIIa	active factor VII
fVIII	factor VIII
fVIIIa	active factor VIII
fIX	factor IX
fIXa	active factor IX
fX	factor X
fXa	active factor X
fXI	factor XI
fXII	factor XII
fXIII	factor XIII
GFP	green fluorescent protein
GMP	good manufacturing procedures

HEK	human embryonic kidney
HIV	human immunodeficiency virus
HPfVIII	high expression B domain-deleted factor VIII
HRS	hours
HSC	hematopoietic stem cell
iPS	induced pluriopotent stem cells
IRES	internal ribosomal entry site
kb	kilobases
kg	kilograms
LEDGF	lens epithelium-derived growth factor
LMO2	lim domain only 2
LOPAC	library of pharmacologically active compounds
LSECs	liver sinusoidal endothelial cells
LTR	long terminal repeat
MFI	mean fluorescent intensity
µg	micrograms
mg	milligrams
miRNA	micro ribonucleic acid
µL	microliter
mL	milliliter
MLV	murine leukemia virus
µM	micromolar
µmol	micromoles

MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MU	milliunits
ng	nanograms
nM	nanomolar
PCR	polymerase chain reaction
PEI	polyethyleneimine
pg	picograms
PGK	phosphoglycerate kinase
PKC	phosphokinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMOL	picomoles
Ψ	psi, packaging sequence
qPCR	quantitative polymerase chain reaction
RNAi	ribonucleic acid interference
RRE	rev response element
SCA-1^{+}	stem cell antigen-1^{+}
SIN	self-inactivating
siRNA	small interfering ribonucleic acid
SIV	simian immunodeficiency virus
TALEN	tal effector nuclease
TF	tissue factor / thromboplastin

TFPI	tissue factor pathway inhibitor
tRNA	transfer ribonucleic acid
TU	transducing units
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TXA_2	thromboxane
U	units
UTR	untranslated region
VSVG	vesicular stomatitis virus glycoprotein
VWF	von Willebrand factor
WAS	Wiskott-Aldrich syndrome
WPRE	woodchuck post-transcriptional regulatory element
X-ALD	X-linked adrenoleukodystrophy
X-SCID	X-linked severe combined immunodeficiency
ZFN	zinc finger nuclease

Chapter 1:

Introduction to Gene Therapeutic Approaches for Hemophilia A

1.1— Hemostasis and Blood Coagulation

Hemostasis is the physiological response to vessel injury in order to prevent blood loss. Normal hemostasis is a two part process consisting of primary and secondary hemostasis (Lippi *et al*., 2009). Primary hemostasis begins immediately after injury with the local contraction of vascular smooth muscle and concludes with the formation of a platelet aggregate plug. During secondary hemostasis, the soft platelet plug is stabilized and vasoconstriction is maintained by the release of serotonin, prostaglandin and thromboxane from the activated platelet.

In the absence of injury, clot formation is prevented by the physical separation of platelets and collagen. The disruption of the endothelial layer, due to injury, eliminates this separation. Local vasoconstriction retards the flow of blood not only preventing extravascular blood loss but also facilitating the interaction between platelets and collagen (**Figure 1.1**). Platelets adhere to collagen at the site of vascular injury both indirectly and directly. At high shear rates associated with small arteries and arterioles, platelets indirectly bind to collagen via von Willebrand factor (VWF). VWF is produced not only by megakaryocytes (Nachman *et al*., 1977) but also by endothelial cells of the vascular wall (Jaffe *et al*., 1974) and is thus found within the plasma as a circulating protein. Once collagen is exposed, VWF binds and becomes immobilized upon undergoing an extended shape conformational change due to sheer forces (Siedlecki *et al*., 1996). Glycoprotein Ib, a component of the multimeric glycoprotein complex (Ib/V/IX) unique to megakaryocytes and platelets (Davi and Patrono, 2007), interacts with VWF bound to collagen. This interaction is unstable with a fast off-rate of

dissociation evident by platelet rolling caused by transient interactions between immobilized VWF and platelet glycoprotein Ib (Savage *et al*., 1996). Despite the transient nature of this initial interaction, it is necessary to decelerate platelets allowing new stable bonds with slower intrinsic binding kinetics to form. These include the binding of immobilized VWF to the platelet integrin $\alpha_{IIb}\beta_3$ (also referred to as glycoprotein IIb/IIIa) and the direct binding of collagen to platelets via the integrin $\alpha_2\beta_1$ (also referred to as glycoprotein Ia/IIa) (Rivera *et al*., 2009). Direct adhesion of platelets to collagen also occurs during levels of low sheer stress within the surrounding medium of veins and arteries. In these vessels, platelets are more numerous making a more amenable scenario for the direct binding of fibrillar collagen to platelets via integrin $\alpha_2\beta_1$ followed by subsequent binding to glycoprotein VI (Surin *et al*., 2008). Glycoprotein VI binding results in a series of intracellular signals providing inside out signals for integrins. At this point integrins can be converted by a conformational change from that of a basal low ligand affinity to a high affinity state (Watson and Nieswandt, 2003).

The adhesion of platelets to collagen results in platelet degranulation. Platelets release the contents of stored granules into the blood, which includes the release of adenosine diphosphate (ADP), thrombin and thromboxane A_2 (TXA_2). These molecules act as mediators recruiting circulating platelets to the site of injury, a process termed platelet activation. Each mediator acts through a G-protein-coupled-receptor in order to increase intracellular calcium. For example, ADP acts through $P2Y_1$ and $P2Y_{12}$ coupled to G_q and G_i respectively. Thrombin signals through PAR1 and PAR4 receptors. TXA_2 binds to the TPα and TPβ receptors coupled to the G proteins G_q, G_{13}, and G_{12}. All interactions

result in an increase in intracellular calcium which activates protein kinase C (PKC). PKC in turn activates phospholipase C (PLC) which activates integrin $\alpha_{IIb}\beta_3$ increasing the affinity for fibrinogen. Platelets then aggregate due to fibrinogen adhesions between neighboring platelets (Kulkarni *et al.*, 2000). VWF can also bind to the integrin $\alpha_{IIb}\beta_3$ to stabilize these interplatelet bridges (Lippi *et al*., 2009), but alone is insufficient for stable platelet aggregation (Ni *et al*., 2000). Platelets are further activated exposing procoagulant phospholipids such as the negatively charged phosphatidylserine and phosphatidylethanolamine (Bevers *et al*., 1982). This provides a surface for the factors of the coagulation cascade to bind as part of secondary hemostasis (specifically the tenase and prothrombinase complex). During secondary hemostasis, the soft platelet plug formed at the conclusion of primary hemostasis is stabilized. Fibrinogen between the aggregated platelets is converted to fibrin at the conclusion of the coagulation cascade which crosslinks to form an insoluble fibrin matrix-like gel.

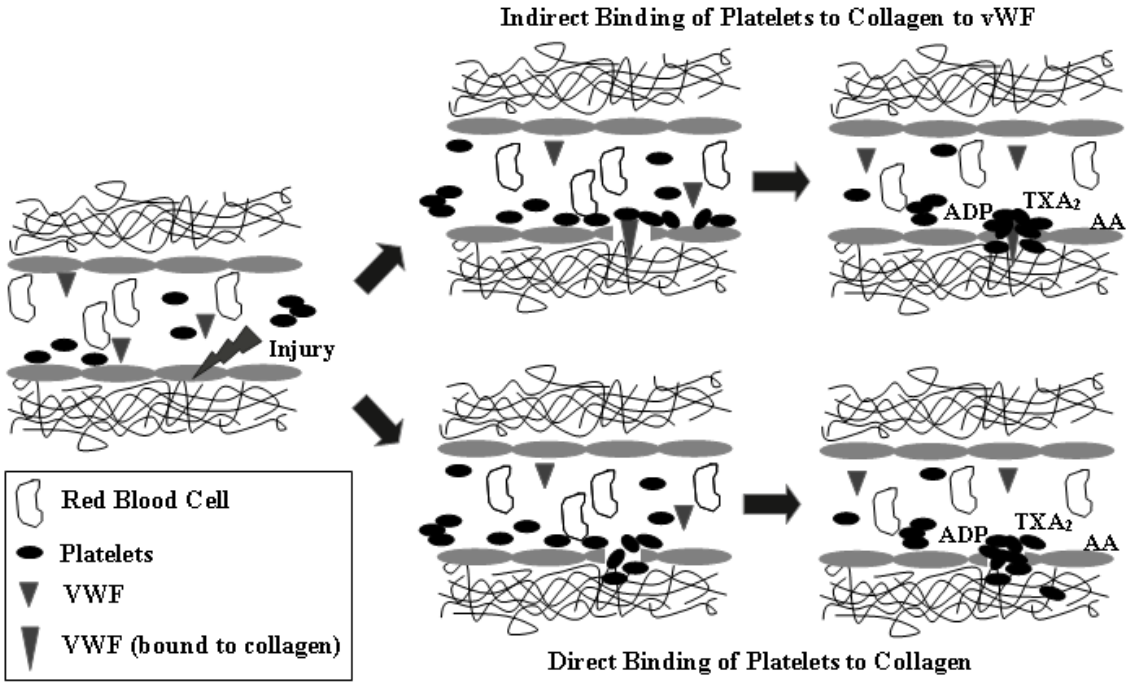


Figure 1.1 – Primary Hemostasis: Platelet Aggregation and Degranulation. Injury exposes the extracellular matrix beneath the endothelial lining of the vessel. Platelets can bind to exposed collagen within the extracellular matrix both indirectly or directly. VWF binds collagen and undergoes a conformational change immobilizing VWF at the site of injury. Platelets bind indirectly to collagen via bound VWF initially via an unstable interaction visible as platelet rolling. This initial interaction decelerates platelets allowing more stable interactions to form which includes binding to VWF as well as direct interactions with collagen.

Abbreviations: VWF (von Willebrand Factor)

Secondary hemostasis involves the process of blood coagulation. Blood coagulation is separated into two proteolytic cleavage cascades denoted as the extrinsic and intrinsic pathways (**Figure 1.2 A**). Both pathways result in the formation of fibrin which becomes cross-linked to each other forming a fibrin clot. The extrinsic pathway is initiated by the interaction between the extravascular tissue factor, thromboplastin (TF) and factor VII (fVII). The importance of these two proteins to the cascade has been confirmed by embryonic lethality within both TF knockouts and fVII knockouts (Toomey *et al*., 1996) (Rosen *et al*., 1997). Under normal conditions, the two proteins are separated by the vascular wall. TF is constitutively expressed by vascular smooth muscle cells (Schecter *et al*., 2000) whereas fVII circulates within the bloodstream as an inactive zymogen after being secreted by the liver. TF and fVII come in contact with each other after the disruption of the vessel endothelium. As a result fVII and TF form a complex which facilitates the cleavage of fVII into an active form, initiating the proteolytic cleavage cascade. Of note, 1% of the secreted fVII circulates as an active form independent of TF. It is thought that this small amount of activated fVII acts as a primer to facilitate timely initiation of the coagulation cascade (Eigenbrot, 2002). However, activated fVII does not possess proteolytic activity unless bound to tissue factor. The complex formed between TF and active fVII (fVIIa) can activate multiple factor X (fX) molecules as well as activating factor IX (fIX) albeit less efficiently resulting in activated fX (fXa) as the initial product (Osterud and Rapaport, 1977) (**Figure 1.2 B**). The initially formed activated fX is sufficient to cleave small amounts of prothrombin to thrombin (also referred to as factor II) (Butenas *et al*., 1997). The small amount of thrombin that is produced by the extrinsic pathway accelerates the coagulation cascade cleaving factor V

(fV) (Mann *et al*., 2003), factor VIII (fVIII) and factor XI (fXI) into their active constituents (Fay, 1988). However, the extrinsic pathway alone is not able to produce substantial amounts of thrombin for the conversion of fibrinogen into fibrin. This is due to the rapid inactivation of the TF-fVIIa-fXa complex by tissue factor pathway inhibitor (TFPI) as well as the inhibition of fXa and thrombin by the serine protease inhibitor, antithrombin (AT) (**See Figure 1.3 for further discussion of anticoagulation**). Thus the second proteolytic cascade of blood coagulation, the intrinsic pathway is vital to the stabilization of the soft platelet aggregate plug.

The intrinsic pathway is initiated *in vitro* by the association of prekallikrein and a negatively charged surface. Here, prekallikrein is converted to kallikrein. Kallikrein in turn activates factor XII (fXII). Activated fXII can then cleave fXI. The role of the previously mentioned cleavage events, otherwise referred to as the contact pathway, in physiological hemostasis is not clear yet. Deficiencies in fXII and prekallikrein do not result in abnormal bleeding *in vivo* (Wuepper, 1973; Colman *et al*., 1975). However these cleavage events have been traditionally included in the waterfall/cascade model (**Figure 1.2 A)** and have thus been included in this dissertation for completeness. The next step in the cascade is the cleavage of fIX) by activated fXI. As stated previously, fXI is cleaved by the small amounts of thrombin produced as a result of the extrinsic pathway prior to inactivation. Thus, the intrinsic pathway is initiated *in vivo* by thrombin and not as a result of the contact pathway. The cleavage of fIX by activated fXI further increases the abundance of activated fIX, since fIX can be initially cleaved by active fVII from the extrinsic pathway (**Figure 1.2 B**). Once fVIII becomes activated (by thrombin),

activated fIX together with activated fVIII and Ca^{2+} form a tenase complex on the membrane of an activated platelet. The activation of fX is dependent on the assembly of the tenase complex. The formation of the tenase complex positions fX to be cleaved by fIX. More than ninety percent of activated fX is generated through the intrinsic pathway (Hockin *et al.*, 2002) (**Figure 1.2 B**). Therefore, the trace amounts of thrombin produced by the extrinsic pathway act to amplify the propagation of activated fX and in turn the propagation of thrombin itself. Thus an inefficient intrinsic pathway as a result of a deficiency in one of the key players such as fVIII or fIX greatly affects hemostasis.

The intrinsic and extrinsic pathways of the coagulation cascade, as part of secondary hemostasis, converge at the formation of the prothrombinase complex composed of the activated cofactor, fV (also by thrombin), and activated fX (**Figure 1.2 A and B**). This complex forms on the surface of activated platelets in the presence of calcium ions. Binding to the platelet membrane localizes the concentration of the products of the coagulation cascade which in this case results in fX cleaving prothrombin yielding thrombin. Thrombin is then released from the membrane to cleave soluble fibrinogen into fibrin between platelets to form an insoluble fibrin matrix like gel. In addition, fibrin then activates factor XIII (fXIII) which forms crosslinks within the fibrin strands increasing the strength and elasticity of the fibrin clot.

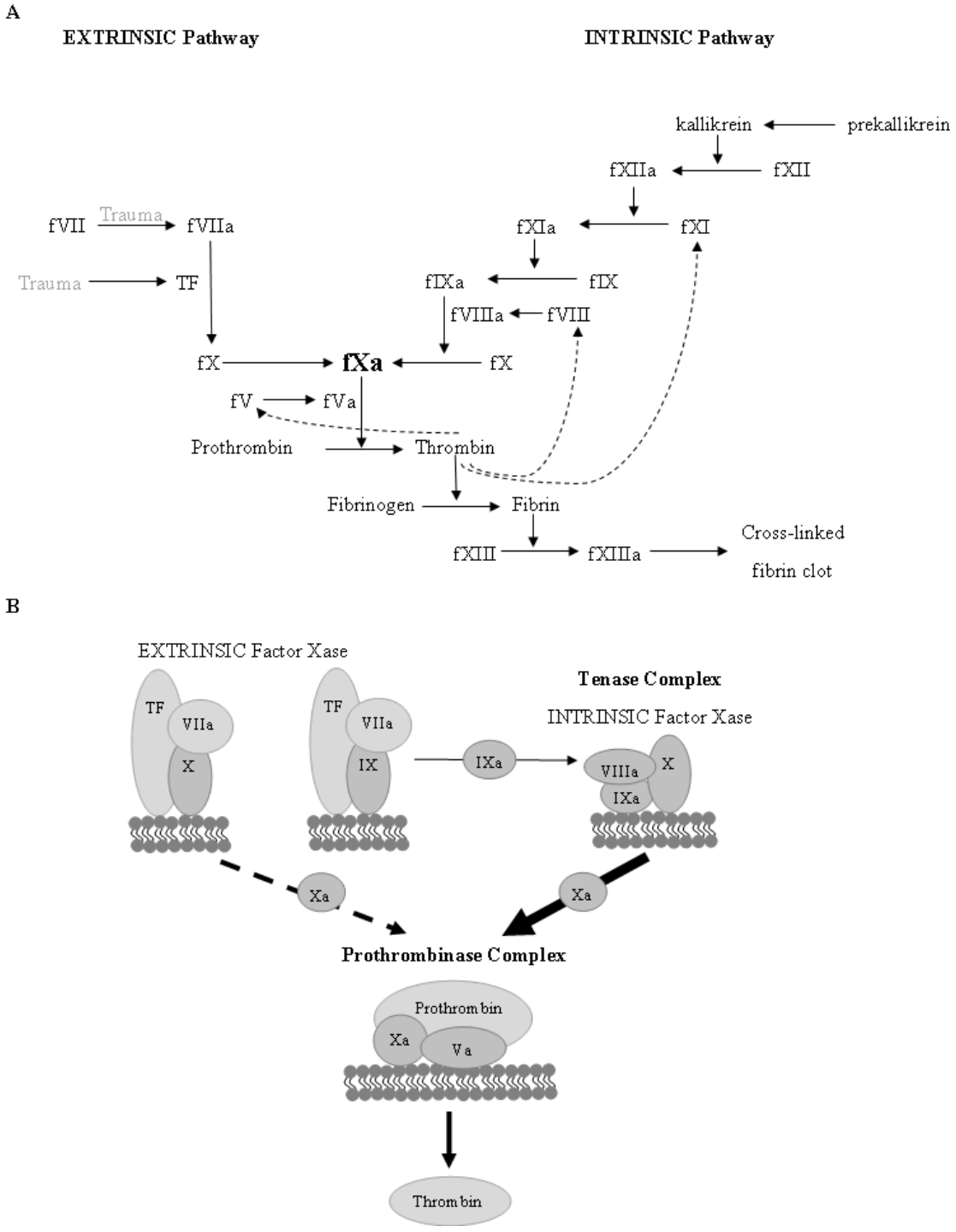


Figure 1.2 – Secondary Hemostasis: Coagulation Cascade. (A) Blood coagulation occurs through a series of cleavage events in which an inactive zymogen is converted into

an active enzyme. The extrinsic pathway is initiated upon trauma exposing tissue factor and activating fVII. (B) Through the extrinsic pathway minute amounts of prothrombin is converted into thrombin, which accelerates the cascade by activating the cofactors fV and fVIII. Activated fVIII interacts with the initial amounts of activated fIX forming a tenase complex which more efficiently converts inactive fX to an active form (as represented by a thick black arrow). In this manner, the intrinsic pathway is the predominant mode whereby thrombin is produced. The cascade culminates with the formation of a stable fibrin clot.

Abbreviations: TF (Tissue Factor).

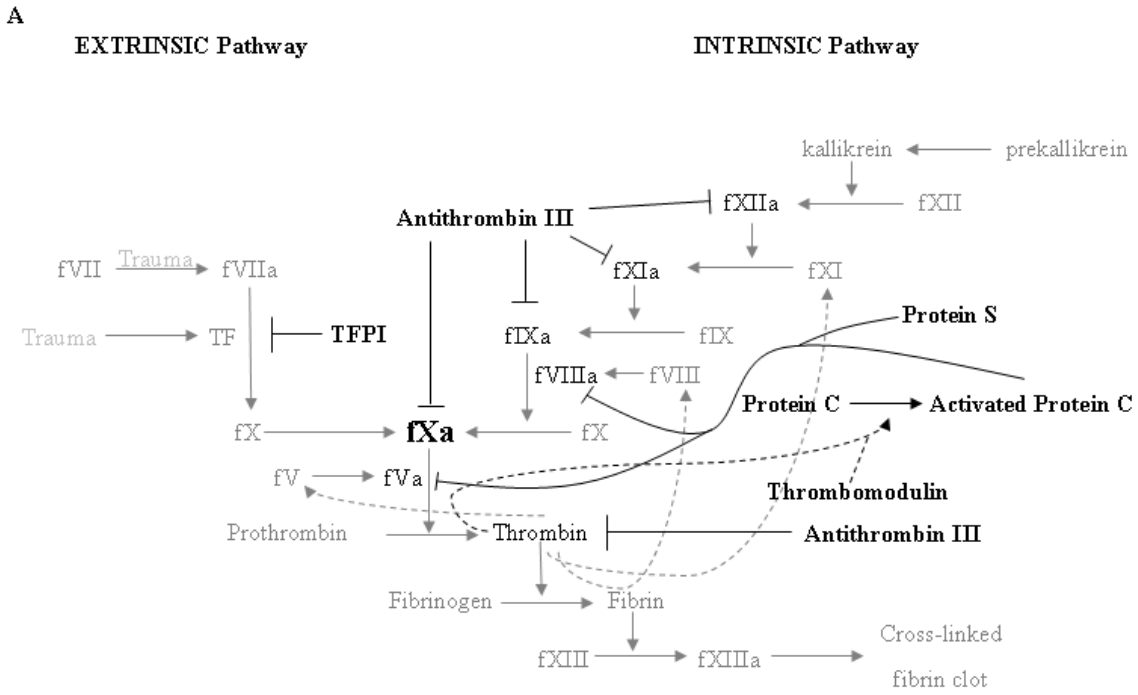


Figure 1.3 – Regulation of the Coagulation Cascade. (A) The process of secondary hemostasis is tightly regulated by the above inhibition factors. The extrinsic arm of the coagulation cascade is inhibited by TFPI acting on the active protease complex consisting of TF, fVIIIa and fX. However, TFPI is present in the bloodstream at low concentrations (approximately 2.5 nmol/L) and thus only delays the anticoagulation process (Novotny *et al*., 1991). TFPI alone is incapable of preventing all activation of FX since the intrinsic Xase complex is unaffected by TFPI. Antithrombin III, on the other hand, is constitutively active and present in the plasma at a higher concentration (approximately 3.2 µmol/L) (Olson *et al*., 1993). As a serine protease inhibitor, antithrombin III inactivates all the procoagulant serine proteases which include thrombin, fIXa, fXa, fXIa and fXIIa. In addition, activated protein C formed as a result of thrombin complexing with thrombomodulin cleaves fVa and fVIIIa, cofactors of the prothrombinase and tenase

(intrinsic Xase) complexes respectively. Thus thrombin production is regulated by both the concentration of procoagulant constituents of the coagulation cascade and by the presence of the aforementioned inhibitors.

Abbreviation: Tissue factor pathway inhibitor (TFPI)

1.2— Hemophilia A: A Deficiency of Factor VIII

Hemophilia A is an X-linked bleeding disorder attributed to the loss of a functional secreted fVIII protein. A deficiency in fVIII prevents the assembly of the tenase complex and thus the generation of activated fX is thwarted. Approximately 1 in every 5,000 males has hemophilia A, equating to approximately 400,000 people across the globe (Doering and Spencer, 2009). The severity of clinical manifestations of hemophilia A depends on the amount of functional fVIII secreted into the bloodstream. Individuals with less than 1% normal fVIII levels are classified as having a severe form of hemophilia A. If left untreated, clinical manifestations of severe hemophilia A include physical disability as a result of repeated spontaneous bleeding occurring predominantly into the joints and muscles. In addition, nonspontaneous bleeds associated with mild trauma are intensified and can culminate into a life-threatening hemorrhage event. Individuals with measurable fVIII between 1-5% of normal are classified as having a moderate form of hemophilia A whereas individuals with levels greater than 5% of normal are considered to have a mild form. As a result, bleeding episodes associated with a mild phenotype are rare and usually nonspontaneous as a result of trauma or surgery. Bleeding episodes associated with a moderate phenotype fall somewhere between that of a mild and severe bleeding tendency.

Hepatocytes and endothelial cells of the liver are believed to be the predominant endogenous producers of fVIII (Wion *et al.*, 1985; Lenting *et al.*, 1998; Kaufman and Pipe, 1999). Within these cells, the 180 kilobase fVIII gene is transcribed into a 9010 base pair (bp) transcript with a short 5' untranslated region (150bp), a large 3'

untranslated region (1806bp), and a 7056 bp open reading frame (**Figure 1.4 A-B**). The open reading frame of the fVIII transcript is translated into a protein containing 2351 amino acids including a 19 amino acid signal peptide that translocates the mature 2332 amino acid polypeptide into the endoplasmic reticulum (Vehar *et al*., 1984) (**Figure 1.4 C**). Within the lumen of the endoplasmic reticulum, fVIII becomes glycosylated (N-linked glycosylation) and interacts with several chaperone proteins (Marquette *et al*., 1995; Swaroop *et al*., 1997; Pipe *et al*., 1998). These interactions have been shown to trap fVIII within the endoplasmic reticulum and thus limit the production of fVIII. However, a portion of fVIII is released from the endoplasmic reticulum and transported through the secretory pathway. Additional processing occurs within the Golgi apparatus which includes modification of the N-linked oligosaccharides previously added in the endoplasmic reticulum, O-linked glycosylation and sulfation of specific tyrosine residues. Once fVIII is accurately processed, fVIII undergoes intracellular proteolysis within the Golgi apparatus. fVIII is comprised of six major structural domains and three small acidic amino acid rich domains sandwiched in between. fVIII can be characterized from the amino terminus as A1-a1-A2-a2-B-a3-A3-C1-C2. Furin recognizes Arg-X-X-Arg motifs within the B domain and cleaves fVIII yielding a 210kDa heavy chain (A1-a1-A2-a2-B) and a 80kDa light chain (a3-A3-C1-C2) (**Figure 1.4 D**). fVIII is secreted into the blood as a heterodimer in which the two chains are noncovalently bound via a Cu^{2+} ion. Once in the blood stream fVIII binds to the plasma protein VWF and circulates as a stable complex.

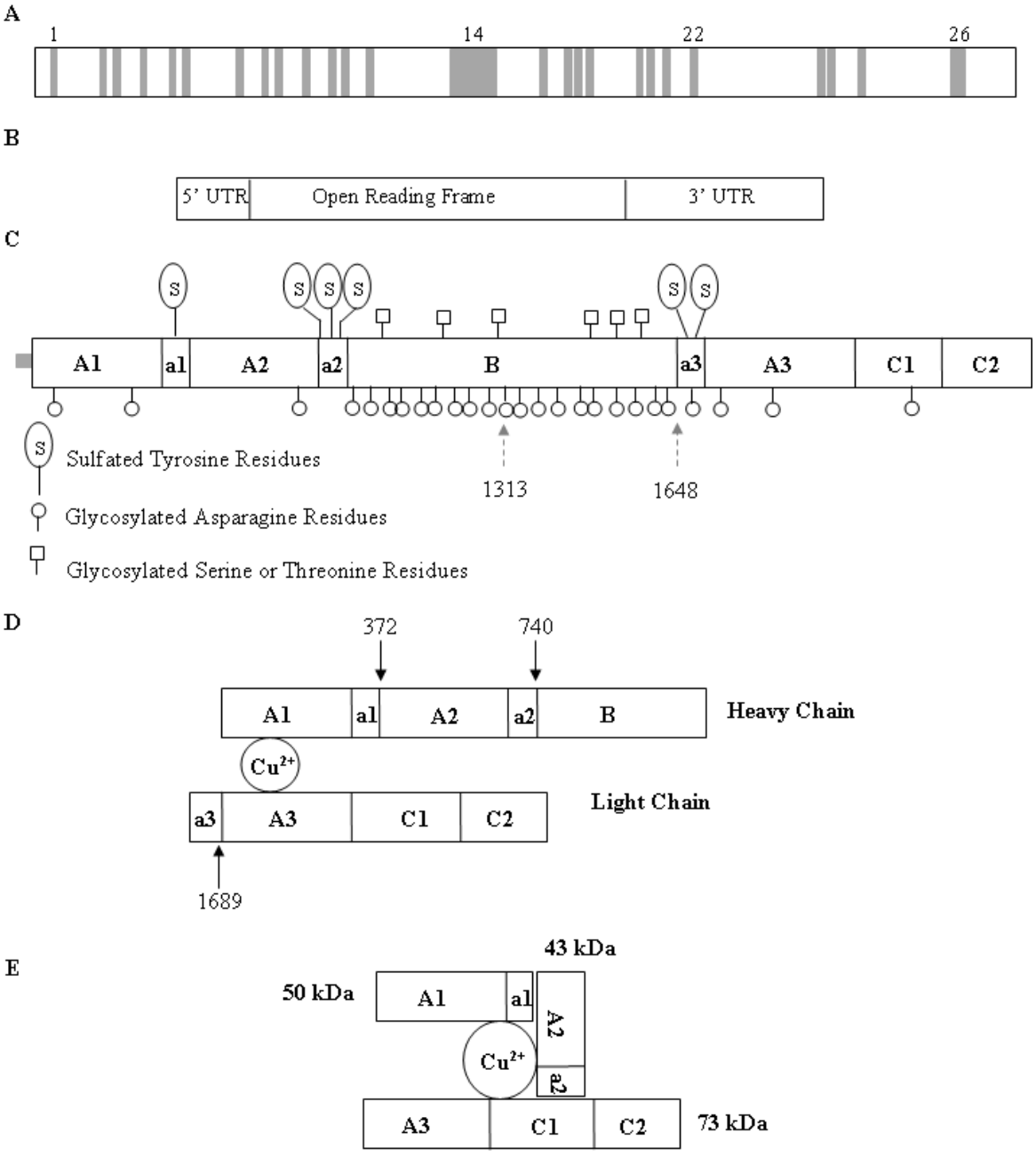


Figure 1.4 – The progression of fVIII from gene to activated protein. (A) The fVIII gene spans 186 kilobases containing 26 exons with lengths ranging from 69 to 3,106 basepairs, the latter correlating to exon 14. (B) The 9 kilobase fVIII transcript contains a 7,056 base pair open reading frame which encodes for (C) the amino acids which make

up the fVIII polypeptide chain, the first 19 of which are act as a signal peptide directing cellular excretion (denoted by the small grey box). A distinct domain structure is exhibited within the mature fVIII protein: A1 (residues 1-336), A2 (373-710), B (741-1648), A3 (1690-2019), C1 (2020-2172) and C2 (2172-2332). The a1, a2 and a3 acidic regions border the major structural domains and contain sulfated tyrosine residues. Sequence homology is shared among all three A domains (30%) as well as among both C domains (40%). The C1 and C2 domains have been found to contain detectable homology with that of proteins which bind glycoconjugates and negatively charged phospholipids (Bhopale and Nanda, 2003). The B domain on the other hand does not exhibit sequence homology with any other known protein. Instead, the B domain contains 19 of the 26 total N-linked glycosylation sites. In addition, the B domain contains sequence motifs recognized by both intracellular and extracellular proteases (Bowen, 2002). fVIII is cleaved intracellularly after Golgi processing at two arginine residues within the B domain, as indicated by arrows. (D) As a result a fVIII heterodimer is produced composed of a heavy chain (A1 and A2 domains, as well as a shortened B domain) and a light chain (A3, C1 and C2 domains) held together by a calcium ion bridge. Further cleavage occurs extracellularly by thrombin at arginine residues within both the heavy and light chains (E) yielding a 50, 43 and 73kDa polypeptide and releasing the remainder of the B domain resulting in FVIII activation (Eaton *et al*., 1986; Lenting *et al*., 1998). Abbreviations: UTR (untranslated region).

Distinct molecular events can result in fVIII deficiency. fVIII deficiency can be the result of limited production or increased clearance. In this scenario, the protein structure of fVIII is normal, but functional activity is dramatically decreased as a result of diminished fVIII concentrations in the blood. One example of this can be observed as a result of VWF unable to bind efficiently to fVIII. A tyrosine residue within the A3 domain has been found to be crucial to the interaction of fVIII and VWF (Leyte *et al*., 1991). The tyrosine residue is normally sulfated, but without sulfation, fVIII and VWF do not bind effectively (Pittman and Kaufman, 1992). In this scenario, the protein structure of fVIII is normal yet concentration and functional fVIII activity is diminished due to a significant reduction in the half-life of fVIII and an increase in the clearance of fVIII from the blood. fVIII deficiency can also be caused by expression of nonfunctional fVIII. Although the concentration of fVIII is normal, the functional activity is less than normal since the protein is intrinsically defective. A series of mutations have been identified as the source of nonfunctional fVIII including inversions, point mutations, deletions, duplications and insertions (Tuddenham *et al*., 1991). In its entirety, the fVIII gene is expansive consisting of 186 kilobases which includes 26 exons (Vehar *et al*., 1984) and is located on the tip of the long arm of the X chromosome (Gitschier *et al*., 1984). Half of all severe hemophilia A cases (20-25% of all hemophilia A cases) are due to a chromosomal mutation in which a segment of the gene is removed and reintegrated 180^{o} from the original orientation, otherwise known as an inversion. Intron 22 and intron 1 are considered inversion hotspots containing long inverted repeats (approximately 500-kb in length) in which intrachromosomal recombination occurs between an identical inverted repeat towards the telomere (Brinke *et al*., 1996; Bagnall *et al*., 2002;

Pezeshkpoor et al., 2012). Inversions originating at intron 22 are more common than inversions found at intron 1 (Lakich *et al*., 1993). The other half of mutations, resulting in severe hemophilia A, are due to large deletions or insertions, duplications, and nonsense mutations. A nonsense mutation is a type of point mutation within a codon that converts the original amino acid translating codon into that of a stop codon, resulting in a truncated nonfunctional protein. A mutation of this magnitude results in a severe form of hemophilia A. However, other types of point mutations are less detrimental and result in a moderate or mild hemophilia A phenotype. These include missense mutations (mutation within a codon resulting in a different amino acid) and mRNA splice-site mutations (mutation disrupting the splicing of the mRNA by either addition or removal of a splice site). More than 80 unique point mutations have been identified within the fVIII gene. Point mutations can be found to be distributed across all of the exons of fVIII (**Table 1.1**). Thus it is not surprising, that point mutations are the most prevalent type of mutation responsible for nearly 50% of all cases of hemophilia A.

Table 1.1 – Recurrent mutations in the fVIII gene

Exon	Domain	Nucleotide Δ	Mutation	Amino Acid Δ	Disease Severity
4	A1	GTG>ATG	Missense	Val>Met	Moderate-Mild
8	A1	CGA>TGA	Nonsense	**Arg**>STOP	Severe
10	A2	GGA>AGA	Missense	Gly>Arg	Moderate-Mild
11	A2	CGG>TGG	Missense	**Arg**>Trp	Mild
11	A2	CGC>TGC	Missense	**Arg**>Cys	Moderate-Mild
11	A2	CGC>CAC	Missense	**Arg**>His	Mild
12	A2	CGC>TGC	Missense	**Arg**>Cys	Moderate-Mild
14	B	TAT>TTT	Missense	Tyr>Phe	Moderate
14	a3	CGC>TGC	Missense	**Arg**>Cys	Moderate
16	A3	CGT>CAT	Missense	**Arg**>His	Moderate
18	A3	CGA>TGA	Nonsense	**Arg**>STOP	Severe
18	A3	CGA>CAA	Missense	**Arg**>Gln	Mild-Moderate
18	A3	CGA>TGA	Nonsense	**Arg**>STOP	Severe
18	A3	CGA>CAA	Missense	**Arg**>Gln	Mild
19	A3	CGG>TGG	Missense	**Arg**>Trp	Severe-Moderate
19	A3	GTG>GCG	Missense	Val>Ala	Moderate
22	A3	CGA>TGA	Nonsense	**Arg**>STOP	Severe
*23	C1	CGA>TGA	Nonsense	**Arg**>STOP	Severe
23	C1	CGT>CAT	Missense	**Arg**>His	Mild-Moderate
23	C1	CGC>TGC	Missense	**Arg**>Cys	Mild
23	C1	CGC>CAC	Missense	**Arg**>His	Moderate
24	C2	CGA>CAA	Missense	**Arg**>Gln	Severe-Moderate
24	C2	CGA>TGA	Nonsense	**Arg**>STOP	Severe
25	C2	TGG>TGT	Missense	Trp>Cys	Mild-Moderate
26	C2	CGA>TGA	Nonsense	**Arg**>STOP	Severe
26	C2	CGA>CAA	Missense	**Arg**>Gln	Mild

Table adapted from (Graw *et al.*, 2005). Mutations were considered to be recurrent if reported at least 10 times in the HAMSTeRs database.
* Most frequent point mutation.

The current treatment of severe hemophilia A consists of repetitive prophylactic administration of recombinant or plasma concentrated fVIII as a means of protein replacement. However, this treatment does not alleviate all the symptoms. Chronic joint pain can be experienced regardless of receiving optimal care (Ide, 2007). In addition, the cost associated with repetitive administration of recombinant fVIII limits the utility of the product to a significant percentage (approximately 75%) of the individuals affected by the disease (Doering and Spencer, 2009). Plasma concentrated fVIII historically has resulted in the transmission of blood borne pathogens (HIV, Hepatitis, etc.) to hemophilia patients and has thus, for the most part, been replaced with the administration of recombinant fVIII. Recombinant fVIII, produced in both Baby Hamster Kidney cells (Miles Inc., West Haven, CT) and Chinese Hamster Ovary cells (Baxter Healthcare Corp., Glendale, CA), is costly because it is difficult to manufacture resulting in periodic product shortages. On average, optimal treatment for an individual with severe hemophilia A can cost $250,000 for recombinant fVIII product per year. Another reason why the current treatment of recombinant fVIII is not optimal is that recombinant fVIII has a short half-life in the circulation requiring repetitive multi-weekly prophylactic administration. Treatment in this manner is extremely inconvenient and can be concerning in regards to patient compliance. Further complicating the current therapy, some individuals who receive this treatment develop an immunogenic response in the form of neutralizing antibodies against the administered fVIII. These inhibitors make managing a bleeding episode in these individuals extremely complicated and therapeutically challenging. Therefore, new therapeutic approaches are being developed to treat hemophilia A.

A monogenic disorder, such as hemophilia A, is a prime candidate for gene therapy for a number of reasons. First, the gene responsible for hemophilia A has been isolated and well characterized, which has led to useful preclinical mouse (Bi *et al.*, 1995) and dog models (Kingdon & Hassell, 1981) as well as a newly described sheep model (Porada *et al.*, 2010). Second, the delivery of fVIII is not restricted to certain cell types. In fact, any tissue with exposure to the vasculature is an appropriate cellular target in that fVIII only needs to be secreted into the bloodstream for effectiveness. This makes a gene therapy approach relatively straightforward and achievable. Third, the therapeutic window is large. Only a moderate increase in fVIII (2-5% of normal equating to 2-5ng ml^{-1}) is required to be therapeutically effective while levels as high as 150% of normal have not been associated with adverse effects such as thrombosis (VandenDriessche *et al.*, 2001). As a result, a number of viral and nonviral delivery strategies have been postulated.

1.3— Pharmacological Principles of Gene Therapy

Within the field of gene therapy, nucleic acids are being used as pharmaceutical agents to treat disease. Currently, there are four different strategies being utilized based on the underlying molecular consequences surrounding the gene mutation (Fischer and Cavazzana-Calvo, 2008). The first and most prominent strategy within the field focuses on restoring a loss-of-function mutation by introducing a functional gene as a means of gene replacement. A second strategy, on the other hand, has directed efforts towards the correction of a gain-of-function mutation. In this scenario, the expression of the mutated

gene is inhibited typically with the use of small interfering RNAs (Pelletier *et al*., 2006). In the same manner, small nuclear RNAs are utilized by the third strategy which attempts to correct an abnormally processed mRNA by hindering incorrect splicing due to the introduction of aberrant splice sites (Gorman *et al*., 1998). This strategy is beneficial in that it restores the proper processing of the mRNA by blocking the aberrant splice sites and forcing the splicing machinery to reselect the correct sites. However, a fourth possibility would be to correct the mutated gene and reverse the manifestations of the disease. This could be accomplished by inducing a double-strand break within or near the mutated gene and providing a correct copy of the gene to be inserted into the host genome by homologous recombination.

Regardless of the strategy chosen, a delivery system is needed to transfer nucleic acids into a cell. This is due to limited transfer efficiency upon treatment of cells with naked DNA. Gene therapy requires sufficient gene transfer in order to provide substantial expression of the therapeutic gene. Although both non-viral and viral methods of delivery have been postulated, the transfer efficiency of viruses has exceeded the potential of non-viral methods possibly due to the natural evolution of viruses as delivery vehicles for nucleic acids into a cell. Therefore, many viral vectors have been considered for gene transfer, including both non-integrating and integrating viral vectors. In addition to requiring high transfer efficiency, successful gene therapy requires a delivery system to be devoid of toxic and immunogenic side effects. Unfortunately, many viral vectors contain potential immune-stimulators resulting in either a T cell-mediated immune response targeting transduced cells or a humoral immune response evident by the

generation of antibodies against viral proteins and/or the transgene product (Zhou *et al*., 2004). Other than these two general properties, characteristics intrinsic to each viral vector dictate their use within specific gene therapy applications determined mainly by the intended cellular target and required duration of expression. The remainder of this section will focus on the intrinsic properties of the available viral vectors with emphasis on their utility for use within hemophilia A gene therapy applications.

Adenoviral vectors were among the first to be used as therapeutic gene transfer vectors in clinical trials as a result of innate advantageous transferring properties. One prime example being that they are not constrained by insert size. The carrying capacity of adenoviral vectors reaches forty-five kilobases allowing for the transfer of large transgenes as well as any number of regulatory elements utilized for expression enhancement or safety. In regards to safety, adenoviral vectors are also appealing as a vector as a member of the non-integrating class of vectors which persist within cells extrachromosomally. Although, there is the risk that the vector genome will be lost upon cell division, non-integrating vectors lack the potential to disrupt the integrity of the genome. In addition, adenoviral vectors are appealing in that they efficiently transduce both dividing and non-dividing cells. If non-dividing cells are targeted then it is possible that long term expression can be achieved as a result of extrachromosomal vector persistence.

However despite these advantages, the continued use of adenoviral vectors has been inhibited by associated acute toxicity. Upon systemic administration of adenoviral

vectors, liver enzymes have been documented to be elevated as a result of the induction of pro-inflammatory cytokines and chemokines directed against viral capsid proteins (Aruda, 2006; Brunetti-Pierri *et al*., 2004; Schnell *et al*., 2001; Muruve, *et al*., 1999; Yang *et al*., 1994). One specific adverse event resulted in the death of an eighteen year old by the name of Jesse Gelsinger who was admitted to a pilot gene therapy study for the treatment of ornithine transcarbamylase deficiency (Raper *et al*., 2003). Upon receiving a systemic injection of adenovirus carrying the missing ornithine transcarbamylase gene, a massive immune response was mounted against the vector. His untimely death could have been prevented had the researchers followed the exclusion protocol outlined prior to patient enrollment. However, despite the cause being associated with the inappropriate conduct of the personnel involved, the death of Jesse Gelsinger resulted in a major setback within the field.

As a result of the innate toxic side effects, adenoviral vectors are constrained to use within neonates as a means to induce tolerance to both the viral vector and the transgene. In this manner, toxicity can be circumvented by capitalizing on the immaturity of the immune system during early development. This was recently performed within hemophilia A mice after intravenous injection of 5×10^{12} vector particles/kg administered at three days of age (Hu *et al.*, 2011). fVIII levels peaked at ~650% of normal on day six, but declined with animal growth as a result of episomal vector loss. The single administration of adenoviral vectors within neonates was not able to achieve sustained therapeutic levels of fVIII. However, tolerance to the transgene and viral capsid proteins was achieved as noted by the lack of adverse effects following subsequent vector

administration (Hu *et al*., 2011). It is worthwhile to note that this therapy could also be beneficial, in the absence of subsequent vector administration, to prevent the development of inhibitors to fVIII which currently complicates protein replacement therapy.

Adeno-associated viral vectors (AAV), on the other hand, are being extensively evaluated for use in gene therapy. The adeno-associated viruses are relatively small viruses, composed of a linear single-stranded DNA genome of approximately 4.6-kb. As of 2009, twelve different serotypes have been isolated from both human and non-human primates (a number which is expected to increase), all of which vary in transduction efficiency and tissue tropism (Youjin and Jun, 2009). Serotype 2 (AAV-2) was the first adeno-associated virus to be sequenced and cloned, and was therefore the first serotype to be used within gene transfer studies (Hermonat and Muzyczka, 1984; Laughlin *et al*., 1983; Samulski *et al*., 1982). Wild-type AAV-2 was found to encode two large open reading frames, composed of the replication (Rep78, Rep68, Rep52, and Rep40) and capsid genes (VP1, VP2, and VP3). To accommodate transgenes, these genes were removed leaving behind the inverted terminal repeats required for replication and packaging into a viron (Youjin and Jun, 2009). Like adenoviral vectors, AAV-2 and other AAV serotypes have the ability to efficiently transduce both non-dividing and dividing cells (Hallek *et al.*, 1998). This quality makes modifying hepatocytes *in vivo* possible, making them a potentially ideal vector for fVIII delivery. However, due to the small size of the genome, the use of AAV is limited in being unable to efficiently package large transgenes like fVIII. In addition, stable integration of the transgene is not possible with adeno-

associated viral vectors. Although, adeno-associated viruses have been documented to integrate stably within a specific region of chromosome nineteen (Surosky *et al*., 1997), this feature has been eliminated by the removal of the replication and capsid genes within the adeno-associated viral vectors used for gene therapy applications. Therefore, as non-integrating vectors, the vector genome remains episomal and could potentially be lost.

In order to provide stable integration of a transgene that can be sustained throughout the life-span of a cell integrating viruses, such as gammaretroviral and lentiviral vectors, have been utilized. However, genomic integration of a viral vector has concerned the gene therapy community because of the possibility of disrupting the integrity of the genome resulting in cellular transformation due to integration of the transgene near an oncogene. This process has been termed insertional mutagenesis, and can cause misregulation of oncogene expression, which could subsequently lead to malignancy. Historically, insertional mutagenesis was debatable due to the lack of data confirming its occurrence. However, solid data has confirmed the possibility of insertional mutagenesis due to γ-retroviruses. First, γ-retroviruses have been found to insert into the genome near promoters of transcriptionally active genes, setting the stage for the disrupted regulation of downstream genes (Wu *et al*., 2003). Second, direct proof of insertional mutagenesis has been documented in human gene therapy clinical trials for the treatment of severe combined immune deficiency syndrome (X-SCID) that used γ-based retroviruses (Hacein-Bey-Abina *et al*., 2003). In the initial X-SCID clinical trials using γ-based retroviruses, of the 20 treated children, 5 have developed a T-cell leukemia-like disorder. The cause of the leukemia has been found to be due to integration of the γ-retrovirus

upstream of the oncogene, LMO2. Integration at this site disrupted the regulation of the oncogene increasing the expression of LMO2. The confirmation of insertional mutagenesis with γ-retroviral vectors halted their use for clinical development for hemophilia.

Unlike γ-retroviruses, no direct evidence for insertional mutagenesis has been documented for recombinant lentiviral based retroviruses. Instead, lentiviral vectors have been promising vectors for the delivery of the fVIII transgene because, like adeno-associated viral vectors, they are able to transduce both dividing and non-dividing cells, (Naldini *et al.*, 1996) but unlike adeno-associated viral vectors are not as constrained by the size of the transgene. Lentiviral vectors have a carrying capacity of approximately 7-kb exogenous DNA. For these reasons, lentiviral vectors are ideal for gene therapy applications for the treatment of hemophilia A. The first lentiviral vectors contained all of the viral genes except for the envelope gene (Naldini *et al.*, 1996). Since then numerous safety measures have been taken to ensure the safety of the virus in the unlikely event of generating a replication competent lentivirus (**Figure 1.5**). Second generation HIV-based vectors removed four of the nine viral genes, eliminating accessory genes (Quinonez & Sutton 2002). As an added precaution second generation lentiviral vectors have been designed as replication incompetent self-inactivating (SIN) vectors. This has been accomplished by removing 133 bps from the 3' long terminal repeat (LTR). LTRs flank viral DNA at both the 3' and 5' ends and are involved in the integration process. During the process of integration, the 3'LTR is copied and becomes the 5'LTR in the integrated sequence, which inactivates the integrated 5'LTR. Further

safety measures resulted in the third generation lentiviral vectors, in which two more viral genes (the tat and the rev genes) were removed from the expression plasmid. To date these third generation lentiviral vectors have been used for gene therapy applications without any adverse complications (Boztug *et al*., 2010; Cartier *et al*., 2010; Cavazzana-Calvo *et al*., 2010; DiGiusto *et al*., 2010).

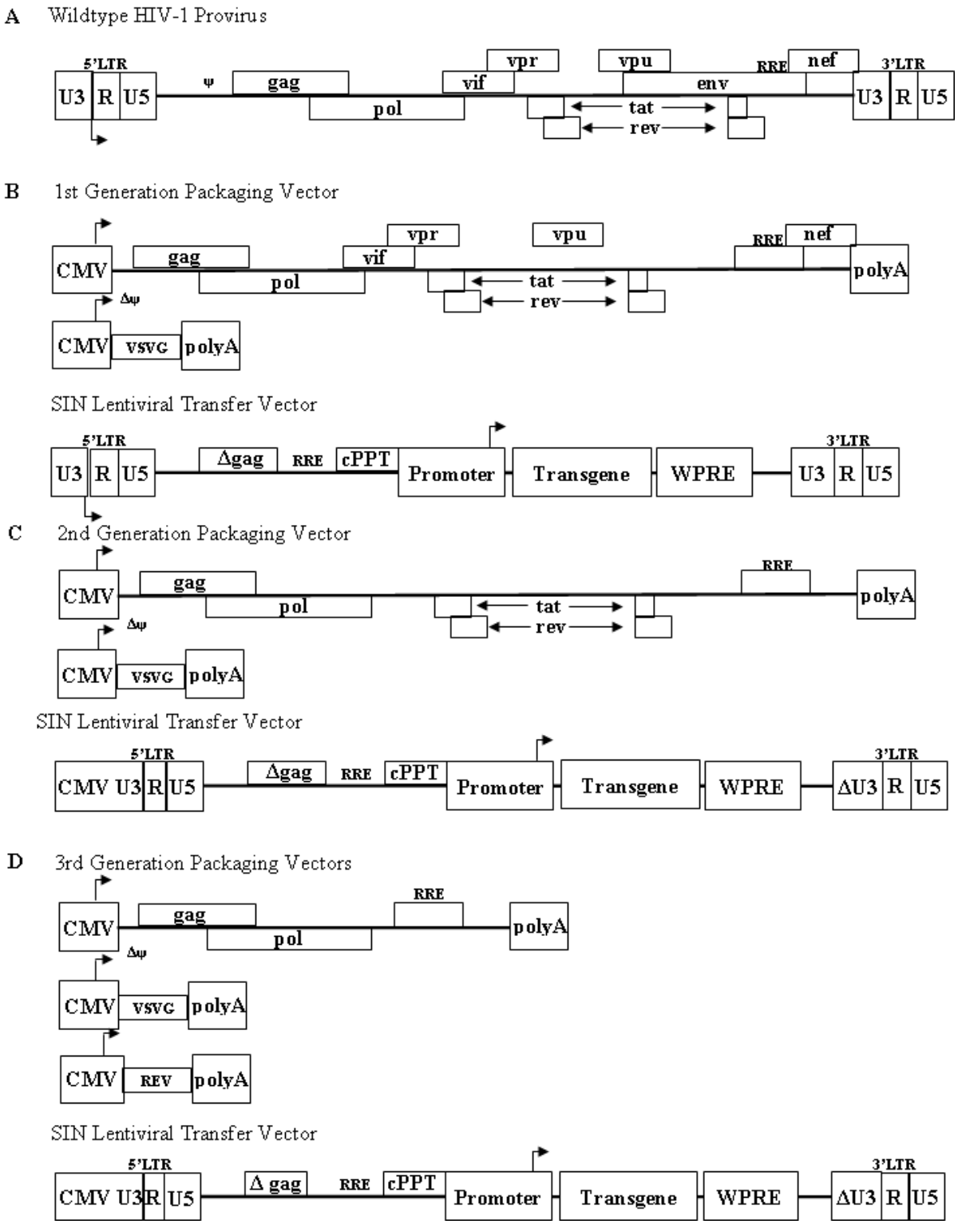


Figure 1.5 – Progression of lentiviral vector packaging systems. (A) Schematic representation of the wild type HIV-1 genome which consists of the structural (gag, pol

and env), regulatory (tat and rev) and accessory genes (vif, vpr, vpu, and nef). (B) A first generation HIV-1 vector system in which the env gene was removed and replaced with the VSVG envelope gene on a separate plasmid. In this manner viral particles have been pseudotyped with a foreign viral glycoprotein. (C) The accessory genes were removed from the second generation HIV-1 vector system. In addition, the LTRs in the transfer vector became self-inactivated due to a deletion in the U3 region of the 3'LTR. (D) Third generation HIV-1 vector systems eliminated the regulatory tat and rev genes. This vector system is tat-independent yet the rev gene is still required and has been placed on a separate plasmid, making this vector system a three plasmid vector system.

1.4— Historical Perspective of Gene Therapy

The first human gene therapy clinical trial occurred in 1989 as a means to treat individuals with advanced metastatic melanoma. Tumor-infiltrating lymphocytes had previously been demonstrated to have lytic properties specific for autologous tumors (Muul *et al.*, 1997; Topalian *et al.*, 1989) that in combination with interuleukin-2 could substantially reduce the size of the tumor (Rosenberg *et al.*, 1988). Yet a handful of questions persisted in regards to their distribution and long-term persistence. Therefore, tumor deposits were excised in order to isolate tumor-infiltrating lymphocytes from each of the five patients admitted in the trial. Following initial expansion, the lymphocytes were modified with a γ-retroviral vector (derived from the Moloney murine leukemia virus) containing a neomycin resistance gene as a marker. No adverse side effects were noted as a result of the gene transfer. In addition, the neomycin resistance gene was detected up to two months following cell infusion (Rosenberg *et al.*, 1990). This study demonstrated successful transfer of a foreign gene to human cells with the use of a retroviral vector. It also alluded to the safety of these vectors as a means to treat other diseases by gene therapy. As a result, preclinical research progressed with the use of similar γ-retroviral vectors for the treatment of monogenic diseases.

In 1990, two children were treated for adenosine deaminase-deficient severe combined immunodeficiency disorder, a congenital disease resulting in frequent opportunistic infections as a result of an impaired immune system. T cells were isolated from each patient, transduced *ex vivo* with a γ-retroviral vector (derived from the Moloney murine leukemia virus) containing the adenosine deaminase gene and readministered back to the

patient (Blaese *et al*., 1995). The effectiveness of the trial is debatable due to continued administration of PEGylated bovine adenosine deamainase as a means of protein replacement therapy during the trial. However, ten years later, the modified cells appear to be persisting in the circulation with one of the children exhibiting 20% gene modified lymphocytes (Muul *et al*., 2003). In addition, no adverse effects were experienced as a result of the γ-retroviral vector utilized, once again providing confidence as to the feasibility and safety of gene therapy with the use of these vectors. However, the two aforementioned trials developed a sense of false security in regards to the safety of a γ-retroviral vector derived from the Moloney murine leukemia virus. This was apparent following a clinical trial for X-linked severe combined immunodeficiency. In this trial, $CD34^+$ bone marrow stem cells were modified *ex vivo* to contain a correct copy of the previously unfunctional γc gene (Hacein-Bey-Adenia *et al*., 2002). Although nearly all twenty patients treated have improved immune functioning as quantified by an increase in T and B cells, five patients developed acute lymphoblastic leukemia due to integration near the proto-oncogene LMO2 (Hacein-Bey-Adenia *et al*., 2003). As a result the clinical use of γ-retroviral vectors derived from the Moloney murine leukemia virus has been restricted. Instead, retroviral vectors derived from an HIV-based lentivirus are being utilized for the modification of $CD34^+$ bone marrow stem cells *ex vivo*. Much clinical progress has been made utilizing this approach for the treatment of X-linked adrenoleukodystrophy, Wiskott-Aldrich Syndrome, β-thalassaemia and AIDS. This has been accomplished without any apparent adverse side effects (Cartier *et al*., 2009; Boztug *et al*., 2010; Cavazzana-Calvo *et al*., 2010; DiGiusto *et al*., 2010).

In regards to hemophilia A, three gene therapy clinical trials to date have been conducted. Each utilized the human cDNA fVIII sequence, but all three differed in the manner whereby the nucleic acid sequence was incorporated into the patients' cells. The first trial by Roth *et al.* (2001) admitted six subjects with severe hemophilia A. Dermal fibroblasts were biopsied from each patient from the upper arm, expanded in tissue culture dishes in a laboratory incubator and electroporated (exposed to a voltage shock in order to perturb the cell membrane layer), resulting in the incorporation of the cDNA sequence into the fibroblast cells. After culturing, stably modified clones were selected and implanted into the omentum of each patient. Transient fVIII expression was observed in three of the patients for up to 6 months, with one patient expressing 4 percent of the normal amount of fVIII twelve weeks after implanting the genetically modified cells. However, the levels of fVIII diminished to less than 0.5% of normal a year later. The second trial included eleven subjects (Powell *et al.*, 2003). Four doses (2.8×10^7, 9.2×10^7, 2.2×10^8, and 4.4×10^8 TU/kg) of a γ-retroviral vector based on the Moloney murine leukemia virus were administered via peripheral vein injection over three consecutive days. No adverse effects were observed throughout the 53 week study. Yet once again only a transient expression of fVIII was achieved. Eight of the patients demonstrated greater than 1% of normal fVIII levels on two or more occasions yet these levels were not sustained throughout the study and could be associated with the administration of exogenous recombinant fVIII. The final trial was performed by GenStar Therapeutics. One patient was injected intravenously with an adenovirus, which contained the full-length human fVIII cDNA. The patient acquired an immune reaction to the virus and the trial was abruptly closed (Berlfein, 2003). Despite promising

preclinical data, hemophilia A gene therapy clinical trials have made little advancement in the field due to limited expression of fVIII (High *et al*., 2005).

1.5— Preclinical Gene Therapeutic Applications to Cure hemophilia A

Large transgenes, such as the fVIII transgene, complicate gene therapy applications using viral vectors by (1) limiting the types of vectors available due to encapsidation limitations and (2) reducing the titer of viral vector that can be produced (Kumar *et al*., 2001; Radcliffe *et al*., 2008; Yacoub *et al*., 2007). The different human cDNA transgene lengths chosen for the clinical trials are a reflection of these limitations. The GenStar Therapeutics trial, unlike the other two trials, utilized an adenovirus which has fewer encapsidation constraints than other viral vectors available for gene transfer. As a result, it was the only trial to utilize the full-length cDNA 7-kb sequence. Incorporating a 7-kb transgene into a retrovirus, on the other hand, results in diminished viral titer making it difficult to achieve a high enough number of viral particles to be effective. Reducing the size of the insert, however, can increase viral titer (Yacoub *et al*., 2007). For this reason, many groups using viral vectors have reduced the size of the cDNA to approximately 4.5-kb. This can be accomplished by removing sequence that encodes the B domain, which has been found to be dispensable for the coagulation activity of fVIII (Toole *et al*., 1986).

As demonstrated above, the complications associated with the large fVIII transgene has led researchers to find innovative ways to apply gene therapy techniques to the transfer of the fVIII transgene. The following sections will discuss the methods that have been

proposed for the pre-clinical testing of gene transfer technologies for fVIII. These methods will be outlined in reference to which vector is being used, since each vector is accompanied with its own set of limitations. Both non-viral and viral vectors will be discussed, highlighting the strategies utilized to overcome the limitations experienced due to the use of a large transgene.

Preclinical use of non-viral vectors for hemophilia A

Unlike viral vectors, non-viral vectors themselves do not risk evoking an immunological response, are less expensive to produce, and are less limited by the size of the transgene. As a result as mentioned above, naked DNA gene transfer was among the first methods to be utilized in a clinical trial for hemophilia A (Roth *et al.*, 2001). However, this strategy results in transient gene expression because of limited uptake by target cells and further limited integration into the genome of these cells (a process that occurs only randomly through non-homologous recombination) (Essner *et al.*, 2005). This issue can be overcome by promoting stable integration into the cell's genome with the use of a transposable element. Transposable elements, although mostly inactive, are found to be littered throughout the human genome (Deininger and Batzer, 2002). In an active form, transposable elements (transposons) have the ability to jump from one location in the genome to another by a "cut and paste" method through the enzyme transposase, which is encoded in the element. In order for a transposon to be utilized as a gene transfer vehicle, two components must be delivered to the target cell, (1) the transgene flanked by inverted repeat/direct repeat elements that are recognized for integration, and (2) a transposase which can be encoded in the same plasmid or in a second plasmid. These plasmids can

be taken up into cells after being complexed to a cationic polymer such as polyethylenimine (PEI). Although transposons can carry an expansive amount of DNA, transposons are still somewhat limited by insert size. Integration efficiency has been shown to decrease with the size of the transgene (Essner *et al*., 2005). This is due to both the difficulties in delivering plasmids containing larger inserts as well as the limitations of the transposase. For example, the transposase enzyme associated with the Sleeping Beauty transposon, the most notable non-viral gene delivery system currently used, is only able to transpose up to 10-kb.

Transposons have been utilized as a non-viral vector for gene therapy of hemophilia A by a number of groups. The Sleeping Beauty transposon system was engineered from an inactive Tc1-like transposable element found in fish (Ivics *et al*., 1997). It has since been used to insert the human B domain deleted fVIII cDNA *in vivo*. fVIII levels remained at ~12% of normal after intravenous plasmid injection through the temporal vein of neonatal hemophilia A mice. However, the presence of inhibitors to fVIII resulted in only partial phenotypic correction (Liu *et al.*, 2006). In order to circumvent the presence of inhibitors, the Largaespada group tolerized neonatal, one day old, hemophilia A mice to fVIII with a facial vein injection of 0.1U/g of recombinant human fVIII. Eight to twelve weeks later, two high pressure tail vein injections of a Sleeping Beauty transposon were administered. As a result, 16% of normal fVIII levels were seen in these mice at eighty-four days after plasmid injection, which was found to be sustained for 6 months. No inhibitors were detected and an improvement in clotting function was noted (Ohlfest *et al.*, 2005).

A high-pressure method of delivery is accomplished by injecting a high volume into the systemic circulation during a short, < 5 min, time frame (termed hydrodynamic injections). In the mouse, this results in DNA uptake followed by expression from the liver. Designed to increase the delivery of DNA to the nucleus of a cell, high-volume high-pressure injection is not yet applicable for humans (Essner *et al*., 2005). Therefore, other methods of transposon delivery are being explored. One method that is being studied is the cell specific delivery of a transposon by encapsulation in a nanocapsule. A recent report by Kren *et al.* (2009) engineered a nanocapsule to contain the Sleeping Beauty transposon system. The nanocapsule was targeted to liver sinusoidal endothelial cells (LSECs) by coating the capsule with an endogenous ligand for the hyaluronan receptor found on LSECs. Inside the nanocapsule a single plasmid was encapsulated containing both a cis-acting transposase as well as the B domain deleted canine fVIII transgene. Eight-week old hemophilia A mice were injected with 25μg of nanocapsule via tail vein injection. fVIII levels were measurable for a total of 11 months.

Preclinical use of Adeno-associated viral vectors for hemophilia A

Hepatocytes have been modified by a number of adeno-associated viral serotypes. For example, AAV-2, after intraportal administration, transferred a canine B domain deleted version of fVIII to liver cells, which resulted in partial phenotypic correction of hemophilia A mice. Although fVIII activity initially peaked to 8% of normal, expression was not sustained, declining to 2% nine months after injection (Sarkar *et al*., 2003). These findings, although confirmed independently by Scallan *et al*. (2003), are

contradictory to similar preclinical and clinical studies for hemophilia B in which therapeutically effective levels of fIX are sustained over time (Manno *et al.*, 2005 and Schuettrumpf *et al.*, 2005). This may be due to the size constraints associated with the AAV vector because fIX is a significantly smaller transgene than fVIII with a cDNA of 2.8-kb. This smaller sequence allows for the inclusion of larger regulatory elements (such as liver specific promoters and enhancers) in the adeno-associated viral vector that are not able to be incorporated after the inclusion of the fVIII cDNA sequence. In the Scallan (2003) and Sarkar (2003) reports, a minipromoter was all that could be incorporated into the AAV-2 vector to promote fVIII expression. Therefore, it was concluded that regulatory elements, that were unable to be included in the AAV-2 vector (due to insert size restraints associated with adeno-associated viral vectors), were required to enhance fVIII expression. A follow up study evaluated additional adeno-associated viral serotypes (AAV-5, AAV-7, and AAV-8) to determine if other serotypes were more efficient at transducing hepatocytes than AAV-2. For the inefficient transfer of large transgenes, it was hypothesized that by increasing hepatocyte transduction, limited expression could be overcome without the inclusion of regulatory expression-enhancement elements. They found that AAV-8 was superior to other serotypes regardless of route of administration (intraportally or intravenously) producing near normal physiological levels of fVIII ($0.58 + 0.2$ IU ml^{-1}) six months post administration at a vector dose of 1×10^{11} vector copies / mouse (Sarkar *et al.*, 2004). A similar comparison was performed by Jiang *et al.* (2006), comparing four serotypes (AAV-2, AAV-5, AAV-6, and AAV-8), in both mice and dogs. In mice, transduction efficiency was found to be least with AAV-5 and greatest with AAV-8. However, for dogs no

substantial difference was observed among the serotypes. Remarkably though, fVIII expression was sustained in some dogs (2 to 5% of normal) for up to three years, resulting in decreased occurrences of spontaneous bleeds (Jiang *et al.*, 2006). This was a significant contribution to the field being the first multiyear report of therapeutic efficacy and safety in a large animal. Albeit, high vector doses of 6×10^{12} and 2.7×10^{13} vector genomes/kilogram were required to yield these subtherapeutic levels of fVIII.

Despite the phenotypic improvements noted above, the inclusion of regulatory elements to the vector for enhancing the expression of fVIII would have been the simplest way to address the limited hepatocyte transduction that was seen with AAV-2. Yet since adeno-associated viral vectors are restricted in their genetic carrying capacity, other avenues had to be evaluated to overcome this limitation. Some reports show packaging of genomes greater than 5-kb in adeno-associated vectors (Alloca *et al*., 2008; Grieger and Samulski, 2005). For example, it is reported that an AAV-8 viral vector was produced containing the B domain deleted human fVIII transgene as well as a full length promoter and enhancer, totaling 5.75-kb (Lu, 2008). However, extensive examination in this report as well as by Wu (2010) clearly showed that inserts ranging from 4.7-kb to 8.7-kb result in heterogeneous virons of varying genome lengths, typically containing truncations at the 5' end. Thus, increasing the size of the adeno-associated vector genome leads to the formation of defective viral particles encapsulating incomplete transgene sequences (Dong *et al.*, 1996).

For these reasons, several groups are attempting to overcome the packaging limitation with the use of two different vectors, one for the heavy chain and one for the light chain of fVIII. This strategy resulted from the demonstration of secretable biologically active fVIII following co-transfection in Chinese hamster ovary cells of two plasmids separating the heavy and light chains (Burke *et al*., 1986 and Yonemura *et al*., 1993). In these cells the two polypeptide chains were able to reconstitute a functional fVIII heterodimer that was secreted into the cellular media. This strategy was first performed in C57BL/6 mice by intraportal administration (Burton *et al*., 1999). These mice, able to secrete endogenous fVIII, were chosen since this strain has already been presented with fVIII and potentially would not elicit an immune response to the fVIII transgene. This would allow for expression to be measured without any contraindications. As a result, greater than physiological levels of fVIII were produced. These results were then extended into the hemophilia A mouse model where therapeutic levels of fVIII were achieved in a dose dependent manner. High levels of transduction were noted with twelve percent of hepatocytes being modified with both vectors. Complicating the issue, a chain imbalance was noted with a 25 to 100 fold excess of light chain. This was found to be due to inefficient translational or posttranslational processing that could not be circumvented by changing the administration ratio of heavy to light chain vectors (Scallan *et al*., 2003). When extended to the hemophilia A dog model, only partial phenotypic correction was achieved, irrespective of adeno-associated serotype used. Although only modest levels of fVIII were observed in the dogs (ranging from 1 to 8% of normal), these levels were found to be sustained for at least two years (Sarkar *et al*., 2006).

Another strategy used to overcome the inability of adeno-associated vectors to deliver large genes is trans-splicing. Trans-splicing attempts to repair the truncated fVIII mRNA *in vivo* by delivering the remaining downstream pre-mRNA. In this strategy, a pre-trans-splicing molecule is delivered by an adeno-associated vector in which complementary mRNA sequences are located at the 5' end of the molecule designed to be spliced with the preexisting truncated fVIII mRNA due to a strong splice site at the 3' end. In this way, a shortened version of the transgene can be delivered *in vivo* to restore the disease phenotype. This method was performed in hemophilia A mice (Chao *et al*., 2003). Hemophilia A mice were created by inserting a neomycin resistance gene into the sixteenth exon of fVIII (Bi *et al*., 1995). As a result truncated fVIII mRNA is still expressed in these mice. By delivering a pre-trans-splicing molecule containing complementary sequence to intron 15, a functional fVIII pre-mRNA was spliced together in vivo resulting in phenotypic correction in eight of the ten injected mice.

Preclinical use of Lentiviral vectors for hemophilia A

Despite these efforts the fact remains that non-integrating adeno-associated viral vectors are unable to stably transduce cells. Therefore, many groups are focusing on the use of integrating vectors. Lentiviral vectors, such as the human immunodeficiency virus (HIV) and the simian immunodeficiency virus (SIV), have been analyzed for their use in gene therapy since 1996 in which Naldini and colleagues revealed the ability of these vectors to overcome the need for cell division during transduction. Being able to transduce both dividing and non-dividing cells gives lentiviral vectors the same advantage as adeno-associated viral vectors. For this reason, lentiviral vectors are likely to make up a second

generation of therapeutic vectors to be tested in clinical trials for the treatment of hemophilia A.

Lentiviral vectors, however, come with a set of limitations (to be discussed in detail in the next section). In particular, lentiviral production is limited. Reduction in titers due to issues associated with transgene size can be overcome by targeting various cell populations *ex vivo* then expanding the genetically modified cells. Therefore, despite diminished titer production, preclinical progress has been made with lentiviral vectors encoding the 4.5-kb B domain deleted fVIII transgene. And, by targeting specific cells, viral transduction can be optimized and enhanced, which further overcomes the reduction in titer due to transgene size. In addition, *ex vivo* modification of cells is considered safer than the *in vivo* delivery of recombinant virus since it eliminates the possible transmission to germline cells, as well as avoiding any systemic toxicity that can result due to direct presentation of the vector particle (Van Damme *et al*., 2004). *Ex vivo* modification also eliminates the issue of modifying antigen-presenting cells, possibly eliminating the development of an immune response to the transgene.

Ex vivo gene therapy for hemophilia A has been analyzed in a broad range of cell types (Viiala *et al*., 2009). Cellular alternatives considered include embryonic stem (ES) cells, bone marrow derived mesenchymal cells, blood outgrowth endothelial cells (BOECs), and hematopoietic stem cells. Stem cells are a reasonable alternative in that they have unlimited replicative potential and contain the ability to differentiate into a wide range of cells. However, the initial use of stem cells in the field of gene therapy resulted in low

expression levels. This was thought to either be due to gene inactivation as a result of extensive differentiation (McIvor, 1987) or an inability to effectively transduce stem cells. Regardless, promising results were published utilizing an inducible system for fVIII in ES cells. However, both ethical and safety concerns have inhibited the continuation of these studies (Kasuda *et al*., 2008). This is due to the controversy surrounding the generation of ES cells from human embryos, as well as the formation of teratomas which arose from undifferentiated ES cells used for insulin production (Fujikawa *et al*., 2005). Fortunately, induced pluripotent stem cells (iPS) appear to be comparable to ES cells and have thus been considered to treat monogenic disorders in order to alleviate the ethical concerns surrounding stem cell usage. iPS cells are derived from adult somatic cells which have been reprogrammed to have stem cell characteristics. Significant progress has been made utilizing iPS cell-based therapy for murine hemophilia A (Xu *et al*., 2009). However, these methods are fairly new and still do not address the potential formation of teratomas. Therefore, many obstacles need to be addressed before this therapy can reach the clinics (for further discussion refer to Liras, 2011). Another cell type, bone marrow derived mesenchymal cells, initially yielded therapeutic levels of fVIII from transduced human mesenchymal cells in immunodeficient mice. Yet fVIII plasma levels deteriorated gradually in spite of the persistence of gene modified cells, suggesting transcriptional repression in this cell type (Van Damme *et al*., 2004).

Human BOECs can be isolated from the peripheral blood of healthy donors and were considered as fVIII gene transfer targets because they express VWF (Jaffe *et al*., 1973;

Wagner *et al.*, 1982). VWF binds with high affinity to fVIII in the circulation and protects fVIII from degradation and uptake by antigen presenting cells (possibly eliminating the development of an immune response to the transgene) (Dasgupta *et al.*, 2007). The expression of fVIII in conjunction with VWF has been demonstrated to enhance efficient transport of fVIII through the secretory pathway (Dorner *et al.*, 1989; Kaufman *et al.*, 1989). Therefore, it was hypothesized that modifying these cells to also express fVIII would result in the secretion of fVIII complexed to VWF, which would result in an increased half-life of fVIII, secreted at sites of injury. After transduction, BOECs expressed high levels of fVIII measured at 1.6 pmol/million cells/24hrs, which lasted for over thirty days in culture. fVIII was found to be stored in the same vesicles as VWF in the BOECs but was not found to be released upon agonist stimulation, unlike VWF. Instead, fVIII appeared to be released in a constitutive manner (van den Biggelaar *et al.*, 2009). When BOECs were implanted into immunocompetent hemophilic mice, fVIII levels were in the therapeutic range for a total of 27 weeks. Afterwards, the levels declined to baseline due to loss of the implanted BOECs (Matsui *et al.*, 2007). Although a benefit for fVIII secretion with VWF was not directly tested, the proof of concept was shown for the expression of fVIII from BOECs.

Similarly, platelets are potentially an ideal source of fVIII expression as specialized secretory cells that endogenously express VWF. In this manner, fVIII expression and stability can be improved by being expressed in conjunction with VWF (Dorner *et al.*, 1989; Kaufman *et al.*, 1989). The following five promoters actively express genes in both megakaryocytes and platelets: GPIIb (αIIb), GPIbα, GPVI, platelet factor 4 and c-

mpl (Shi and Montgomery, 2010). However among this list, the GPIIb and the GPIbα promoters have been utilized the most to direct expression of transgenes to platelets. This is due to the high level of expression exhibited by the GPIIb and GPIbα promoters yielding surface densities of approximately 80,000 and 25,000 copies per platelet respectively (Debili *et al.*, 1992; Wagner *et al.*, 1996).

Two separate lines of transgenic mice were produced in which the B-domain deleted human fVIII cDNA was expressed solely in platelets as directed by the GPIIb and GPIbα promoter (Yarovoi *et al.*, 2003; Shi *et al.*, 2006). In both cases, fVIII was found to colocalize with VWF in the α-granules of platelets by immunofluorescent microscopy. The activity of the fVIII in platelets expressed from the GPIIb platelet specific promoter was assessed by a chromogenic assay. Utilizing platelet lysates as well as plasma samples following agonist-induced platelet activation, fVIII activity was measured to be near or slightly above 0.7mU / 10^8 platelets which was able to correct the hemophilia A phenotype as determined by a tail-clip assay (Shi *et al.*, 2006). Similarly, fVIII levels produced as a result of expression directed from the GPIbα promoter were able to correct the phenotype of hemophilia A knockout mice following carotid artery injury. The occlusion time was equivalent to that achieved with 20% normal fVIII levels (Yarovoi *et al.*, 2003). The results of these reports served as proof-of principle analyses in that it suggested that expression of platelet-derived fVIII by the GPIIb and the GPIbα promoter is efficient for the correction of the hemophilia A phenotype. To address if VWF is necessary for fVIII storage in the α-granules of platelets, the transgenic GPIbα were crossed onto a VWF^{null} background. Despite the lack of VWF, fVIII was localized in the

α-granules and was effective at making an improvement in the phenotype of hemophilia A fVIII knockout mice (Yarovoi *et al.*, 2005). In addition, fVIII in the α-granules of platelets was demonstrated to be protected from inhibitory antibodies to fVIII by 6-fold (Gewirtz *et al*., 2008). The restriction of fVIII to platelets has also been observed to serve as a protective measure in regards to the production of inhibitory antibodies to the fVIII transgene. No inhibitory antibodies were observed following a bone marrow transplantation from the transgenic GPIIb mice into fVIII knockout mice that yielded similar levels of fVIII activity and phenotypic correction (Shi *et al.*, 2006). For these reasons, this same group designed a lentiviral vector to transduced bone marrow mononuclear cells as a source of HSCs. The GPIIb promoter was incorporated within the vector as an internal promoter directing the expression of the B-domain deleted human fVIII transgene (Shi *et al.*, 2007). In order to assess the effectiveness of platelet-derived fVIII in the presence of inhibitory antibodies to human fVIII, recipient mice were preimmunized with fVIII prior to transplantation by receiving weekly intravenous injections of recombinant human fVIII (50U/kg). Despite the presence of inhibitors to fVIII, long-term engraftment of the genetically modified HSCs was established following both myeloblative (1100cGy) and nonmyeloblative conditioning regimens (660cGy). Chromogenic assays revealed active fVIII within platelets that was sustained over time that resulted in phenotypic correction of hemophilia A (Shi *et al.*, 2008). A similar report was published by the same group recently in which minor changes were made to the transduction/transplantation protocol. Instead of utilizing bone marrow mononuclear cells, sca-1^+ cells were isolated and transplanted into lethally and sublethally irradiated recipient mice. As compared to a parallel non-inhibitor model, fVIII activity was similar

and found to be sustained over time at approximately 1.56 mU/10^8 platelets. Phenotypic correction was found to be achieved at these levels by an electronically induced femoral injury and tail-clip assay. In addition, nonmyeloblative conditioning regimens were used which included busulfan (25-12.5 mg/kg at two and one day prior to transplantation) and anti-(murine)-thymocyte globulin (10mg/kg at two days prior to transplantation) treatment. fVIII activity was undetectable in busulfan alone treated recipients, however the inclusion of anti-(murine)-thymocyte globulin to the conditioning regimen boosted the level of fVIII to that of 1.08 mU/10^8 platelets (Kuether *et al.*, 2012). Therefore, an HSC gene therapy protocol utilizing a lentiviral vector encoding the GPIIb internal promoter is a viable option for the treatment of individuals with hemophilia A with pre-existing inhibitors to fVIII.

The utilization of platelet-specific promoters for directed expression of fVIII is a plausible tool to be incorporated into an HSC gene therapy application for the treatment of hemophilia A. The GPIIb and the GPIbα platelet-specific promoters have demonstrated efficient expression of fVIII confined in the α-granules of platelets in the absence of vesicular injury. In both cases, curative levels of fVIII were produced as was evident by phenotypic correction of hemophilia A fVIII knockout mice. However, ectopic expression of fVIII from platelets has been associated with clot instability as a result of temporal and physical maldistribution of fVIII (Neyman *et al.*, 2008). In order for fVIII to be available for the process of secondary hemostasis, platelet degranulation has to occur, a process that begins at the base of the clot and slowly spreads throughout the clot. Thus fVIII is released from platelets in an unconcerted manner. This may be

the reason for the increased rate of embolism observed upon phenotype analysis following both a cuticular bleeding model and a cremaster laser arteriole/venule injury model (Greene *et al.*, 2010). Stability of a clot needs to be addressed before these promoters can be utilized in the clinic.

Another cell target, which has been considered, is skeletal muscle cells. Skeletal muscles cells are an ideal target for *in vivo* gene transfer in that skeletal muscle cells are terminally differentiated and provide a consistent source of fVIII persisting throughout the lifetime of an individual. For these reasons, Jeon *et al*. (2010) injected 10^7 lentiviral particles intramuscularly into the thigh of rats and found plasma fVIII levels to increase slightly above that of control mice for up to 4 weeks before deteriorating. Despite the need for follow up studies in order to achieve therapeutic levels of fVIII with this strategy, the results suggested that the *in vivo* administration of a lentivirus targeted at skeletal muscle cells may be an effective strategy for the treatment of hemophilia A.

Most *in vivo* strategies, however, are aimed at targeting hepatocytes, an endogenous producer of fVIII. Lentivirus is administered via either the portal vein or intravenously. Unfortunately this strategy in the case of fVIII (a protein with extensive immunogenic properties), results in the development of anti-fVIII antibodies due to the possible transduction of antigen-presenting cells. To overcome this, a miRNA sequence was incorporated downstream of the WPRE sequence which would prevent expression in hematopoietic cells (including cells which make up the immune system). This method was found to be very effective in eliminating a fIX immune response (Brown *et al.*,

2007), but alone was unable to do so with fVIII. Instead the fVIII-miRNA lentivirus had to be pseudotyped with the baculovirus envelope glycoprotein GP64, which has been shown to restrict transduction away from hematopoietic stem cells (Schauber *et al*., 2004). Combined, the miRNA incorporation and the GP64 pseudotyping were able to restrict fVIII expression to the liver, eliminating the presence of inhibitors, while resulting in about 9% of normal levels of fVIII (0.1U ml^{-1}), which was sustained in mice for a total of 60 weeks (Matsui, 2011). This study was significant in that it modified the current *in vivo* lentiviral gene transfer of fVIII making it safer by restricting expression in the liver.

In contrast to focusing on transferring the missing or malfunctioning gene, constructs that encode proteins that can bypass the missing protein can be used to overcome the difficulties associated with the size of the transgene. In the case of hemophilia A, a smaller gene such as fVII can be used to bypass the need for fVIII. fVII is an extrinsic pathway coagulation factor that along with thromboplastin initiates the blood coagulation proteolytic cleavage cascade (**Figure 1.2**) and has been shown in a recombinant form to be an alternative treatment for hemophilia A (Jurlander *et al*., 2001). For these reasons, Ohmori *et al*. (2008) transduced HSCs *ex vivo* with a SIV-based lentiviral vector encoding an activated form of fVII expressed from a platelet specific promoter (the GPIbα promoter). As a result, fVII was found to localize to the cell surface following platelet activation in transplanted fVIII-deficient mice. Due to species-specific interactions, the murine TF was unable to interact with the human form of fVII resulting in unimproved hemophilia conditions in the fVIII-deficient mice. However, when a

murine fVII was incorporated into the SIV-based lentiviral vector, the clot time and rate of clot formation were significantly reduced, decreasing the mortality rate after tail clipping (Ohmori *et al*., 2008). In addition, fVII was recently incorporated into an adeno-associated vector (AAV-8) being able to overcome the encapsidation limitations of the vector due to the shorter size of fVII. Yet it was shown that in hemophilic dogs, large doses of vector were required to be efficacious (Margaritis *et al*., 2009). Therefore, a bioengineered fVII variant with enhanced intrinsic activity was recently utilized in order to reduce the dose of *in vivo* administered vector. However, adverse thrombotic effects were observed in treated mice which limits enthusiasm for this therapy (Margaritis *et al*., 2011).

In our laboratory, variations of the fVIII transgene have been studied extensively. It was found that expression was increased up to 100 fold utilizing a B domain deleted porcine fVIII transgene (Doering *et al*., 2002). High level fVIII expression was sustained for ten months in hemophilia A mice following transplantation of MSCs modified to contain the porcine fVIII sequence (Gangadharan *et al*., 2006). A subsequent study revealed that high level fVIII expression could be achieved even after low-toxicity pretransplantation conditioning (Ide *et al*., 2007). Together these studies demonstrated the ability of high-expression porcine sequence elements to function *in vivo* for the correction of fVIII deficiency in the context of *ex vivo* HSC genetic modification. Human/porcine fVIII chimeras were then constructed for the purpose of determining which porcine fVIII domains were responsible for the increased expression. The chimera constructs revealed sequences in the A1 and A3 domains to be responsible for enhanced secretion of porcine

fVIII (Doering *et al*., 2004, Dooriss *et al.*, 2009). These findings resulted in construction of a high expression chimeric transgene that was utilized to genetically modify HSCs *ex vivo* via lentiviral vectors. The transplantation of these cells resulted in therapeutic levels of fVIII in hemophilia A mice (Doering *et al*., 2009). The chimeric fVIII transgene holds much promise for the gene therapy field in regards to the treatment of hemophilia A.

1.6— Limitations of Gene Therapy

Since the birth of the concept of gene therapy in the early seventies, the field has experienced tremendous highs and lows. A significant number of individuals have experienced clinical benefit as a result of gene therapy. However, these advancements have been overshadowed by highly publicized setbacks. News feeds associated with the death of Jesse Gelsinger, an eighteen year old admitted in a gene therapy trial at the University of Pennsylvania, described the journey of gene therapy as one that has been tainted with a history of hype and unfulfilled promises. This statement was made despite successful reports in the same year from an X-SCID clinical trial in Europe demonstrating improved immune systems in children. It is important to note that this setback and others including the appearance of insertional mutagenesis as a result of the retrovirus utilized in the X-SCID clinical trial have been treated with sensitivity and resulted in improvements in the current understanding of the technology and methods used during gene replacement. The public perception of gene therapy, in my opinion, has been affected by the disparaging reports unfairly represented by the media. Sadly, the scientific community is not immune to these misconceptions. Upon deciding to join a gene therapy laboratory, a number of my colleagues questioned my involvement in a

"dying field." However, these concerns were raised prior to progress made in the field which has resulted in successful gene therapy trials for hemophilia B, AIDS, cancer, X-SCID, adenosine deaminase deficiency, Wiskott-Aldrich syndrome, β-thalassemia and adrenoleukodystrophy (**Table 1.2**).

Table 1.2 – Clinically Beneficial Gene Therapy Trials for Monogenic Diseases

Disease	Vector	Treated	Outcome
X-SCID	γ-retrovirus	20	Full or nearly full correction in 17 patients Normal T-cell subset counts Normal T-cell-mediated immune functions Five developed T-cell leukemia Four in remission following chemotherapy (Cavazzana-Calvo *et al.* 2000; Hacein-Bey-Abina *et al*., 2008)
ADA-SCID	γ-retrovirus	40	Increase of lymphocyte counts Improvement of cellular and humoral responses Enzyme replacement therapy halted in 29 patients No leukemic or oncogenic event (Aiuti *et al*., 2009; Gaspar *et al*., 2011)
WAS	γ-retrovirus	10	Significant clinical benefit Resolution of hemorrhagic diathesis, eczema, autoimmunity and predisposition to recurrent infections One patient developed T-cell leukemia (Boztug *et al.*, 2010)
X-ALD	SIN-lentivirus	2	Cerebral demyelination arrested No clonal dominance or leukemic / oncogenic event (Cartier *et al., 2012)*
β-thalassemia	SIN-lentivirus	1	Stable hemoglobin levels One-third contains vector encoded β-globin Transfusion independent No leukemic / oncogenic event (Cavazzana-Calvo *et al*., 2010)
Hemophilia B	AAV-8	6	FIX levels 2-11% of normal Four discontinued FIX protein replacement therapy Other two had longer spans between prophylaxis At highest vector dose, slight and transient liver-enzyme levels which normalized after glucocorticoid treatment (Nathwani *et al*., 2011)

Abbreviations: X-SCID (X-linked severe combined immunodeficiency), ADA-SCID (adenosine deaminase deficient severe combined immunodeficiency), WAS (Wiskott-Aldrich Syndrome), X-ALD (X-linked adrenoleukodystrophy)

In the field of gene therapy, hemophilia A and B are among the most extensively researched monogenic diseases. Hemophilia A and B are X-linked bleeding disorders attributed to the loss of fVIII and fIX respectively. Hemophilia B is not as prevalent as hemophilia A affecting approximately 1 in 30,000 males. However the size of the fIX transgene is significantly smaller than fVIII with a cDNA of 2.8-kb. The adeno-associated viral (AAV) vector is able to withstand the small size of the fIX transgene which poses as a safer vector existing predominantly as a concatemer of episomes extrachromosomally in cells. Minimal integration with the use of an AAV-based vector reduces the risk of insertional mutagenesis. In addition, long-term expression can be maintained if non-dividing cells are targeted. Since the liver is the main physiological site of fIX synthesis, liver-directed gene transfer of fIX poses an ideal strategy for the treatment of hemophilia B. The first clinical trial for hemophilia B utilized the most prevalent serotype of AAV, AAV-2 which was infused via the hepatic artery. However, this trial presented a number of obstacles to the therapy including the possibility of vertical transmission as a result of transient vector DNA present in the sperm of subjects. This obstacle appeared to be dose-related and could potentially be diminished by the use of lower doses of administered vector. The presentation of humoral and cellular immunity to the vector itself, however, presented itself as the greatest obstacle to be overcome. A number of subjects admitted to the trial prior to admittance contained neutralizing antibodies to AAV, a consequence of utilizing a vector derived from a human pathogen. These antibodies blocked the transduction of target cells and have resulted in the modification of participation requirements for future trials utilizing an AAV-based vector. In addition, hepatocytes that were transduced were eliminated by

memory T cells due to the presentation of AAV capsid-derived antigens resulting in transient fIX expression. Since the first trial, serotypes of AAV have been identified that are capable of transducing hepatocytes more effectively (AAV-8) and thus eliminate the destruction of transduced hepatocytes due to diminished T cell activation. Therefore, a clinical trial was performed utilizing an AAV-8 vector. In addition potential improvements were made to the vector in regards to transduction, the AAV-8 vector was engineered to overcome the limiting transduction step of AAV (the conversion of the single-stranded genome to that of a double-stranded AAV genome) as a self-complementary, double-stranded AAV-8 vector. A total of six subjects with severe hemophilia B were included in the trial and all six subjects experienced levels of fIX above that of a therapeutically relevant increase in fIX (Nathwani *et al*., 2011). The increase in fIX in these subjects drastically changed their lifestyle. Yet more importantly, this was the first trial for hemophilia B that demonstrated sustained levels of fIX representing a significant advancement in the field of gene therapy. Improvements are still needed before this therapy can become widespread, since patients admitted into the trial are still receiving infusions of fIX. Preclinical reports are surfacing in which the specific activity of fIX is increased due to improvements in the transgene. It is likely that the next generation of clinical trials for hemophilia B will include the use of an enhanced fIX transgene.

Gene therapy protocols for the treatment of Hemophilia A, on the other hand, have been more difficult to manage due to the large size of fVIII. The B domain deleted fVIII cDNA is 4.5-kb leaving very little room for the inclusion of regulatory elements to be

packaged in an AAV-based vector. Nonetheless, preclinical attempts have been made to replace the fVIII gene with the use of an AAV-based vector (Burton *et al*., 1999; Chao *et al*., 2003). However when translated to large animal models, therapeutic levels of fVIII can only be achieved upon administration of vector ten-fold higher than the highest dose administered thus far in humans. It is likely that a dose this high will not be tolerated in humans due to the induction of an immune response to the AAV-based vector. For this reason and due to the limited carrying capacity of adeno-associated viral vectors, a lentiviral based vector has been considered for gene therapy applications for the treatment of hemophilia A. The carrying capacity of lentiviral vectors reaches 7-kb, large enough to encompass the B domain deleted fVIII cDNA and relevant promoters. A lentiviral vector is an ideal choice for the genetic modification of HSCs *ex vivo*. However, the utilization of a lentiviral vector does not come without obstacles. First, the production of lentiviral vectors containing an HP-fVIII transgene is limited in HEK-293T cells. For a yet unknown reason, lentiviral titers from vectors containing an HP-fVIII transgene are considerably lower than lentiviral vector titers containing a GFP transgene. Consistently HIV-based lentiviral vectors containing a GFP transgene produced by transient transfection in HEK-293T cells yield titers of approximately 10^8 while vectors containing the HP-fVIII transgene yield titers of 10^7. It has been suggested that the decrease in viral titer correlates to the size of the transgene. The larger the transgene the less viral particles produced (Kumar *et al*., 2001; Yacoub *et al*., 2007). This obstacle may need to be overcome in order to produce a significant amount of vector for a gene therapy protocol aimed at treating a substantial portion of the population affected by hemophilia A. Compounding upon this obstacle is the inefficiency of a lentiviral vector at

transducing HSCs. In theory, only one HSC would need to be modified and engrafted in order to provide a life-long cure to hemophilia A. However the target $CD34^+$ population includes both stem cells and early progenitors of the stem cell. It is possible that the early progenitors present in the $CD34^+$ population are more readily transduced by the lentiviral vector than the pure stem cell, monopolizing the transduction of the lentiviral vector. The sole modification of early progenitors would not attribute to expression of the HP-fVIII transgene over a life-time. Instead, any benefit observed as a result of gene therapy would only be transient due to the shortened persistence of early $CD34^+$ progenitors as compared to the self-renewing stem cell. Therefore, the modification of self-renewing $CD34^+$ stem cells is vital to the success of a gene therapy protocol aimed at modifying HSCs. For this reason, much emphasis in the field has been placed on delineating methods whereby the transduction efficiency of HSCs can be improved. This dissertation has focused on the obstacles presented by the use of a lentiviral vector in a gene therapy protocol designed for the treatment of hemophilia A.

Chapter 2:

Pharmacological Evaluation of Lentiviral Vector Optimized for fVIII Expression

2.1— Introduction

As previously mentioned in chapter 1, hemophilia A is a monogenic disease caused by mutations in the gene encoding fVIII, resulting in the inability to properly form a clot. Hemophilia A is a prime candidate for gene therapy in that only a moderate increase in fVIII (2-5% of normal equating to 2-5 ng ml^{-1}) is required to be therapeutically effective. In addition, the current treatment, consisting of repetitive prophylactic administration of recombinant fVIII as a means of protein replacement, is expensive, invasive and does not always result in patient compliance. Further complicating the current therapy, 30% of individuals with severe and moderately severe hemophilia develop an immunogenic response in the form of neutralizing antibodies against the administered fVIII (Ehrenforth *et al*., 1992). These inhibitors make managing a bleeding episode extremely complicated. Therefore, new therapeutic approaches are needed to treat hemophilia A. fVIII gene therapy attempts to rectify the presence of a mutant F8 gene with either the addition of a functional gene or correction of the original gene. In the case of gene addition, delivery of the fVIII gene is not restricted to a certain cell type because, theoretically, any tissue with exposure to the vasculature is suitable as a cellular target. In addition, the therapeutic window is large, as fVIII levels as high as 150% of normal have not been associated with adverse effects such as thrombosis (VandenDriessche *et al*., 2001). As a result, a number of viral and non-viral delivery strategies have been postulated.

Despite promising preclinical data, hemophilia A gene therapy clinical trials have not progressed past phase 1 trials due to limited expression of fVIII (Roth *et al*., 2001; Powell *et al*., 2003; Berlfein, 2003). Although each trial was unique in regards to the cell

type modified and the viral vector used, all trials yielded less than therapeutic levels of fVIII. To overcome low level transgene expression, we recently compared several high-expression fVIII transgene sequences and demonstrated enhanced expression of a B domain deleted porcine fVIII sequence, both *in vitro* and *in vivo* (Ide *et al*., 2007; Doering *et al*., 2007; Dooriss *et al*., 2009). Upon comparison of a series of hybrid human/porcine cDNAs, the domains responsible for the high expression characteristics of porcine fVIII were identified as the A1 and A3 domains (Doering *et al*., 2004). These findings resulted in the construction of a high expression human/porcine transgene. The bioengineered construct was then introduced via lentiviral vectors into hematopoietic stem cells (HSC) *ex vivo* and used to effectively treat fVIII knockout mice with hemophilia A, yielding therapeutic levels of fVIII (Doering *et al*., 2009). Further optimization resulted in the inclusion of the porcine C1 domain and three alanine substitutions in the A2 domain in order to reduce immunogenicity (Healey *et al*., 2009; Lubin *et al*., 1997). The final high expression B domain deleted fVIII transgene (HP-fVIII) contains human A2 and C2 domains in addition to porcine A1, A3 and C1 domains. This HP-fVIII has been shown to maintain the high expression characteristics of porcine fVIII (Doering *et al*., 2009).

Lentiviral vectors are promising vectors for the delivery of the fVIII transgene because they provide stable integration and are able to transduce both dividing and non-dividing cells (Naldini *et al*., 1996). Lentiviral vectors, unlike adeno-associated viral vectors, are less constrained by the size of the transgene. Vector size constraints are an issue for fVIII gene therapy since the B domain-deleted fVIII cDNA is approximately 4.4-kb (for

review see Johnston *et al*., 2011). For these reasons, lentiviral vectors are reasonable for gene therapy applications aimed at the treatment of hemophilia A utilizing *ex vivo* modification of HSCs. In lentiviral vectors, a woodchuck post-transcriptional regulatory element (WPRE) is routinely added to the 3' end of the transgene. The inclusion of this sequence is for increased transgene expression, as it has been demonstrated that a two to fivefold increase in expression is achieved with a WPRE sequence, which in part is due to an increased export of mRNA and possibly due to facilitating transcript processing (Zuffery *et al*., 1999; Brun *et al.*, 2003; Gonzalea-Murillo *et al.*, 2010). However, a recent report showed that enhanced transgene expression in the presence of a WPRE sequence was dependent on the promoter and cell line used, where in some instances the inclusion yielded no increase or decreased expression (Gonzalea-Murillo *et al*., 2010; Klein *et al*., 2006). In addition, the WPRE codes for the first 60 amino acids of the hepadnavirus X protein, a protein that has been linked to oncogenesis (Kingsman *et al*., 2005). Therefore, the function of the WPRE appears to be more complex than originally assumed and may need to be evaluated in conjunction with individual transgenes, which has not been done for fVIII.

Previous studies have focused on optimizing the fVIII transgene for enhanced fVIII expression (for review see Doering and Spencer, 2009), but the components of the viral vector system have not been as well characterized. The studies presented in this chapter focuses on optimizing the lentiviral vector for virus production, transduction efficiency, and transgene expression with the use of a fVIII transgene that has been bioengineered

for high level fVIII expression (HP-fVIII). The optimized lentiviral vector was utilized in *ex vivo* HSC transduction studies to determine *in vivo* HP-fVIII expression.

2.2— Materials and Methods

Reagents

Dulbecco's Modified Eagle's medium (DMEM)/F-12, Aim V medium and StemPro-34 serum-free medium were purchased from Invitrogen Life Technologies (Carlsbad, CA). Heat-inactivated fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). Penicillin-streptomycin solution was purchased from Mediatech (Manassas, VA). Cell transfections were performed with polyethyleneimine purchased from Fisher Scientific (Pittsburg, PA). Plasmids utilized for viral preparation were isolated from bacterial stocks utilizing QIAGEN Hispeed midiprep plasmid kits (Valencia, CA). Nucleic acid isolation kits were purchased from QIAGEN. Integration events were analyzed using a qPCR SYBR Green Low Rox master mix from Thermo Fisher Scientific (Waltham, MA) an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) and oligonucleotide primers synthesized by Integrated DNA Technologies (Coralville, IA). HP-fVIII RNA was quantified utilizing fVIII RNA standards generated with an mMessage mMachine kit (Ambion, Austin, TX). Northern blots were performed with the digoxigenin nonradioactive nucleic acid-labeling and detection system (Roche, Indianapolis, IN). Human fVIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KS). Automated activated partial thromboplastin (APTT) reagent was purchased from BioMérieux (Durham, NC). Clotting times were measured using an ST art

Coagulation Instrument (Diagnostica Stago, Asnieres, France). Anti-pfVIII and anti-hfVIII monoclonal antibodies were a kind gift of Dr. Pete Lollar (Aflac Cancer Center and Blood Disorder Services, Emory University, Atlanta, GA). Sca-1^+ cells were isolated using magnetic separation columns purchased from Miltenyi Biotec (Auburn, CA). Exon 16-disrupted hemophilia A mice have been previously described (Bi *et al*., 1995). All antibodies for flow cytometry were purchased from BD Pharmingen (San Diego, CA).

Vector production

Expression plasmids containing the HP-fVIII or eGFP gene along with the necessary packaging plasmids were transiently transfected into HEK-293T cells utilizing polyethylenimine (6 µg PEI/1 µg DNA). A 2:1:1 ratio of expression plasmid to packaging plasmids (expression plasmid:psPAX2:pVSVG) was used to manufacture research-grade HIV-based lentiviral vectors in the LentiMax production system. Research-grade SIV-based lentiviral vectors were manufactured using a 1.3:1:1:1.6 ratio of expression plasmid to packaging plasmids (expression plasmid:pCAG4:pVSVG:pSIV). One day after transfection, the media was replaced with DMEM-F12 containing 10 % fetal bovine serum and 1 % penicillin/streptomycin. Conditioned medium from the HEK-293T viral producing cells was collected for the following three days, passed through 0.45 µmol l^{-1} filter and stored at -80^{o}C until concentration. Virus was concentrated by velocity sedimentation upon centrifugation at 10,000g (4^{o}C) overnight. Viral pellets were resuspended in 1/100th of the original volume of StemPro media, and filtered through a 0.22 µmol l^{-1} filter. Viral titer was

assessed on HEK-293T cells with increasing vector volumes by real-time quantitative PCR (qPCR) seventy-two hours after viral addition. Unconcentrated viral supernatant had titers ranging from $5x10^5$ to $2x10^6$ transducing units ml^{-1} (TU ml^{-1}). Concentrated virus ranged from $5x10^7$ to $1x10^8$ TU ml^{-1}. Virus was stored in 1 ml aliquots at -80^{o}C.

qPCR is a highly sensitive reaction that could overestimate viral titer if residual plasmid remained as a consequence of viral preparation. Therefore, plasmid contamination as a was assessed in order to confirm the viral titers generated by qPCR. Virus was pretreated with 50 units of benzonase per ml of virus for 15 minutes as previously described (Sastry *et al*., 2004). Prior to viral transduction, 100,000 HEK-293T cells were plated in a six well tissue culture treated plate and given 24 hours to adhere to the plate. At the time of viral addition, approximately 200,000 HEK-293T cells were transduced with viral containing media with or with benzonase treatment. DNA was isolated from the cells over time resulting in DNA samples at 24, 48, 72, 240 and 288 hours post transduction. Each time point was performed in triplicate. qPCR analysis was performed on the DNA isolated from each condition. Transgene copies/cell were similar despite benzonase treatment yielding a fold difference near one suggests a lack of plasmid contamination in the viral containing media (**Table 2.1**).

Table 2.1 – The effect of benzonase treatment on copy number analysis

Hours Post Transduction	Fold Difference
24	0.729
48	0.668
72	0.805
240	1.012
288	0.818

Transgene copies/cell were determined at the indicated hours post viral addition by qPCR analysis. The fold difference was determined by dividing the transgene copies/cell from the nontreated wells by the transgene copies/cell of the benzonase treated wells.

Measurement of HP-fVIII transgene copy number

Total genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacture's protocol for cultured cells. DNA was quantified with a spectrophotometer at an absorbance of 260 nm. To determine transgene copy number, 50 ng of each sample was added to a 25 µl real-time quantitative PCR reaction containing 1x SYBR green PCR master mix (Thermo Fisher Scientific, Surrey UK) and 0.01 µM forward and reverse primers. HP-fVIII specific primers annealing to the A1 porcine domain were utilized: forward primer, 5'- CAG GAG CTC TTC CGT TGG -3' at position 164 and reverse primer, 5'- CTG GGC CTG GCA ACG C -3' at position 239. C_t values for each sample were compared to C_t values produced from plasmid standards of known copy quantities. The equivalent copy number was then divided by 8333, the predicted number of genome equivalents in 50 ng of DNA.

To assess the appropriate time following transduction in which to perform the *in vitro* copy number analyses, a time course analysis of copy number was performed in EU1, BHK-M and HEK-293T cells. Approximately 250,000 EU1 (**Figure 2.1 A**) and BHK-M cells (**Figure 2.1 B**) were transduced with SIV-based lentiviral vectors at an MOI of 5. DNA was isolated throughout the analysis as indicated. Each time point was performed in triplicate. Transgene copies/cell were determined by qPCR as previously described. Grey arrows represent when cells were passaged. A gradual decay in copy number is apparent in both EU1 and BHK-M cells that stabilizes 72 hours post transduction (**Figure 2.1 A-B**). The decay observed is consistent with the loss of non-integrated vector over time. A similar protocol was used to assess copy number over time in K562 cells

(performed previously by Kerry Dooriss). A similar decay profile was apparent in K562 cells as was observed in EU1 and BHK-M cells (data not shown).

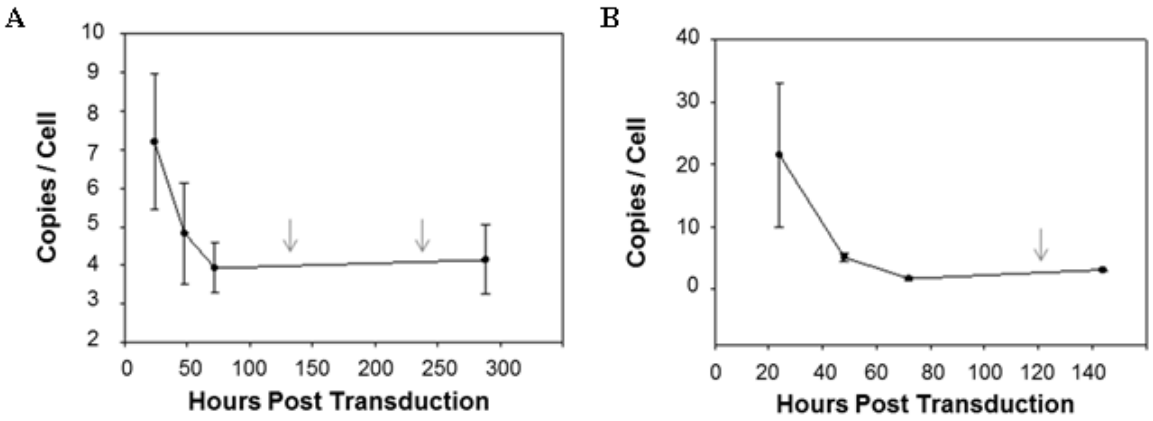


Figure 2.1 Copy number analysis over time reveals loss of non-integrated lentiviral vector by seventy-two hours following viral transduction. (A) EU1 and (B) BHK-M cells were transduced with an SIV-based lentiviral vector at an MOI of 5. DNA was isolated at the indicated time points and assessed for transgene copy number by qPCR. Each time point was performed in triplicate.

Measurement of HP-fVIII transcript expression

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacture's protocol for animal cells 72 hours post transduction. RNA was then quantified spectrophotometrically at an absorbance of 260 nm. HP-fVIII transcripts were measured by quantitative RT-PCR (qRT-PCR) utilizing a porcine fVIII RNA standard as previously described (Doering *et al.*, 2002). qRT-PCR reactions were carried out in 25 µl containing 1x SYBR green PCR master mix, 300 µM forward and reverse primers, 12.5 units MultiScribe, 10 units RNase inhibitor, and 5 ng of sample RNA. Reactions containing the porcine fVIII RNA standard also included 5 ng of yeast tRNA, mimicking the RNA environment of the sample RNA. The oligonucleotide primers utilized for the qRT-PCR reaction annealed in the A2 domain of the fVIII cDNA sequence at positions 1897-1917 for the forward primer (5'-ATGCACAGCATCAATGGCTAT-3') and at positions 2044-2063 for the reverse primer (5'-GTGAGTGTGTCTTCATAGAC-3'). One-step real-time qRT-PCR was performed by incubation at 48^{o}C for 30 min for reverse transcription followed by one cycle at 95^{o}C for 10 min and 40 amplification cycles of 90^{o}C for 15 sec and then at 60^{o}C for 1 min. Postreaction dissociation was performed to confirm single-product amplification. C_t values for each sample were compared to C_t values produced from the porcine fVIII standards having known transcript quantities.

Measurement of HP-fVIII activity from cell lines

fVIII activity was measured from the supernatant of cells cultured in AIM V media for 24 h before the assay as previously described (Doering *et al.*, 2002). In short, the APTT reagent-based one stage coagulation assay was performed in duplicate for each

supernatant on a ST art Coagulation Instrument in human fVIII-deficient plasma. The clot time for each sample was compared to a standard curve based on dilutions of FACT.

Animals

Exon-16 deleted hemophilia A mice were originally obtained from Dr. Leon Hoyer (Holland Laboratories, American Red Cross, Rockville, MD) by Pete Lollar. The colony has been maintained at Emory University (Atlanta, GA) since 1998. B6.SJL (CD45.1) mice were acquired from Dr. David Archer (Emory University, Atlanta, GA). Both strains were maintained at the animal care facility of Emory University. All procedures were approved by the Institutional Animal Care Committee at Emory University.

Isolation and transduction of murine stem cell antigen-1+ cells

Whole bone marrow was flushed from the femurs and tibias of 8- to 10-week-old CD45.1 mice and then subjected to positive immunomagnetic bead selection. The isolated sca-1^{+} cells were cultured at a density of 10^{6} cells per ml in StemPro media supplemented with L-glutamine (29 μg ml^{-1}). For 3 days before transduction, the cells were stimulated with murine stem cell factor (100 ng ml^{-1}), murine interleukin-3 (20 ng ml^{-1}), human interleukin-11 (100 ng ml^{-1}), and human Flt-3 ligand (100 ng ml^{-1}). Cells were transduced twice (MOI 15), 8 hours apart, and transplanted via tail-vein injection the following day into lethally irradiated (11*Gy* TBI using a Gammacell 40 Exactor) 8- to 10-week-old recipient hemophilia A mice (CD45.2). Blood was collected from the transplanted mice every two weeks retro-orbitally and fVIII was measured using an ELISA specific for HP-fVIII. The ELISA only detects properly folded HP-fVIII in that

the primary antibody detects the heavy chain (human A2 domain) and the secondary antibody detects the light chain (porcine A3 domain). In addition, HP-fVIII was detected from the plasma using a commercially available chromogenic substrate assay (COATEST SP FVIII) as previously described (Doering *et al*., 2007).

Statistical analysis

Results are presented as mean ± standard deviation. A student's t-test was used to evaluate a significance of difference between two groups. For comparisons between more than two groups, a one way ANOVA was used. A p value less than 0.05 was considered to be statistically significant.

2.3— Results

2.31— Assessment of the WPRE sequence

Assessment of the requirement for a WPRE element for the production of virus containing an HP-fVIII transgene

A recent report revealed that enhanced transgene expression as a result of a WPRE is dependent on both the promoter and cell line used, and should thus be assessed for individual transgene scenarios (Kingsman *et al*., 2005). The WPRE was evaluated in the context of an optimized HP-fVIII transgene. Both SIV and HIV expression plasmids were constructed with and without a WPRE sequence (**Figure 2.2 A**) and used to produce recombinant viral vector from HEK-293T cells. Virus, with and without a WPRE, was generated in triplicate under identical conditions. Unconcentrated viral supernatant then was used to transduce HEK-293T cells to assess viral titer. In this manner, viral

production was analyzed from the context of a highly transducible cell line. DNA was extracted from the transduced cells and analyzed for viral copy number by qPCR. For both lentiviral vectors, assuming equal transduction efficiency, the absence of a WPRE did not affect viral production ($P = 0.436$ for SIV and $P = 0.309$ for HIV) (**Figure 2.2 B**).

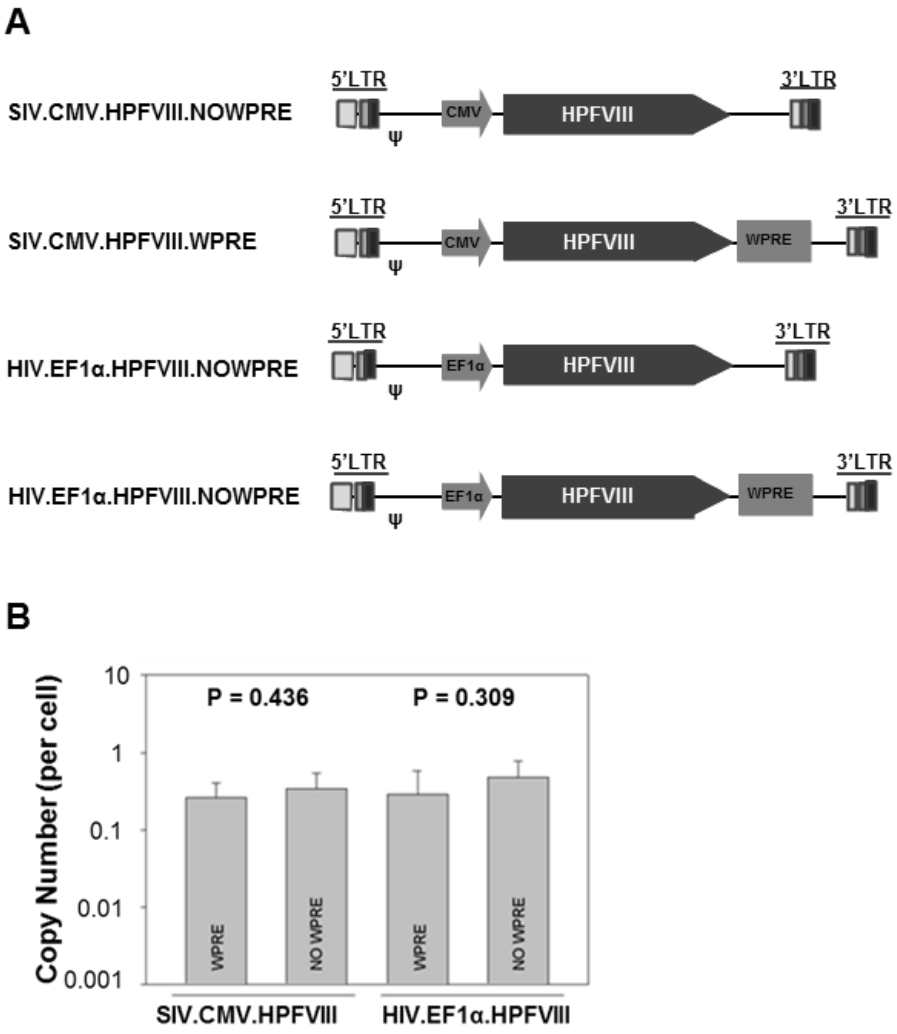


Figure 2.2 Evaluation of the WPRE sequence in SIV and HIV vector systems in regards to viral production. (A) Schematic representation of SIV- and HIV-based lentiviral vectors. (B) Recombinant virus was produced under identical conditions (n = 3). Unconcentrated viral supernatant (0.5 ml) was used to transduce HEK-293T cells. Seventy-two hours later, genomic DNA was isolated from the transduced cells and used to assess viral copy number by qPCR. Each bar represents the mean ± standard deviation of three wells measured in duplicate. *P* values were derived from a student's *t* test.

Evaluation of the expression of the HP-fVIII transgene from lentiviral vectors containing a WPRE element

Two independent internal promoters and vector systems were used to evaluate the effects of a WPRE on expression, a human elongation factor-1 alpha (EF1α) promoter in an HIV lentiviral system and a cytomegalovirus (CMV) promoter in a SIV lentiviral system. The HP-fVIII expression constructs were transiently transfected into HEK-293T cells. Forty-eight hours later, HP-fVIII expression was quantified using an APTT reagent-based one-stage coagulation assay. Comparison of HP-fVIII expression showed that the WPRE did not enhance expression of the transgene driven from either a CMV ($P = 0.757$) internal promoter or an EF1α ($P = 0.405$) (**Figure 2.3 A**). The lack of effect was further analyzed in a hematopoietic context in which two hematopoietic cell lines (EU1 and K562 cells) were transduced with the SIV-based lentiviral vector system. HP-fVIII expression was assessed at both the transcript and protein level. To correct for transduction efficiency, transcript and fVIII activity levels were normalized to copy number for each viral preparation. No enhancement in HP-fVIII transcripts was observed when a WPRE was included in the lentiviral vector (**Figure 2.3 B**). As shown in **Figure 2.3 C**, a similar observation was noted for the activity of expressed HP-fVIII from transduced K562 cells when normalized to copy number. However, significantly more activity was observed from EU1 cells that were transduced with a SIV viral vector devoid of a WPRE sequence than those that were transduced with a SIV virus containing a WPRE sequence ($P = 0.009$) (**Figure 2.3 C**). This relative enhancement was not observed upon normalization of HP-fVIII activity to transcripts (**Figure 2.3 D**). We have previously demonstrated a correlation between fVIII transcript number and fVIII activity (Doering *et al.*, 2009).

However, less correlation has been documented between copy number and fVIII expression and would account for the lack of enhancement seen as a result of HP-fVIII activity normalization to transcripts (Spencer *et al*., 2011).

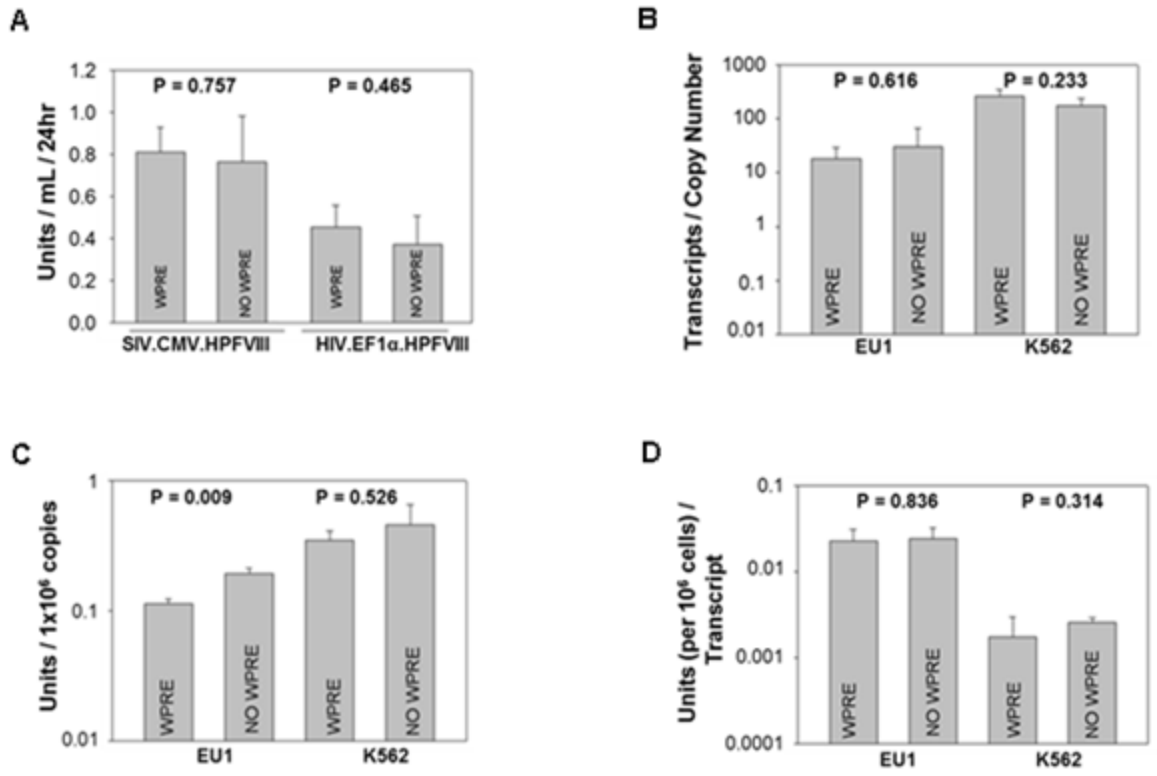


Figure 2.3 Evaluation of the WPRE sequence in regards to HP-fVIII expression. (A) To assess HP-fVIII activity, the expression plasmids were transiently transfected into HEK-293T cells. HP-fVIII activity was detected by an APTT-reagent based one-stage coagulation assay 48 hours post transfection. Twenty-four hours prior to the APTT-reagent based one-stage coagulation assay, conditioned media was replaced with AIM-V (serum-free). Each bar represents the mean ± standard deviation of six wells measured in duplicate. (B) An SIV-based lentivirus also was used to assess HP-fVIII expression in EU1 and K562 cells (MOI 1). Seventy-two hours post-transduction, genomic DNA and total RNA were isolated from the cells. HP-fVIII transcripts and HP-fVIII copy number was assessed by qRT-PCR and qPCR, respectively. (C) HP-fVIII activity was assessed 24 hour after exchange of conditioned media with serum-free AIM-V media by an

APTT-reagent based one-stage coagulation assay. (D) HP-fVIII activity was normalized to transcript levels. Each bar represents the mean ± standard deviation of three wells measured in duplicate. *P* values were derived from a student's *t* test.

Quantification of transcriptional read-through as a result of the presence of a WPRE sequence

It was suggested that the WPRE sequence provides a protective feature in viral vectors by safe guarding against insertional mutagenesis by reducing transcriptional read-through (Higashimoto, 2007). However, none of the constructs tested contained a transgene comparable to the length of HP-fVIII. Therefore, I designed constructs in which an internal ribosomal entry site (IRES) was placed after the 3' viral long terminal repeat (LTR) so that the green fluorescent protein (eGFP) could be expressed only as a result of transcriptional read-through (**Figure 2.4 A**). The IRES eGFP constructs, produced with and without a WPRE, were transiently transfected into HEK-293T cells and assessed for eGFP expression (96 hours after transfection) (**Figure 2.4 B and 2.4 C**). Although minimal transcriptional read-through was apparent, no statistically significant difference was found in the amount of transcriptional read-through when a WPRE sequence was present as compared to when it was removed ($P = 0.905$) (**Figure 2.4 C**). This observation was noted despite similar expression of HP-fVIII ($P = 0.891$) (**Figure 3.4 D**). Taken together, these data demonstrate that the WPRE does not enhance viral titer, transduction or HP-fVIII expression, and led to the removal of the WPRE element from both the HIV-based and SIV-based lentiviral vectors in subsequent studies.

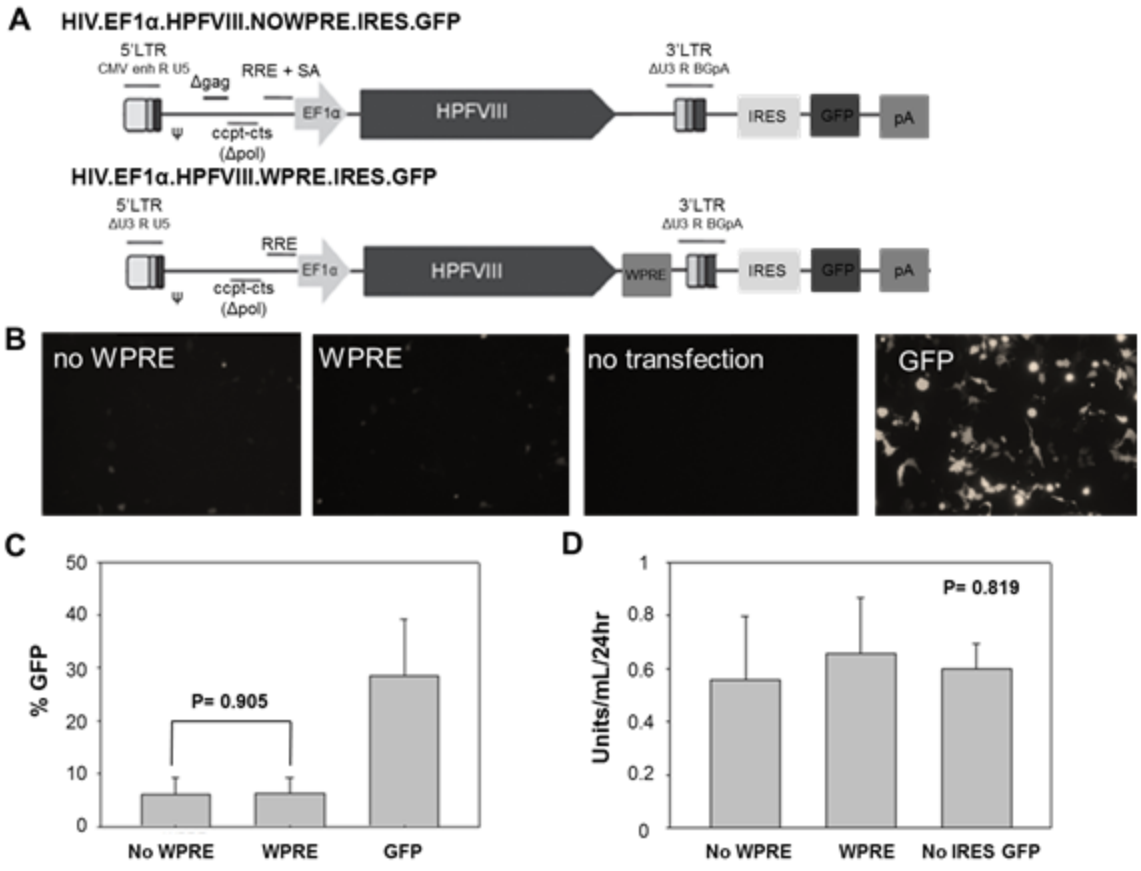


Figure 2.4 Evaluation of transcriptional read-through in the absence of a WPRE sequence. (A) Schematic representation of HIV-based expression plasmids transiently transfected into HEK-293T cells. (B) Fluorescent microscopy images taken 96 hours after transfection. (C)Transcriptional read-through was quantified by flow cytometry. (D) HP-fVIII activity was assessed 24 hours after exchange of conditioned media with serum-free AIM-V media by an APTT-reagent based one-stage coagulation assay. Each bar represents the mean ± the standard deviation of three wells measured in duplicate. *P* values were derived from a one-way ANOVA.

2.32— Comparison of three lentiviral vectors

Comparison of the transduction efficiency and HP-fVIII expression of SIV- and HIV-based lentiviral vectors containing the HP-fVIII transgene

To determine if there are inherent differences in HP-fVIII expression between SIV and HIV gene transfer systems, SIV- and HIV-based expression plasmids were produced in which the only differences were derived from the vector system itself (**Figure 2.5 A**). The HP-fVIII transgene was expressed in both lentiviral vectors from the EF1α internal promoter. Expression plasmids were used to produce virus from HEK-293T cells under identical conditions three separate times. Virus was quantified by assessing viral titer using 1 ml unconcentrated viral supernatant, which was added to the highly transducible HEK-293T cell line as a baseline for comparison. Viral copy number was determined utilizing qPCR with primers specific for the HP-fVIII transgene and compared between SIV- transduced and HIV- transduced cells. Unconcentrated viral vector titers were significantly higher with the SIV vector system ($P = 0.014$) (**Figure 2.5 B**). Recombinant SIV and HIV then was used to transduce BHK-M, K562 and HEK-293T cells at a MOI 5. Seventy-two hours after viral addition, transduction efficiency was measured by analyzing the copy number of HP-fVIII by qPCR. As previously observed in the lab, BHK-M and K562 cells are transduced less efficiently with lentiviral vectors than HEK-293T cells, as noted by lower HP-fVIII copy numbers (**Figure 2.5 C**). Comparison of the two vector systems in cell lines, on the other hand, did not show any difference with respect to the transduction efficiency in both K562 cells ($P = 0.837$) and HEK-293T cells ($P = 0.120$) (**Figure 2.5 C**). Identical copy numbers in HEK-293T cells were observed and expected as these cells were used to calculate viral titer (**Figure 2.5 C**). However, a

slight difference was seen with BHK-M cells. Greater copy numbers were noted when a SIV-based vector was used to transduce BHK-M cells compared with the HIV-based vector ($P = 0.045$).

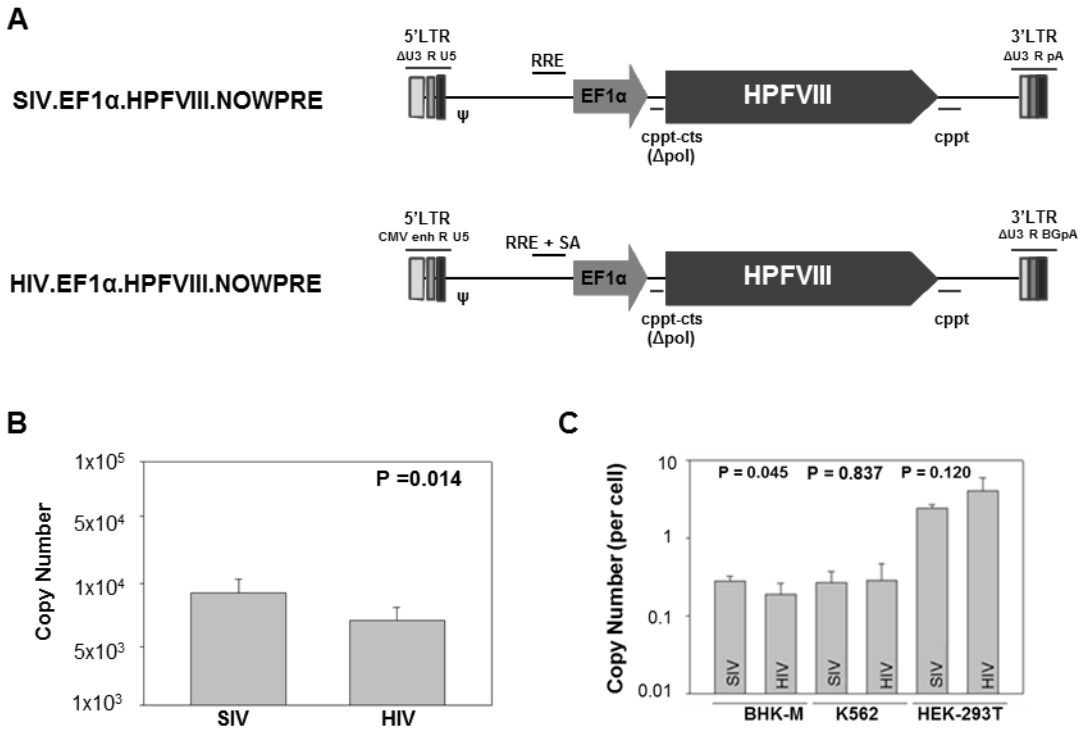


Figure 2.5 Comparison of SIV- and HIV-vector systems encoding HP-fVIII. (A) Schematic representation of the SIV and HIV expression plasmids generated to encode HP-fVIII. (B) Recombinant virus was produced under identical conditions (n = 3) and titered on HEK-293T cells. (C) Each viral preparation was utilized to transduce two wells of BHK-M, K562 and HEK-293T cells at an MOI of 5. Seventy-two hours post-transduction, genomic DNA was isolated and assessed for copy number by qPCR. *P* values were derived from a student's *t* test.

RNA was extracted and fVIII transcripts were quantified using qRT-PCR from the BHK-M, K562 and HEK-293T cells transduced with either SIV- or HIV-based vectors. BHK-M cells were included in this analysis as a high-expressing fVIII cell line. It was expected that the expression of the transgene would be similar between the two viral vectors, as similar MOIs were used and similar copy numbers were determined. However, there was enhanced HP-fVIII RNA expression by the SIV system, which was evident in both BHK-M ($P < 0.001$) and K562 ($P = 0.019$) cell lines (**Figure 2.6 A and 2.6 B**). Enhanced HP-fVIII RNA levels were confirmed by Northern blot analysis (**Figure 2.6 D upper panel**). Supernatants from the transduced cells were used to assess HP-fVIII activity by an APTT reagent-based one-stage coagulation assay. HP-fVIII activity was significantly increased for BHK-M ($P < 0.001$) and K562 ($P = 0.006$) cells transduced with SIV (**Figure 2.6 C**). In BHK-M and K562 cells, greater RNA levels led to greater protein production. This is consistent with previous findings that showed a strong correlation between RNA and fVIII activity (Doering *et al*., 2009). Based on this set of data, the SIV-based vector system was selected for further studies.

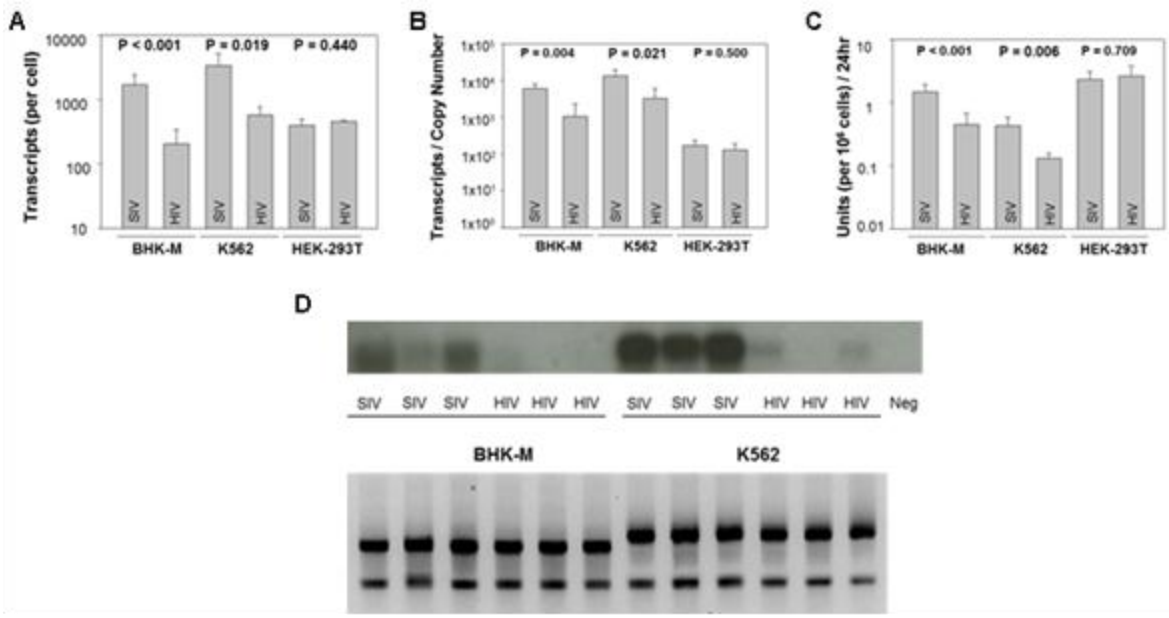


Figure 2.6 Comparison of SIV- and HIV-vector systems encoding HP-fVIII. (A) Seventy-two hours post-transduction, total RNA was isolated and transcript levels were quantified by qRT-PCR. (B) Transcript levels were normalized to copy number. (C) Twenty-four hours prior to isolation, the conditioned media was exchanged for serum-free AIM-V in order to assess the HP-fVIII activity by an APTT reagent-based one-stage coagulation assay. Bars represent the mean ± the standard deviation of three sets of virus each added to two wells, while each well was measured in duplicate. (D) Enhanced transcript levels were confirmed by Northern Blot analysis. Equivalent amounts of ribosomal RNA were apparent among each sample (lower panel). *P* values were derived from a student's *t* test.

Comparison of the transduction efficiency of SIV- and HIV-based lentiviral vectors containing an eGFP transgene

In order to determine if enhanced expression from the SIV-based lentiviral vector was specific to the HP-fVIII transgene, expression plasmids were constructed in which both SIV- and HIV-based lentiviral vectors contained the eGFP transgene (**Figure 2.7 A**). In both gene transfer systems, the eGFP transgene was driven from the EF1α internal promoter. In this manner differences in eGFP expression could be assessed as a consequence of differences derived from the vector system itself. As described earlier, expression plasmids were used to produce virus from HEK-293T cells under identical conditions. Following ultracentrifugation, virus was concentrated and quantified by viral titer using flow cytometry. Titers were comparable among the two viral preparations yielding TU ml^{-1} near 2×10^7.

Transduction efficiency was assessed in BHK-M, K562 and HEK-293T cells. Approximately 200,000 cells were transduced with either the SIV- or HIV-based lentiviral vector at an MOI of 1. The percentage of eGFP positive cells was quantified by flow cytometry. The transduction efficiency of the HIV vector system was greatest with an eGFP transgene in K562 ($P < 0.001$) and HEK-293T cells ($P < 0.001$) (**Figure 2.7 B**). Conversely, the SIV vector system exhibited greater transduction efficiency in BHK-M cells ($P = 0.017$) (**Figure 2.7 B**).

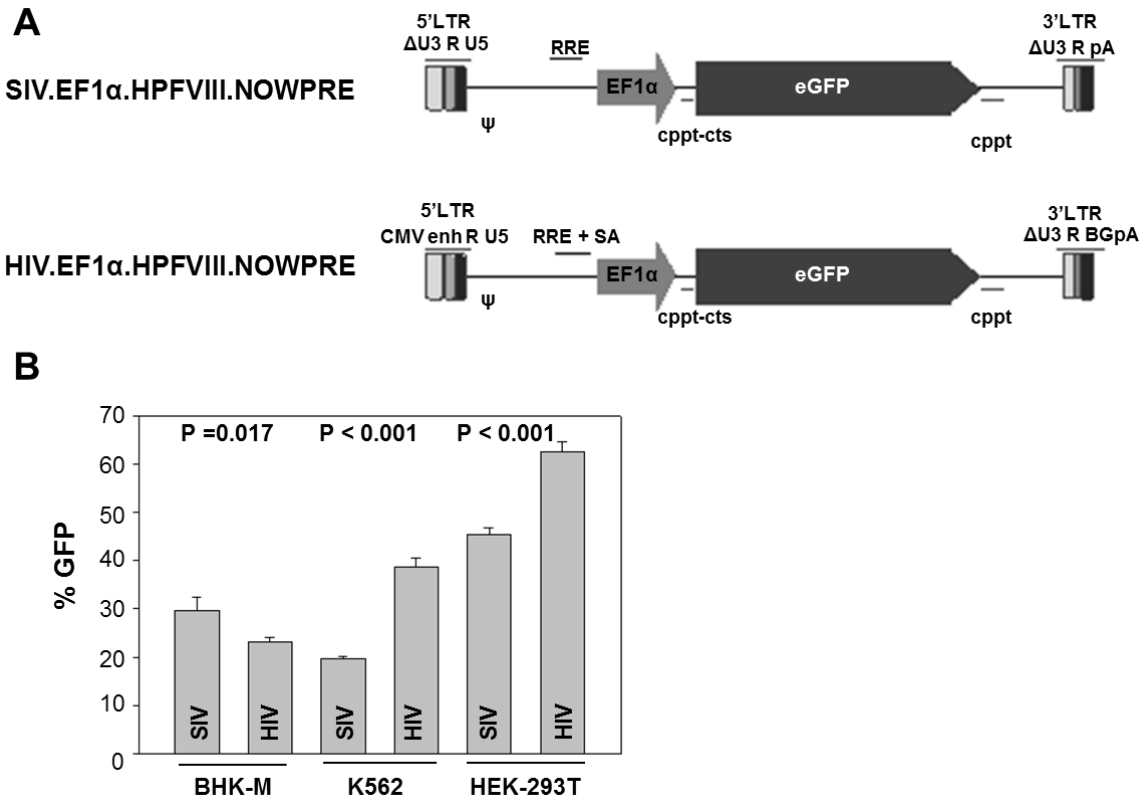


Figure 2.7 Comparison of SIV- and HIV- vector systems encoding eGFP. (A) Schematic representation of the SIV and HIV expression plasmids used to produce recombinant virus containing the eGFP transgene. (B) Single viral preparations were used to transduce three wells of BHK-M, K562 and HEK-293T cells at an MOI of 1. Transduction efficiency was assessed seventy-two hours following transduction by flow cytometry. *P* values were derived from a student's *t* test.

Comparison of the transduction efficiency of an SIV and EIAV-based lentiviral vectors encoding an eGFP transgene

Alternative non-primate lentiviruses have been proposed as vectors for gene transfer. Specifically, a vector has been derived from the equine infectious anemia virus (EIAV) (Olsen, 1998). The EIAV-based vector was previously optimized for eGFP expression (O'Rourke, *et al.* 2005) and obtained as a generous gift from Manji Patel of the University of North Carolina. The optimized EIAV-based vector contained an internal promoter consisting of elements from the CMV and β-actin promoter (Cβ) and included a WPRE sequence (**Figure 2.8 A**).

Transduction efficiencies were compared between the SIV- and EIAV-based lentiviral vectors in sca-1^+ cells. Since expression is not being compared between the two lentiviral vectors, only that of transduction efficiencies, different promoters governing expression of eGFP between the two lentiviral vectors is irrelevant. Sca-1+ cells were isolated from a hemophilia A mouse and stimulated with cytokines for 3 days. Cell counts during stimulation exhibited cell growth indicative of typical sca-1^+ cell functioning (data not shown). Sca-1^+ cells were transduced at an MOI of 20. Seventy-two hours after transduction the sca-1^+ cells were analyzed for eGFP by fluorescent microscopy images (**Figure 2.8 B**) and flow cytometry. Sca-1^+ cells transduced with an SIV-based lentiviral vector yielded 10% GFP positive cells, whereas cells transduced with an EIAV-based lentiviral vector yielded 1.4% GFP positive cells. To confirm the superior transduction efficiency of SIV to EIAV, the titer of the EIAV-based lentiviral vector was reevaluated on HEK-293T cells. Due to the differences in titering protocols,

the EIAV-based lentiviral vector was shown to be tenfold less than originally designated with a titer of $4.6x10^{9}$ TU ml^{-1}. It is predicted that a ten-fold difference would not increase the percentage of GFP positive cells above that of the SIV-based lentiviral vector, suggesting that the EIAV-based lentiviral vector is not superior.

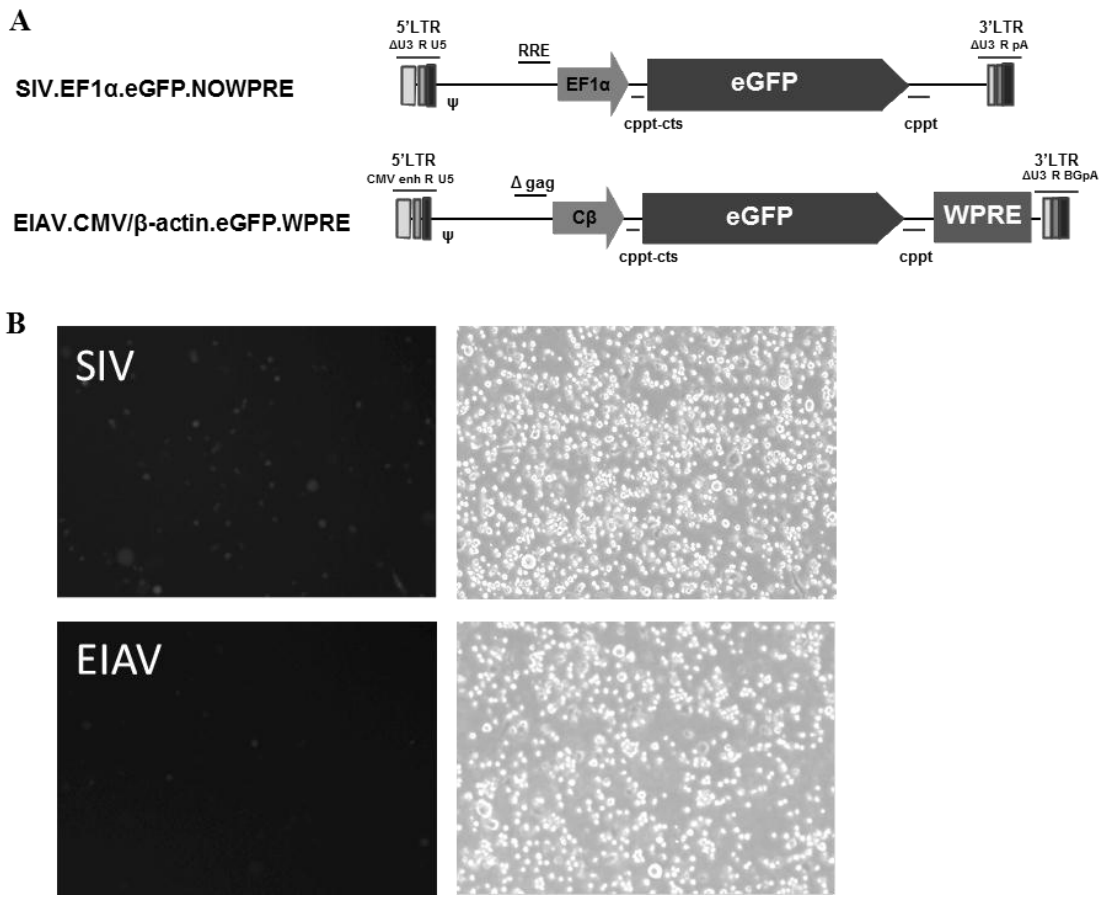


Figure 2.8 Comparison of SIV- and EIAV- based vector systems encoding eGFP. (A) Schematic representation of the SIV and EIAV expression plasmid used for viral preparations in HEK-293T cells. (B) Fluorescence microscopy images were taken prior to flow cytometry. Images depict increased transduction efficiency of an SIV-based vector.

2.33— Analysis of three internal promoters

Self-inactivating (SIN) vectors require internal promoters for transgene expression due to the inactivation of the viral 5' long terminal repeat (LTR) upon transfer of the U3 deletion during integration. A balance between adequate transgene expression and the elimination of transactivation of nearby genes must be maintained by the internal promoter. Three ubiquitous heterologous promoters, varying in enhancer activity, were evaluated in transduced HEK-293T cells. The human EF1α promoter, the CMV promoter, and the yeast phosphoglycerate kinase (PGK) promoter were incorporated into the SIV expression plasmid and used to produce virus under identical conditions (**Figure 2.9 A**). Viral titer was determined for each vector by qPCR. Vector then was added to HEK-293T cells at an MOI of 3. HEK-293T cells, although unable to accurately depict the transcription profile of HP-fVIII in sca-1^+ cells, were chosen due to the inherent superior transduction capabilities as compared to the hematopoietic EU1 and K562 cell lines. Transgene expression was greatest when driven by the CMV promoter and least by the PGK promoter as evaluated by the level of HP-fVIII transcripts from transduced cells (**Figure 2.9 B**). In addition, HP-fVIII activity normalized to copy number showed that HP-fVIII activity is greatest when expression is driven by the CMV promoter and least by the PGK promoter (**Figure 2.9 C**).

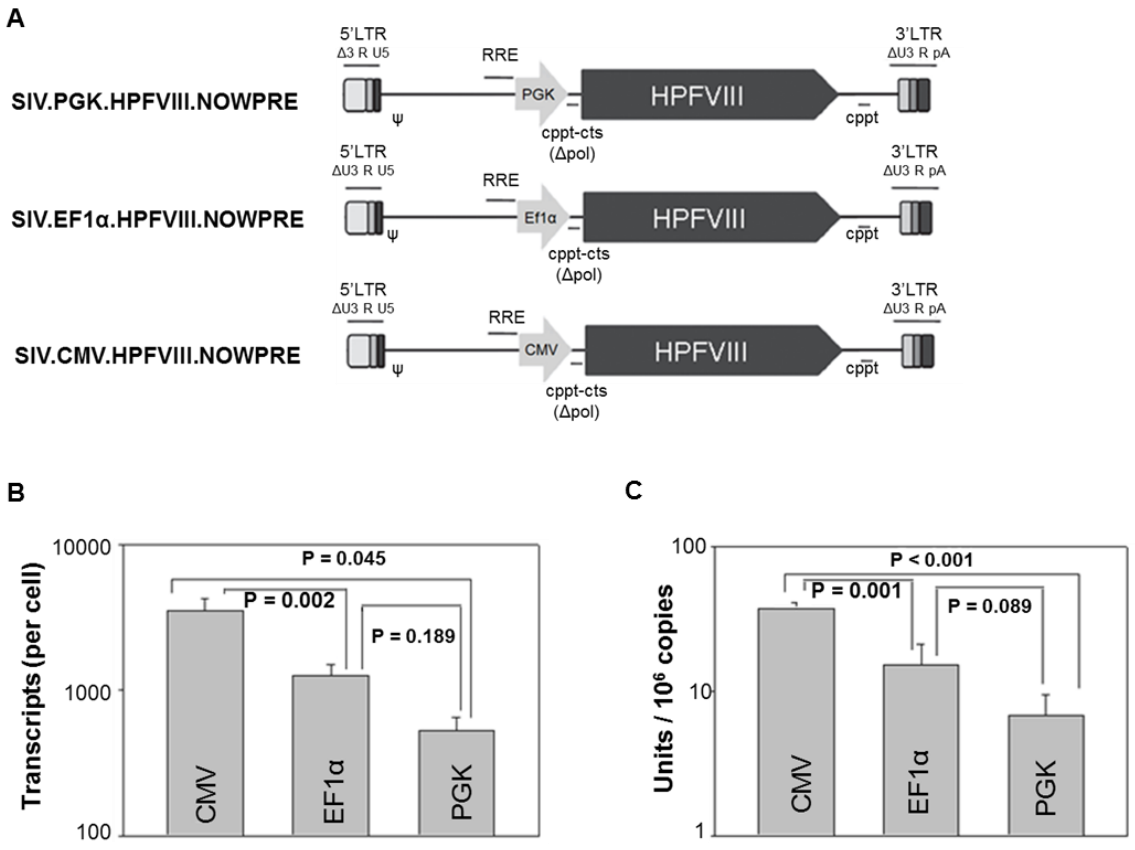


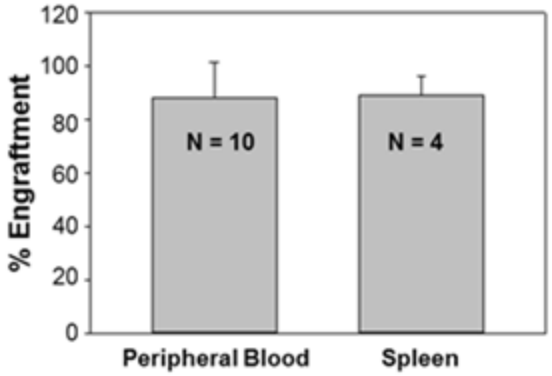
Figure 2.9 Effects of various internal promoters on HP-fVIII activity *in vitro*. (A) Schematic representation of SIN SIV-based expression vectors constructed with the PGK, EF1α or CMV internal promoter to make recombinant lentivirus. (B) HEK-293T cells were transduced at an MOI of 3. Seventy-two hours post-transduction, total RNA and genomic DNA were isolated from the cells and quantified by qPCR for transcript levels. (C) Twenty-four hours before isolation, the conditioned media was exchanged for serum-free AIM-V to assess HP-fVIII activity, which was normalized to copy number to correct for transduction efficiency. Each bar represents the mean ± the standard deviation of three wells. *P* values were determined by a one way ANOVA.

2.34— Hematopoietic stem cell gene therapy for hemophilia A utilizing the optimized lentiviral vector

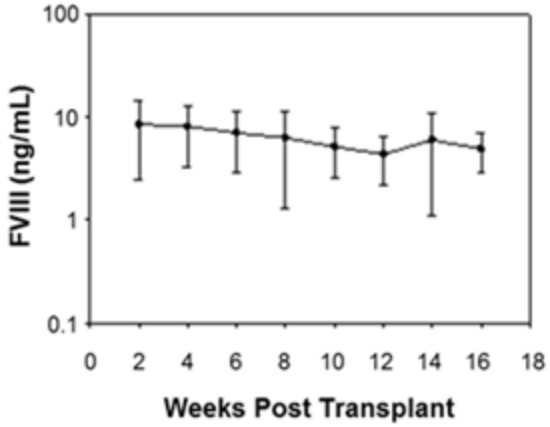
Based on the above analyses, an SIV-based lentiviral vector containing the HP-fVIII transgene expressed from a CMV promoter without the inclusion of a WPRE was predicted to be optimal for HP-fVIII expression *in vitro*. This construct then was evaluated *in vivo* in hemophilia A mice. Sca-1^+ cells were isolated from CD45.1 mice and transduced with the optimized SIV vector encoding HP-fVIII. CD45.1 sca-1^+ cells were transduced twice, 24 h between transductions, at an MOI of 15 each time, which resulted in 30-60% transduction efficiency with similar eGFP encoding vectors. However, with fVIII-containing vectors, only 3-10% is expected as previous findings showed a ten-fold lower transduction efficiency when using a vector encoding fVIII as compared to eGFP (data not shown, Doering *et al*., 2009). Transduction protocols were designed to ensure that transduced cells would only contain, on average, one or fewer copies of the vector. The transduced sca-1^+ cells then were transplanted into lethally irradiated (11 *Gy* split dose total body irradiation) hemophilia A mice. Three months after transplantation, donor cell engraftment was measured by flow cytometry. Average engraftment in the peripheral blood and spleen was shown to be approximately 90% (**Figure 2.10 A**). Every two weeks HP-fVIII levels were quantified by an ELISA assay. HP-fVIII levels persisted for the duration of the study (4 months) with a range between 2 and 20 ng ml^{-1} (**Figure 2.10 B**). A Coatest activity assay showed a similar HP-fVIII expression profile (**Figure 2.10 C**). Copy numbers in six mice remained below the detectable level of five percent gene-modified cells by qPCR with one each having 0.07,

0.10, 0.11 and 0.14 vector copies per genome. No correlation between copy number and HP-fVIII expression was observed in these mice.

A


120
100
80
60
40
20
0
% Engraftment
N = 10
N = 4
Peripheral Blood
Spleen

B


100
10
1
0.1
FVIII (ng/mL)
0 2 4 6 8 10 12 14 16 18
Weeks Post Transplant

C

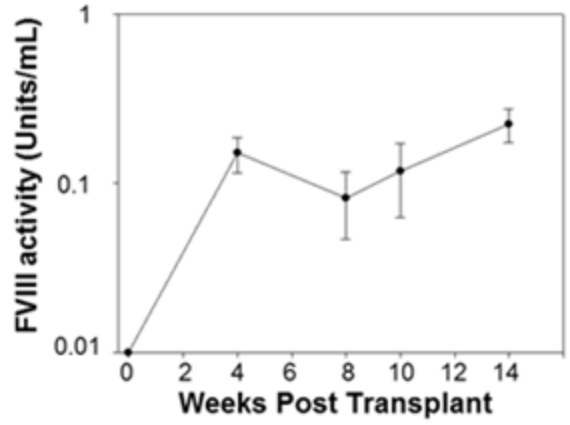

1
0.1
0.01
FVIII activity (Units/mL)
0 2 4 6 8 10 12 14
Weeks Post Transplant

Figure 2.10 *In vivo* expression of the optimized vector in transplanted hemophilia A mice. Sca-1^{+} cells were isolated from CD45.1 mice and transduced with the optimized vector with an MOI of 15 (x 2). Approximately 1 x 10^{6} cells were transplanted into Hemophilia A mice (CD45.2) (n = 14). (A) Engraftment was measured from peripheral blood and splenocytes of three mice and was assessed three months after transplant by flow cytometry. (B) HP-fVIII expression levels were assessed in the mice every two weeks following transplantation by an ELISA. (C) A chromogenic test (Coatest assay) was used to assess HP-fVIII activity in a subset of mice (n = 3).

2.4— Discussion

To date, three clinical trials have been initiated for gene therapy applications to treat hemophilia A (Roth *et al.*, 2001; Powell *et al.*, 2003; Berlfein *et al.*, 2003). However, each trial failed to yield sustained therapeutic levels of human fVIII. To overcome the obstacle of limited expression, a human/porcine fVIII hybrid has been constructed exhibiting expression 19-fold higher than human B domain-deleted fVIII (Doering *et al.*, 2009). Further optimization of the transgene resulted in a theoretically less immunogenic high expression fVIII transgene. With the production of an extensively optimized transgene, efforts can now be directed towards optimizing the viral vector for transgene delivery and expression.

A number of viral vectors have been considered for the modification of cells including both non-integrating and integrating vectors. Non-integrating vectors, such as the adenoviral and adeno-associated viral vectors, exist in cells extrachromosomally and are limited by potential vector genome loss. Yet, adenoviral vectors and adeno-associated viral vectors are appealing in that they efficiently transduce both dividing and non-dividing cells. With respect to the treatment of hemophilia A, adenoviral vectors have been utilized in neonates to produce tolerance (Hu *et al.*, 2011; Hu and Lipshutz, 2011) but currently are less clinically desirable due to toxic side effects experienced in clinical trials (Aruda, 2006). However, adeno-associated viral vectors are being extensively evaluated for use in gene therapy, especially for hemophilia B. Unfortunately, adeno-associated viral vectors are limited by their genetic carrying capacity. The vector cassette associated with the 4.4-kb of the B domain deleted fVIII cDNA, including regulatory

elements, is at and above the carrying capacity of an adeno-associated viral vector for the treatment of hemophilia A. Several groups are attempting to overcome this limitation by separating the genetic payload, that is, the heavy chain and light chain of fVIII, into two different vectors (Hu and Lipshutz, 2011; Scallan *et al*., 2003; Sarkar *et al*., 2006). Other groups are attempting to minimize the regulatory elements by utilizing smaller internal promoters (Scallan *et al*., 2003; Sarkar *et al*., 2004; Jiang *et al*., 2006; Lu *et al*., 2008; Sabatino *et al*., 2011).

Several groups have focused on the use of integrating viral vectors such as lentiviral vectors. Lentiviral vectors are suitable vectors for hemophilia A gene therapy applications in that they (1) stably integrate in the host genome, (2) are able to transduce quiescent cells and (3) can encapsulate large transgenes such as HP-fVIII. Although insertional mutagenesis was observed clinically with the use of integrating γ-retroviral vectors (Wu *et al*., 2003; Hacein-Bey-Abina *et al*., 2003), to date similar issues have not been observed with lentiviral vectors (Cartier *et al*., 2009; Cavazzana-Calvo *et al*., 2010; Biffi et al., 2011). This may be due to integration site preferences between the two vectors. In addition, clinically used lentiviral vectors contain safety measures that are now routinely incorporated, including a 133-bp deletion in the U3 region of the 3' LTR that self-inactivates the vector, creating a replication incompetent or SIN vector (Miyoshi *et al*., 1998; Iwakuma *et al*., 1999), (for review see Pauwels *et al*., 2009). Comparative studies have shown that SIN lentiviral vectors are less oncogenic than γ-retroviruses (Montini *et al*., 2006; Montini *et al*., 2009).

A WPRE often is incorporated into lentiviral vectors since the demonstration of two to fivefold enhanced eGFP and luciferase expression (Zuffery *et al*., 1999; Brun *et al*., 2003; Gonzalea-Murillo *et al*., 2010). However, a recent report showed that the enhanced expression was dependent on the promoter and cell line used, and in some scenarios, the presence of a WPRE resulted in a decrease in transgene expression (Klein *et al*., 2006). It is apparent that the WPRE is more complex than originally assumed and requires individual transgene evaluation. With the HP-fVIII transgene, the WPRE was found to be negligible in regards to viral production, transgene expression and transcriptional read-through. For our high expression construct, there appears to be no benefit to include a WPRE. Thus, based on these results confirmed by Gabriela Denning at Expression Therapeutics, the WPRE was removed from the clinical vector. However, it may be useful for lower expressing fVIII constructs and should be tested in conjunction with each.

The SIV- and HIV-based vector systems were also analyzed in this study in regards to HP-fVIII expression. The production of SIV and HIV lentiviral vectors were optimized separately. Using standard production conditions, higher viral titers routinely were achieved with the SIV system compared with HIV. After each viral preparation was quantified with respect to viral titer, transduction at identical MOIs yielded integration events that were not statistically different in K562 and HEK-293T cells. However, BHK-M and K562 cells transduced with SIV expressed HP-fVIII more efficiently than those cells transduced with identical amounts of HIV. This suggests that although SIV and

HIV integrated at similar levels, the integration events of SIV may be in regions of the genome that promoted greater expression than those regions where HIV integrated. This potential difference requires further study to test this hypothesis. It is interesting that this same phenomenon was not observed with an eGFP transgene and that the enhanced expression of HP-fVIII also was not seen with HEK-293T cells. This suggests a potential transgene and cell type specificity of SIV enhancement. This data also suggests that SIV is, at a minimum, as efficient at gene transfer and expression of HP-fVIII as HIV. In addition, SIV was found to be superior to an EIAV-based lentiviral vector in regards to transduction efficiency. SIV-based vectors may be a safer option for gene therapy applications to combat hemophilia A since a number of adults with hemophilia are HIV-1 positive after receiving plasma-derived fVIII infusion products before HIV testing became routine. The use of HIV vectors can raise safety concerns due to possible recombination events of the vector and wild-type HIV. SIV, on the other hand, is less likely to recombine due to sequence differences between HIV and SIV.

Different internal promoters also were tested as a component of the expression vector for HP-fVIII expression. SIN-lentiviral vectors require internal promoters to direct expression of the transgene due to the inactivation of the 5' LTR during integration. This safety feature removes the enhancer element of the LTRs preventing transactivation of nearby genes. Therefore, the internal promoter must be sufficiently strong to provide adequate expression of the transgene without having the capacity to transactivate genes nearby. The CMV promoter was superior to the EF1α and PGK promoters at expressing HP-fVIII *in vitro* using the SIV-based vector system. The enhanced expression from the

CMV promoter *in vitro* is not unexpected as the CMV promoter contains the strongest enhancer among the three internal promoters. However, with the stronger enhancer activity, the transactivation of nearby genes is a concern with the use of CMV, as well as the possibility of methylation-induced inactivation of the promoter. In hemophilia A mice under limiting transduction protocols using an SIV vector without a WPRE, the CMV promoter directed expression of HP-fVIII at therapeutically relevant levels.

In summary, several fVIII transgenes have been studied extensively for use in gene therapy applications of hemophilia A. HP-fVIII overcomes low-level expression obstacles while theoretically reducing the immunogenicity. Although the transgene has been fairly well described, the expression vector has not been as well characterized. In the studies presented in this chapter, a lentiviral vector was optimized for HP-fVIII expression. Under the conditions tested, the SIV-based lentiviral backbone was found to be more effective for HP-fVIII expression than an HIV-based lentiviral backbone. In the SIV-based lentiviral backbone, the CMV internal promoter was shown to drive HP-fVIII expression efficiently, but it is realized that this promoter may have complications due to possible transactivation and promoter inactivation. The WPRE was found to be unnecessary and was removed from the lentivector. *In vivo* data show that the optimized vector provides sustained HP-fVIII expression in hemophilia A mice, supporting its further development for hemophilia A.

Chapter 3:

Assessment of Alternative Approaches to Lentiviral Production and Transduction

3.1— Introduction

Upon identifying a lentiviral that is optimized for HP-fVIII expression, two limitations associated with the utilization of lentiviral vectors for the modification of HSCs in order to treat hemophilia A were addressed. The first limitation to be confronted is that of the reduced titer apparent with the addition of a large transgene such as fVIII in the lentiviral vector. It is apparent that as the size of the transgene increases the production of vector decreases (Kumar *et al.*, 2001; Yacoub *et al.*, 2007). Thus an alternative approach to lentiviral production was assessed as an attempt to combat this limitation. The second limitation is that of the inherent reduction in infectivity of HSCs to lentiviral vectors. This has been observed clinically in recent gene therapy clinical trials (Cartier *et al.*, 2009; Boztug *et al.*, 2010; Cavazzana-Calvo *et al.*, 2010; DiGiusto *et al.*, 2010). In one particular report as low as 1% gene corrected $CD34^+$ cells were found to be engrafted (DiGiusto *et al.*, 2010). In the case of an HSC gene therapy protocol for hemophilia A, one in which a growth advantage is not expected, greater transduction efficiencies will be needed to achieve therapeutic potential.

Phenotypically mixed viral particles are routinely formed as a result of the infection of a cell with two or more separate viruses (Zavada, 1982). This observation along with the separation of the three retroviral components (gag-pol, viral genome and envelope) onto separate plasmid constructs (refer to **Figure 1.4**) led to the development of pseudotyping methods for lentiviral vectors used in the context of gene therapy. The first alternatively pseudotyped HIV based viral vector contained the amphotropic glycoprotein of the murine leukemia virus (MLV) (Page *et al.*, 1990). This study followed the demonstration

of phenotypic mixing between wild-type HIV and MLV virons (Chesbro *et al*., 1990; Spector *et al*., 1990). The range of host cells transduced by a virus is dependent on the interaction of a viral envelope glycoprotein with specific cell surface receptors. Therefore, other glycoproteins were subsequently used to expand the host range of HIV-based lentiviral vectors (Cronin *et al*., 2005).

The vesicular stomatitis virus glycoprotein (VSVG) gives viral vectors the broadest of host-cell range by interacting with what has historically been thought to be an abundant ubiquitous component of the cellular plasma membrane. The receptor was postulated to be phosphatidyl serine, phosphatidyl inositol or GM3 ganglioside (Schlegel *et al*., 1983, Mastromarino *et al*., 1987; Conti *et al*., 1988). Phosphatidylserine, however, has since been ruled out as the cell surface receptor for VSVG (Coil and Miller, 2004) being attributed solely to a post-binding step of viral entry (Coil and Miller, 2005). Regardless of the exact surface receptor utilized by VSVG, three groups in the same year independently expanded the host range of an HIV-based lentiviral vector by successfully pseudotyping the vector with VSVG (Akkina *et al*., 1996; Naldini *et al*., 1996; Reiser *et al*., 1996). In addition to expanding the host range, another advantage to pseudotyping viral vectors with VSVG is that these vectors can withstand the g-forces of ultracentrifugation in order to yield concentrated vector with high viral titers. Despite the advantageous properties associated with pseudotyping lentiviral vectors with VSVG, VSVG expression is toxic in most cells. Accumulation of VSVG at the surface of HEK-293T cells leads to syncytia formation followed by cell death (Burns *et al*., 1993). As a result, several failed attempts have been made to produce stable packaging cell lines for

the manufacture of VSVG pseudotyped lentivirus. This is mainly due to diminished cell survival. For this reason, the production of lentiviral vectors pseudotyped with VSVG is limited by a transient transfection procedure in HEK-293T cells. By this method, virus can only be produced for a total of three days before the cells become inviable. In addition, vector production in this manner is not ideal being unable to offer a nonvariable source of recombinant virus that can be extensively characterized prior to use in human clinical trials.

Another limitation encountered in the field of gene therapy includes a diminished susceptibility of the hematopoietic stem cell (HSC) to *ex vivo* genetic manipulation. Lentiviral vectors pseudotyped with VSVG, regardless of an increased host range, do not transduce HSCs efficiently. This phenomenon appears to be intrinsic to HSCs being that HSCs are also resistant to wild-type HIV infection regardless of the presence of the CXCR4 cell surface receptors (Von Laer *et al*., 1990; Weichold *et al*., 1998; Lee *et al*., 1999; Shen *et al*., 1999). The exact mechanism responsible for HSC resistance to HIV remains elusive. Therefore, much effort has been directed towards increasing lentiviral transduction of HSCs. One such effort was performed with the use of RNAi technology. p21, a cell cycle checkpoint protein, was transiently silenced in $CD34^+$ hematopoietic stem and progenitor cells. As a result HIV lentiviral transduction was enhanced 2- to 4-fold (Zhang *et al*., 2005). Initially, p21 was chosen as a target being a cyclin-dependent kinase inhibitor necessary for the maintenance of HSC quiescence (Cheng *et al*., 2000). It was stipulated that high efficient gene transduction is cell cycle dependent (Sutton *et al*., 1999). However, the enhancement in HIV lentiviral transduction due to p21 silencing

was independent of a direct effect on cell cycling. Instead, p21 was found to immunoprecipitate with the HIV preintegration complex and increase the abundance of 2-LTR circles indicative of abortive chromosomal HIV integration. In this manner, p21 was suggested to block HIV viral infection by disturbing the endogenous function of HIV integrase (Zhang *et al*., 2007). These reports proposed introducing siRNA to p21 in preclinical transduction protocols. Current methods include HSC pre-stimulation with cytokine cocktails that have been demonstrated to enhance transduction (Santoni *et al*., 2006; Millington *et al*., 2009) but typically result in a loss of gene modified cells following transplantation potentially due to diminished multipotentiality of the HSCs (Zhang *et al*., 2005). RNAi technology, on the other hand, does not disrupt the multipotentiality of HSCs and could potentially be a more advantageous addition to preclinical transduction protocols. In this manner, the multipotentiality of HSCs could remain undisrupted while being more susceptible to transduction.

The studies presented in this chapter depict attempts to overcome some of the previously mentioned pitfalls plaguing the field of gene therapy. VSVG pseudotyped lentivirus production is limited to a transient transfection procedure in which virus is produced for only 3 days. In order to avoid toxicity associated with the overexpression of VSVG in HEK-293T cells, an alternative cell line was assessed for recombinant lentiviral production. In addition, lentiviral transduction enhancement was attempted using two separate methods based off previous reports.

3.2— Materials and Methods

Spinoculation

Spinoculation was performed on K562 and sca-1+ cells in 24 well plates coated with retronectin (1µg µl^{-1}). Cells were plated at a density of 10^5 cells per well and transduced by spinoculation in minimal volume (300 µl) in the presence of polyberene. Spinoculation was performed at 1000g for 2 hours. The effect of spinoculation on lentiviral transduction of K562 cells was assessed with recombinant virus containing either a GFP or HP-fVIII transgene. Transduction with a GFP containing recombinant lentivirus was assessed by flow cytometry, while the effect of spinoculation on the transduction of a recombinant lentivirus containing the HP-fVIII was assessed by the APTT reagent-based one stage coagulation assay as described below.

In vitro fVIII expression

fVIII activity was measured from the supernatant of cells transduced previously with a lentiviral vector containing the high expressing HP-fVIII transgene. The cells were cultured in serum-free media for 24 hours before the assay as previously described in chapter 2. In short, the APTT one stage coagulation assay was performed in duplicate for each supernatant on a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France) in human fVIII-deficient plasma (George King Biomedical, Overland Park, KS). The clot time for each sample was compared to a standard curve based on dilutions of pooled normal citrated human plasma (George King Biomedical, Overland Park, KS).

Isolation of murine stem cell antigen-1^+ (sca-1^+) cells

fVIII knockout mice with hemophilia A due to disruption of exon 16 (as previously described in Bi *et al*, 1995) were treated with 5-FU (150 mg kg^{-1}) intraperitoneally two days prior to bone marrow isolation. The bone marrow was extracted from the femurs and tibias of 9-week old hemophilia A mice upon being flushed with PBS (supplemented with 2% FBS). The flushed bone marrow was then filtered through a 0.7 µmol l^{-1} filter and pelleted at 300g for 5 minutes. Red blood cells were then lysed for 10 minutes with the addition of RBC lysis buffer (Sigma, St. Louis, MO, USA). The remaining cells of the bone marrow were then assessed for purity by flow cytometry utilizing a Ly-6A/E antibody (clone E13-161.7) conjugated to FITC (BD Pharmingen, San Diego, CA). The purity of the population was found to be 85% positive for sca-1^+. Upon confirmation, the sca-1^+ cells were cultured at a density of 10^5 cells per well in a 24 well tissue culture treated plate previously coated with retronectin (1µg $µl^{-1}$). Sca-1^+ cells were cultured in StemPro media supplemented with L-glutamine (29µg mL^{-1}), murine stem cell factor (100ng mL^{-1}), murine interleukin-3 (20ng mL^{-1}), human interleukin-11 (100ng mL^{-1}), and human Flt-3 ligand (100ng mL^{-1}). Transduction began the same day as isolation in the presence of polyberene. Cells were transduced twice, 12 hours apart by spinoculation at a MOI of 15. An *in vivo* assessment of spinoculation was made by transplanting $4x10^5$ sca-1^+ cells via tail-vein injection into lethally irradiated (11*Gy* TBI using a Gammacell 40 Exactor) 8-week-old recipient hemophilia A mice (n = 3). Transplantation occurred 4 days after initial sca-1^+ isolation, 2 days following the second transduction. Blood was collected from the transplanted mice every two weeks beginning one week after transplantation via retro-orbital capillary insertion. HP-fVIII was measured using an

ELISA specific for HP-fVIII. The ELISA only detects properly folded HP-fVIII in that the primary antibody detects the heavy chain (human A2 domain) and the secondary antibody detects the light chain (porcine A3 domain).

Colony-forming Unit Assay

Colony-forming cell assay was performed per manufacturer's protocol (Stemcell Technologies, MethoCult® GF M3434 Optimum with EPO). Briefly, a 10X concentration ($2x10^4$ cells/mL) of sca-1^+ cells was prepared in Iscove's MDM with 2% FBS. A total of 0.3 mL of 10X cell mixture was then added to 3 mL of MethoCult® and mixed by vortexing. MethoCult®/cell mixture was allowed to sit for 5 minutes to allow bubbles to rise followed by dispensing 1.1 mL onto 35 mm dishes in duplicate using a 16 gauge blunt-end needle. The methylcellulose was evenly distributed into the dish by rotation and dishes were placed in a 100 mm culture dish alongside a third dish containing sterile water to maintain proper humidity. Cultures were incubated at 37^oC in 5% CO^2 for 14-16 days for optimal CFC growth. CFU-GM colonies were counted at 5X under an inverted microscope using a 60 mm gridded dish. GFP-positive colonies were scored under fluorescence microscopy.

3.3— Results

3.31— Toxicity of VSVG in BHK-M cells

Lentiviral vector is predominantly produced by transient transfection of the highly transfectable HEK-293T cells. This method of vector production is sufficient to produce vector titers appropriate for laboratory use. However, vector production is limited to 3

days due to toxicity associated with the accumulation of VSVG at the surface of HEK-293T cells (Burns *et al.*, 1993). This drawback has prevented the generation of a stable lentiviral vector-producing HEK-293T cell line in which clinical-grade vectors would ideally be manufactured. BHK-M cells are a suitable alternative for viral production as a highly transfectable cell line. In addition, BHK-M cells are already being used clinically for a portion of the commercial production of recombinant fVIII. BHK-M cells are able to efficiently synthesize recombinant fVIII with all the proper post-translational modifications (**see Figure 1.2**). And, if not susceptible to VSVG toxicity, the production abilities of BHK-M cells could be used to generate substantial amounts of lentiviral vector or even be used to create a packaging cell line. For this reason, the toxicity of VSVG was first assessed in BHK-M cells.

As a transfectable unit, PEI condenses DNA into positively charged particles in order to be brought into the cell via endocytosis upon binding anionic cell surface residues. Once inside the cell, the amines are protonated resulting in osmotic swelling of the vesicle which then bursts releasing the DNA into the cytoplasm. However, PEI is also very cytotoxic and could be the cause of the cell death. Therefore, PEI concentrations were varied to determine the extent of toxicity in BHK-M cells. At a concentration of 3.5 µg ml^{-1}, transfection was not achieved as observed by a lack of expression of GFP (**Figure 3.1 A**). A concentration of 17.67 µg ml^{-1} PEI, however, was tolerable by BHK-M cells (**Figure 3.1 B**). Concentrations higher than 17.67 µg ml^{-1} were followed by a decrease in cell number suggesting cytotoxicity at these higher concentrations (**Figure 3.1 C-E**).

Thus, the optimal concentration of PEI transfection in BHK-M cells appears to be less than 17.67 μg ml^{-1} PEI.

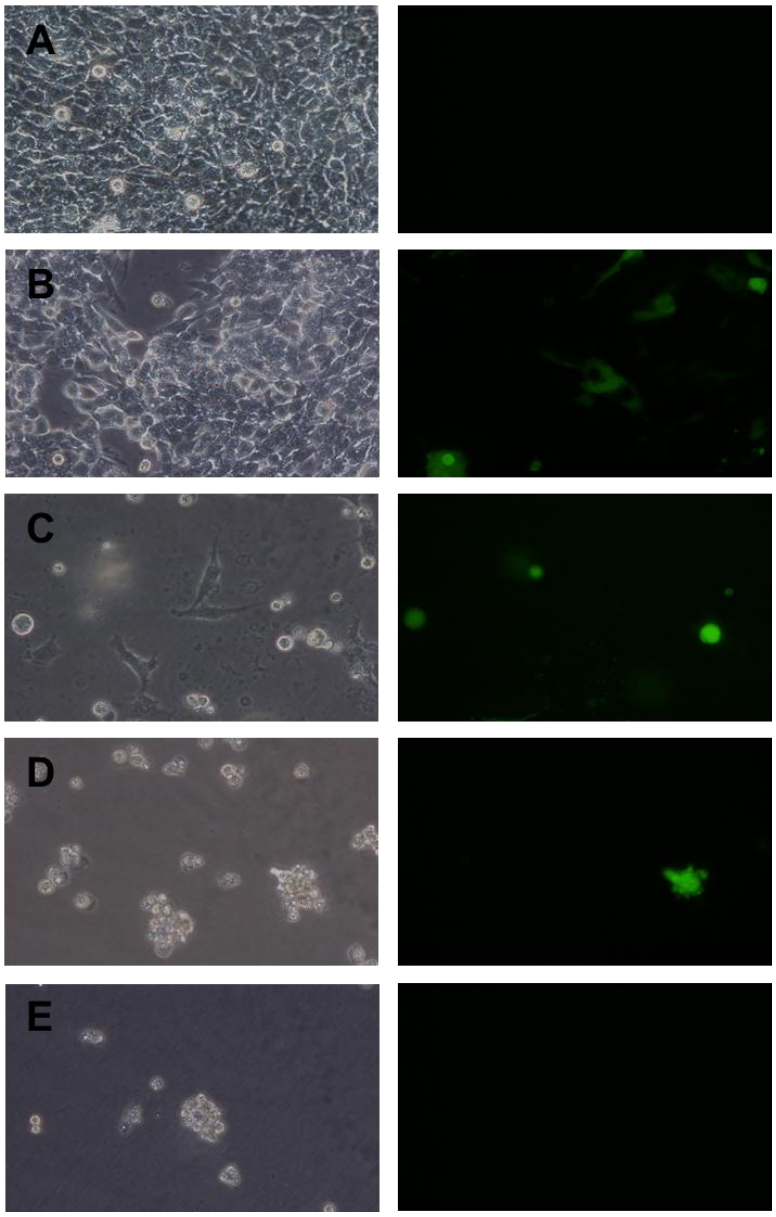


Figure 3.1 PEI toxicity assessment in BHK-M cells. A GFP encoding plasmid was transfected in BHK-M cells in order to assess transfection efficiency by fluorescence microscopy. The following concentrations of PEI were in order to determine the optimal concentration of PEI for BHK-M cellular transfection: (A) 3.5 µg ml^{-1} PEI (B) 17.67 µg ml^{-1} PEI (C) 35.34 µg ml^{-1} PEI (D) 176.7 µg ml^{-1} PEI (E) 353.4 µg ml^{-1} PEI. Images were taken seventy-two hours after transient transfection.

The plasmid containing the VSVG envelope protein was transiently transfected into BHK-M cells using the transfection reagent polyethylenimine (PEI). VSVG plasmid concentrations ranged from 0 to 3200 ng. Fluorescence microscopy images were taken seventy-two hours following transfection in order to assess cell viability (**Figure 3.2 A-F**). Although each well initially contained the same quantity of cells, less cells were adherent at the higher concentrations (**Figure 3.2 D-F**) of VSVG as compared to the well undergoing a mock transfection (**Figure 3.2 A**). In some instances syncytia formation is apparent (**Figure 3.2 F**). This suggests that VSVG is toxic to BHK-M cells. The BHK-M fluorescent images were compared to similar images taken on HEK-293T cells assessed for VSVG toxicity in the same manner. At concentrations as high as 3200 ng, no syncytia formation was observed and cell numbers appeared to be similar to the mock transfection control (**Figure 3.3 A-F**).

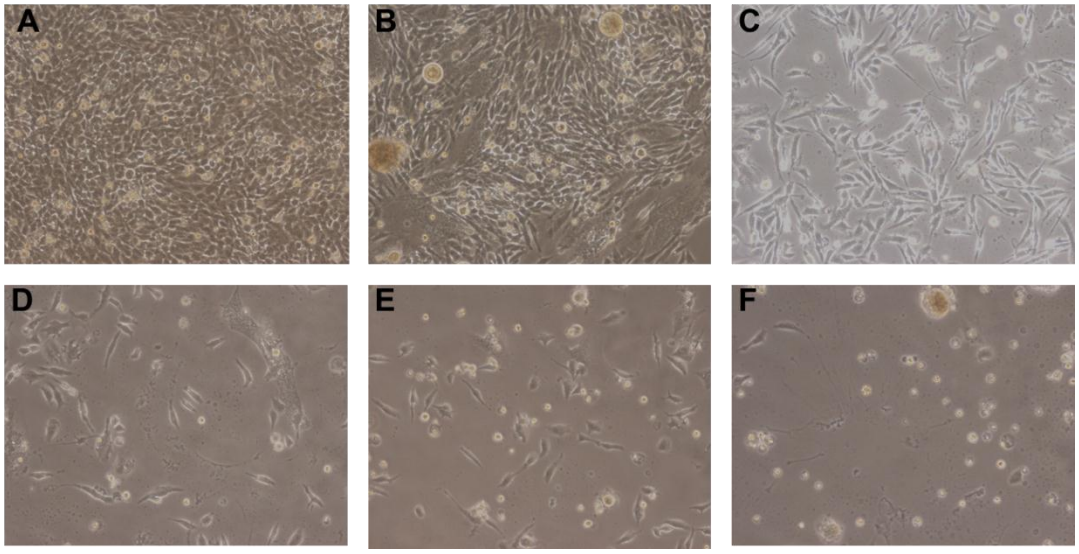


Figure 3.2 VSVG toxicity assessment in BHK-M cells. The VSVG containing lentiviral production plasmid was transiently transfected into BHK-M cells at the following concentrations: (A) no vsvg (B) 200 ng VSVG (C) 400 ng VSVG (D) 800 ng VSVG (E) 1600 ng VSVG (F) 3200 ng VSVG. 6 µg ml^{-1} PEI was used for the transfection to reduce toxicity associated with the transfection agent.

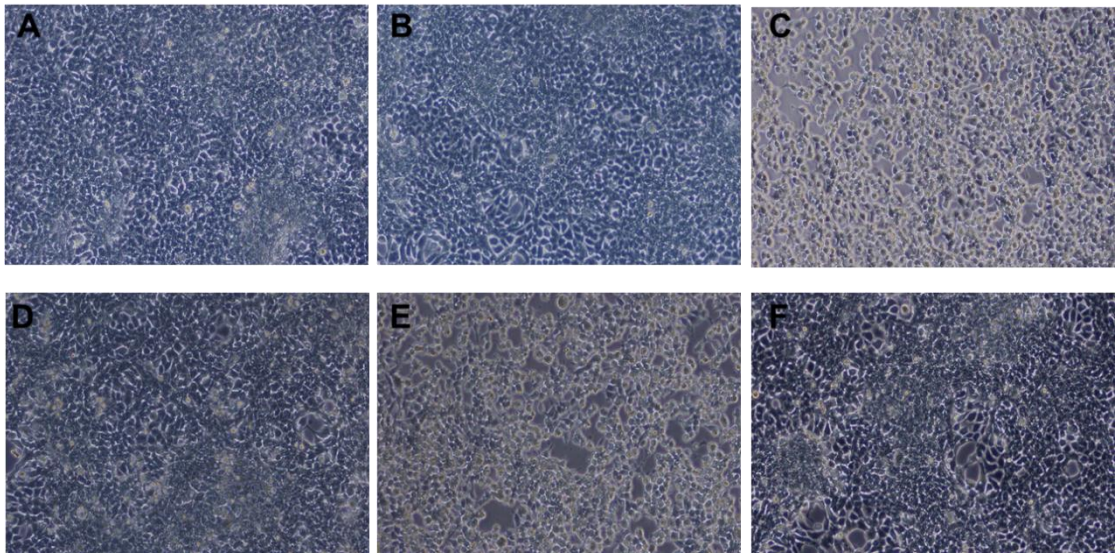


Figure 3.3 VSVG toxicity assessment in HEK-293T cells. VSVG containing plasmid was added to HEK-293T cells at the following concentrations utilizing the transfection reagent PEI: (A) no VSVG (B) 200 ng VSVG (C) 400 ng VSVG (D) 800 ng VSVG (E) 1600 ng VSVG (F) 3200 ng VSVG. Microscopic images were taken seventy-two hours after transfection.

3.32— Effect of alternatively pseudotyping an HIV-based lentiviral vector

Due to the toxicity associated with VSVG in BHK-M cells, three alternative envelopes were assessed for the production of an HIV-based lentiviral vector encoding a GFP transgene in BHK-M cells. Virus was produced by transient transfection in 10cm well plates. Transfection efficiency was assessed seventy-two hours later by fluorescence microscopy. The expression of GFP was similar among all wells despite the concentration or the viral envelope utilized (**Figure 3.4 A-C**). The only exception is that of the BHK-M cells transfected with a VSVG encoding plasmid. As expected, the expression of VSVG diminished BHK-M cell viability as observed by a decrease in BHK-M cells per well in addition to the presence of syncytia formation (**Figure 3.4 D**). Overall, the transfection efficiency appeared to be very low. Conditioned media containing recombinantly produced virus was used to transduce HEK-293T cells to determine viral infectivity. Viral transduction was assessed seventy-two hours after viral addition by examining the presence of GFP positive cells via fluorescence microscopy. GFP expression was not observed in the HEK-293T cells treated with the alternatively pseudotyped viruses (**Figure 3.5 A-D**). This would suggest that the alternatively pseudotyped viruses were not adequately produced, probably due to low transfection efficiency.

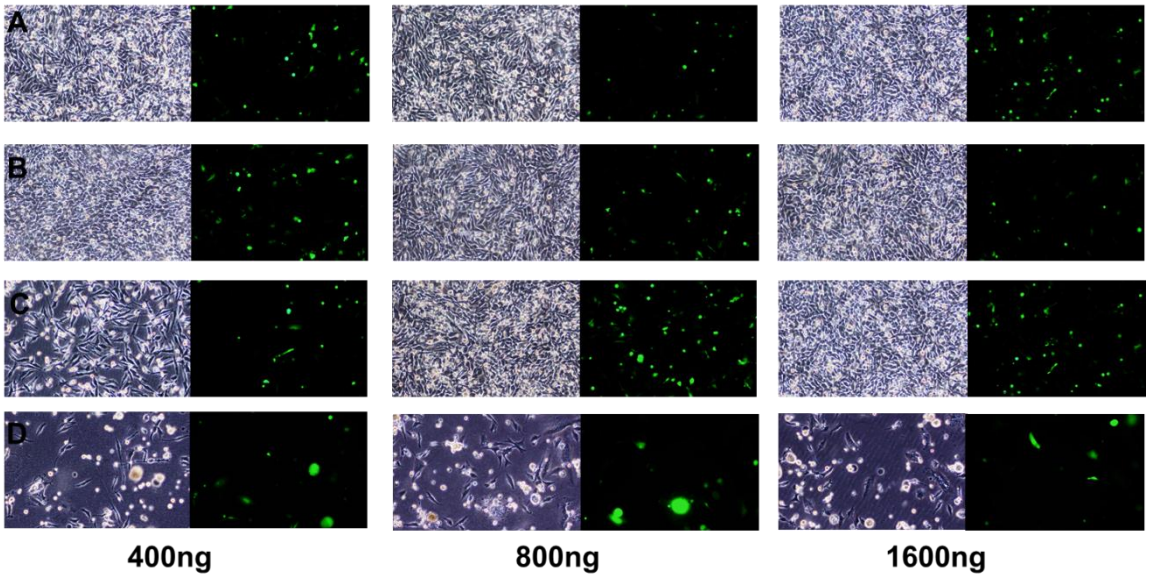


Figure 3.4 Transfection efficiency assessment. BHK-M cells were used to make recombinant HIV-based virus containing a GFP transgene. Four separate envelope plasmids were transfected into the BHK-M cells in order to produce alternatively pseudotyped recombinant virus. Three different concentrations of the (A) Eco (B) pHit (C) 10A1 and (D) VSVG envelope plasmids were transfected into the BHK-M cells using PEI.

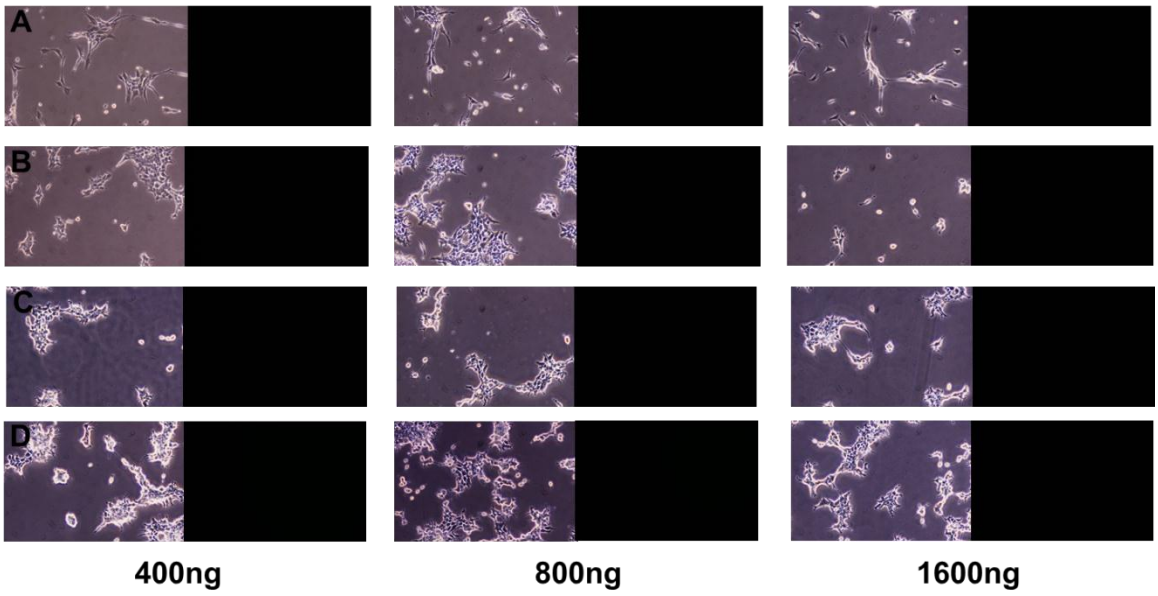


Figure 3.5 Viral infectivity assessment of alternatively pseudotyped HIV-based lentiviral vectors. Conditioned medium containing viral particles was added to HEK-293T cells. Virus was pseudotyped with either (A) Eco (B) pHIT (C) 10A1 or (D) VSVG glycoproteins. In addition, three different concentrations of the envelope plasmid were used to produce the alternatively pseudotyped recombinant virus. Fluorescence microscopy images were taken seventy-two hours after viral addition.

3.33— Knocking down p21

It has been suggested that lentiviral transduction can be enhanced by silencing p21. An HIV-based lentiviral vector was used to transduce human bone marrow-derived $CD34^+$ cells in which p21 had been silenced using RNAi technology. As a result, a 2- to 4-fold increase in GFP positive cells was observed (Zhang *et al*., 2005). An enhancement of that magnitude could be useful in the context of HSC gene therapy for hemophilia A. Therefore, siRNA specific to p21 was obtained and preliminary experimentation was performed.

K562 cells were plated in a 96 well plate at a density of $5x10^4$ cells / well. p21 was silenced by transfecting p21 siRNA into cells using the HiPerfect Transfection Reagent. The K562 cells were transduced forty-eight hours later with an HIV-based lentiviral vector encoding a GFP transgene at an MOI of 1 in the presence of polyberene. Transduction was assessed seventy-two hours after viral addition by flow cytometry. An MOI of 1 transduced approximately 60% of the K562 cells. No significant difference was observed between K562 cells treated with p21 siRNA and nonspecific siRNA (**Figure 3.6 A**). However, a slight increase in mean fluorescent intensity (MFI) was observed in K562 cells treated with p21 siRNA (**Figure 3.6 B**). This suggested a potential to enhance lentiviral transduction of K562 cells, which may not be seen utilizing an MOI of 1. For this reason, the experiment was repeated using lower MOIs. However, no enhancement in transduction efficiency was noted as seen by the percentage of positive GFP cells (**Figure 3.7 A, C, E**). It is unclear why an increase in MFI as seen

with an MOI of 1 (**Figure 3.6 B**) was not observed at the lower MOIs (**Figure 3.7 B, D, F**).

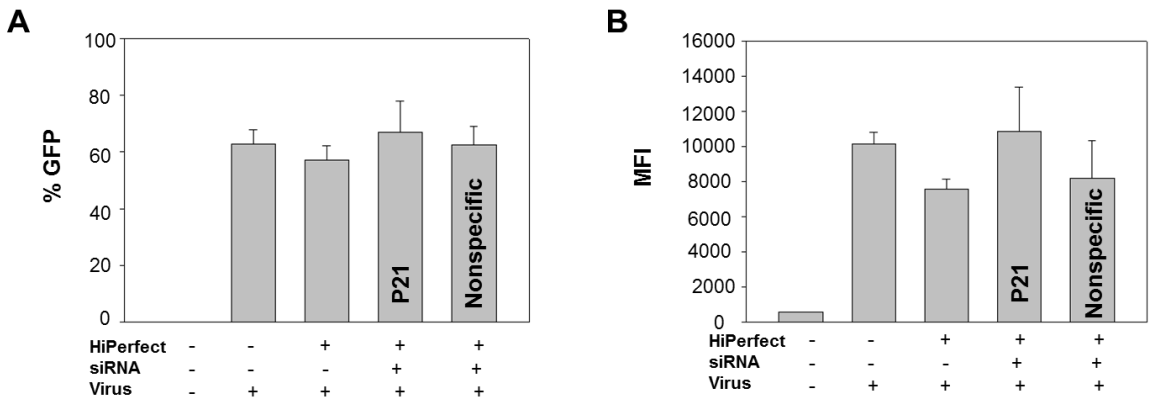


Figure 3.6 Utilizing p21 siRNA to enhance lentiviral transduction. (A) K562 cells were treated with 150nM siRNA (either specific for p21 or nonspecific as a control). An HIV-based lentiviral vector encoding a GFP transgene was used to transduce the cells forty-eight hours after siRNA treatment. Transduction efficiency was assessed seventy-two hours later by flow cytometry. As a result, the percentage of GFP positive cells was quantified and (B) the intensity of fluorescence for each positive cell was recorded as mean fluorescent intensity. A mock transfection control was included (no siRNA just HiPerFect Reagent) as well as an untransduced and an untransfected control.

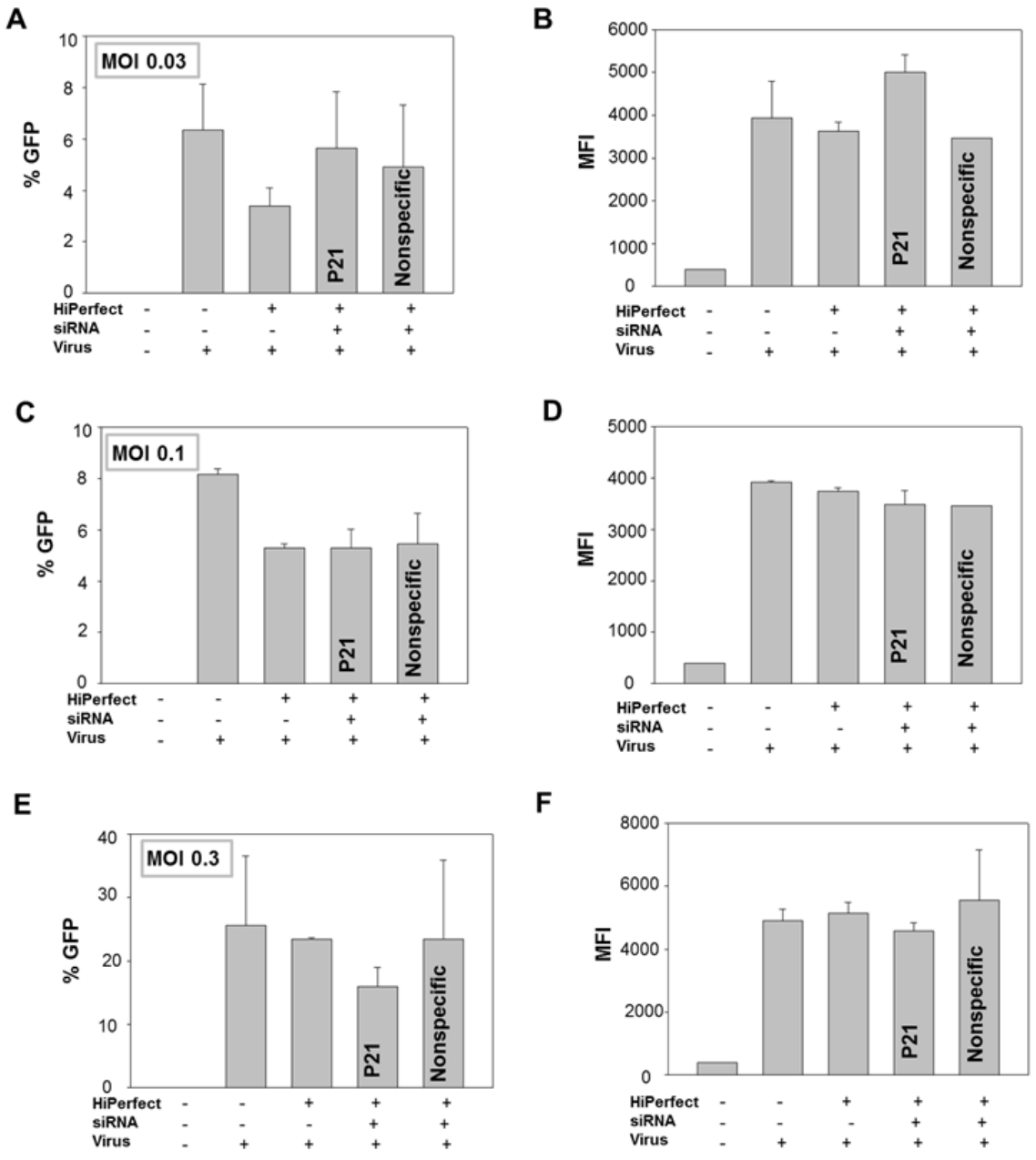


Figure 3.7 The effect of p21 silencing to enhance lentiviral transduction. p21 was silenced forty-eight hours prior to lentiviral transduction. An HIV-based lentiviral vector encoding a GFP transgene was added to K562 cells at three different MOIs as indicated: (A) (B) 0.03. (C) (D) 0.1 and (E) (F) 0.3. Transduction efficiency was quantified by flow cytometry seventy-two hours after viral addition.

3.34— Utilizing spinoculation as a means for enhancing sca-1$^+$ transduction

A recent report demonstrated therapeutic levels of fVIII as high as 225 mU mL^{-1} in hemophilia A mice (Ramezani *et al.*, 2011). Few apparent differences were found between the *in vivo* protocol used in the report and the one used in chapter 2. In both cases, sca-1$^+$ cells were transduced with an SIV-based lentiviral vector containing an enhanced fVIII transgene engineered for high expression. In addition, both protocols included transplanting a limited number of cells in hemophilia A mice after being transduced at a low MOI. Despite the similarities between the protocols, drastically different results were observed. Ramezani *et al*. (2011) achieved average fVIII levels equating to 23% of normal fVIII as compared to an average of 4.5% of normal fVIII observed in chapter 2.

One main difference between the protocols was the addition of a spinoculation step via centrifugation immediately following viral addition to sca-1$^+$ cells. For this reason, the effect of spinoculation was evaluated in regards to the transduction efficiency of the optimized vector outlined in chapter 2. Preliminary analysis was performed *in vitro* using the myeloid erythroid-leukemic K562 cell being that K562 cells are an immortalized cell line as compared to sca-1$^+$ cells obtained upon isolation from the bone marrow. In addition, initial analyses included the use of an SIV-based lentiviral vector containing the GFP transgene in order to assess transduction efficiency rapidly by flow cytometry.

K562 cells were transduced at an MOI of 15 and either immediately spun at 1000g for 2 hours or placed in the incubator overnight (n = 3). In addition, three separate wells were

transduced by spinoculation on two consecutive days at an MOI of 15 each day. Transduction was assessed over time by quantifying the percentage of GFP positive cells in each well. However, an MOI of 15 yielded approximately 100% GFP positive cells in the absence of spinoculation, eliminating the possibility of seeing any enhancement in transduction as a result of spinoculation (**Figure 3.8 A**). However, an enhancement in mean fluorescent intensity (MFI) was noted suggesting some benefit to including a spinoculation step in the transduction protocol ($p = 0.0055$ at 5 days post transduction; $p = 0.0332$ at 7 days post transduction as assessed by the Student's t test between the no spinoculation group and the spinoculation group) (**Figure 3.8 B**). This could be either due to enhanced expression of GFP from the cells transduced by spinoculation or due to an increase in integrated vector copies due to enhanced viral transduction. To determine if the increase in MFI was due to enhanced viral transduction, the analysis was repeated utilizing $1/3^{rd}$ the amount of virus equating to a lower MOI of 5. In the first week, the percentage of GFP positive cells was significantly increased from an average of 68.6% to 89.2% as a result of including a spinoculation step in the transduction protocol ($p = 0.0398$ one week after transduction) (**Figure 3.9 A**). This enhancement was maintained throughout the analysis suggesting an enhancement in lentiviral transduction due to spinoculation. A comparison between the single transduction group and the group transduced twice by spinoculation revealed no significant enhancement in percentage of GFP positive cells over time (**Figure 3.9 A**). However, a significant increase in MFI was observed in the double transduction group as compared to the single transduction group (**Figure 3.9 A**). Once again, this suggests some benefit to transducing K562 cells twice by spinoculation.

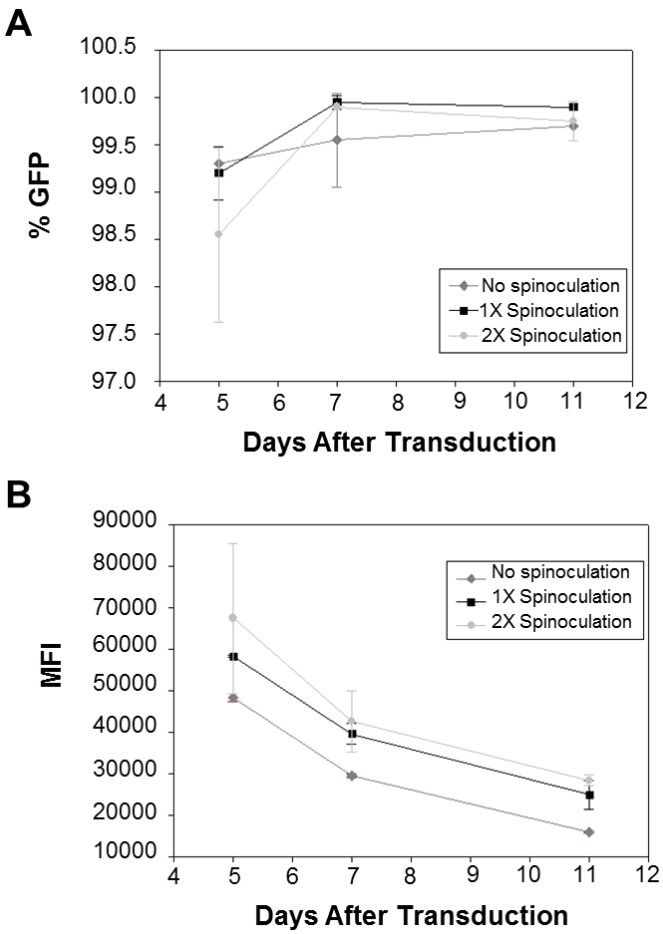


Figure 3.8 The effect of spinoculation on transduced K562 cells with an MOI of 15. (A) An SIV-based lentiviral vector containing a GFP transgene was utilized to transduce K562 cells at an MOI of 15. Transduction was assessed over time by flow cytometry. The percentage of GFP positive cells was compared between K562 cells without spinoculation (dark grey circles), with spinoculation (black squares). K562 cells were transduced twice under spinoculation conditions as a positive control (grey diamonds). (B) The mean fluorescent intensity of the GFP positive cells was quantified by flow cytometry.

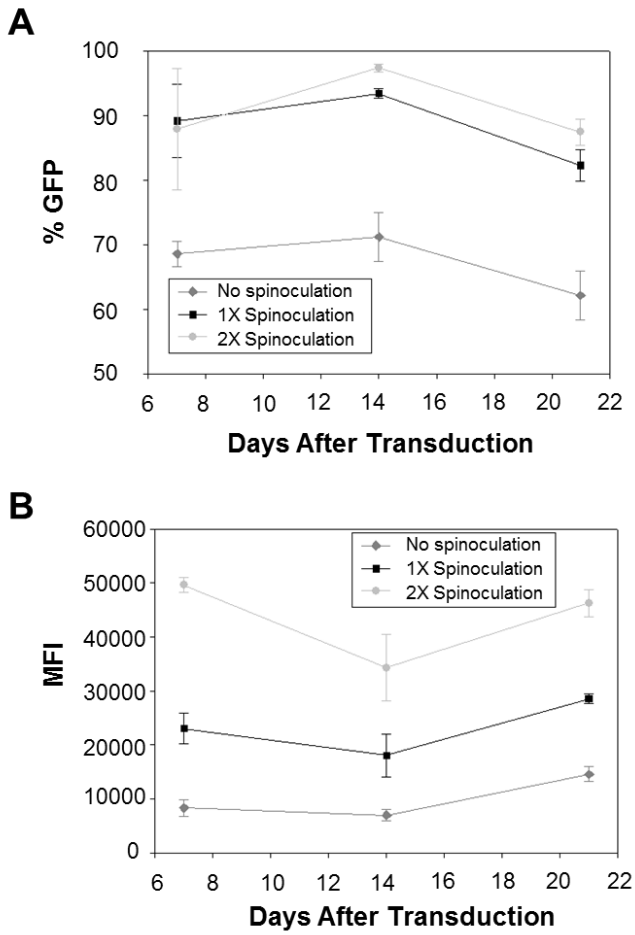


Figure 3.9 The effect of spinoculation on transduced K562 cells with an MOI of 5. (A) Transduction of K562 cells by an SIV-based lentiviral vector containing a GFP transgene (MOI 5) was assessed by flow cytometry. The percentage of GFP positive cells was determined over time and compared among K562 cells without spinoculation (dark grey circles), with spinoculation (black squares) and transduced twice under spinoculation conditions as a positive control (grey diamonds). (B) In the same manner, the mean fluorescent intensity of the GFP positive cells was quantified by flow cytometry.

The enhancement in lentiviral transduction due to spinoculation was then assessed for the optimized lentiviral vector containing the HP-fVIII transgene outlined in chapter 2. K562 cells were transduced with an SIV-based lentiviral vector expressing HP-fVIII from the CMV internal promoter at an MOI of 15. HP-fVIII protein levels were assessed overtime by an APTT reagent-based one stage coagulation assay. K562 cells that underwent spinoculation consistently expressed HP-fVIII at greater levels for a total of eleven days compared to the K562 cells that were not spun after viral addition ($p = 0.003$ three weeks after transduction as determined by a Student's t test) (**Figure 3.10**). This data suggests that SIV-based lentiviral transduction with an HP-fVIII transgene can be enhanced by spinoculation.

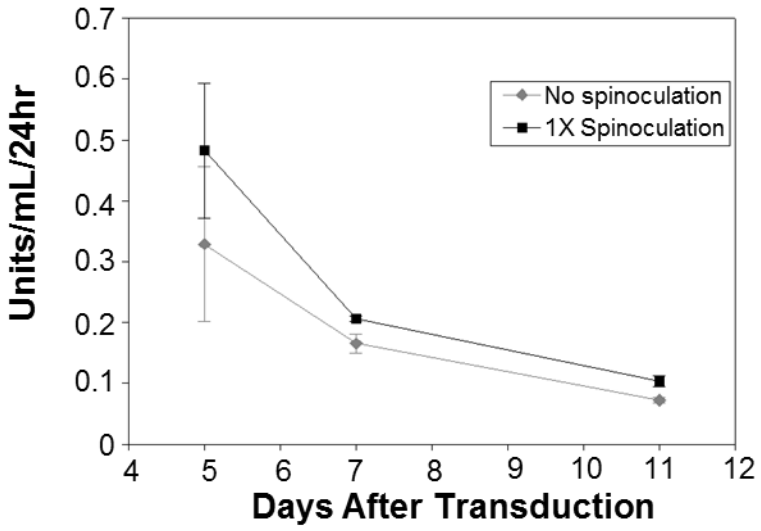


Figure 3.10 HP-fVIII expression overtime in K562 cells. Spinoculation was assessed following transduction of K562 cells with an SIV-based lentiviral vector containing a high-expressing HP-fVIII hybrid transgene expressed from the CMV internal promoter. K562 cells were transduced at an MOI of 15. Immediately following viral addition the cells were either spun for 2 hours at 1000g (black squares) or returned to the incubator (gray circles).

Sca-1^+ cells represent the hematopoietic stem and progenitor population in the mouse. In order to assess the effect of spinoculation on these target cells, sca-1^+ cells were isolated from the bone marrow of the tibias and femurs of 5-FU treated hemophilia A mice. Following isolation, the sca-1^+ cells were transduced with an SIV-based lentiviral vector encoding the GFP transgene. Recombinant virus was added so as to yield an MOI of 15. Transduction was assessed by fluorescent microscopy (**Figure 3.11 A**) and flow cytometry 9 days after transduction. The percentage of GFP positive cells increased from 35% to 68.8% suggesting that SIV-based lentiviral transduction of sca-1^+ can be enhanced by spinoculation (**Figure 3.11 B**). A similar enhancement in transduction was observed in the sca-1^+ cells transduced by spinoculation as was observed in K562 cells.

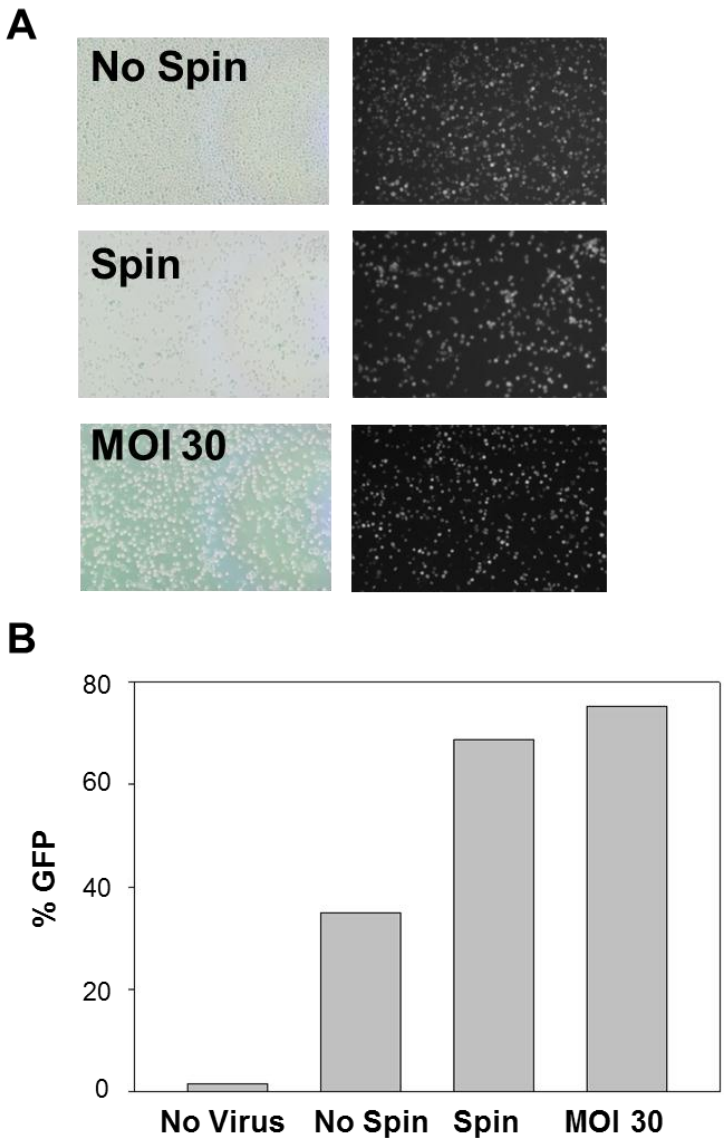


Figure 3.11 The effect of spinoculation on sca-1$^+$ cells. (A) Sca-1$^+$ cells were isolated from hemophilia A mice and transduced with an SIV-based lentiviral vector encoding GFP. Fluorescence microscopy images were taken prior to quantification (B) by flow cytometry.

Due to the results above, an attempt was made to translate these findings *in vivo*. Sca-1^+ cells were isolated and transduced the same day with the optimized lentiviral vector, an SIV-based lentiviral vector containing the HP-fVIII transgene with expression directed from a CMV internal promoter. An identical transduction protocol was utilized as above (**Figure 3.12 A**). Four days after isolation $4x10^5$ cells per mouse were transplanted into lethally irradiated (11*Gy* TBI using a Gammacell 40 Exactor) 8-week-old recipient hemophilia A mice following total body irradiation (n = 3). Despite encouraging preliminary *in vitro* results, minimal HP-fVIII was observed one week after transplantation as assessed by an ELISA assay (**Figure 3.12 B**). In addition, only one mouse remained in the cohort three weeks after transplantation. Although 4×10^5 cells were transplanted per mouse, the fatalities may be due to limited engraftment as a result of transplanting less than optimal sca-1^+ cells. This could also explain the lack of HP-fVIII detected in these mice. A follow-up methyl cell assay confirmed a reduction in sca-1^+ viability following spinoculation (**Table 3.1**).

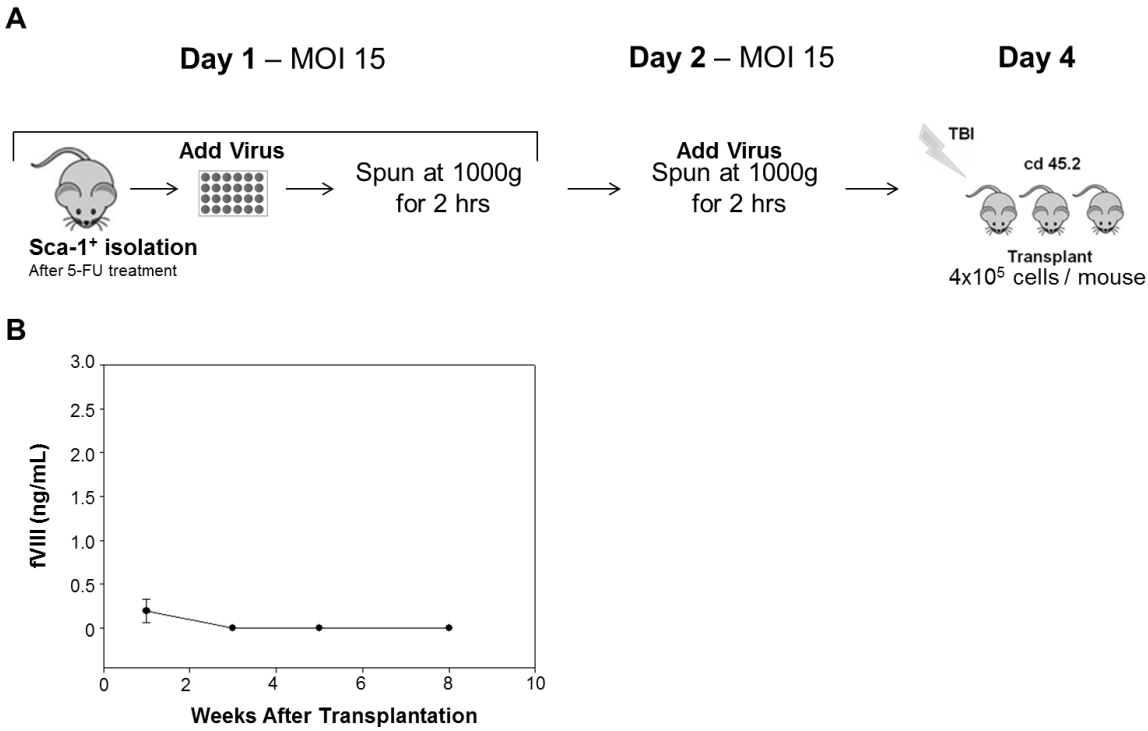


Figure 3.12 The effect of adding spinoculation to the transduction protocol. (A) The transduction protocol was performed as outlined schematically. (B) HP-fVIII was quantified from the peripheral blood of the transplanted mice every two weeks starting one week after transplantation. An ELISA assay was used to quantify HP-fVIII.

Table 3.1 – Sca-1^{+} viability following spinoculation

	Colonies	GFP
Spinoculation	+	
No Spinoculation	+++	++

+ <10 colonies
++ 10-30 colonies
+++ > 30 colonies

3.4— Discussion

In the field of gene therapy, lentiviral vector production is constrained to a transient transfection procedure. HEK-293T cells are predominantly used as a highly transfectable cell line. However, VSVG expression in HEK-293T cells yields syncytia formation followed by a loss in cell viability. Therefore, vector production in this manner is limited. In addition, a transient transfection procedure is not an ideal manufacturing procedure for vector to be utilized in human clinical trials. A stable vector producing cell line would be more appropriate being able to be scaled up and scrutinized as a nonvariable source of vector. For these reasons, BHK-M cells were assessed as an alternative vector producing cell line. Before attempting to create a stable producing cell line, the plasmids required for lentiviral vector production were transfected transiently in order to assess the ability of BHK-M cells to produce a recombinant lentiviral vector. BHK-M cells, however, experienced toxicity associated with VSVG expression. Cell viability was significantly diminished and syncytia formation was evident. In comparison to HEK-293T cells, BHK-M cells appear to be more susceptible to VSVG toxicity. Specifically, HEK-293T cells were found to withstand VSVG expression as a result of transfecting 3200 ng of the envelope plasmid as opposed to the 200 ng threshold observed in BHK-M cells. The increased toxicity in BHK-M cells was not found to be associated with the transfection reagent PEI. Therefore, BHK-M cells are not a viable alternative for VSVG pseudotyped lentiviral production and should not be utilized to generate a stable vector producing cell line.

Many envelopes are currently being evaluated in the field of gene therapy. Since VSVG pseudotyped lentiviral production is not attainable in BHK-M cells, three alternative envelopes to VSVG (Eco, pHit and 10A1), were acquired and assessed for viral production. No apparent toxicity was observed due to the expression of the Eco, pHit or 10A1 glycoproteins. This confirmed a lack of PEI toxicity experienced by BHK-M cells. In addition, cell viability appeared to be unaltered during viral production, potentially overcoming the limitation in days in which lentivirus can be secreted and collected. However, the conditioned medium containing the secreted recombinant virus was unable to sufficiently transduce HEK-293T cells. Recombinant lentivirus virus was not efficiently produced from BHK-M cells. Thus, an improvement was not made to the current HEK-293T transient transfection procedure currently used to produce lentiviral vector. However, if transfection efficiency can be increased vector production may be enhanced in BHK-M cells.

A second limitation in the field of gene therapy is the inability to overcome the inherent resistance of HSCs to lentiviral infectivity. Low transduction efficiencies have been observed clinically and in many cases greater transduction will be required to achieve a therapeutic effect. Therefore, two methods were assessed for the ability to enhance lentiviral transduction. A previous report revealed enhanced expression of bone-marrow derived $CD34^+$ stem and progenitor cells due to the silencing of p21. p21 was suggested to interact with the preintegration complex of HIV inhibiting the integration of the provirus into the genome of the host cell. Silencing p21 would then provide a mechanism in which provirus integration could proceed uninhibited. Therefore, the siRNA to p21

utilized in the report was acquired and assessed on the transduction of K562 cells as an initial indicator of hematopoietic cellular transduction. Three different MOIs were utilized to transduce K562 cells. However, no enhancement in HIV-based lentiviral transduction was observed. This result may be due to cell-type specific pleiotropic effects of p21. For example, depending on cell type, p21 has been attributed to both the inhibition and the progression through the cell cycle. In addition, an opposing role for p21 has been reported in macrophages in regards to HIV infection. p21 levels were found to be upregulated in macrophages as a result of HIV infection and were unable to repress the replication of HIV in this cell type. Therefore, a pleiotropic effect of p21 may account for the lack of enhancement noted in K562 cells following p21 siRNA treatment. However, insufficient knockdown of p21 cannot be eliminated since knockdown was not confirmed.

The use of RNAi technology as a means to enhance lentiviral transduction was ideal in that the effects were transient and were unable to disrupt the multipotentiality of HSCs as is the case with the use of cytokine cocktails. However without an observable enhancement in K562 cells another means of enhancing transduction is required. Spinoculation has been utilized by other groups in the field in the preclinical transduction protocol of sca-1^+ cells. Sca-1^+ cells represent the hematopoietic stem and progenitor population of the mouse. A mechanism providing lentiviral enhancement in this cell could potentially be extrapolated to the $CD34^+$ human bone marrow-derived hematopoietic stem and progenitor cell. Therefore, spinoculation was evaluated for its inclusion into the current transduction protocol utilized in our laboratory. Spinoculation

was found to enhance the lentiviral transduction of K562 cells regardless of the inclusion of a GFP or fVIII transgene. Similarly, the transduction of sca-1^+ cells was enhanced 2-fold with the inclusion of a spinoculation step. However, when included into an *in vivo* transplantation transduction protocol, spinoculation at 1000g for 2 hours diminished the viability of the sca-1^+ cells as evidenced by a lack of hematopoietic reconstitution and gene expression in the lethally irradiated mice. Although spinoculation improved lentiviral transduction, the addition of a spinoculation step did not improve the transduction protocol.

The studies presented in this chapter represent a series of attempts to overcome two of the major hurdles present in the field of gene therapy. Lentiviral production limitations were not able to be overcome by utilizing BHK-M cells as an alternative vector producing cell line. In addition, the transduction protocol was unable to be improved in regards to lentiviral transduction efficiency of the HSC. Silencing p21 did not enhance the transduction efficiency of an HIV-based lentiviral vector. Spinoculation, although able to enhance the percentage of cells infected by the lentiviral vector was unable to disrupt the viability of the stem cell. Therefore, new methods are needed in order to overcome these limitations confronted by the field of gene therapy such as that presented in the next chapter.

Chapter 4:

Pharmacologic Enhancement of Lentiviral Transduction

4.1— Introduction

Hematopoietic stem cells (HSCs) are an attractive cellular target for *ex vivo* gene therapy applications for the correction of several inherited diseases. The reason for their attractiveness is that HSCs have the ability to self-renew as well as differentiate into all lineages of the hematopoietic system. In addition, the use of HSCs is well characterized and these cells are routinely used clinically. However despite their appeal, limited transduction efficiency has been observed in clinical trials (Cartier *et al*., 2009; Boztug *et al*., 2010; Cavazzana-Calvo *et al*., 2010; DiGiusto *et al*., 2010). For example, in a recent phase I clinical trial using HSC gene therapy to treat X-linked adrenoleukodystrophy, $CD34^+$ cells modified with a self-inactivating (SIN) HIV-based lentiviral vector *ex vivo* achieved genetic modification in only 9-14% of blood cells after transplant (Cartier *et al*., 2009). Similarly, in a clinical trial for AIDS, transduction efficiencies were reported to be as low as 1% vector copies per genome in engrafted $CD34^+$ cells (DiGiusto *et al*., 2010). In many scenarios, especially one in which a growth advantage is not expected, greater transduction efficiencies will be needed to achieve therapeutic potential.

Further complicating the issue of limited transduction, high titer viral preparations are not always possible, especially in a scenario in which a large transgene or expression cassette is being utilized. As insert size increases the lentiviral particles recovered by ultracentrifugation have been shown to decrease (Kumar *et al*., 2001; Yacoub *et al*., 2007). A prime example of this can be observed when considering gene therapy applications for the treatment of hemophilia A (for review see Johnston *et al*, 2011). Even after significant reductions in the size of the cDNA encoding fVIII, low viral titer

has been observed (Radcliffe *et al*., 2008). This can limit the amount of virus available to acquire desired multiplicity of infections (MOI) that may be necessary to achieve high transduction efficiencies. Therefore, new strategies would be useful to maximize the transduction efficiency of HSCs.

Multiple attempts have been made towards increasing lentiviral transduction through the use of small molecules and cytokines. In addition, retronectin was shown to enhance the transduction of c-kit^{+} Lin^{-} bone marrow cells 3-fold (Lee *et al.*, 2009) and is thought to act as a bridge for the co-localization of stem cells and viral particles. Small molecules such as ABC transport inhibitors and proteasome inhibitors also have been used with some success (Davis *et al*., 2004; Leuci *et al*., 2011). Both classes of compounds were found to enhance lentiviral transduction of CD34^{+} cells. Similar effects are observed following pre-stimulation with cytokines (Santoni *et al*., 2006). However, a series of comparisons were performed in order to determine an optimal clinically relevant lentiviral transduction protocol using various cytokine combinations and concentrations as well as combining pre-stimulation with the use of retronectin. Overall, low levels of gene-modified cells were observed (Millington *et al*., 2009).

To overcome the transduction barrier, a high-throughput screen was performed to identify compounds that enhance lentiviral transduction. Among the positive compounds identified by the screen were camptothecin and etoposide, which have been shown previously to be enhancers of lentiviral transduction (Groeschel and Bushman, 2005). However, as topoisomerase inhibitors they also are known to cause DNA strand breaks

(Pommier *et al.*, 2010). A potentially safer compound identified was phorbol 12-myristate 13-acetate (PMA). PMA stimulates protein kinase C (PKC), a family of closely related serine/threonine kinases involved in signal transduction pathways regulating an array of cellular processes. The PKC family consists of 10 isoforms classified into three subfamilies according to which secondary messengers are required for PKC activation. Two of the subfamilies, the conventional (PKCα, PKCβ_I, PKCβ_{II}, and PKCγ) and novel PKCs (PKCδ, PKCθ, PKCε, and PKCη) require diacylglycerol (DAG) for activation. PMA, an analog of DAG, activates isoforms in these two subfamilies and associates at the same binding site as DAG in the C1 regulatory domain (Stahelin *et al.*, 2005) (Steinberg, 2008). In the current study, PMA was confirmed as a positive enhancer of lentiviral transduction, enhancing the transduction of hematopoietic cell lines and human $CD34^+$ cells.

4.2— Materials and Methods

Lentiviral Vector Production

SIV-based and HIV-based lentiviral vectors pseudotyped with VSVG were produced from HEK-293T cells upon transient co-transfection utilizing polyethylenimine (6 µg PEI / 1 µg DNA) (Fisher Scientific, Pittsburg, PA). HIV-based lentiviral vectors were manufactured utilizing the LentiMax production system which consists of a 2:1:1 ratio of expression plasmid to packaging plasmids (expression plasmid:psPAX2:pVSVG). A ratio of 1.3:1:1:1.6 of expression plasmid to packaging plasmids (expression plasmid:pCAG4:pVSVG:pSIV) was used to manufacture SIV-based lentiviral vectors. The media was replaced twenty-four hours after transfection with DMEM/F-12

(Invitrogen Life Technologies, Carlsbad, CA) containing 10 % fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1 % penicillin / streptomycin (Mediatech, Manassas, VA). No antibiotics were present during the time of transfection. For the following three days, conditioned medium was collected from the HEK-293T viral producing cells, passed through 0.45 μmol l^{-1} filter and stored at -80^{o}C until concentration by velocity sedimentation upon centrifugation at 10,000 x *g* (4^{o}C) overnight. Viral pellets were resuspended in 1/100th of the original volume of StemPro media (Invitrogen life technologies, Carlsbad, CA), and filtered through a 0.22 μmol l^{-1} filter. Viral titer was assessed on HEK-293T cells with increasing vector volumes by real-time quantitative PCR seventy-two hours after viral addition. Virus was stored in 1mL aliquots at -80^{o}C.

High-throughput Screening

Compounds from the LOPAC^{1280} library (Sigma, St. Louis, MO, USA) were screened in 384 well tissue culture treated clear bottom plates. Each well contained 10^4 K562 cells cultured in phenol-red free DMEM/F-12 media. For each plate, two columns of sixteen wells were used as a baseline for fluorescence containing only K562 cells and virus at an MOI of 0.5. Another set of two columns was used as a positive control containing K562 cells transduced at an MOI of 5. The remaining wells contained K562 cells treated with one compound per well three hours prior to viral addition. Compounds were added at a concentration of 20 μM. Virus was added at an MOI of 0.5. The following day, media in the wells was exchanged for fresh DMEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin / streptomycin. Fluorescence intensity was assessed seventy-

two hours following viral transduction with the use of a fluorescent imager, the Image Xpress. Compounds able to demonstrate a fold increase of 2.5 or higher than the average fluorescence emitted from the baseline columns were identified as positive hits. The positive hits were then confirmed utilizing ten different doses of the compound under the same conditions as the initial screen. Doses ranged from 0.1 μM to 60 μM. Compounds that were able to demonstrate a fold increase in fluorescence intensity a second time were denoted as positive hits.

Culture, treatment and transduction of K562, EU1, BHK-M, HEK-293T, NIH-3T3 and U937 cell lines

K562, EU1, BHK-M, HEK-293T, NIH-3T3 and U937 cell lines were cultured in DMEM/F-12 supplemented with 10% FBS and 1% penicillin / streptomycin. Cells were plated in twenty-four well tissue culture plates and treated with PMA (Fisher Scientific, Pittsburg, PA). Prior to treatment, PMA was dissolved in DMSO to yield an initial concentration of 10 mg mL^{-1}. Subsequent dilutions were performed in DMEM/F-12 supplemented with 10% FBS and 1% penicillin/streptomycin. Following a two hour treatment with PMA, cells were transduced in minimal volume (300 μL) with a lentiviral vector in the presence of polybrene (8 ng μL^{-1}). Transduction efficiency was assessed seventy-two hours by flow cytometry unless otherwise noted. The percentage of GFP positive cells was compared to the percentage of GFP positive cells from cells transduced in the absence of PMA.

Isolation, treatment and transduction of murine stem cell antigen-1^+ cells

The femurs and tibias of 8 to 10 week old exon 16-disrupted hemophilia A mice (as previously described in Bi *et al*, 1995) were flushed with PBS supplemented with 2% FBS. The flushed bone marrow was then subjected to positive immunomagnetic bead selection using magnetic separation columns purchased from Miltenyi Biotec (Auburn, CA) as previously described (Gangadharan *et al.,* 2006). The isolated sca-1^+ cells were then assessed for purity by flow cytometry utilizing a Ly-6A/E antibody (clone E13-161.7) conjugated to FITC (BD Pharmingen, San Diego, CA). The purity of the population was found to be 96% positive for sca-1^+. Upon confirmation, the sca-1^+ cells were cultured at a density of 10^6 cells per mL in StemPro media supplemented with L-glutamine (29µg mL^{-1}) and stimulated for three days with murine stem cell factor (100ng mL^{-1}), murine interleukin-3 (20ng mL^{-1}), human interleukin-11 (100ng mL^{-1}), and human Flt-3 ligand (100ng mL^{-1}). Following stimulation, sca-1^+ cells were plated onto a twenty-four well tissue culture treated plate at a density of $2x10^6$ cells per well and treated with PMA for two hours. Sca-1^+ cells were then transduced in the presence of 8ng μL^{-1} polybrene with a lentivirus containing the GFP transgene. Transduction efficiency was assessed by flow cytometry seventy-two hours following transduction.

Isolation, treatment and transduction of $CD34^+$ cells

$CD34^+$ cells were purchased from AllCells (Emeryville, CA). Prior to arrival, $CD34^+$ cells were mobilized from human bone marrow and isolated from the peripheral blood of 3 individual subjects. Flow cytometry was used to assess the purity of the sample which ranged between 97 and 99 percent of the population found to be positive for CD34. Cells

were shipped over night on dry ice and upon arrival stimulated for 24 hours with recombinant human Flt-3 ligand (300ng mL^{-1}), human recombinant stem cell factor (300ng mL^{-1}), human recombinant thrombopoietin (100ng mL^{-1}), human interleukin-3 (60ng mL^{-1}). $CD34^+$ cells were cultured in GMP serum-free stem cell growth medium (Cell Genix, Portsmouth NH) supplemented with 1% penicillin / streptomycin. After stimulation, the purity of the population was confirmed by flow cytometry. Upon confirmation, $CD34^+$ cells were cultured in a twenty-four well tissue culture treated plate at $2x10^6$ cells per well and treated with PMA for two hours prior to transduction. $CD34^+$ cells were transduced in minimal volume (300 µL) and initially assessed for GFP seventy-two hours following transduction by flow cytometry. The continued purity and transduction efficiency of the $CD34^+$ cells was assessed up to twelve days following transduction.

Colony-forming Unit Assay

Colony-forming cell assay was performed per manufacturer's protocol (Stemcell Technologies, MethoCult® H4035 Optimum without EPO). Briefly, a 10X concentration ($2x10^4$ cells/mL) of $CD34^+$ cells was prepared in Iscove's MDM with 2% FBS. A total of 0.3 mL of 10X cell mixture was then added to 3 mL of MethoCult® and mixed by vortexing. MethoCult®/cell mixture was allowed to sit for 5 minutes to allow bubbles to rise followed by dispensing 1.1 mL onto 35 mm dishes in duplicate using a 16 gauge blunt-end needle. The methylcellulose was evenly distributed into the dish by rotation and dishes were placed in a 100 mm culture dish alongside a third dish containing sterile water to maintain proper humidity. Cultures were incubated at 37^oC in

5% CO^2 for 14-16 days for optimal CFC growth. CFU-GM colonies were counted at 5X under an inverted microscope using a 60 mm gridded dish. GFP-positive colonies were scored under fluorescence microscopy.

fVIII Copy Number Analysis

Copy number analysis was performed as outlined in chapter 2. In short, were harvested by centrifugation at 300g for 5 minutes and used to isolate total genomic DNA using the DNeasy® Blood & Tissue Kit (QIAGEN, Valencia, CA). A quantitative PCR reaction was performed utilizing primers specific for the high expressing chimeric fVIII transgene. Ct values for each sample were compared to Ct values produced from plasmid standards of known copy quantities. The equivalent copy number was then divided by 8333, the predicted number of diploid genome equivalents in 50 ng of DNA assuming a haploid genome weight of 3 pg.

fVIII expression

fVIII expression was measured as previously outlined in chapter 2 from the supernatant of cells. In short, the cells were cultured in serum-free media for 24 hours prior to the activated partial thromboplastin reagent-based one stage coagulation assay. The analysis was performed in duplicate for each supernatant on a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France) in human fVIII-deficient plasma (George King Biomedical, Overland Park, KS). The clot time for each sample was compared to a standard curve based on dilutions of pooled normal citrated human plasma (George King Biomedical, Overland Park, KS).

Statistical analysis

All values are reported as mean ± standard deviation. A student's t-test was used for comparisons between two groups. For comparisons between more than two groups, a one way ANOVA was used. Significance was defined as a p value of less than 0.05.

4.3— Results

4.31— High-throughput screen identifies lead compounds

Protocol Optimization of a High-Throughput Screen

In collaboration with Emory's Chemical Biology Discovery Center, a high-throughput screen was performed to identify compounds that could enhance the lentiviral transduction of hematopoietic cells. A library of 1280 pharmacologically active compounds (LOPAC^{1280}) was screened. This library was chosen because it covers the major drug target classes and impacts most cellular processes. Although the screen was performed to determine compounds that could enhance the transduction of HSCs, the myeloid erythroid-leukemic K562 cell line was chosen as an initial indicator of enhanced transduction, as K562 cells are less difficult to culture than the cytokine requiring murine sca-1$^+$ or human CD34$^+$ cells. In addition, a lentivirus containing the GFP transgene was used in order to assess transduction efficiency in a high-throughput manner with the use of a fluorescent imager, the Image Xpress.

The screening protocol was optimized in regards to culturing conditions and endpoint time frame. In order to determine the appropriate culturing condition, a range of K562

cellular densities was tested in a 144 well tissue culture treated clear bottom plate. For each density, cells were transduced with recombinant lentivirus (n = 6) at increasing MOIs. Fluorescent intensity was then assessed seventy-two hours following transduction and compared to nontransduced wells. As shown in **Figure 4.1 A**, the culture of 10^4 cells per well produced the greatest separation in relative fluorescent intensity among wells transduced with increasing MOIs. Therefore the cellular density of 10^4 K562 cells/well yielded an appropriate condition in which to measure transduction enhancement. In order to assess the appropriate endpoint of the screen, 10^4 K562 cells were plated per well and virus was added to each well at increasing amounts (n = 8). Transduction efficiency was assessed at multiple time points after transduction. Well to well variability improved 72 hours following transduction as compared to an assessment at 48 hours (data not shown). In this manner, an appropriate assay window was produced in order to assess enhanced transduction as a fold-increase from the baseline of an MOI of 0.5 (**Figure 4.1 A**). Upon identifying an optimal culturing condition and post transduction read out time for the screen, a total cell image was taken with the use of a Hoechst dye. Minute holes were found in the monolayer of K562 cells that may be the cause for variability among wells (**Figure 4.1 B**). However a distinct difference in fluorescence is clearly noted between an MOI of 0.5 and 4 (**Figure 4.1 C**). This obvious enhancement in fluorescence validates the effectiveness of the screen in being able to determine enhanced transduction as a result of the addition of more virus. It is postulated that a similar increase could be observed as a result of the presence of a molecular compound that enhances transduction.

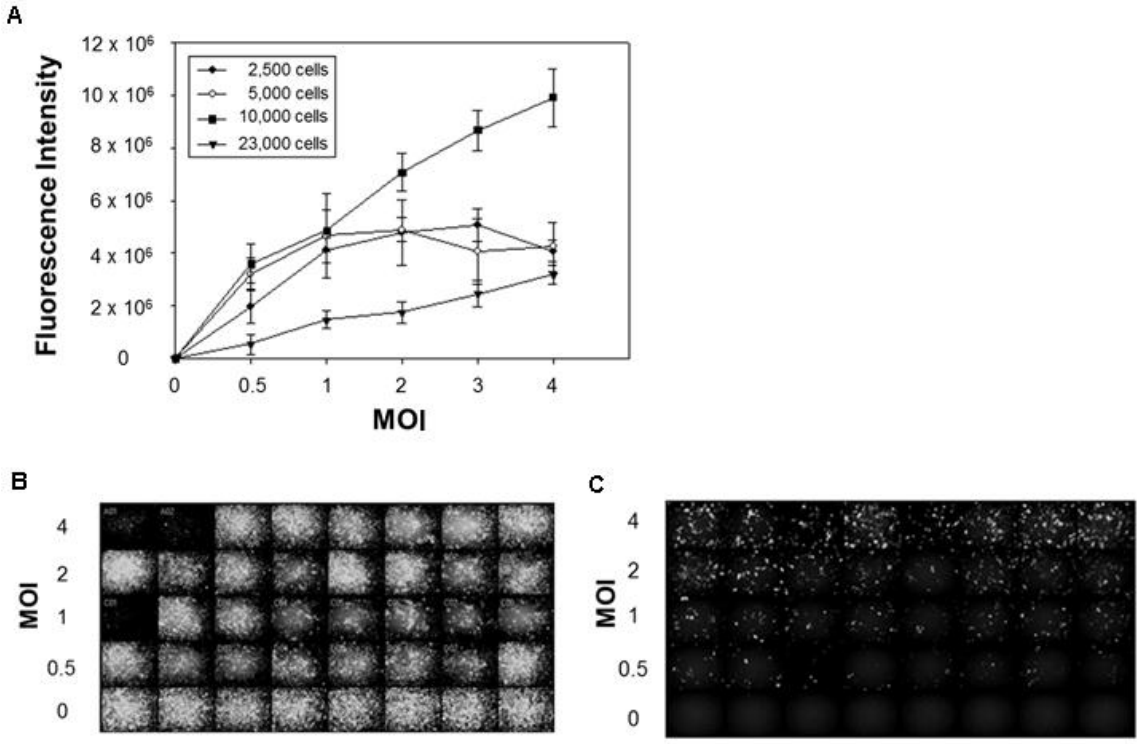


Figure 4.1 Optimization of the high-throughput screening protocol. (A) In order to identify the appropriate cell density at which to yield an appropriate assay window, K562 cells were plated at varying densities (n = 6) and transduced with MOIs increasing from 0 to 4. Fluorescence intensity was quantified seventy-two hours following transduction with the use of a fluorescence imager, the Image Xpress. (B) Cells were stained with a Hoechst dye. (C) Representative fluorescence microscopy image demonstrating observable increases in GFP positive cells as a result of increasing MOI.

High-Throughput Screen

The high-throughput screen was then performed in a tissue culture treated plate in which 10^4 K562 cells were plated per well. The compounds were added at a concentration of 20 μM three hours before viral addition at an MOI of 0.5. Media was exchanged the following day and fluorescence was assessed seventy-two hours following transduction. Fluorescence intensity was compared to baseline fluorescence intensity as determined in the first two columns of the plate, which only contained K562 cells transduced at an MOI of 0.5 (**Figure 4.2 A**). As a positive control, the last two columns of the plate were transduced with 8-fold more virus to confirm increase in fluorescence intensity (**Figure 4.2 A**). Compounds that yielded a fold induction of 2.5 or higher over baseline fluorescence were denoted as positive hits (for example, H22 and O5 of **Figure 4.2 A**) while obvious autofluorescent compounds were not further analyzed (for example, L10 of **Figure 4.2 A**). The screen was performed for both an SIV-based and an HIV-based lentiviral vector. For the SIV-based lentiviral vector, thirty-four compounds were identified that yielded a fold induction in fluorescent intensity of 2.5 or higher. Similarly, thirty-two compounds were identified with the use of an HIV-based lentiviral vector.

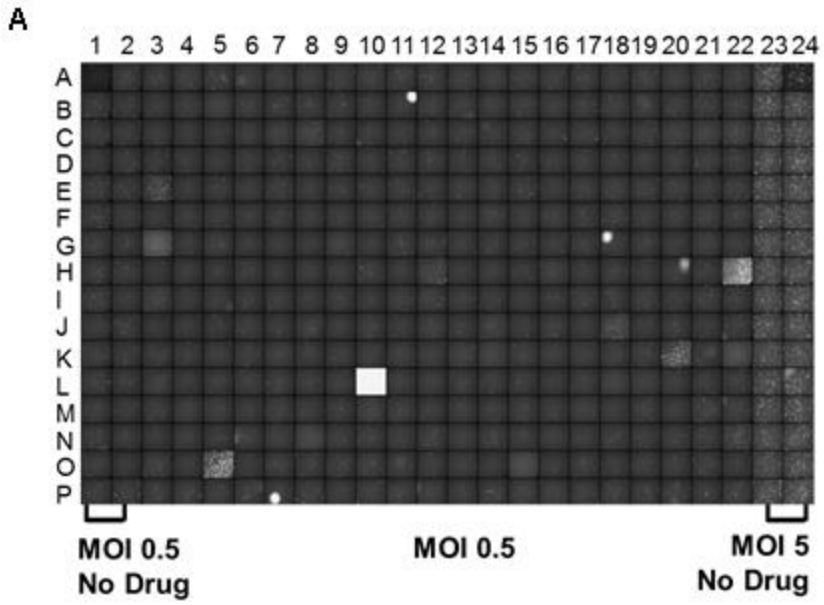


Figure 4.2 Visual representation of the high-throughput screen. (A) Sample fluorescence microscopy image of 1 of 4 plates run during the high-throughput screen. Columns are denoted numerically from 0 to 24. Rows are denoted alphabetically from A to P. The first and last two columns did not contain test compounds. Columns 1 and 2 acted as a baseline from which to assess fold-induction in fluorescent intensity whereas columns 23 and 24 contained K562s transduced with 10 times more virus (positive control). Well L10 represents an autofluorescent compound. Wells H22 and 05 represent compounds identified as positive hits yielding a fold induction greater than 2.5 from that of the first two column means.

4.32— High-throughput screen identifies false positives

Forskolin: False positive

As an FDA approved drug, forskolin was included in the LOPAC^{1280} library. Forskolin was initially identified as a positive hit of the high-throughput screen, which yielded a 6.8 fold induction in fluorescence. A 10-point dose response was then performed utilizing the optimized K562 cellular culturing condition and the optimized endpoint time frame. Forskolin was added at the indicated doses (**Figure 4.3 A**) 3 hours prior to viral transduction at an MOI of 0.5. The results of the 10-point dose assay were promising in that a similar fold induction (7-fold) in fluorescence was observed at 20 μM Forskolin, the concentration used in the initial screen. In addition, a dose response was observed indicating the potential of forskolin to enhance lentiviral transduction (**Figure 4.3 A**). During the validation process, the assay was repeated in a twenty-four well tissue culture treated plate and compared to the initial assessment performed at the screening center in which a three hundred eighty-four well plate was used. K562 cells were plated at a density of 10^5 cells/well and treated with the indicated doses of forskolin in triplicate. An SIV-based lentiviral vector containing a GFP transgene was added to each well at an MOI of 0.5. The percentage of GFP positive K562 cells was quantified by flow cytometry seventy-two hours following viral transduction. However despite the initial observation of forskolin-induced enhanced transduction in a high-throughput format, an increase in GFP positive cells was not able to be reproduced (**Figure 4.3 B**).

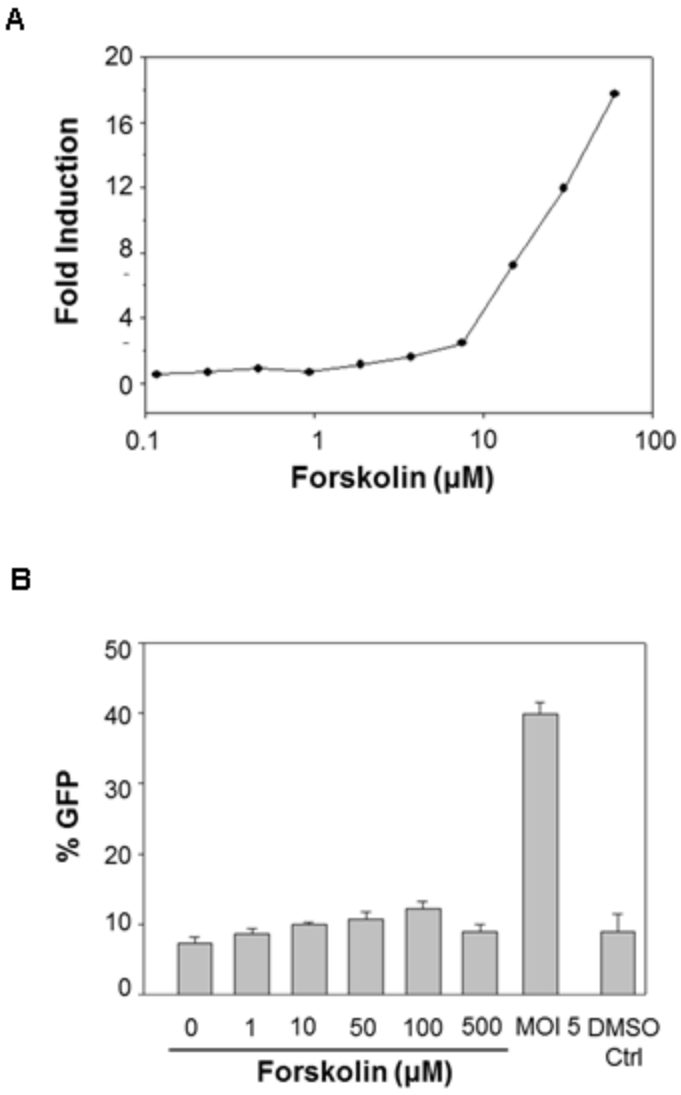


Figure 4.3 Forskolin as an example of a false positive identified from the high-throughput screen. (A) A 10-point dose response curve was performed in the screening center. K562 cells were plated at a cell density of 10^4 cells/well and treated with forskolin at the indicated concentrations for 3 hours prior to viral addition (MOI of 0.5). (B) To validate the 10-point dose response a second dose response analysis was performed utilizing the same concentrations as the screening center with the addition of 500 μM forskolin (n = 3). A twenty-four well plate was seeded with K562 cells at a density of 10^5 cells/well and treated with forskolin at the indicated concentrations and

transduced at an MOI of 0.5. Transduction efficiency was assessed by flow cytometry seventy-hours following viral addition. A DMSO control was added in which the maximum amount of DMSO (used to solubilize forskolin) present in the highest dose of forskolin was added prior to viral addition.

SB 205384: False Positive

The high-throughput screen included the $GABA_A$ partial agonist, denoted as SB 205384. Initially, SB 205384 yielded a fold induction of 7.5 in fluorescence intensity as compared to the control wells transduced in the absence of compound. The 10-point dose response curve performed at the screening center suggested that the enhancement seen in transduction with SB 205384 on K562 cells had yet to be observed at its fullest (**Figure 4.4 A**). Therefore, greater concentrations of SB 205384 were tested on K562 cells plated in a 24 well plate at a density of 10^5 cells/well. K562 cells were treated with SB 205384 at the indicated concentrations (**Figure 4.4 B - C**). Minimal virus was added to each well either in the presence (**Figure 4.4 B**) or absence (**Figure 4.4 C**) of polyberene. Transduction was assessed seventy-two hours later by flow cytometry. Although concentrations greater than 100 μM were used no enhancement in transduction was observed (**Figure 4.4 B - C**). The enhancement in transduction observed at the screening center was unable to be repeated identifying SB 205384 as a false positive.

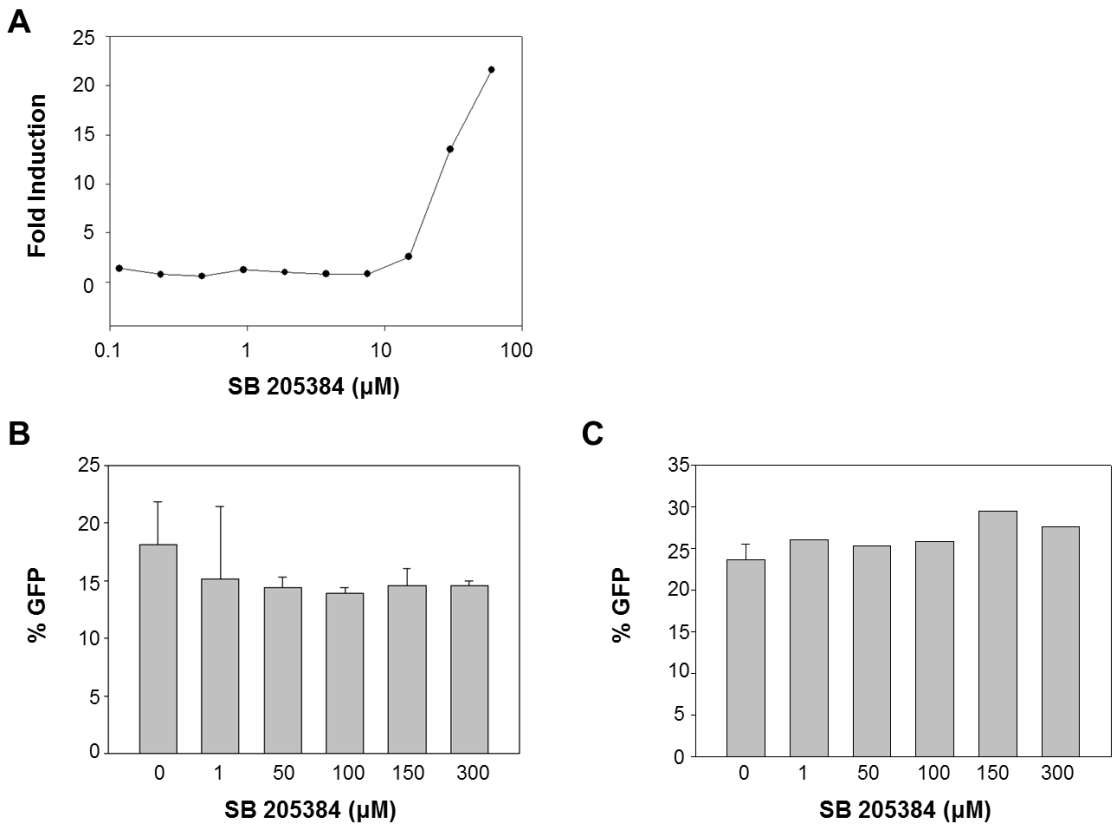


Figure 4.4 SB 205384: a false positive identified from the high-throughput screen. (**A**) In the screening center, a 10-point dose response curve of SB 205384 was performed in order to verify SB 205384 as a positive hit. As previously described above, 10^4 K562 cells were plated per well and treated with SB 205384 at the indicated concentrations for 3 hours prior to viral addition (MOI of 0.5). Fluorescence intensity was measured using the Image Xpress seventy two hours later in which the treated wells were compared to the non-treated wells. (**B**) Similarly, SB 205384 was analyzed in the Spencer laboratory in a dose dependent manner utilizing the same concentrations as the screening center with the addition of 150 and 300 μM SB 205384. Virus was added immediately following compound addition either in the presence or (**C**) absence of polybrene. Transduction efficiency was assessed by flow cytometry seventy-two hours following viral addition.

Cytosine-1-β-D-arabinofuranose hydrochloride: False positive

Cytosine-1-β-D-arabinofuranose hydrochloride was denoted as a positive hit from the screen yielding a 3.1 fold induction in fluorescence intensity from the baseline fluorescence observed from the non-treated wells of the high-throughput screen. The compound was included in the LOPAC screen as an FDA approved drug. Clinically, cytosine-1-β-D-arabinofuranose hydrochloride is used to treat leukemia. As a pyrimidine analog, cytosine-1-β-D-arabinofuranose hydrochloride becomes incorporated into the DNA during synthesis and inhibits cycling through the S phase resulting in apoptosis.

Following the initial high-throughput screen, a 10-point dose response analysis on cytosine-1-β-D-arabinofuranose hydrochloride was performed at the screening center. Fluorescence intensity was increased in a fold dependent manner from the baseline fluorescence of non-treated wells at concentrations up to 15 μM (**Figure 4.5 A**). Concentrations of cytosine-1-β-D-arabinofuranose hydrochloride higher than 15 μM demonstrated a decrease in fluorescence intensity. For this reason, lower concentrations were used to verify cytosine-1-β-D-arabinofuranose hydrochloride as an enhancer of lentiviral transduction in the Spencer laboratory. Cytosine-1-β-D-arabinofuranose hydrochloride was added to K562 cells plated in a 24 well plate at a density of 10^5 cells / well at the indicated concentrations (**Figure 4.5 B - C**). Minimal virus was added the treated and non-treated K562 cells immediately following treatment either in the presence (**Figure 4.5 B**) or absence (**Figure 4.5 C**) of polyberene. The enhancement observed at the lower concentrations of the 10-point dose response analysis performed at the screening center was not able to be repeated.

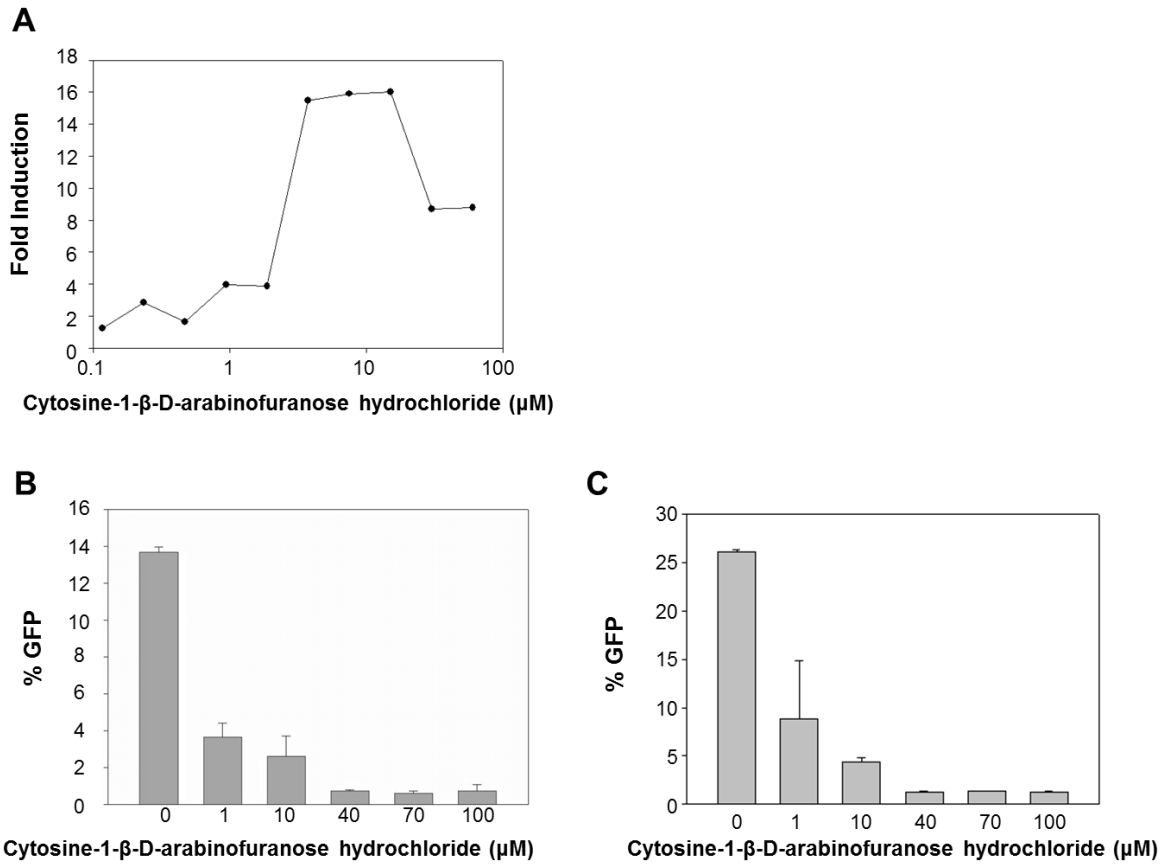


Figure 4.5 Cytosine-1-β-D-arabinofuranose hydrochloride: a false positive of the high-throughput screen. (A) The result of a 10-point dose response assay performed at the screening center for the compound Cytosine-1-β-D-arabinofuranose hydrochloride. The analysis was performed on K562 cells (plated at a density of 10^4 cells/well) treated with the indicated concentrations for 3 hours prior to viral addition (MOI of 0.5). The Image Xpress was used to determine fold induction in fluorescence intensity seventy-two hours after viral addition. (B) Cytosine-1-β-D-arabinofuranose hydrochloride was evaluated in a similar fashion in the Spencer laboratory. K562 cells were plated in 24 well plates at a density of 10^5 cells/well and treated at concentrations similar to those used by the screening center (n = 2). Transduction was assessed by flow cytometry seventy-two hours after viral addition in the presence or (**C**) absence of polybrene.

4.33— High-throughput screen identifies previously confirmed enhancers

Camptothecin: True positive

Camptothecin, etoposide, and taxol were also identified from the screen as positive hits yielding a fold increase in fluorescent intensity of 3.2, 2.6, and 2.7 respectively. Encouragingly, all 3 compounds were previously shown to enhance the transduction of HEK-293T and HeLa cells (Groeschel and Bushman, 2005). In the current study, camptothecin was confirmed as an enhancer of lentiviral transduction of HEK-293T cells. Increasing concentrations of camptothecin were added to HEK-293T cells while simultaneously being transduced with an SIV-based lentiviral vector containing a GFP transgene. Each concentration was performed in triplicate. Transduction efficiency was determined seventy-two hours following transduction by quantifying the percent of GFP positive cells by flow cytometry. As observed by Groeschel and Bushman (2005), transduction efficiency peaked at 0.4 μM camptothecin (**Figure 4.6 A**). Higher concentrations of camptothecin resulted in diminished transduction efficiency, which may be a result of toxicity to the cells. Camptothecin was found to inhibit cell division at concentrations as low as 3 nM (**Figure 4.6 B**), which is likely due to cell cycle arrest in the G2/M phase (Groeschel and Bushman, 2005).

In addition to confirming a previous report identifying camptothecin as a positive enhancer of HEK-293T cell lentiviral transduction, K562 cells were analyzed in order to validate camptothecin as a positive effector of transduction of hematopoietic cells. A similar protocol was performed in which increasing concentrations of camptothecin were added to the cells in triplicate while being transduced with identical amounts of lentiviral

vectors. In this analysis, both HIV and SIV-based lentiviral vectors were used. Similar transduction efficiency curves were noted for both lentiviral vectors. K562 cells yielded a peak in transduction efficiency at 0.08 μM (**Figure 4.6 A**). An inhibition in cellular division as a result of camptothecin was observed with K562 cells as was observed with HEK-293T cells (**Figure 4.6 C**). Because camptothecin and other cytotoxic agents identified in the screen induce DNA damage, they are not considered ideal compounds for clinical use.

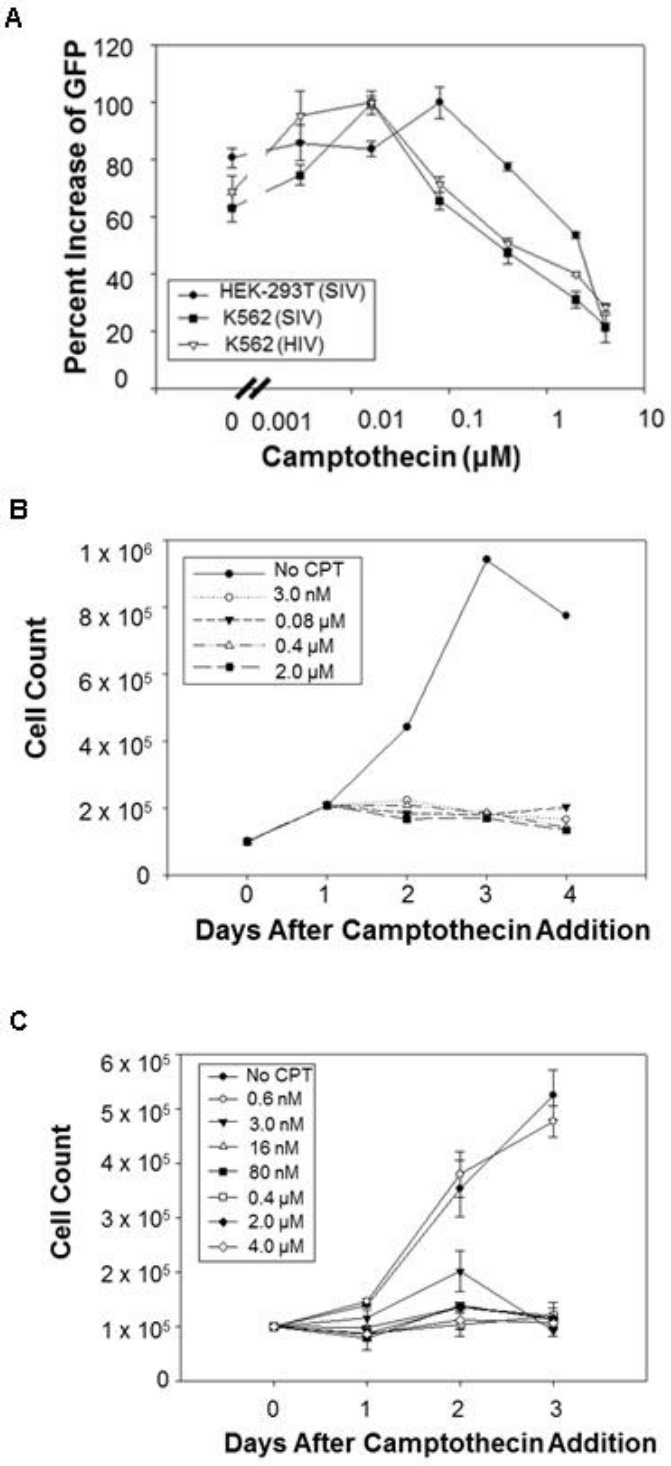


Figure 4.6 Camptothecin enhances lentiviral transduction. (A) In a twenty-four well plate, K562 cells were plated at a density of 10^5 cells/well and treated with the indicated doses of camptothecin (n = 3). K562 cells were transduced with either an SIV-based

(solid squares) or HIV-based (open triangles) lentiviral vector at an MOI of 5. In the same manner, $5x10^4$ HEK-293T cells were plated in a twenty-four well plate twenty-four hours prior to transduction yielding approximately 10^5 cells/well (solid circles). The percentage of GFP positive cells was assessed by flow cytometry seventy-two hours following transduction. (B) HEK-293T cell counts were performed throughout the experiment in which only viable cells were counted after Trypan Blue staining. (**C**) Viable K562 cell counts were obtained up to 3 days after treatment with camptothecin.

4.34— Enhancement of the Positive Hit, PMA is cell dependent

PMA as an enhancer of K562 lentiviral transduction

PMA is an analog of diacylglycerol and is able to bypass the signal transduction pathway leading to PKC activation. PMA was identified from the SIV-based screen as an enhancer of lentiviral transduction and yielded the highest increase in fluorescence intensity observed, which was a 10 fold-increase from the baseline fluorescence. The compound was confirmed as a positive enhancer of lentiviral transduction in a 10-point dose response testing assay. At high nanomolar concentrations, a morphological change is evident (**Figure 4.7 A**), similar to that previously reported (Papayannopoulou *et al*., 1982). Decreasing the concentration of PMA to 2.5 nM diminished this effect (**Figure 4.7 A**). In addition, at the lower doses of PMA a dose response was observed indicating the potential of PMA to enhance lentiviral transduction. This was observed with both the use of an SIV-based (**Figure 4.7 B**) and an HIV-based lentiviral vector (**Figure 4.7 C**). Although no morphological change occurs with PMA treatment at 2.5 nM, a 3.5-fold enhancement in transduction is apparent (**Figure 4.7 B**). Cell proliferation was found to be halted in a dose-dependent manner being completely inhibited at 2.5 nM and higher concentrations (**Figure 4.7 D**). In addition, a time course analysis was performed in order to determine an optimal time at which to add virus after PMA incubation. Virus was added either simultaneously or after a 2, 4, 8, 24 or 48 hour incubation. The analysis was performed under similar culturing conditions in triplicate. Consistently, a 2 hour pre-incubation yielded the greatest enhancement in viral transduction as apparent by the percentage of GFP positive cells determined by flow cytometry seventy-two hours following transduction (**Figure 4.7 E**).

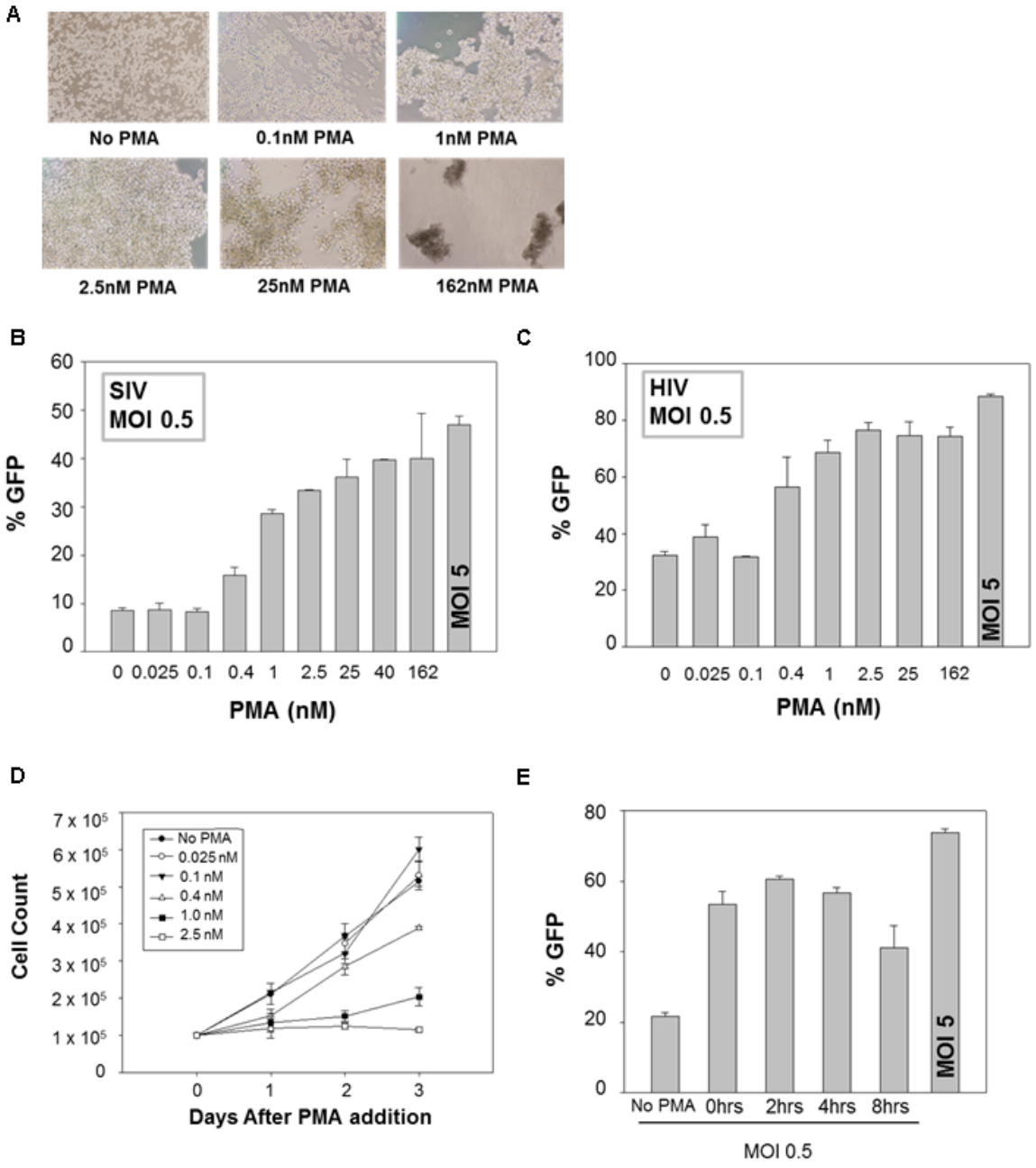


Figure 4.7 PMA enhances lentiviral transduction of K562 cells. (A) Representative microscopy images reveal a morphological change in K562 cells occurring in a dose-dependent manner with PMA treatment. (B) K562 cells were plated at 10^5 cells/well in a twenty-four well plate and treated with PMA at the indicated doses (n = 3) prior to transduction with either an SIV-based or an (C) HIV-based lentiviral vector at an MOI of

0.5. Seventy-two hours post-transduction was assessed by flow cytometry of GFP positive cells. As a positive control, virus was added at an MOI of 5 to the K562 cells in the absence of PMA. (D) Viable cell counts were performed up to 3 days after PMA treatment utilizing a Trypan Blue exclusion assay. (E) In the same manner, a time course analysis was performed in which K562 cells were treated with 162 nM PMA for the indicated hours prior to transduction with an SIV-based lentiviral vector. Transduction efficiency was assessed seventy-two hours later.

PMA enhances lentiviral transduction of EU1 cells

A second human hematopoietic cell line, EU1 cells, which are derived from a B cell acute lymphoblastic leukemia, was analyzed for the effect of PMA. Cells were plated in a twenty-four well tissue culture treated plate at a density of 10^5 cells/well and transduced at an MOI of 5. The analysis was performed with both an SIV-based and an HIV-based lentiviral vector. The cells were treated with PMA at the indicated dose (**Figure 4.8 A**) 2 hours prior to transduction and compared to cells transduced under the same conditions in the absence of PMA. Flow cytometry was performed seventy-two hours following transduction in which the percentage of GFP positive cells was quantified. Non-transduced cells cultured in the presence of PMA were also analyzed as a negative control. Fluorescence microscopy images taken seventy-two hours following transduction demonstrated morphologically unchanged EU1 cells following culture with PMA up to 25 nM and an enhancement in GFP positive EU1 cells was observed by fluorescent microscopy (data not shown). The enhancement in lentiviral transduction of EU1 cells treated with PMA was confirmed by flow cytometry (**Figure 4.8 A**). Using an SIV-based lentiviral vector, transduction of EU1 cells was enhanced almost 3-fold yielding a percentage of GFP positive cells greater than that achieved by adding three times the amount of virus (**Figure 4.8 A**). The percentage of GFP positive cells increased from an average of 43% in the absence of PMA to 75% in the presence of PMA when an HIV-based lentiviral vector was used (**Figure 4.8 B**). As was apparent with K562 cells, EU1 cell proliferation was inhibited by PMA in a dose-dependent manner (**Figure 4.8 C**).

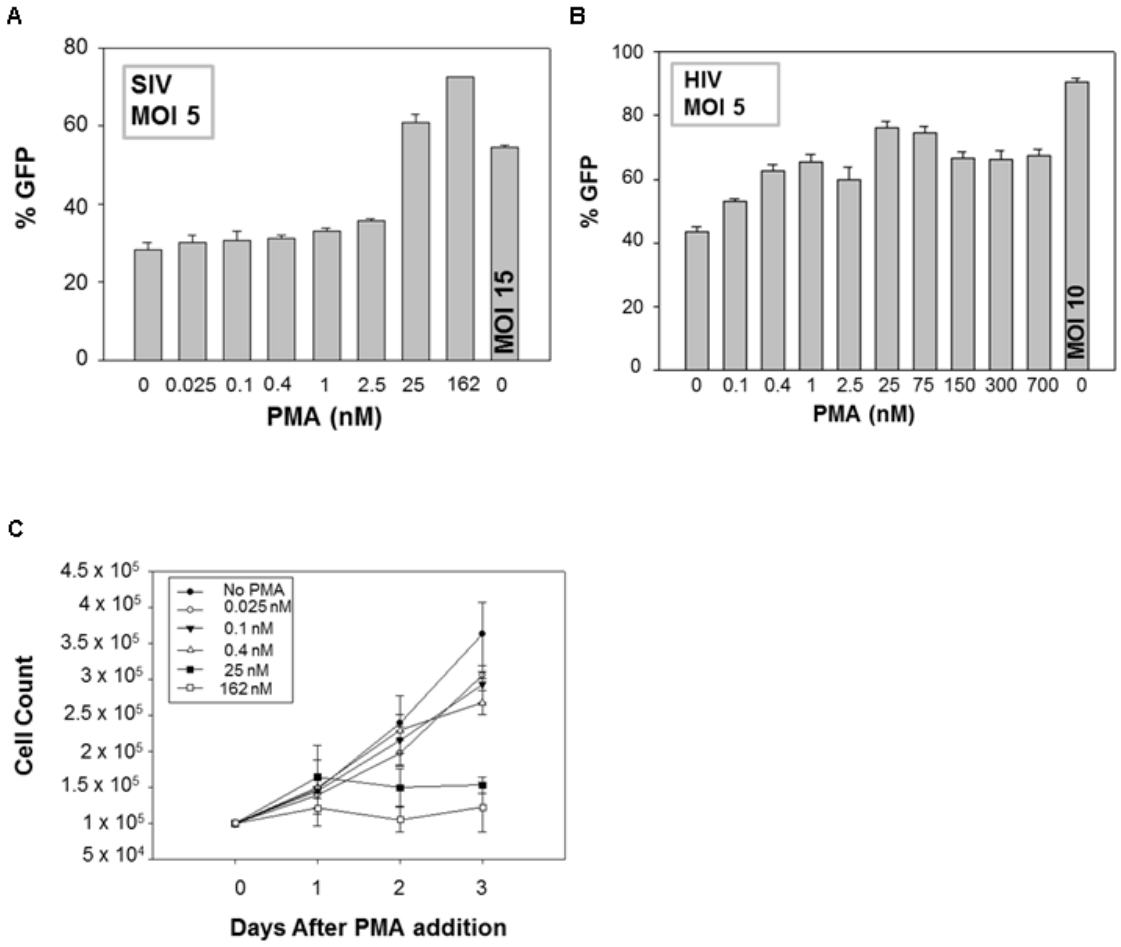


Figure 4.8 PMA enhances the transduction of EU1 cells. (A) EU1 cells were treated with PMA at the indicated doses after being plated at 10^5 cells/well in a twenty-four well plate (n = 3). Transduction was performed 2 hours later at an MOI of 5 with an SIV-based or an (B) HIV-based lentiviral vector. Transduction efficiency was quantified by determining the percentage of GFP positive cells seventy-two hours after transduction by flow cytometry. (C) Viable cell counts were performed throughout the assay up to three days after PMA treatment by a Trypan Blue exclusion assay.

PMA enhances the transduction of a lentiviral vector containing a high-expressing fVIII transgene

A high-expression B domain deleted human/porcine fVIII hybrid was previously used to effectively cure a murine model of hemophilia A using HSC transplantation gene therapy (Doering, 2009). This high-expressing fVIII transgene was incorporated into an HIV-based lentiviral vector (**Figure 4.9 A**) and used in this study to assess the enhancement properties of PMA with the use of a therapeutic transgene. K562 cells were plated at a density of $5x10^5$ cells per well of a twenty-four well plate and transduced at an MOI of 5 (n = 3). The K562 cells were transduced following a 2 hour PMA incubation and compared to K562 cells not transduced in the presence of PMA. Media was exchanged twenty-four hours prior to a one-stage coagulation assay which was performed 12 days following transduction. FVIII activity was significantly greater from K562 cells treated with 10 nM PMA prior to transduction ($p = 0.002$) (one way ANOVA)(**Figure 4.9 B**). DNA was isolated from the transduced cells and assessed for vector copies per genome by qPCR. Enhanced transduction was noted for K562 cells treated with 10 nM PMA prior to transduction as shown by an increase in vector copies per genome ($p = 0.003$) (one way ANOVA)(**Figure 4.9 C**).

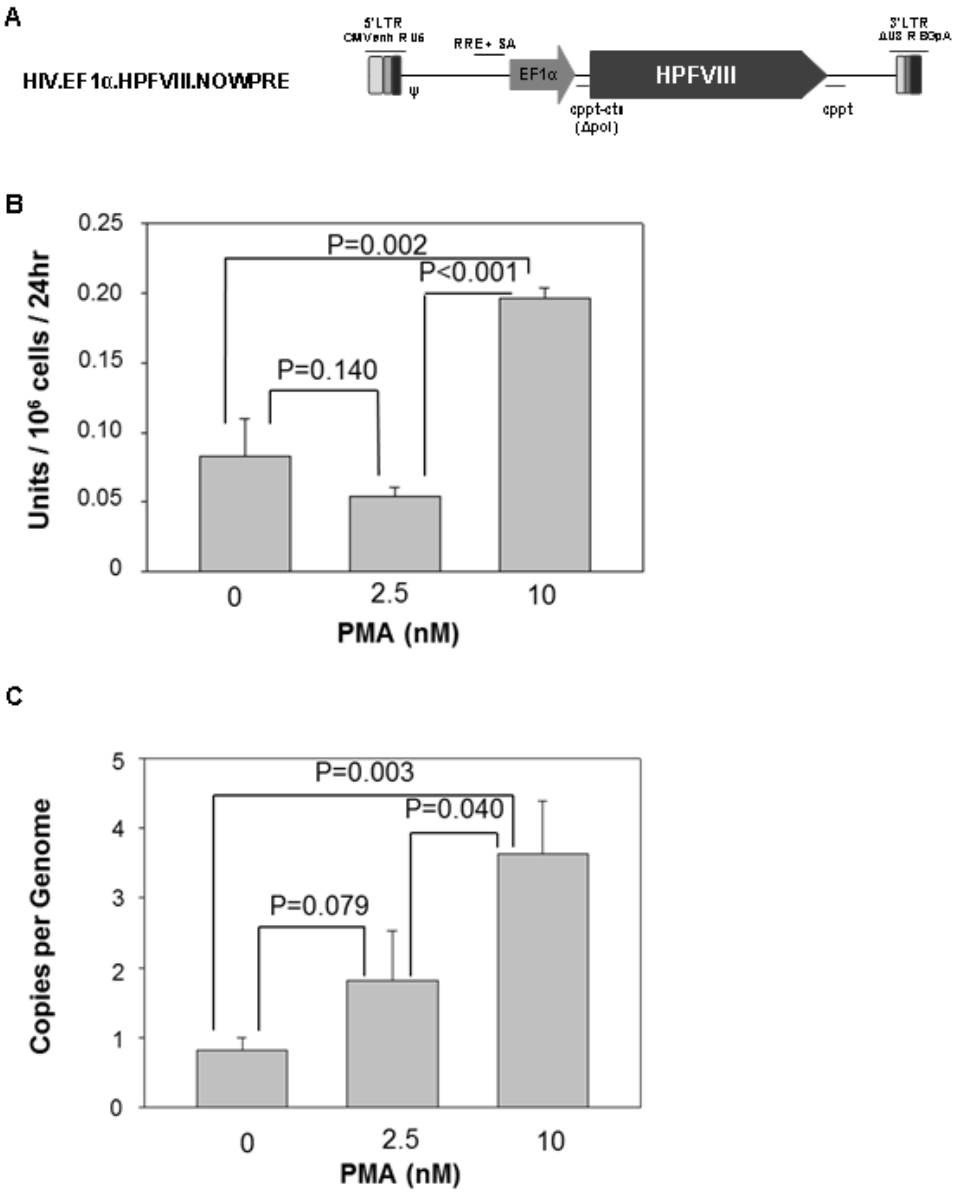


Figure 4.9 PMA enhances the transduction of a lentiviral vector encoding a high-expressing fVIII transgene. (A) Schematic representation of the HIV-based lentiviral vector utilized to transduce K562 cells. (B) fVIII expression was quantified by an aPTT reagent-based one-stage coagulation assay. Twenty-four hours prior to isolation, the conditioned media was exchanged for serum-free AIM-V in order to assess the fVIII activity. Bars represent the mean ± the standard deviation of three transduced wells with each well measured in duplicate. (C) DNA was isolated twelve days following

transduction and assessed for integrated vector copies per genome by qPCR. Bars represent the mean ± the standard deviation of 3 transduced wells measured in duplicate. A one way ANOVA was utilized to determine the significance between groups treated with and without PMA.

Abbreviations: HPFVIII, high expressing porcine/human chimeric fVIII; EF1α, elongation factor 1α.

The effect of PMA on U937, HEK-293T, NIH-3T3 and BHK cell lentiviral transduction

A panel of cell lines was assessed for the effect of PMA on lentiviral transduction. Included in the panel was a third hematopoietic cell type, the U937 cell line originating from a monocytic lymphoma. As with the K562 and EU1 cell lines, 10^5 cells were transduced per well in a twenty-four well tissue culture treated plate. The analysis was performed with both an SIV-based (MOI of 5) and an HIV-based lentiviral vector (MOI of 1). The cells were treated with PMA at the indicated dose (**Figure 4.10 A**) 2 hours prior to transduction and compared to cells transduced under the same conditions in the absence of PMA. Flow cytometry was performed seventy-two hours following transduction in which the percentage of GFP positive cells was quantified. Non-transduced control cells cultured in the presence of PMA were also analyzed as a negative control. No enhancement was noted even upon increasing the concentration of PMA to 700 nM. This observation was noted with both SIV and HIV-based lentiviral vectors (**Figure 4.10 A**). Despite the lack of enhancement, cell proliferation was inhibited over time in a dose-dependent manner (data not shown).

Three adherent cell lines were also included in the panel to be analyzed. HEK-293T (human embryonic kidney cells), NIH-3T3 (mouse fibroblasts) cells, and BHK (baby hamster kidney cells) were plated at a density of $2.5x10^4$ cells per well of a twenty-four well tissue culture treated plate a day prior to transduction. Cells approached $5x10^4$ the following day and were transduced at an MOI of 0.5 with either SIV or HIV-based lentiviral vectors. Cells were treated with the indicated doses of PMA two hours prior to transduction. Transduction was assessed by fluorescence microscopy and quantified by

flow cytometry seventy-two hours after transduction. The percentage of GFP positive cells as compared to those cells transduced with a lentivirus in the absence of PMA. No enhancement was observed upon addition of PMA in HEK-293T cells transduced with an SIV-based or an HIV-based lentiviral vector (**Figure 4.10 B**). A similar observation was apparent for NIH-3T3 cells (**Figure 4.10 C**). As expected, the transduction efficiency of BHK cells was less than that observed with HEK-293T and NIH-3T3 cells at the same MOI. The percentage of GFP positive cells following PMA treatment was not enhanced in BHK cells with either SIV-based and HIV-based lentiviral vectors (**Figure 4.10 D**). In addition, proliferation of HEK-293T, NIH-3T3 or BHK cells was not inhibited in the presence of PMA (data not shown).

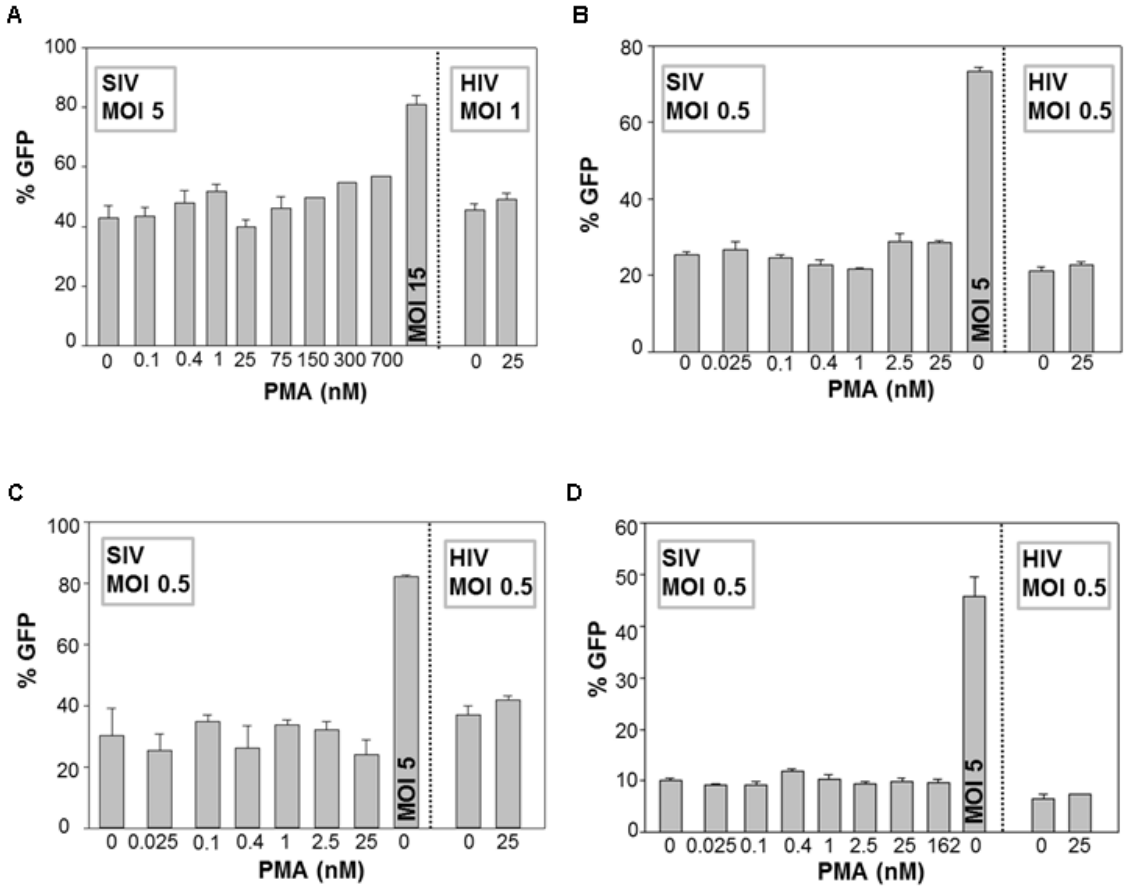


Figure 4.10 Assessment of the effect of PMA on a panel of cell lines. (A) U937 (B) HEK-293T (C) NIH-3T3 and (D) BHK-M cells were treated with the indicated doses of PMA. Two hours later, the cells were transduced with either an SIV-based or an HIV-based lentiviral vector (n = 3). Transduction efficiency was assessed by flow cytometry seventy-two hours after transduction.

PMA effect on murine stem cell antigen-1^+ cells

Murine preclinical models of genetic disease are common. Sca-1^+ cells represent the HSC early and late progenitor population. PMA was tested on sca-1^+ cells isolated from the tibia and femurs of C57BL/6 mice. Sca-1^+ cells were stimulated with cytokines for 3 days. Cell counts during stimulation showed cell growth indicative of typical sca-1^+ cell functioning (data not shown). Following stimulation, $2x10^5$ cells were plated per well of a twenty-four well tissue culture treated plate and treated with PMA at the indicated doses 2 hours prior to transduction. The percentage of GFP positive cells was quantified by flow cytometry seventy-two hours later. Despite increasing the concentration of PMA to 1 μM, no enhancement was observed with sca-1^+ cells transduced with an SIV-based lentiviral vector in the presence of PMA as compared to those cells transduced in the absence of PMA (**Figure 4.11 A**). A similar observation was noted with the use of an HIV-based lentiviral vector (data not shown). In addition, no inhibition of cell proliferation was observed at concentrations up to 100 nM (**Figure 4.11 B**).

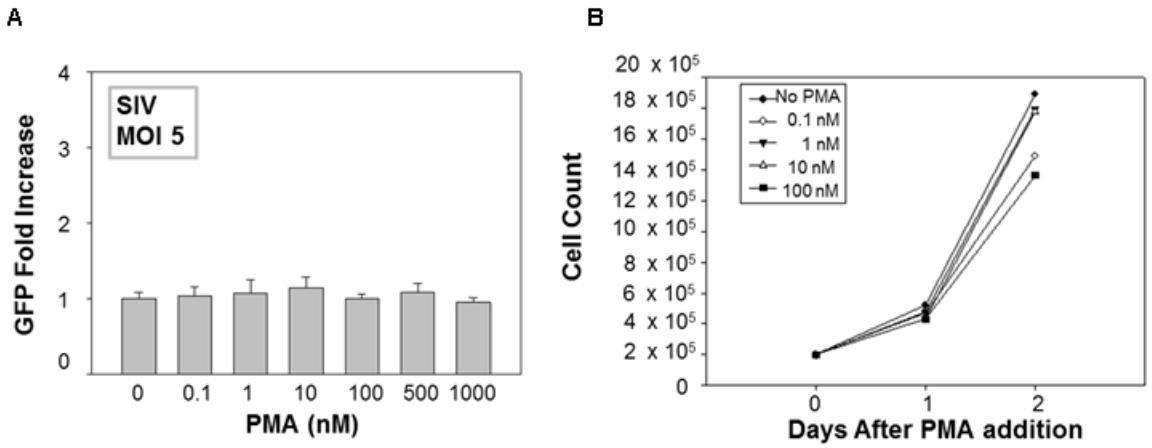


Figure 4.11 Lack of PMA effect on murine hematopoietic stem and progenitor cells. (A) Sca-1^+ cells isolated from the femurs and tibias of C57BL/6 mice were stimulated with cytokines for 3 days prior to PMA treatment. Sca-1^+ cells were treated with PMA at the indicated doses for a total of 2 hours prior to viral transduction at an MOI of 5 with an SIV-based lentiviral vector encoding GFP (n = 3). Transduction efficiency was quantified seventy-two hours following transduction by flow cytometry. (B) Viable cell counts were obtained up to 2 days following PMA treatment utilizing a Trypan Blue exclusion assay.

PMA effect on bone marrow-derived $CD34^+$ cells

The most important clinical target to be evaluated in the presence of PMA is the $CD34^+$ cell population which includes hematopoietic stem and early progenitor cells. Similar protocols as above were used to assess the effect of PMA on $CD34^+$ cells mobilized and isolated from human peripheral blood. $CD34^+$ cells were plated in twenty-four well tissue culture treated plate at a density of $2x10^5$ cells per well. Following a 2 hour incubation with PMA at the indicated doses, cells were transduced with either an SIV-based or an HIV-based lentiviral vector at an MOI of 20. Transduction efficiency was initially assessed seventy-two hours following transduction and quantified by flow cytometry. With both an SIV (**Figure 4.12 A**) and HIV-based lentiviral vector (**Figure 4.12 B**), the percentage of GFP positive cells was increased from 5% to greater than 20% in the presence of PMA. A significant enhancement in lentiviral transduction was observed at as low as 1 nM PMA (**Figure 4.12 A-B**). Initial assessments were performed at seventy-two hours, however prolonged culture revealed similar percentages of GFP positive cells 5 days after transduction (**Figure 4.12 C**). In order to assess longer term transgene expression, the $CD34^+$ cells were cultured for a total of 2 weeks. Over time, the percentage of $CD34^+$ cells positive for GFP was found to have diminished. Yet the fold-induction as a result of PMA treatment was preserved (**Figure 4.12 D**). In addition, $CD34^+$ cells were found to retain the CD34 and CD45 markers after culture with PMA. Flow cytometry was performed seventy-two hours after the cells were transduced with the HIV-based lentiviral vector. Cells not treated with PMA were 97.2% positive for CD34 and CD45 while cells treated with PMA were found to be 95.6% (0.1 nM PMA) and 93.0% (1 nM PMA) positive for both markers (**Figure 4.12 E**). In regards to cellular

proliferation, PMA was found to inhibit cellular proliferation of $CD34^+$ cells over the expanse of 4 days (**Figure 4.12 F**). In addition, $CD34^+$ cells were transduced with a clinical grade HIV-based lentiviral vector following a 2 hour incubation with 1nM PMA. $CD34^+$ cells treated with PMA were found to exhibit colony forming units (CFU) 2-fold greater than those cells not treated with PMA however this is in the range of standard error as outlined in the manufacturer's protocol (**Figure 4.12 G**). Total DNA was extracted from the colonies and analyzed for copy number by real-time quantitative PCR utilizing primers that bind to the rev responsive element of HIV. In the absence of PMA, the copies/cell averaged 0.06 whereas the number of copies/cell for the PMA treated colonies averaged 0.29.

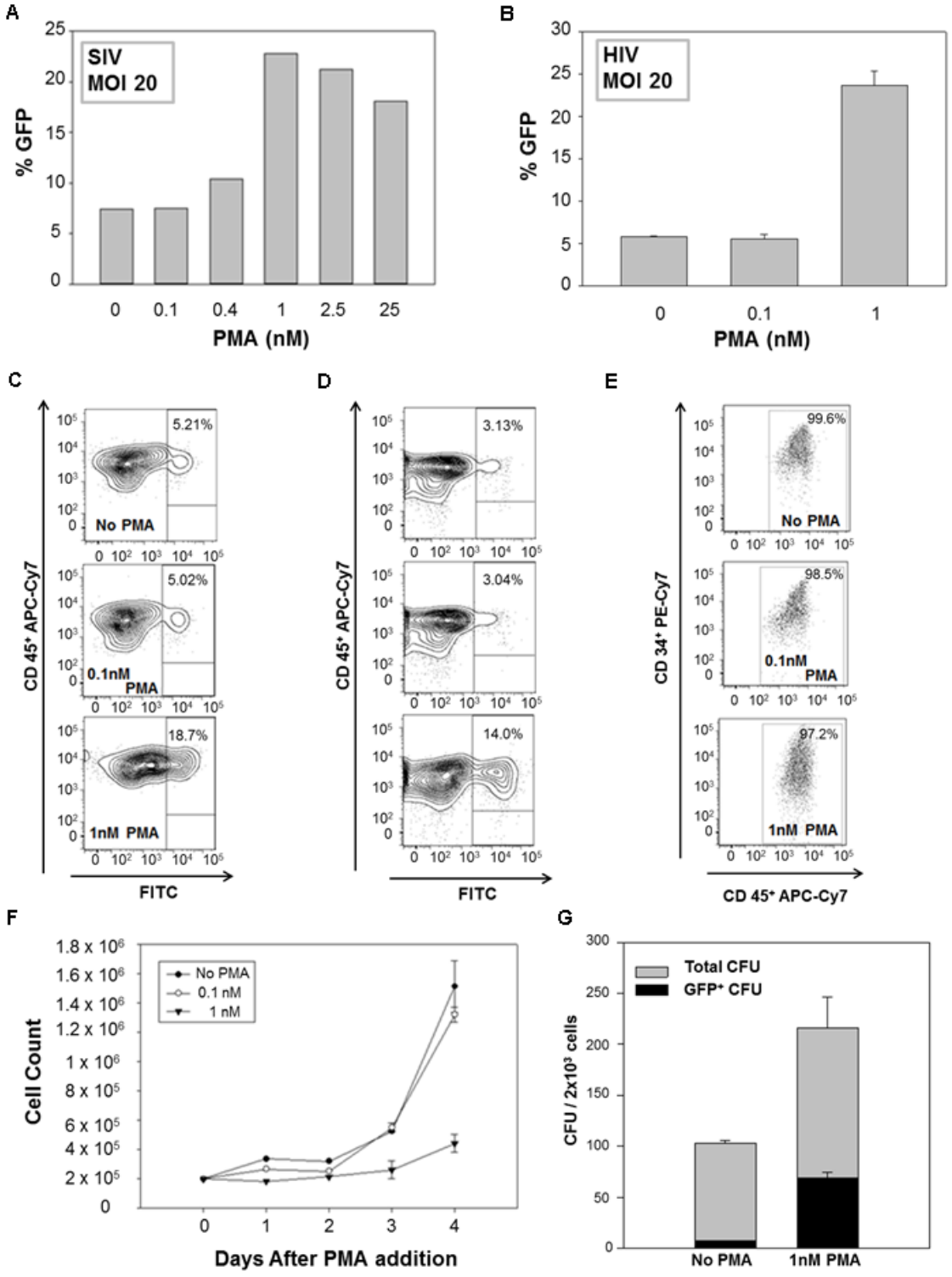


Figure 4.12 PMA enhances lentiviral transduction of CD34$^+$ cells. (A) Mobilized CD34$^+$ cells isolated from human peripheral blood were treated with PMA at the

indicated doses for 2 hours prior to viral addition at an MOI of 20 with an SIV-based and (B) an HIV-based lentiviral vector (n = 3). The percentage of GFP positive cells was quantified seventy-two hours after transduction assessing transduction efficiency in the presence of PMA. The persistence of gene modification was assessed by flow cytometry 5 (C) and 13 days (D) following PMA treatment. Anti-CD45 antibody conjugated to APC-CY^7 was used to identify the population. (E) The persistence of CD34 and CD45 markers was assessed by flow cytometry seventy-hours following PMA treatment. Anti-CD34 antibody conjugated to PE-Cy7 was used. (F) Viable cell counts were performed up to 4 days following PMA treatment by a Trypan Blue Exclusion assay. (G) In addition, the appearance of CFUs was quantified from $CD34^+$ cells transduced with a clinical grade HIV-based lentiviral vector encoding a codon-optimized GFP transgene. Fluorescence microscopy was used to determine genetically-modified colonies.

4.35— Mechanism for PMA effect on K562 cells

As a phorbol ester, PMA binds to the C1 domain of PKC resulting in PKC translocation and activation (Stahelin *et al.*, 2005; Steinberg, 2008). Eight PKC isoforms are activated by PMA and have been implicated in an array of cellular processes. The lack of enhanced lentiviral transduction in HEK-293T, NIH-3T3, BHK, U937 and sca-1+ cells may be due to the different PKC isoform found in these cells as compared to those found in K562, EU1 and $CD34^+$ cells. However, PMA may be acting on more than just PKC. Approximately 50 amino acids located in the C1 domain are responsible for PMA/DAG binding (Ono *et al.*, 1989; Kazanietz *et al.*, 1994, 1995; Quest *et al.*, 1994). These amino acids have been found to constitute similar C1 domains on nonkinase phorbol ester receptors which include chimaerins (a family of Rac GTPase activating proteins), RasGRPs (exchange factors for Ras/Rap1), and Munc13 isoforms (scaffolding proteins involved in exocytosis) (Ron and Kazanietz, 1999; Kazanietz, 2000). Further analysis is needed to assess the mechanism by which PMA is enhancing lentiviral transduction.

Enhanced Transduction as a result of DNA damage

To access DNA damage as a result of PMA treatment, DNA fragmentation was measured by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. By this method, a free 3'-hydroxyl residue generated during DNA fragmentation is labeled by a biotinylated nucleotide via the enzyme terminal deoxynucleotidyl transferase. The biotinylated nucleotides are then quantified with the use of a streptavidin-FITC conjugate by flow cytometry.

In this manner, K562 cells were treated with PMA for 6, 4, 2 or zero hours prior to enzyme and biotinylated nucleotide addition. As a positive control, K562 cells were treated with nuclease for thirty minutes to generate DNA breaks in every cell. Immediately following the labeling reaction, flow cytometry was performed in order to quantify the amount of incorporated biotinylated nucleotides at the site of a DNA nick with the use of a streptavidin-FITC conjugated antibody. Extensive DNA fragmentation was observed in the nuclease treated K562 cells, in that seventy-six percent were positive for FITC (**Figure 4.13**). However, less than 1% of the K562 cells treated with PMA were found to be positive for FITC. In addition, no significant difference was observed between the PMA treated and non-treated K562 cells (**Figure 4.13**). Therefore, breakage in the DNA does not appear to be increased as a result of PMA treatment.

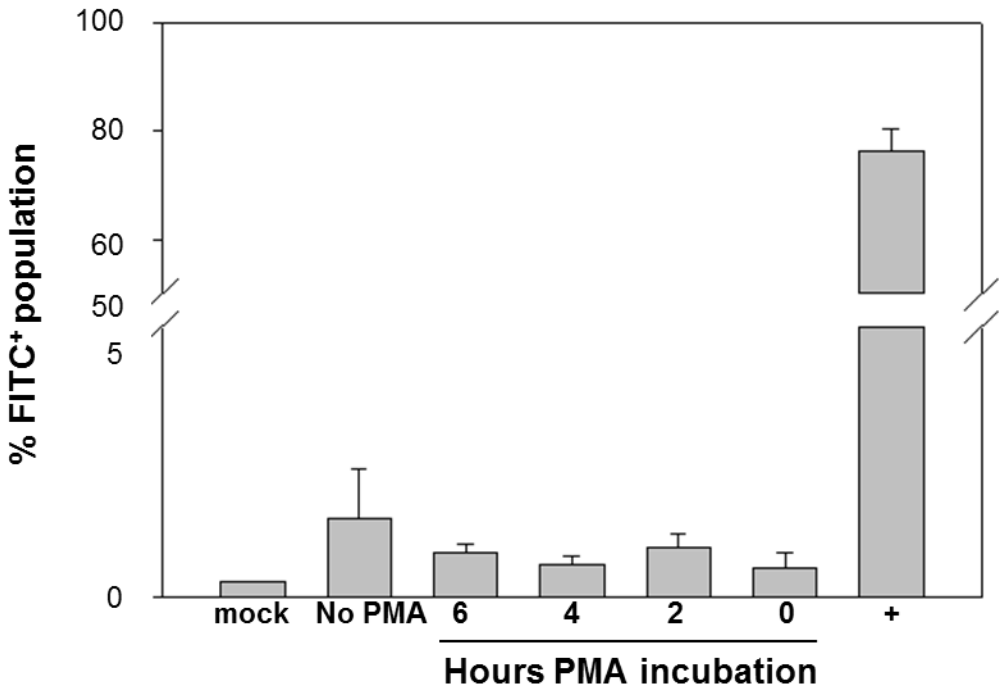


Figure 4.13 Assess of DNA fragmentation as a result of PMA treatment. A TUNEL assay was performed in order to quantify the amount of DNA nicks present as a result of PMA treatment. K562 cells were plated at a density of 10^6 cells / well in a 6 well plate and treated with 10nM PMA at the indicated times (n = 4). Cellular pellets were acquired and fixed with a 3.7% formaldehyde solution. DNA nicks were labeled with biotinylated nucleotides by the enzymatic activity of terminal deoxynucleotidyl transferase. As a mock control, the terminal deoxynucleotidyl transferase enzyme was omitted. Quantification of the incorporated nucleotides was performed with the addition of a streptavidin-FITC antibody measured by flow cytometry. In addition to the mock control, a subset of K562 cells was treated with nuclease prior to the labeling reaction as a positive control in order to fragment the DNA. Non PMA-treated K562 cells were utilized as a baseline for comparison.

Enhanced Transduction as a result of PKC activation

In order to assess the involvement of PKC in the transduction enhancing process of PMA on K562 cells, a PKC inhibitor was utilized. Calphostin C targets the regulatory domain of PKC inhibiting translocation and activation. With an IC_{50} of 50nM, K562 cells were treated with calphostin C (50nM) for 4 hours prior to PMA addition (10 nM). Two hours following PMA addition, an SIV-based lentivirus encoding the GFP transgene was used to transduce the treated K562 cells at an MOI of 0.5. Flow cytometry was performed seventy-two hours later to assess transduction efficiency. The average percentage of K562 cells positive for GFP as a result of PMA treatment averaged 76.9 percent while K562 cells pretreated with 50 nM calphostin C prior to PMA treatment averaged 45.4 percent. A significant reduction in the enhancement achieved by the addition of PMA was inhibited following calphostin C treatment at 50 nM (**Figure 4.14 A**). This suggests that PKC is involved in the mechanism whereby which PMA enhances lentiviral transduction in K562 cells. To determine which isoform of PKC was involved, inhibitors selective for PKC isoforms were utilized in the same manner as calphostin C. K562 cells were treated with the inhibitors for 4 hours prior to PMA treatment for 2 hours. After treatment, cells were transduced with a GFP containing lentivirus at an MOI of 0.5. Transduction was determined seventy-two hours after viral addition by flow cytometry. (**Figure 4.14 B**)

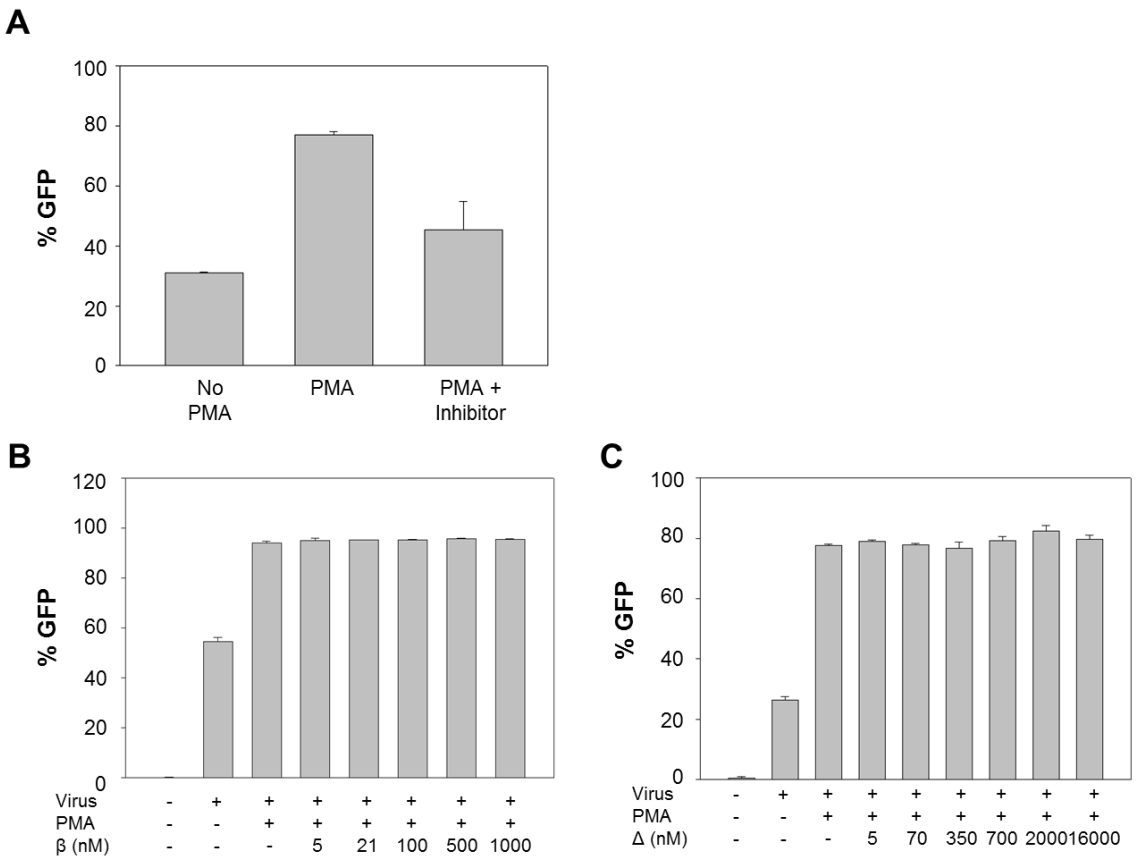


Figure 4.14 Use of a PKC inhibitor as a means to determine the mechanism whereby which PMA enhances the lentiviral transduction of K562 cells. (A) K562 cells were treated with the 50 nM calphostin C for 4 hours prior to PMA treatment at 10 nM. An SIV-based lentiviral vector encoding a GFP transgene was used to transduce the K562 cells at an MOI of 0.5 two hours following PMA treatment. Transduction efficiency was assessed by flow cytometry seventy-two hours after viral addition. In the same manner, K562 cells were treated with selective inhibitors specific for the (B) β_2 (N-(2-ethyl-2H-1,2,3-triazol-4-yl)-2-[4-(4-methoxypyrimidin-2-yl)-1H-pyrazol-1-yl]acetamide) isoform and (C) the Δ (1-(3-methylphenyl)-5-{[(2-methylphenyl)amino]methylene}-2-thioxodihydro-4,6(1H,5H)-pyrimidinedione) isoform of PKC for 4 hours prior to PMA treatment at 10 nM.

Assessment of LEDGF expression as a result of PMA treatment

HIV infection has been found to be expedited by the transcriptional co-activator identified as the lens epithelium-derived growth factor (LEDGF). LEDGF interacts with the lentiviral integrase facilitating integration of the transgene into the genome of the target cell. LEDGF contains a zinc-finger in the N-terminal region of the protein which has been determined to bind to cellular DNA. Despite as the name infers, LEDGF is ubiquitously expressed at all stages of life (Nishizawa *et al.*, 2001). Yet its role in development is significant, being that mice born with an inactivated LEDGF gene die perinatally upon experiencing severe skeletal abnormalities (Sutherland *et al.*, 2006). However, the complete biological function of LEDGF remains unknown.

LEDGF expression was evaluated in the context of PMA due to a report demonstrating LEDGF interaction with HIV integrase. In this manner, LEDGF directs viral integration into the genome more efficiently (Ciuffi, 2008; Shun *et al.*, 2008). Thus K562 cells were treated with PMA and assessed for the levels of LEDGF protein by Western blot analysis. LEDGF protein levels were not increased in K562 cells as a result of PMA treatment (**Figure 4.15 A-B**). This suggests that an enhancement in lentiviral transduction as a result of PMA treatment is not due to increased levels of LEDGF.

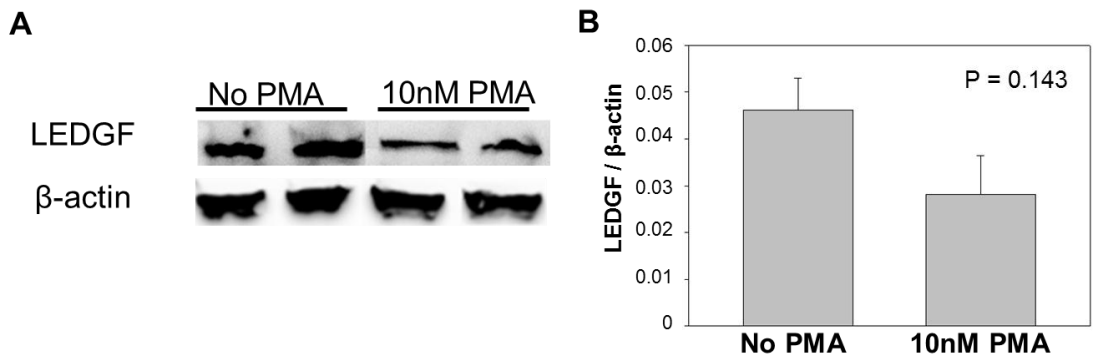


Figure 4.15 Western Blot detecting LEDGF levels following PMA treatment. (A) K562 cells were treated with PMA (10 nM) and harvested 12 hours later for protein. Protein concentration was analyzed by a BCA assay. Equivalent amounts of protein (75 µg) were added to each lane of an SDS-PAGE gel. Following transfer to a 0.2 micron membrane, LEDGF and β-actin levels were detected by a chemiluminescent Western Blot. (B) The p75 LEDGF and β-actin bands were quantified using ImageJ. The p75 LEDGF band was normalized to β-actin as graphically. A t-test was utilized to determine significance.

4.4— Discussion

Limiting transduction of hematopoietic stem cells has been observed clinically with the use of lentiviral vectors (Cartier *et al*., 2009; Boztug *et al*., 2010; Cavazzana-Calvo *et al*., 2010; DiGiusto *et al*., 2010). This obstacle is compounded among other things by the loss of modified cells after transplantation. This was observed in a previous HSC gene therapy phase I clinical trial designed to treat X-linked adrenoleukodystrophy. Prior to transplantation, 50% of the $CD34^+$ population was genetically modified, but only 9-14% of blood cells were found to be positive after transplantation (Cavazzana-Calvo *et al*., 2010). This discrepancy could either be a result of culturing parameters *ex vivo* that may significantly reduce the repopulation potential of $CD34^+$ cells (Kustikova *et al*., 2009) or due to the difficulty surrounding the transduction of pure HSCs as opposed to differentiated progenitor cells in the $CD34^+$ population. Enhancing the transduction of HSCs would prove beneficial for many gene therapy applications that utilize HSCs for gene delivery.

A library of 1280 pharmacologically active compounds was screened for the purpose of identifying enhancers of SIV and HIV-based lentiviral transduction. One apparent shortcoming of the performed high-throughput screen would be that the $LOPAC^{1280}$ library was not entirely inclusive. The proteasome inhibitor, MG132, was not a part of the library and thus not included in the screen despite the previous demonstration of MG132 to enhance lentiviral transduction of $CD34^+$ cells and T lymphocytes (Leuci, *et al*., 2011). Camptothecin, etoposide and taxol, on the other hand, were included in the $LOPAC^{1280}$ library. All three compounds have been previously identified to enhance the

transduction of an HIV-based lentiviral vector (Groeschel and Bushman, 2005). In the high-throughput screen, these 3 compounds were noted as positive hits. However, validation of camptothecin as a positive hit independent of the high-throughput screen showed an enhancement in the transduction of K562 cells with both a SIV and an HIV-based lentiviral vector. Thus camptothecin represents a false negative of the HIV-based lentiviral vector screen. Therefore, false negatives are an issue for identifying compounds with the use of a high-throughput screen as well as false positives, such as described for forskolin, SB 205384 and cytosine-1-β-D arabinofuranose hydrochloride.

Despite the ability of camptothecin or other topoisomerase inhibitors to enhance lentiviral transduction, these compounds are not ideal for clinical use because they induce DNA damage (Pommier *et al*., 2010). Therefore, our focus was on non-DNA damaging agents such as PMA. PMA, another positive hit from the high-throughput screen, represents a potentially safer compound than topoisomerase inhibitors. Initial assessment was performed with K562 cells in which a 4-fold increase in modified cells was apparent when culturing with PMA prior to transduction. This increase was achieved at a concentration as low as 2.5 nM PMA, where no morphological change was evident (Papayannopoulou *et al*., 1983). On the other hand, inhibition of cellular proliferation, on the other hand, was observed at 2.5 nM PMA.

In addition to enhancing the transduction efficiency of K562 cells, PMA also enhanced the transduction of the human hematopoietic cell line, EU1. EU1 cells are derived from the lymphoid lineage as opposed to K562 cells which are of the myeloid lineage. Once

again, an inhibition of cellular proliferation was apparent following PMA treatment, suggesting that inhibition of the cell cycle might be a method whereby PMA is exhibiting its effects. This is consistent with the observation that G_2/M arrest was responsible for the increased efficiency of HIV infection in HEK-293T, HeLa and IMR90 cells treated with camptothecin (Groschel and Bushman, 2005). The exact method whereby arresting cellular proliferation results in a favorable cellular condition for lentiviral gene transfer is unknown. Transduction could be enhanced at one or possibly multiple steps during lentiviral infection. If transduction is enhanced at the entry phase, a simple explanation would be to conclude that G_2/M arrest provides a greater surface area for which a lentivirus can attach being that the surface area of a cell stalled prior to division is expected to be 2-fold (Mitchison, 2003). However, this scenario is unlikely in that transduction was not enhanced with U937 cells or cytokine deprived sca-1^+ cells in which proliferation was inhibited (data not shown). A more likely explanation would be that a molecular event is occurring in the cell as a result of PMA treatment resulting in a cellular change more conducive to lentiviral infection.

As a phorbol ester, PMA binds to the C1 domain of PKC resulting in PKC translocation and activation (Stahelin *et al.*, 2005; Steinberg, 2008). Eight PKC isoforms are activated by PMA and have been implicated in an array of cellular processes. The lack of enhanced lentiviral transduction in HEK-293T, NIH-3T3, BHK, U937 and sca-1+ cells may be due to the different PKC isoform found in these cells as compared to those found in K562, EU1 and $CD34^+$ cells. However, PMA may be acting on more than just PKC. Approximately 50 amino acids located in the C1 domain are responsible for PMA/DAG

binding (Ono *et al.*, 1989; Kazanietz *et al*., 1994, 1995; Quest *et al*., 1994). These amino acids have been found to constitute similar C1 domains on nonkinase phorbol ester receptors which include chimaerins (a family of Rac GTPase activating proteins), RasGRPs (exchange factors for Ras/Rap1), and Munc13 isoforms (scaffolding proteins involved in exocytosis) (Ron and Kazanietz, 1999; Kazanietz, 2000). Further analysis is needed to assess the mechanism by which PMA is enhancing lentiviral transduction.

A novel finding of this study is the ability of PMA to enhance the lentiviral transduction of $CD34^+$ cells, a clinical target of gene therapy applications. Transduction was enhanced 3-fold with both an SIV-based and an HIV-based lentiviral vector, where at a concentration as low as 1 nM PMA increased the percentage of modified cells from 7 to 22 percent. This 3-fold difference remained apparent two weeks after transduction. Cells treated with PMA retained the CD34 and CD45 markers in a similar fashion to cells not treated with PMA. In addition, these cells were able to give rise to a subset of lineage committed cells as evidenced by the ability of $CD34^+$ cells treated with PMA to produce colonies in methylcellulose. Similar to the enhancement in transgene copy number using a fVIII transgene and K562 cells, the increase in percentage of GFP positive cells observed with PMA treatment of $CD34^+$ cells is due to an enhancement in lentiviral transduction as opposed to simply an enhancement in GFP expression. Additional studies are needed to determine the engraftment potential of $CD34^+$ cells following treatment with PMA.

Despite recent preclinical and some clinical progress with the use of HSC gene therapy, the transduction efficiency of HSCs remains a critical hurdle. Limited transduction often results in limiting expression of the transgene, which could result in little to no therapeutic benefit. In the current study, a high-throughput screen identified PMA as an enhancer of lentiviral transduction. Furthermore, PMA was shown to enhance the transduction of hematopoietic cell lines from both the myeloid and lymphoid lineages. PMA enhanced the transduction of a clinically relevant population of hematopoietic stem and progenitor cells. PMA has been approved for use in a phase I clinical trial to treat hematological malignancies in which the doses used (ie systemic µM concentrations) were tolerable without any apparent renal, hepatic or hematological toxicity establishing the feasibility of utilizing phorbol esters in humans (Strair *et al.*, 2002). Therefore, the use of PMA clinically to enhance the transduction of $CD34^+$ cells *ex vivo* is a viable option.

Chapter 5:

Conclusions and Future Perspectives

5.1— Conclusions

Optimization of the Lentiviral Vector

To date, three hemophilia A gene therapy clinical trials have been initiated. However the results of all three trials resulted in limited transient fVIII levels unable to circumvent the bleeding episodes experienced in these patients. Limited fVIII expression demonstrated in these trials could potentially be due to the inefficient transport of the human fVIII cDNA from the endoplasmic reticulum to the Golgi apparatus. fVIII is retained in the endoplasmic reticulum via a resident protein chaperone, BiP, which binds to a hydrophobic cluster in the A1 domain. In addition, efficient secretion of fVIII has been associated with carbohydrate-facilitated transport via the protein, lectin mannose binding-1 (LMAN1) which binds mannose residues attached to the B domain posttranslationally. With this in mind, several high-expression fVIII transgene sequences have been postulated in order to overcome the low level transgene expression. We recently compared these sequences to that of a B domain deleted porcine fVIII sequence. Enhanced expression of the B domain deleted porcine fVIII sequence was demonstrated both *in vitro* and *in vivo* (Ide *et al*., 2007; Doering *et al.*, 2007; Dooriss *et al*., 2009). The responsible domains for the high expression characteristics of porcine fVIII were found to be attributed to the A1 and A3 domains (Doering *et al.*, 2004). In addition, the immunogenicity of fVIII was found to be reduced by the addition of the porcine C1 domain and three alanine substitutions in the A2 domain (Healey *et al*., 2009; Lubin *et al*., 1997). Thus a hybrid human/porcine B domain deleted fVIII transgene containing the human A2 and C2 domains and the porcine A1, A3 and C1 domains was constructed (HP-fVIII). The bioengineered construct contains 91% human amino acid sequence and

has been shown to maintain the high expression characteristics of porcine fVIII (Doering *et al*., 2009).

As outlined above, much effort has been focused towards the optimization of the fVIII transgene. However, little attention has been directed towards the lentiviral vector itself in regards to fVIII expression. For this reason, the components of the lentiviral vector were evaluated and optimized for enhanced expression of fVIII. Three components were specifically assessed as a part of the lentiviral vector (1) the WPRE sequence (2) the lentiviral backbone and (3) the internal promoter.

The WPRE, although originally observed to increase the expression of GFP, was not found to exert any effect on the expression of HP-fVIII. A panel of cell lines was assessed for lentiviral vectors differing only in the presence or absence of a WPRE sequence. For all cell lines considered, no increase in HP-fVIII was observed at both the transcript and protein level. In addition, the WPRE was not found to be beneficial in regards to the amount of transcriptional read-through observed. Thus without any benefit to HP-fVIII expression or safety, the WPRE was eliminated from the lentiviral vector. Of note prior to these studies, the inclusion of a WPRE sequence in a gene therapy viral vector was considered a standard operation. Direct evidence confirming enhanced transgene expression as a result of a WPRE sequence was not required by the Recombinant DNA Advisory Committee prior to clinical application. However, due to this report as well as that of Klein *et al.* (2006) and Kingsman *et al.* (2005) it has been

advised that the utility of the WPRE sequence be scrutinized more closely in regards to enhanced transgene expression and safety

The second component of the lentiviral vector to be evaluated was the lentiviral backbone itself. A total of three different lentiviral backbones were evaluated. As a result, the SIV-based lentiviral vector was found to be superior in regards to HP-fVIII expression. Interestingly, this was not the case with a GFP transgene. The reason for this discrepancy is unknown. It is possible that the integrase associated with the SIV-based lentiviral vector has an integration profile that favors the expression of a large transgene such as HP-fVIII. However an integration analysis will need to be performed in order to affirm such a hypothesis. If this is the case, this information could be useful for future gene therapy applications for hemophilia A utilizing non-viral vectors able to integrate the transgene at specific sites in the genome such as zinc finger nucleases and tal effector nucleases. These non-viral vectors could be designed to incorporate the fVIII transgene at safe harbors or locations in the genome conducive to efficient fVIII expression. However, the technology associated with these non-viral vectors is not advanced enough for human application. Therefore the use of an SIV-based lentiviral vector for the treatment of hemophilia A by gene therapy is a viable first-generation clinical vector. However before this can become a reality, clinical-grade SIV-based lentiviral vectors will need to be produced in large quantities as is currently available for HIV-based lentiviral vectors. Until this is accomplished, an HIV based lentiviral vector is a suitable choice to begin treating individuals with hemophilia A via gene therapy and could serve as a substitute first-generation clinical vector. The SIV-based lentiviral vector may then serve

as a second-generation vector aimed at treating the subpopulation of individuals with hemophilia A living with AIDS (estimated to be between 6000 to 10,000 in the United States alone) (Meier, 1996). A large portion of adults with hemophilia A are HIV-1 positive due to receiving plasma derived fVIII as a means of protein replacement therapy prior to routine testing of blood-borne pathogens from donated blood products. Therefore, an SIV-based lentiviral vector may be a safer lentiviral vector since utilizing an HIV-based lentiviral vector in these individuals could potentially result in unsafe recombination events between the vector and wild-type HIV in the genome. Regardless of the limitations associated with the production of a clinical-grade SIV-based lentiviral vector, the SIV-based lentiviral backbone was used to analyze the third component of the lentiviral vector, the internal promoter.

Three ubiquitous promoters were assessed. In terms of HP-fVIII expression, the CMV internal promoter was found to be most efficient in HEK-293T cells. This result was not unexpected due to the strong enhancer found in the CMV promoter. However, the use of a CMV internal promoter clinically is constrained by the potential transactivation of nearby genes as a result of the strong enhancer found in the CMV promoter as compared to the enhancers associated with the EF1α and PGK internal promoters. This raises safety concerns surrounding the use of a CMV promoter in humans. In addition, the CMV promoter has been associated with methylation-induced inactivation which could result in transient expression of HP-fVIII. Therefore, further analysis may need to be performed in order to determine a more suitable internal promoter for clinical gene therapy applications designed for the treatment of hemophilia A. This may include the

clinically applicable EF1α internal promoter, demonstrating levels of HP-fVIII in HEK-293T cells greater than that of the PGK internal promoter. However, a larger panel of internal promoters including cell-specific promoters needs to be evaluated. Regardless of the drawbacks to utilizing a CMV internal promoter, as a result of these studies, a lentiviral vector was optimized for HP-fVIII expression and demonstrated therapeutic levels of HP-fVIII in a fVIII deficient mouse model. The results of this study was used to help delineate a lentiviral vector that received approval by the Recombinant DNA Advisory Committee (**Figure 5.1**).

Gene Therapy for the Treatment of Hemophilia A

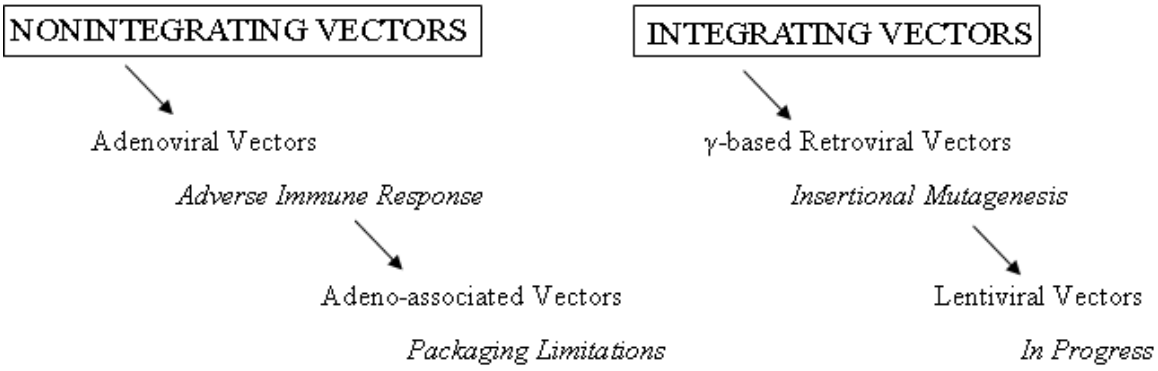


Figure 5.1 Viral vectors proposed for gene therapy protocols for the purpose of treating hemophilia A. Two types of viral vectors have been proposed for gene transfer, the nonintegrating and integrating vectors. Adenoviral vectors were one of the first vectors to be used clinical to treat hemophilia A. However, the trial was abruptly halted due to an adverse immune response to the vector. Thus adeno-associated vectors have been utilized preclinically to transfer the fVIII gene to the liver of mice. Yet encapsidation limitations are the major setback associated with the use of adeno-associated vectors for hemophilia A. Integrating vectors, on the other hand, provide a means for stable integration. The γ-based retroviral vectors were the first retroviral vectors to be utilized in the field. However, their use in the context of hemophilia A has been hindered by the presentation of insertional mutagenesis as seen in the clinical trial for X-SCID. The lentiviral vector, able to package a large transgene such as fVIII, has a safer integration profile than the γ-based retroviral vectors. In addition, the transfer of fVIII has been achieved preclinically in a hemophilia A mouse in which therapeutic levels were observed. Thus, a lentiviral vector is an appropriate vector to use for the treatment of hemophilia A.

Alternate Lentiviral Production Protocol

The delineation of an optimized lentiviral vector for HP-fVIII expression was demonstrated above. However, the production limitations associated with such a lentiviral vector remain a concern in the field. Lentiviral production is currently confined to a transient transfection procedure in HEK-293T cells due to the toxicity associated with the VSVG viral envelope. Lentiviral vectors are pseudotyped with the VSVG envelope protein in order to increase the range of host cells capable of being genetically modified. However, the VSVG protein accumulates at the cell surface of HEK-293T cells forming syncytia and ultimately leading to diminished cell viability. HEK-293T cells are viable for approximately three days following the onset of VSVG expression. Thus the generation of a stable packaging cell line for the mass production of VSVG-pseudotyped lentiviral vectors has yet to be delineated. Production of lentiviral vectors in this manner would be ideal for lentiviral vectors utilized in human clinical trials, in that the lentiviral vector could not only be produced in mass quantities but could also come from an extensively characterized nonvariable source. In order to establish a stable lentiviral packaging cell line an alternative cell line was analyzed for VSVG pseudotyped lentiviral production. BHK-M cells were chosen as a highly efficient clinically applicable protein producing cell line. The production of lentivirus was assessed in BHK-M cells following transient transfection. However, it was apparent that VSVG was more toxic in BHK-M cells than in HEK-293T cells. This observation is probably due to an increase in VSVG expression in BHK-M cells as compared to HEK-293T cells. Thus BHK-M cells are not a viable option for the production of lentiviral vectors pseudotyped

with VSVG. Therefore, other avenues will need to be explored in order to overcome the production limitation of lentiviral vectors, especially those encompassing large transgenes such as fVIII. This includes establishing a stable producing cell line in which VSVG expression is induced. This would diminish toxic side effects associated with the expression of VSVG until lentiviral production is required. In addition, alternative nontoxic envelopes may be utilized.

Enhancement of HSC transduction by a lentiviral vector

Another obstacle encountered in the field of gene therapy with the use of a lentiviral vector is the inefficient transduction of HSCs by lentiviral vectors. HSCs are innately resistant to lentiviral transduction by a yet unknown mechanism. However, the transduction of a self-renewing and fully capable differentiating $CD34^+$ cell is pertinent to the success of *ex vivo* gene therapy applications for the treatment of hemophilia A. Therefore, several attempts were made to enhance the transduction efficiency of lentiviral vectors.

The most progress was made as a result of a high-throughput screen. 1280 compounds were screened as a part of the LOPAC library. Three previously identified enhancers of lentiviral transduction were fortuitously included in the LOPAC library. The high-throughput screen confirmed all three compounds as enhancers of HIV-based lentiviral transduction as well as identifying the compounds as enhancers of SIV-based lentiviral transduction. However, these compounds induce DNA damage and are not ideal for clinical use. PMA, on the other hand, was also identified as an enhancer of lentiviral

transduction as a positive hit of the high-throughput screen yet not found to induce DNA damage. Initially, PMA was found to enhance the transduction of K562 cells. However, further analysis revealed that PMA was also able to enhance the transduction of the hematopoietic EU1 and most importantly the clinically therapeutic target, the $CD34^+$ population of hematopoietic stem and early progenitor cells. PMA was not able to enhance all cell lines assessed. The adherent HEK-293T and BHK-M cells, as well as the hematopoietic U937 and $sca\text{-}1^+$ cell line were resistant to the positive effects of PMA on lentiviral transduction. The discrepancy associated with the positive and negative effects of PMA is postulated to be due to the specific isoform of PKC prevalent in each individual cell type. However, at this time it is unclear which isoform PMA is acting on.

The ability of a universal PKC inhibitor (Calphostin C) to prevent the effect of PMA does suggest that PKC is the route in which PMA enhances transduction. Although the involvement of PKC in the mechanism whereby PMA enhances transduction has only been experimentally portrayed in K562 cells, potentially this same pathway is being utilized in EU1 and $CD34^+$ cells. PKC activation depending on the cell type can result in a multitude of downstream effects. The identification of this pathway will aid in the incorporation of PMA into a clinical transduction protocol.

The incorporation of a molecule that behaves in the same manner as PMA into a transduction protocol would ensure adequate genetic manipulation. Since PMA was revealed to enhance the transduction of the clinically relevant $CD34^+$ population of hematopoietic stem and progenitor cells, it is likely that transduction would be efficient

enough to result in adequate expression of a transgene such as fVIII. In regards to safety, reports following a phase I clinical trial designed for the treatment of hematological malignancies revealed that PMA was well tolerated following administration of PMA at μM concentrations without the appearance of any renal, hepatic or hematological toxicity (Strair *et al*., 2002). Therefore, PMA can act as a lead compound for the determination of a chemical with improved potency and/or selectivity for enhanced lentiviral transduction of the clinically relevant $CD34^+$ cells. This compound can then be utilized in a gene therapy clinical trial (**Figure 5.2**). However, it is likely that the transduction protocol will include such a compound during a second-generation clinical trial.

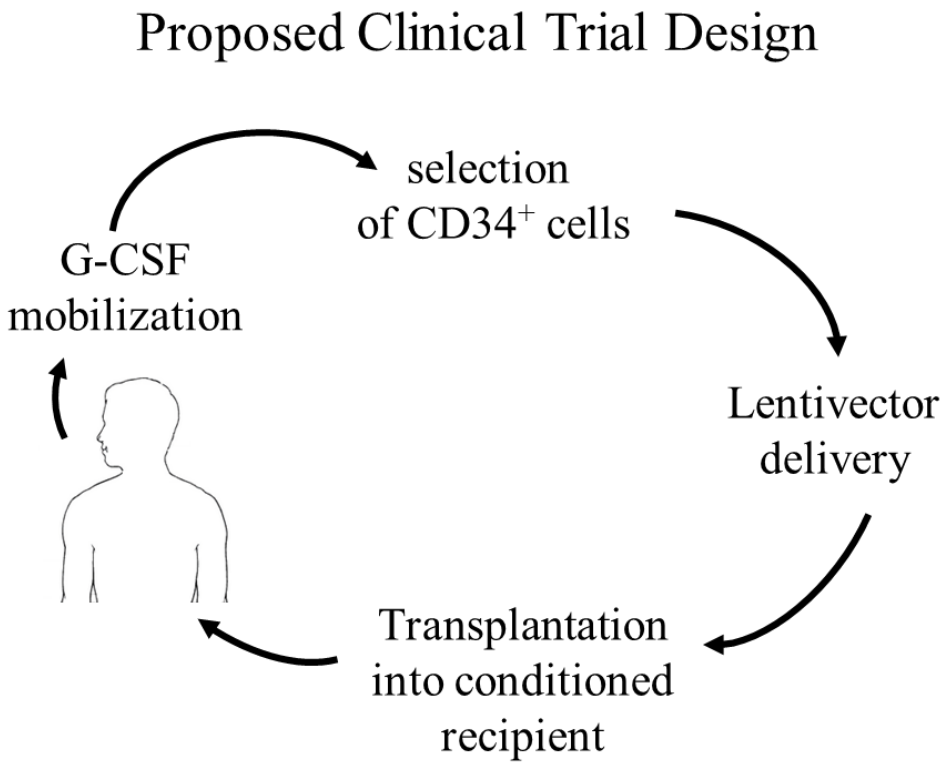


Figure 5.2 Schematic of gene therapy protocol for the treatment of hemophilia A. A clinical trial to be conducted at Emory University has been reviewed and approved by the Recombinant DNA Advisory committee. In addition, the protocol which includes the components involved have been deemed as a viable candidate for further development following a preinvestigational new drug and biosafety review. Following submission of and approval from the institutional review board and investigational new drug application, subjects with hemophilia A will be admitted into a gene therapy clinical trial in which $CD34^+$ cells will be mobilized from the hematopoietic stem cell compartment by G-CSF administration. G-CSF stimulates the bone marrow to produce and release granulocytes and stem cells into the bloodstream. Upon collection, the $CD34^+$ cells will be genetically modified *ex vivo* with a first-generation HIV-based lentiviral vector

lacking a WPRE sequence while encoding the high expressing human/porcine fVIII transgene expressed from the clinically approved EF1α internal promoter. The $CD34^+$ cells will then be removed from the viral containing medium prepping the cells for transplantation back into the patient in order to deliver a renewable supply of fVIII.

Abbreviation: G-CSF (Granulocyte colony-stimulating factor)

5.2— Future Perspectives

In my opinion, the field of gene therapy is migrating away from utilizing integrating viral based vectors as a means of gene replacement. This is mainly due to the risk of insertional mutagenesis. Although insertional mutagenesis has not been observed clinically with the use of lentiviral vectors (Cartier *et al*., 2009; Cavazzana-Calvo *et al*., 2010; Biffi *et al*., 2011), the potential of the integrated lentiviral cDNA to activate a protooncogene or disrupt a tumor suppressor gene is still a major concern in the field. One avenue explored as a means to eliminate insertional mutagenesis has capitalized on the fact that lentiviruses and lentiviral vectors, in addition to integrating in the genome, produce circular extrachromosomal forms of DNA. Two types of circular episomes can be formed. The first consists of 2 adjacent long terminal repeats (LTR) formed as a result of nonhomologous end joining of the linear reverse transcribed viral cDNA. The second type of circular episome contains one LTR following homologous recombination. As with AAV-based vectors, gene transfer of this nature is lost overtime as cells divide due to a lack of origin of replication in the 1- and 2-LTR episomal circles. However, these circular viral episomes may persist in terminally differentiated cells such as hepatocytes. For this reason, integrase-defective lentiviral vectors have been established in order to eliminate the potential risk of insertional mutagenesis associated with integration.

The lentiviral integrase protein consists of three domains, two zinc-finger binding domains (one in the N-terminal region which binds the viral DNA and another in the C-terminal region of the protein which binds the host DNA) and a catalytic core domain. Three amino acids of the core domain have been found to be highly conserved among the

retroviral integrase proteins and have thus been denoted as the catalytic triad. Therefore, the majority of the missense mutations introduced to produce an integrase-defective lentiviral vector occur at one of these three amino acids. These mutations prevent the integration of the transgene and if utilized to modify non-dividing cells can potentially persist for the lifetime of the host.

Integrase-defective lentiviral vectors could be an ideal route of transferring the fVIII gene into hepatocytes, the endogenous producer of fVIII. The route of administration would be similar to that of an AAV-based vector containing fIX for hemophilia B recently performed clinically. Likewise, insertional mutagenesis would not be a concern since the genome would not be disrupted by an integrase-defective lentiviral vector. But unlike AAV-based vectors, an integrase-defective lentiviral vector would not be constrained by the size of fVIII. Thus, integrase-defective lentiviral vectors encompass all of the advantages that AAV-based vectors offer while providing a means to overcome their shortcomings. However, as is a major concern in most gene therapy applications, the level of fVIII expression driven from the 1- and 2-LTR episomal circles is questionable. fVIII will need to be equivalent if not superior to those applications utilizing a lentiviral vector. Unfortunately, the integrase-defective lentiviral vector is not able to overcome the production limitations associated with lentiviral vectors. Up to this point, integrase-defective lentiviral vector titers for the most part have been reported to be similar to those of integrating lentiviral vectors (Saenz *et al.*, 2004; Nightingale *et al.*, 2006; Negri *et al.*, 2007; Coutant *et al.*, 2008). However, some reports have indicated lower titers than those

of integrating lentiviral vectors (Vargas *et al*., 2004; Philippe *et al*., 2006; Apolonia *et al*., 2007).

Integrase-defective lentiviral vectors are promising tools for providing a safer method of achieving gene transfer of fVIII. However, another method in which safer integration can be achieved is by utilizing a vector that provides a means for site-directed integration. Integrating viruses, such as the HIV and SIV lentiviruses, integrate into the host genome in a somewhat nonspecific manner, showing preference for active transcriptional units (Mitchell *et al*., 2004). Although this is a safer integration profile than that of the γ-retroviral vector utilized in the X-SCID clinical trial which favored integration near transcription start sites, an ideal vector would integrate at a specific location in the genome recognized as a safe harbor. This can be achieved with the use of either zinc finger nucleases or tal effector nucleases.

Zinc finger nucleases (ZFN) are fusion proteins comprised of a nonspecific FokI endonuclease domain and a zinc finger DNA-binding domain. Integration specificity comes from the ZFN DNA-binding domain which can be engineered to recognize a specific sequence. With 3-6 zinc finger DNA-binding domains arranged in tandem, a 9-18 base-pair target site can be achieved since each domain recognizes three base pairs. When two FokI endonuclease domains dimerize, a double strand break is formed. This double strand break can then be repaired naturally by the cell via nonhomologous end joining or homologous recombination. In the case of homologous recombination, the cell uses the undamaged sister chromatid to repair the break by copying the sequence across

the break. However, an exogenous donor DNA template consisting of a transgene flanked by homologous regions could also be used to repair the double strand break while at the same time transferring the gene encoding fVIII into the cell (Porteus, 2011).

Tal effector nucleases (TALENs) perform site-directed integration of a transgene in a similar manner as ZFNs. Like ZFNs, TALENS contain a FokI endonuclease domain fused with a DNA-binding domain. To achieve integration at a specific location in the genome of the target cell, the DNA-binding domain takes advantage of the ability of TAL effector proteins to specifically recognize a DNA sequence. TAL effector proteins contain a series of repeating subunits each thirty-four amino acids in length. The amino acid residues at position twelve and thirteen determine the recognition of a single nucleic acid. These residues can be modified in each repeat to engineer a TAL effector that is able to recognize a specific piece of DNA (Moscou *et al*.., 2009).

Both ZFNs and TALENs have been utilized to target endogenous disease-related mammalian genes (Urnov *et al*., 2005; Zou *et al*., 2009; Connelly *et al*., 2010; Mussolino *et al*., 2011). However, the frequency of gene targeting with the use of these nucleases is not yet substantial enough in primary patient-derived cells. In addition, toxic side effects have been observed with the use of ZFNs (Porteus and Baltimore, 2003). This is probably due to the production of off-target double-strand breaks which is not only concerning in regards to cytotoxicity but would also pose questions as to the safety profile of a ZFN. TALENs, on the other hand, have been shown to be less toxic and may

be a more ideal platform to accomplish site-directed gene targeting (Mussolino *et al*., 2011).

Until these technologies are improved, the utilization of a lentiviral vector for the treatment of hemophilia A is appropriate (**Figure 5.2**). One of the major limitations with the utilization of lentiviral vectors for *ex vivo* HSC modification has potentially been overcome by the results of this dissertation. Lentiviral transduction of $CD34^+$ cells has been enhanced by treatment prior to viral addition with the phorbol ester, PMA. The mechanism whereby PMA enhanced transduction has been delineated to be a result of PKC. Before PMA can be clinically applicable as a part of the gene therapy protocol for hemophilia A, the effects on integration will need to be adequately explored in regards to safety. Enhancing the transduction of $CD34^+$ cells with PMA is unfavorable if the likelihood of an insertional mutagenic event is increased as a consequence of an integration preference towards transcription start sites. This has yet to be analyzed. In addition, the effect of PMA on the multipotentiality of $CD34^+$ cells will need to be addressed. As demonstrated in chapter 4, $CD34^+$ cells treated with PMA produced colonies in methycellulose indicative of having the potential to give rise to lineage committed cells. However, the colonies were not specifically analyzed for the exact lineage that the $CD34^+$ cells differentiated into. Without this assessment, it is unclear whether the PMA treated $CD34^+$ cells have maintained the ability to differentiate into all hematopoietic lineages necessary for reconstitution and not just a subset. However, transplantation of PMA treated $CD34^+$ cells into irradiated NOD-SCID mice could answer this question as well as confirming that PMA does not affect the transplantation

efficiency of $CD34^+$ cells. Transducing the $CD34^+$ cells with a GFP containing virus prior to transplantation would allow one to identify lineage committed transplanted $CD34^+$ cells by identifying GFP positive cells that also contain lineage specific markers by flow cytometry. In this manner, the multipotentiality of the PMA treated $CD34^+$ cells could be assessed as well as confirming their persistence overtime indicative of a lack of effect of PMA on transplantation efficiency.

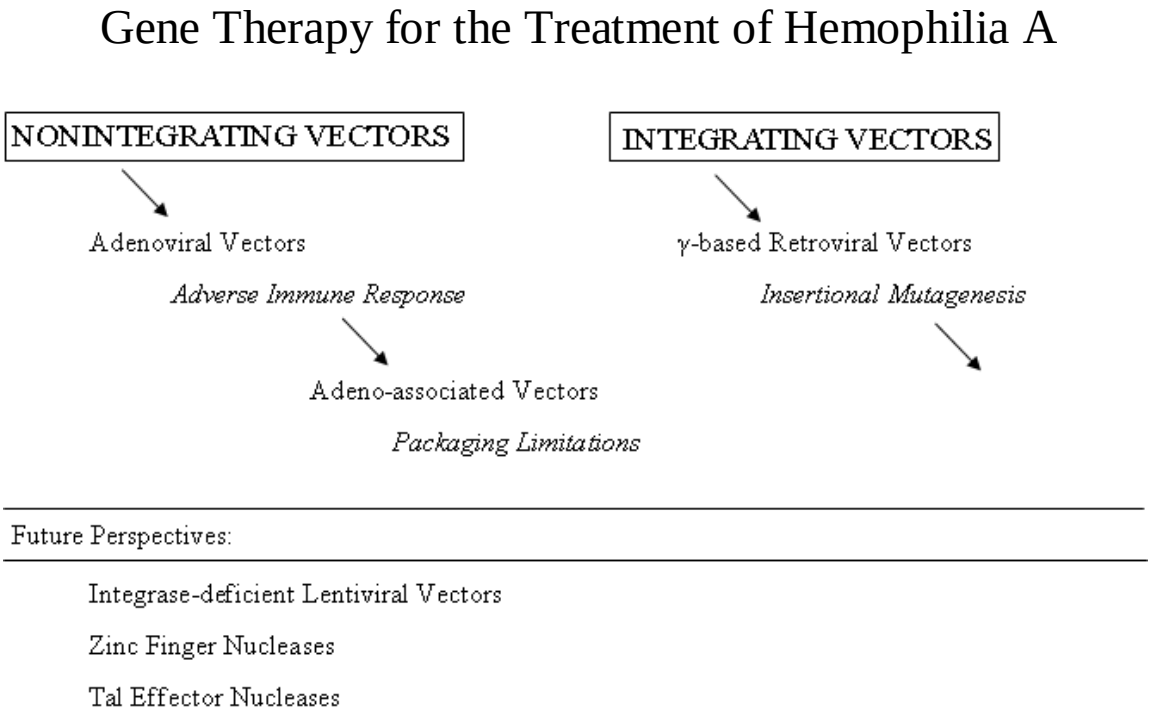


Figure 5.3 Modes of gene transfer proposed for the treatment of hemophilia A. Future perspectives include the use of an integrase-deficient lentiviral vector in which the transgene would remain episomal in the target cell. In addition, site-directed integration by zinc finger nucleases and tal effector nucleases will be utilized in the future providing a safer integration profile. However, until these technologies are improved the use of a lentiviral vector in a gene therapy protocol is an appropriate mode to transfer fVIII for the treatment of hemophilia A.

Limited expression of fVIII as apparent in all previous clinical attempts to treat hemophilia A with gene therapy has been potentially overcome by the construction of a high-expression chimeric fVIII transgene. HP-fVIII was used to genetically modify hematopoietic stem cells and effectively treat hemophilia A mice in order to overcome the low expression barrier. Prior to advancing to the clinic, the gene transfer system was optimized by analyzing the various components of the self-inactivating lentiviral vectors in terms of viral production, transduction efficiency and transgene expression. The data outlined in this dissertation was used to help develop a clinical vector that was recently approved by the Recombinant DNA Advisory Committee. In addition to optimizing the lentiviral vector, the obstacle associated with the resistance of HSCs to lentiviral infection was overcome by PMA as identified from a high-throughput screen. Notably, the viral transduction of $CD34^+$ cells was enhanced from 7% to >20%. PMA enhancement seems to correlate with an inhibition in cell division. Further analysis will be needed before PMA can be incorporated into a gene therapy protocol. Otherwise, an efficient lentiviral vector and protocol for the treatment of hemophilia A has been delineated and is currently being evaluated by the FDA for a phase I clinical trial to treat and possibly cure hemophilia A.

REFERENCES

Aiuti A, Cattaneo F, Galimberti S, Benninghoff U, Cassani B, Callegaro L, Scaramuzza S, Andolfi G, Mirolo M, Brigida I, Tabucchi A, Carlucci F, Eibl M, Aker M, Slavin S, Al-Mousa H, Ghonaium A, Ferster A, Duppenthaler A, Notarangelo L, Wintergerst U, Buckley RH, Bregni M, Marktel S, Valsecchi MG, Rossi P, Ciceri F, Miniero R, Bordignon C, Roncarolo MG. 2009. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*. 360:447-58.

Akkina RK, Walton RM, Chen M.L, Li QX, Planelles, V, Chen IS. 1996. High-efficiency gene transfer into $CD34^{+}$ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J. Virology*. 70:2581-2585.

Allocca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D, Kim SR, Maguire A, Rex TS, Di Vicino U, Cutillo L, Sparrow JR, Williams DS, Bennett J, Auricchio A. 2008. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invst.* 118:1955-1964.

Apolonia L, Waddington SN, Fernandes C, Ward NJ, Bouma G, Blundell MP, Thrasher AJ, Collins MK, Philpott NJ. 2007. Stable gene transfer to muscle using non-integrating lentiviral vectors. *Molecular Therapy*. 15:1947-1954.

Aruda V. 2006. Toward gene therapy for hemophilia A with novel adenoviral vectors: success and limitations in canine models. *J. Thromb. Haemost*. 4:1215-7.

Bagnall RD, Waseem N, Green PM, Giannelli F. 2002. Recurrent inversion breaking

intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood*. 99:168-174.

Berlfein J. 2003. Clinical trial update. *Hemaware*. 8:52-3.

Bevers E, Comfurius P, Zwall R. 1982. Changes in membrane phospholipid distribution platelet activation. *Biochim Biophs Acta.*736:57-66.

Bhopale G, Nanda R. 2003. Blood coagulation factor VIII: An overview. *J. Biosci.* 28: 783-789.

Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr. 1995. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nature Genetics*. 10:119-21.

Biffi A, Bartolomae CC, Cesana D, Cartier N, Aubourg P, Ranzani M, Cesani M, Benedicenti F, Plati T, Rubagotti E, Merella S, Capotondo A, Sgualdino J, Zanetti G, von Kalle C, Schmidt M, Naldini L, Montini E. 2011. Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood*. 117:5332-9.

Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, Greenblatt JJ, Rosenberg SA, Klein H, Berger M, Mullen CA, Ramsey WJ, Muul L, Morgan RA, Anderson WF. 1995. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science*. 270:475-480.

Boztug K, Schnmidt M, Schwarzer A, Banerjee PP, Díez IA, Dewey RA, Böhm M,

Nowrouzi A, Ball CR, Glimm H, Naundorf S, Kühlcke K, Blasczyk R, Kondratenko I, Maródi L, Orange JS, von Kalle C, Klein C. 2010. Stem-cell therapy for Wiskott-Aldrich syndrome. *N.Engl. J. Med.* 363: 1918-27.

Bowen DJ. 2002. Haemophilia A and haemophilia B: molecular insights. *Molecular Pathology*. 55:1-18.

Brinke A, Tagliavacca L, Naylor J, Green P, Giangrande P, Giannelli F. 1996. Two chiameric transcription units result from an inversion breaking intron 1 of the factor VIII gene and a region reportedly affected by reciprocal translocations in T-cell leukaemia. *Hum Mol Genet.* 5:1945-1951.

Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A, Baccarini A, Lazzari G, Galli C, Naldini L. 2007. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nature Technology*. 25:1457-1467.

Brun S, Faucon-Biguet N, Mallet J. 2003. Optimization of transgene expression at the posttranscriptional level in neural cells: implications for gene therapy. *Molecular Therapy*. 7:782-9.

Brunetti-Pierri N, Palmer DJ, Beaudet AL, Carey KD, Finegold M, Ng P. 2004. Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Human Gene Therapy*. 15:35-46.

Burns JC, Friedmann T, Driever W, Burrascano M. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *PNAS*. 90:8033-8037.

Butenas S, DiLorenzo ME, Mann KG. 1997. Ultrasensitive fluorogenic substrates for serine proteases. *Journal of Thrombosis and Haemostasis*. 78:1193-1201.

Burton M, Nakai H, Colosi P, Cunningham J, Mitchell R, Couto L. 1999. Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. *Proc Natl Acad Sci U S A*. 96:12725-30.

Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, Vidaud M, Abel U, Dal-Cortivo L, Caccavelli L, Mahlaoui N, Kiermer V, Mittelstaedt D, Bellesme C, Lahlou N, Lefrére F, Blanche S, Audit M, Payen E, Leboulch P, l'Homme B, Bougnéres P, von Kalle C, Fischer A, Cavazzana-Calvo M, Aubourg P. 2009. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science. 326:818-23.

Cartier N, Hacein-Bey-Abina S, Von Kalle C, Bougnères P, Fischer A, Cavazzana-Calvo M, Aubourg P. 2010. Gene therapy of x-linked adrenoleukodystrophy using hematopoietic stem cells and a lentiviral vector. *Bull Acad Natl Med*. 194:255-64.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*. 288:669-672.

Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F, Down J, Denaro M, Brady T, Westerman K, Cavallesco R, Gillet-Legrand B, Caccavelli L, Sgarra R, Maouche-Chrétien L, Bernaudin F, Girot R, Dorazio R, Mudler GJ, Polack A, Bank A, Soulier J, Larghero J, Kabbara N, Dalle B, Gourmel B, Socie G,

Chrétien, S, Cartier N, Aubourg P, Fischer A, Cornetta K., Galacteros F, Beuzard Y, Gluckman E, Bushman F, Hacein-Bey-Abina S, Leboulch P. 2010. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature*. 467:318-22.

Chao H, Mansfield SG, Bartel RC, Hiriyanna S, Mitchell LG, Garcia-Blanco MA, Walsh CE. 2003. Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing. *Nat Med*. 9:1015-1019.

Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden DT. 2000. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science*. 287:1804-1808.

Chesebro B, Wehrly K, Maury W. 1990. Differential expression in human and mouse cells of human immunodeficiency virus pseudotyped by murine retroviruses. *J. Virology*. 64:4553-4557.

Ciuffi A. 2008. Mechanisms governing lentivirus integration site selection. *Current Gene Therapy*. 8:419-429.

Coil DA, and Miller AD. 2004. Phosphatidylserine is not the cell surface receptor for vesticular stomatitis virus. *J. Virol.* 78:10920-10926.

Coil DA, and Miller AD. 2005. Enhancement of enveloped virus entry by phosphatidylserine. *J. Virol.* 79:11496-11500.

Colman RW, Bagdasarian A, Talamo RC, Scott CF, Seavey M, Guimaraes JA, Pierce JV, Kaplan AP. 1975. Williams trait. Human kiniongen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with

abnormalities of the Hageman factor-dependent pathways. *J. Clin. Invest.* 56:1650-1662.

Connelly JP, Barker JC, Pruett-Miller S, Porteus MH. 2010. Gene correction by homologous recombination with zinc finger nucleases in primary cells from a mouse model of a generic recessive genetic disease. *Molecular Therapy*. 18:1103-1110.

Conti C, Mastromarino P, Ciuffarella MG, Orsi N. 1988. haracterization of rat brain cellular membrane components acting as receptors for vesicular stomatitis virus. *Arch. Virol.* 99:261-269.

Coutant F, Frenkiel MP, Despres P, Charneau P. 2008. Protective antiviral immunity conferred by a nonintegrative lentiviral vector-based vaccine. *Plos One*. 3:3973.

Cronin J, Zhang X, Reiser J. 2005. Altering the tropism of lentiviral vectors through pseudotyping. Curr. Gene Ther. 5:387-398.

Dasgupta S, Repessé Y, Bayry J, Navarrete AM, Wootla B, Delignat S, Irinopoulou T, Kamaté C, Saint-Remy JM, Jacquemin M, Lenting PJ, Borel-Derlon A, Kaveri SV, Lacroix-Desmazes S. 2007. VWF protects FVIII from endocytosis by dendritic cells and subsequent presentation to immune effectors. *Blood*. 109:610-620.

Davi G, and Patrono C. 2007. Platelet activation and atherothrombosis. *New England Journal of Medicine*. 357:2482-2494.

Davis BM, Humeau L, Slepushkin V, Binder G, Korshalla L, Ni Y, Ogunjimi EO,

Chang LF, Lu X, and Dropulic B. 2004. ABC transporter inhibitors that are substrates enhance lentiviral vector transduction into primitive hematopoietic progenitor cells. *Blood*. 104:364-373.

Debili N, Issaad C, Masse JM, Guichard J, Katz A, Breton-Gorius J, Vainchenker W. 1992. Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation. *Blood*. 80:3022-3035.

Deininger P. and Batzer M. 2002. Mammalian Retroelements. *Genome Research*. 12: 1455-1465.

DiGiusto DL, Krishnan A, Li L, Li H, Li S, Rao A, Mi S, Yam P, Stinson S, Kalos M, Alvarnas J, Lacey SF, Yee JK, Li M, Couture L, Hsu D, Forman SJ, Rossi JJ, Zaia JA. 2010. RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. *Sci. Transl. Med*. 2:36-43

Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. 2002. High level expression of recombinant porcine coagulation factor VIII. *J Biol Chem*. 277:38345-9.

Doering C, Parker E, Healey J, Craddock H, Barrow R, Lollar P. 2002. Expression and characterization of recombinant murine FVIII. *Thromb Haemost.* 88:450-458.

Doering C, Healey JF, Parker ET, Barrow RT, Lollar P. 2004. Identification of porcine coagulation factor VIII domains responsible for high level expression via enhanced secretion. *J Biol Chem*. 279:6546-6552.

Doering CB, Gangadharan B, Dukart HZ, Spencer HT. 2007. Hematopoietic stem cells encoding porcine factor VIII induce pro-coagulant activity in hemophilia A mice with pre-existing factor VIII immunity. *Molecular Therapy*. 15:1093-9.

Doering CB, Denning G, Dooriss K, Gangadharan B, Johnston JM, Kerstann KW, McCarty DA, Spencer HT. 2009. Directed engineering of a high-expression chimeric transgene as a strategy for gene therapy of hemophilia A. *Molecular Therapy*. 17:1145-54.

Doering CB, and Spencer HT. 2009. Advancements in gene transfer-based therapy for hemophilia A. *Expert Rev Hematol*. 2:673-83.

Dooriss KL, Denning G, Gangadharan B, Javazon EH, McCarty DA, Spencer HT. Doering CB. 2009. Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A. *Human Gene Therapy*. 20:465-78.

Dorner AJ, Wasley LC, Kaufman RJ. 1989. Increased synthesis of secreted proteins induces Expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol Chem*. 264(34):2062-2067.

Eaton D, Rodriguez H, Vehar G. 1986. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulation activity. *Biochemistry*. 25:505-512.

Ehrenforth S, Kreuz W, Scharrer I, Kornhuber B. 1992. Factor VIII inhibitors in haemophiliacs. *Lancet* 340:253.

Eigenbrot C. 2002. Structure, function, and activation of coagulation factor VII. *Curr Protein Pept Sci*. 3:287-299.

Emi N, Friedmann T, Yee JK. 1991. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *Journal of Virology*. 65:1202-1207.

Essner JJ, McIvor RS, Hackett PB. 2005. Awakening gene therapy with Sleeping Beauty transposons. *Current Opinion in Pharmacology*. 5:513-519.

Fay PJ. 1988. Subunit structure of thrombin-activated human factor VIIIa. *Biochim Biophys Acta*. 952:181-190.

Fischer A. and Cavazzana-Calvo M. 2008. Gene therapy of inherited diseases. *Lancet.* 371:2044-2047.

Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. 2005. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol*. 166:1781-91.

Gangadharan B, Parker E, Ide L, Spencer HT, Doering C. 2006. High level expression of porcine FVIII from genetically modified bone marrow-derived stem cells. *Blood.* 34:461-475.

Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Zhang F, Adams S, Bjorkegren E, Bayford J, Brown L, Davies EG, Veys P, Fairbanks L, Bordon V, Petropoulou T, Kinnon C, Thrasher AJ. 2011. Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Sci Transl Med*. 3:97ra80.

Gewirtz J, Thornton MA, Rauova L, Poncz M. 2008. Platelet-delivered factor VIII provides limited resistance to anti-factor VIII in hibitors. *J. Thromb. Haemost.* 6:1160-1166.

Gitschier J, Wood W, Goralka T, Wion K, Chen E, Eaton D, Vehar G, Capon D, Lawn R. 1984. Characterization of the human factor VII gene. *Nature*. 312:326-330.

Gonzalez-Murillo A, Lozano ML, Alvarez L, Jacome A, Almarza E, Navarro S, Segovia JC, Hanenberg H, Guenechea G, Bueren JA, Rio P. 2010. Development of lentiviral vectors with optimized transcriptional activity for the gene therapy of patients with Fanconi anemia. *Human Gene Therapy*. 21:623-30.

Gorman L, Suter D, Emerick V, Schumperli D, Kole R. 1998. Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs. *Proceedings of the National Academy of Sciences.* 95:4929-4934.

Greene TK, Wang C, Hirsch JD, Ahai L, Gewirtz J, Thornton MA, Miao HZ, Pipe SW, Kaufman RJ, Camire RM, Arruda VR, Kowalska MA, Poncz M. 2010. In vivo efficacy of platelet-delivered, high specific activity factor VIII variants. *Blood.* 116:6114-6122.

Groeschel B. and Bushman F. 2005. Cell Cycle Arrest in G2/M Promotes Early Steps of Infection by Human Immunodeficiency Virus. *Journal of Virology*. 79:5695–5704.

Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M. 2002. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N. Engl. J. Med*. 18:1185-93.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P. 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 302:415-9.

Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier

E, Caccavelli L, Delabesse E, Beldjord K, Asnafi V, MacIntyre E, Dal Cortivo L, Radford I, Brousse N, Sigaux F, Moshous D, Hauer J, Borkhardt A, Belohradsky BH, Wintergerst U, Velez MC, Leiva L, Sorensen R, Wulffraat N, Blanche S, Bushman FD, Fischer A, Cavazzana-Calvo M. 2008. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest.* 118:3132-3142.

Hallek M, Girod A, Braun-Falco M, Wendtner CM, Bogedain C, Hörer M. 1998. Recombinant adeno-associated virus vectors. *IDrugs*. 1:561-573.

Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. 2009. The comparative immunogenicity of human and porcine factor VIII in haemophilia A mice. *Thrombosis and Haemostasis*. 102:35-41.

Hermonat PL. and Muzyczka N. 1984. Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue cultured cells. *Proceedings of the National Academy of Sciences*. 81:6466-6470.

Higashimoto T, Urbinati F, Perumbeti A, Jiang G, Zarzuela A, Chang LJ, Kohn DB, Malik P. 2007. The woodchuck hepatitis virus post-transcriptional regulatory element reduces readthrough transcription from retroviral vectors. *Gene Therapy*. 14:1298-1304.

High K. 2005. Gene transfer for hemophilia: can therapeutic efficacy in large animals be safely translated to patients? *J Thromb Haemost.* 3:1682-1691.

Hockin MF, Jones KC, Everse SJ, Mann KG. 2002. A model for the stoichiometric regulation of blood coagulation. *Journal of Biological Chemistry*. 277:18322-33.

Hu C, Cela RG, Suzuki M, Lee B, Lipshutz GS. 2011. Neonatal helper-dependent

adenoviral vector gene therapy mediates correction of hemophilia A and tolerance to human factor VIII. *Proceedings of National Academy of the Sciences*. 108:2082-7.

Hu C. and Lipshutz GS. 2012. AAV-based neonatal gene therapy for hemophilia A: long-term correction and avoidance of immune responses in mice. *Gene Therapy*. 2012; e-pub ahead of print 12 January 2012; doi:10.1038/gt.2011.200

Ide LM, Gangadharan B, Chiang KY, Doering CB, Spencer HT. 2007. Hematopoietic stem-cell gene therapy of hemophilia A incorporating a porcine factor VIII transgene and nonmyeloablative conditioning regimens. *Blood*. 110:2855-63.

Iwakuma T, Cui Y, Chang LJ. 1999. Self-inactivating lentiviral vectors with U3 and U5 modifications. Virology. 261:120-32.

Jaffe EA, Hoyer LW, Nachman RL. 1973. Synthesis of antihemophnilic factor antigen by cultured human endothelial cells. *J. Clin, Invest.* 52:2757-2764.

Jaffe EA, Hoyer LW, Nachman, R.L. 1974. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci.* 71:1906-1909.

Jeon HJ, Oh TK, Kim OH, Kim ST. 2010. Delivery of factor VIII gene into skeletal muscle cells using lentiviral vector. *Yonsei Med J.* 51:52-7.

Jiang H, Lillicrap D, Patarroyo-White S, Liu T, Qian X, Scallan CD, Powell S, Keller T, McMurray M, Labelle A, Nagy D, Vargas JA, Zhou S, Couto LB, Pierce GF. 2006. Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood*. 108:107-15.

Johnston J, Doering, CB, Spencer HT. 2011. Gene Therapy Strategies Incorporating

Large Transgenes. In: Chunsheng Kang (ed). Gene Therapy - Developments and Future Perspectives. InTech: Croatia, pp 121-142.

Jurlander B, Thim L, Klausen NK, Persson E, Kjalke M, Rexen P, Jørgensen TB, Østergaard PB, Erhardtsen E, Bjørn SE. 2001. Recombinant activated factor VII (rFVIIa): characterization, manufacturing, and clinical development. *Semin Thromb Hemost*. 27:373-84.

Kasuda S, Kubo A, Sakurai Y, Irion S, Ohashi K, Tatsumi K, Nakajima Y, Saito Y, Hatake K, Pipe SW, Shima M, Yoshioka A. 2008. Establishment of embryonic stem cells secreting human factor VIII for cell-based treatment of hemophilia A. *J Thromb Haemost*. 6:1352-9.

Kaufman RJ, Wasley LC, Davies MV, Wise RJ, Israel DI, Dorner AJ. 1989. Effect of von Willebrand factor coexpression on the synthesis and secretion of factor VIII in Chinese hamster ovary cells. *Mol Cell Biol*. 9(3):1233-42.

Kaufman RJ. and Pipe SW. 1999. Regulation of factor VIII expression and activity by von Willebrand factor. *Thromb Haemost*. 82:201-208.

Kazanietz MG, Bustelo XR, Barbacid M, Kolch W, Mischak H, Wong G, Pettit BR, Bruns JD, Blumberg PM. 1994. Zinc finger domains and phorbol ester pharmacophore. Analysis of binding to mutated form of protein kinase C zeta and the vav and c-raf proto-oncogene products. *J. Biol Chem*. 269:11590-11594.

Kazanietz MG, Barchi JJ Jr., Omichinski JG, Blumberg PM. 1995. Low affinity binding of phorbol esters to PKC and its cysteine-rich region in the absence of phospholipids. *J. Biol. Chem*. 270, 14679-14684.

Kazanietz, MG. 2000. Eyes wide shut: protein kinase C isozymes are not the only

receptors for the phorbol ester tumor promoters. *Mol Carcinog*. 28, 5-11.

Kingdon HS, and Hassell TM. 1981. Hemophilic dog model for evaluating therapeutic effectiveness of plasma protein fractions. *Blood.* 58:868-872.

Kingsman SM, Mitrophanous K, Olsen JC. 2005. Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE). *Gene Therapy*. 12:3-4.

Klein R, Ruttkowski B, Knapp E, Salmons B, Gunzburg WH, Hohenadl C. 2006. WPRE-mediated enhancement of gene expression is promoter and cell line specific. *Gene*. 372:153-61.

Kuether EL, Schroeder JA, Fahs SA, Cooley BC, Chen Y, Montgomery R, Wilcox DA, Shi Q. 2012. Lentivirus-mediated platelet gene therapy of murine hemophilia A with pre-existing anti-factor VIII immunity. *J Thrombosis and Haemostasis*. 10:1570-1580.

Kulkarni S, Dopheide S, Yap C, Ravanat C, Freund M, Mangin P, Heel KA, Street A, Harper IS, Lanza F, Jackson SP. 2000. A revised model of platelet aggregation. *J Clin Invest.* 105:783-791.

Kumar M, Keller B, Makalou N, Sutton RE. 2001. Systemic determination of the packaging limit of lentiviral vectors. *Human Gene Therapy*. 12:1893-905.

Kustikova OS, Schiedlmeier B, Brugman MH, Stahlhut M, Bartels S, Li Z, Baum C. 2009. Cell-intrinsic and vector-related properties cooperate to determine the incidence and consequences of insertional mutagenesis. *Mol. Ther*. 17:1537-47.

Lakich D, Kazazian HH Jr., Antonarakis SE, Gitschier J. 1993. Inversions disrupting

the factor VIII gene are a common cause of severe haemophilia A. *Nat Genet*. 10:236-241.

Laughlin CA, Tratschin JD, Coon H, Carter BJ. 1983. Cloning of infectious adenoassociated virus genomes in bacterial plasmids. *Gene*. 23:65-73.

Lee B, Ratajczak J, Doms RW, Gewirtz AM, Ratajczak MZ. 1999. Coreceptor/chemokine receptor expression on human hematopoietic cells:biological implications for human immunodeficiency virus-type 1 infection. *Blood*. 93:1145-1156.

Lee HJ, Lee YS, Kim HS, Kim YK, Kim JH, Jeon SH, Lee HW, Kim S, Miyoshi H, Chung HM, Kim DK. 2009. Retronectin enhances lentivirus-mediated gene delivery into hematopoietic progenitor cells. *Biologicals*. 37:203-209.

Lenting PJ, van Mourik JA, Mertens K. 1998. The life cycle of coagulation factor VIII in view of its structure and function. *Blood*. 92:3983-3996.

Leuci V, Mesiano G, Gammaitoni L, Cammarata C, Capellero S, Todorovic M, Jordaney N, Circosta P, Elia A, Lesnikova M, Georges G, Piacibello W, Fagioli F, Cignetti A, Aglietta M, Sangiolo D. 2011. Transient proteasome inhibition as a strategy to enhance lentiviral transduction of hematopoietic $CD34^{+}$ cells and T lymphocytes: Implications for the use of low viral doses and large-size vectors. *J. Biotechnology*. 156:218-226.

Leyte A, van Schijndel H, Niehrs C, Hunger W, Verbeet M, Mertens K, van Mourik J. 1991. Sulphation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. J Biol Chem. 266:740-746.

Lippi G, Favaloro E, Franchini M, Guidi G. 2009. Milestones and perspectives in coagulation and hemostasis. *Semin Thromb Hemost.* 35:9-22.

Lu H, Chen L, Wang J, Huack B, Sarkar R, Zhou S, 2008. Complete correction of hemophilia A with adeno-associated viral vectors containing a full-size expression cassette. *Human Gene Therapy*. 19:648-54.

Lubin IM, Healey JF, Barrow RT, Scandella D, Lollar P. 1997. Analysis of the human factor VIII A2 inhibitor epitope by alanine scanning mutagenesis. *J Biol Chem*. 272:30191-5.

Mackman N, Tilley R, Key N. 2007. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arterioscler Throm. Vasc Biol.* 27:1687-1693.

Mann KG. and Kalafatis M. 2003. Factor V: a combination of Dr Jekyll and Mr Hyde. *Blood*. 101:20-30.

Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, Ozelo MC, Hoots K, Blatt P, Konkle B, Dake M, Kaye R, Razavi M, Zajko A, Zehnder J, Rustagi PK, Nakai H, Chew A, Leonard D, Wright JF, Lessard RR, Sommer JM, Tigges M, Sabatino D, Luk A, Jiang H, Mingozzi F, Couto L, Ertl HC, High KA, Kay MA. 2005. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med.* 12:42-247.

Margaritis P, Roy E, Aljamali MN, Downey HD, Giger U, Zhou S, Merricks E, Dillow A, Ezban M, Nichols TC, High KA. 2009. Successful treatment of canine hemophilia by continuous expression of canine FVIIa. *Blood.* 113:3682-3689.

Margaritis P, Roy E, Faella A, Downey HD, Ivanciu L, Pavani G, Zhou S, Bunte RM,

High KA. 2011. Catalytic domain modification and viral gene delivery of activated factor VII confers hemostasis at reduced expression levels and vector doses in vivo. *Blood.* 117:3974-3982.

Marquette K, Pittman D, Kaufman R. 1995. A 110 amino acid region within the A1-domain of coagulation factor VIII inhibits secretion from mammalian cells. *Thromb Haemost.* 72:155-158.

Mastromarino P, Conti C, Goldoni P, Hauttecoeur B, Orsi N. 1987. Characterization of membrane components of the erythrocyte involved in vesicular stomatitis virus attachment and fusion at acidic pH. *J. Gen. Virol.* 68:2359-2369.

Matsui H, Shibata M, Brown B, Labelle A, Hegadorn C, Andrews C, Hebbel RP, Galipeau J, Hough C, Lillicrap D. 2007. Ex vivo gene therapy for hemophilia A that enhances safe delivery and sustained in vivo factor VIII expression from lentivirally engineered endothelial progenitors. *Stem Cells.* 25:2660-2669.

Matsui H, Hegadorn C, Ozelo M, Burnett E, Tuttle A, Labelle A, McCray PB Jr, Naldini L, Brown B, Hough C, Lillicrap D. 2011. A microRNA-regulated and GP64-pseudotyped lentiviral vector mediates stable expression of FVIII in a murine model of Hemophilia A. *Mol Therapy*. 19:723-730.

McIvor RS, Johnson MJ, Miller AD, Pitts S, Williams SR, Valerio D, Martin DW Jr, Verma IM. 1987. Human purine nucleoside phosphorylase and adenosine deaminase: gene transfer into cultured cells and murine hematopoietic stem cells by using recombinant amphotropic retroviruses. *Mol Cell Biol.* 7:838-46.

Meier B. 1996. "Blood, Money and AIDS: Hemophiliacs Are Split; Liability Cases Bogged Down in Disputes". *The New York Times*. 6-11-1996.

Millington M, Arndt A, Boyd M, Applegate T, Shen S. 2009. Towards a clinically relevant transduction protocol for primary human $CD34^{+}$ hematopoietic stem/progenitor cells. *Plos One*. 4:1-10.

Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR, Bushman FD. 2004. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *Plos One*. 2:1127-1137.

Mitchison JM. 2003. Growth during the cell cycle. Int. Rev. Cytol. 226:165-258.

Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. 1998. Development of a self-inactivating lentivirus vector. *Journal of Virology*. 72(10):8150-7.

Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C. 2006. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol*. 24:687-96.

Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, Benedicent F, Sergi LS, Ambrosi A, Ponzoni M, Doglioni C, Di Serio C., von Kalle C, Naldini L. 2009. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *Journal of Clinical Investigation*. 119:964-75.

Moscou MJ. and Bogdanove AJ. 2009. A simple cipher governs DNA recognition by TAL effectors. *Science*. 326:1501.

Muller MJ. and Baer HP. 1983. Relaxant effects of forskolin in smooth muscle: Role of cyclic AMP. *Naunyn- Schmiedeberg's Archives of Pharmacology*. 322:78-82.

Muruve DA, Barnes MJ, Stillman IE, Libermann TA. 1999. Adenoviral gene

therapy leads to rapid induction of multiple chemokines and acute neutrophil dependent hepatic injury in vivo. *Human Gene Therapy*. 10:965-976.

Mussolino C, Morbitzer R, Lütge F, Dannemann N, Lahaye T, Cathomen T. 2011. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Research.* 39:9283-9293.

Muul LM, Spiess PJ, Director EP, Rosenberg SA. 1987. Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma . *J Immunol*. 138:989–95.

Muul LM, Tuschong LM, Soenen SL, Jagadeesh GJ, Ramsey WJ, Long Z, Carter CS, Garabedian EK, Alleyne M, Brown M, Bernstein W, Schurman SH, Fleisher TA, Leitman SF, Dunbar CE, Blaese RM, Candotti F. 2003. Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. *Blood.* 101:2563-2569.

Nachman R, Levine R, Jaffe EA. 1977. Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *J. Clin. Invest.* 60:914-921.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 272:263-7.

Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, Chowdary P, Riddell A, Pie AJ, Harrington C, O'Beirne J, Smith K, Pasi J, Glader B, Rustagi P, Ng CY, Kay MA, Zhou J, Spence Y, Morton CL, Allay J, Coleman J, Sleep S, Cunningham JM, Srivastava D, Basner-Tschakarjan E, Mingozzi F,

High KA, Gray JT, Reiss UM, Nienhuis AW, Davidoff AM. 2011. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med.* 365:2357-2365.

Negri DR, Michelini Z, Baroncelli S, Spada M, Vendetti S, Buffa V, Bona R, Leone P, Klotman ME, Cara A. 2007. Successful immunization with a single injection of non-integrating lentiviral vector. *Molecular Therapy.* 15:1716-1723.

Neto M, Lunardi C, Rodrigues G, Bendhack L. 2011. Vasodilatation induced by forskolin involves cyclic GMP production. *J. Biopysical Chem.* 2:373-379.

Neyman M, Gewirtz J, Poncz M. 2008. Analysis of the spatial and temporal characteristics of platelet-delivered factor VIII-based clots. *Blood.* 112:1101-8.

Ni H, Denis C, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. 2000. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest.* 106:385-392.

Nightingale SJ, Hollis RP, Pepper KA, Petersen D, Yu XJ, Yang C, Bahner I, Kohn DB. 2006. Transient gene expression by nonintegrating lentiviral vectors. *Molecular Therapy.* 13:1121-1132.

Nishizawa Y, Usukura J, Singh DP, Chylack LT Jr, Shinohara T. 2001. Spatial and temporal dynamics of two alternatively spliced regulatory factors, lens epithelium-derived growth factor (ledgf/p75) and p52, in the nucleus. *Cell Tissue Res.* 305:107-14.

Novotny WF, Brown SG, Miletich JP, Rader DJ, Broze GJ Jr. 1991. Plasma antigen levels of the lipoprotein-associated coagulation inhibitor in patient samples. *Blood.* 78:387-393.

Ohmori T, Ishiwata A, Kashiwakura Y, Madoiwa S, Mitomo K, Suzuki H, Hasegawa M, Mimuro J, Sakata Y. 2008. Phenotypic correction of hemophilia A by ectopic expression of activated factor VII in platelets. *Molecular Therapy*. 16:1359-1365.

Olsen JC. 1998. Gene transfer vectors derived from equine infectious anemia virus. *Gene Therapy*. 5:1481-1487.

Olson ST, Bjork I, Shore JD. 1993. Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. *Methods Enzymol*. 222:525-559.

Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U, Nishizuka Y. 1989. Phorbol ester binding to protein kinase C requires a cysteine-rich zing-finger-like sequence. *Proc Natl Acad Sci USA*. 86, 4868-4871.

O'Rourke JP, Olsen JC, Bunnell BA. 2005. Optimization of equine infectious anemia derived vectors for hematopoietic cell lineage gene transfer. *Gene Therapy*. 12:22-29.

Osterud B. and Rapaport, S.I. 1977. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. *Proceedings of the National Academy of Sciences*. 74:5260-5264.

Page KA, Landau NR, Littman DR. 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J. Virol*. 64:5270-5276.

Papayannopoulou T, Nakamoto B, Yokochi T, Chait A, and Kannagi R. 1983. Human

erythroleukemia cell line (HEL) undergoes a drastic macrophage-like shift with TPA. *Blood*. 62:832-845.

Pauwels K, Gijsbers R, Toelen J, Schambach A, Willard-Gallo K, Verheust C, Debyser Z, Herman P. 2009. State-of-the-art lentiviral vectors for research use: risk assessment and biosafety recommendations. *Current Gene Therapy*. 9:459-74.

Pelletier R, Caron SO, Puymirat J. 2006 RNA based gene therapy for dominantly inherited diseases. *Current Gene Therapy*. 6:131-146.

Pezeshkpoor B, Rost S, Oldenburg J, El-Maarri O. 2012. Identification of a third rearrangement at Xq28 that causes severe hemophilia A as a result of homologous recombination between inverted repeats. *J Thromb Haemost.* 10:1600-1608.

Philippe S, Sarkis C, Barkats M, Mammeri H, Ladroue C, Petit C, Mallet J, Serguera C. 2006. Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. *Proc Natl Acad Sci.* 103:17684-17689.

Pipe S, Morris J, Shah J, Kaufman R. 1998. Differential interaction of coagulation factor VIII and Factor V with protein chaperones calnexin and calreticulin. *J Biol Chem*. 273:8537-8544.

Pittman D, and Kaufman R. 1992. Identification and functional importance of tyrosine sulfate residues within recombinant factor VIII. *Biochemistry*.31:3315-3325.

Pommier Y, Leo E, Zhang H, and Marchand C. 2010. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chem. and Biol*. 17:421-433.

Porada CD, Sanada C, Long CR, Wood JA, Desai J, Frederick N, Millsap L, Bormann C, Menges SL, Hanna C, Flores-Foxworth G, Shin T, Westhusin ME, Liu W, Glimp H, Zanjani ED, Lozier JN, Pliska V, Stranzinger G, Joerg H, Kraemer DC,

Almeida-Porada G. 2010. Clinical and molecular characterization of a re-established line of sheep exhibiting hemophilia A. *J Thromb Haemost*. 8:276-285.

Porteus MH and Baltimore D. 2003. Chimeric nucleases stimulate gene targeting in human cells. *Science*. 300:763.

Powell JS, Ragni MV, White GC, 2nd, Lusher JM, Hillman-Wiseman C, Moon TE, Cole V, Ramanathan-Girish S, Roehl H, Sajjadi N, Jolly DJ, Hurst D. 2003. Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood*. 102:2038-45.

Quest AF, Bardes ES, Bell RM. 1994. A phorbol ester binding domain of protein kinase C gamma. Deletion analysis of the Cys2 domain defines a minimal 43-amino acid peptide. *J. Biol. Chem*. 269:2961-2970.

Quinonez R. and Sutton R. 2002. Lentiviral vectors for gene delivery into cells. *DNA and Cell Biology*. 21:937-951.

Radcliffe P, Sion CJM., Wilkes EJ, Custard EJ, Beard GL, Kingsman SM, Mitrophanous K.A. 2008. Analysis of factor VIII mediated suppression of lentiviral vector titres. *Gene Therapy*. 15:289-297.

Ramezani A, Zweier-Renn LA, Hawley RG. 2011. Factor VIII delivered by haematopoietic stem cell-derived B cells corrects the phenotype of haemophilia A mice. *Thromb Haemost*. 105:676-87.

Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML. 2003 Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol. Genet. Metab*. 80:148-158.

Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. 1996 Transduction of nondividing cells using pseudotyped defective high-titer HIV type I particles. *PNAS*. 93:15266-15271.

Rivera J, Lozano M, Navarro-Nunez L, Vincente V. 2009. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*. 94:700-711.

Ron D, and Kazanietz MG. 1999. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J*. 13:1658-1676.

Rosen E, Chan J, Idusogie E, Clotman F, Vlasuk G, Luther T, Jalbert L, Albrecht S, Zhong L, Lissens A. 1997. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature*. 390:290-294.

Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA. (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: a preliminary report . *N. Engl. J. Med*. 319:1676–80.

Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, Merino M, Culver K, Miller AD, Blaese RM, Anderson WF. (1990) Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med*. 323:570-8.

Roth DA, Tawa NE, Jr., O'Brien JM, Treco DA, Selden RF. 2001. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *New England Journal of Medicine* 344:1735-42.

Sabatino DE, Lange AM, Altynova ES, Sarkar R, Zhou S, Merricks EP, Franck HG,

Nichols TC, Arruda VR, Kazaian HH Jr. 2011. Efficacy and safety of long-term prophylaxis in severe hemophilia A dogs following liver gene therapy using AAV vectors. *Molecular Therapy*. 19:442-9.

Saenz DT, Loewen N, Peretz M, Whitwam T, Barraza R, Howell KG, Holmes JM, Good M, Poeschla EM. 2004. Unintegrated lentivirus DNA persistence and accessibility to expression in nondividing cells: analysis with class I integrase mutants. *Journal of Virology*. 78:2906-2920.

Samulski RJ, Berns KI, Tan M, Muzyczka N 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proceedings of the National Academy of Sciences*. 79:2077-2081.

Santoni de Sio FR., Cascio P, Zingale A, Gasparini M, Naldini L. 2006. Proteasome activity restricts lentiviral gene transfer into hematopoietic stem cells and is down-regulated by cytokines that enhance transduction. *Blood*. 107:4257-4265.

Sarkar R, Xiao W, Kazazian HH Jr. 2003. A single adeno-associated virus (AAV)-murine factor VIII vector partially corrects the hemophilia A phenotype. *J Thromb Haemost*. 1:220-6.

Sarkar R, Tetreault R, Gao G, Wang L, Bell P, Chandler R, *et al.* 2004. Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood*. 103:1253-60.

Sarkar R, Mucci M, Addya S, Tetreault R, Bellinger DA, Nichols TC, Kazazian HH Jr. 2006. Long-term efficacy of adeno-associated virus serotypes 8 and 9 in hemophilia a dogs and mice. *Human Gene Therapy*. 17:427-39.

Sastry L, Xu Y, Cooper R, Pollok K, Cornetta K. 2004. Evaluation of plasmid DNA removal from lentiviral vectors by benzonase treatment. *Human Gene Therapy*. 15:221-226.

Savage B, Saldivar E, Ruggeri Z. 1996. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 84:289-297.

Scallan CD, Liu T, Parker AE, Patarroyo-White SL, Chen H, Jiang H, Vargas J, Nagy D, Powell SK, Wright JF, Sarkar R, Kazazian HH, McClelland A, Couto LB. 2003. Phenotypic correction of a mouse model of hemophilia A using AAV2 vectors encoding the heavy and light chains of FVIII. *Blood*. 102:3919-26.

Scallan CD, Lillicrap D, Jiang H, Qian X, Patarroyo-White SL, Parker AE, Liu T, Vargas J, Nagy D, Powell SK, Wright JF, Turner PV, Tinlin SJ, Webster SE, McClelland A, Couto LB. 2003. Sustained phenotypic correction of canine hemophilia A using an adeno-associated viral vector. *Blood*. 102:2031-7.

Schauber CA, Tuerk MJ, Pacheco CD, Escarpe PA, Veres G. 2004. Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. *Gene Therapy*. 11:266-275.

Schecter A, Spirn B, Rossikhina M, Giesen P, Bogdanov V, Fallon J, Fisher E, Schnapp L, Nemerson Y, Taubman M 2000. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res*. 87:126-132.

Schlegel R, Tralka TS, Willingham MC, Pastan I. 1983. Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site? *Cell*. 32:639-646.

Schneider S, Nuschele S, Wixforth A, Gorzelanny C, Alexander-Katz A, Netx R. 2007. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proceedings of National Academy of Sciences*. 104:7899-903.

Schnell MA, Zhang Y, Tazelaar J, Gao GP, Yu QC, Qian R, Chen SJ, Varnavski AN, LeClair C, Raper SE, and Wilson JM. 2001. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Molecular Therapy*. 5:708-22.

Schuettrumpf J, Herzog RW, Schlachterman A, Kaufhold A, Stafford DW, Arruda VR. 2005. Factor IX variants improve gene therapy efficacy for hemophilia B. *Blood*. 105:2316-2323.

Shen H, 1999. Intrinsic human immunodeficiency virus type 1 resistance of hematopoietic stem cells despite coreceptor expression. *Journal of Virology*. 73:728-737.

Shi Q, Wilcox DA, Fahs SA, Weiler H, Wells CW, Cooley BC, Desai D, Morateck P, Gorski J,Montgomery R. 2006. Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J. Clin. Invest*. 116:1974-1982.

Shi Q, Fahs SA, Wilcox DA, Kuether E, Morateck P, Mareno N, Weiler H, Montgomery R. 2008. Syngenic transplantation of hematopoietic stem cells that are genetically modified to express factor VIII in platelets restores hemostasis to hemophilia A mice with preexisting FVIII immunity. *Blood*. 112:2713-2720.

Shi Q, and Montgomery R. 2010. Platelets as delivery systems for disease treatments. *Advanced Drug Delivery Reviews*. 62:1196-1203.

Shun MC, Botbol Y, Li X, Di Nunzio F, Daigle JE, Yan N, Lieberman J, Lavigne M, Engelman A. 2008. Identification and characterization of PWWP domain residues critical for LEDGF/p75 chromatin binding and human immunodeficiency virus type 1 infectivity. *Journal of Virology*. 82:11555-11567.

Siedlecki CA, Lestini B, Kottke-Marchant K, Eppell S, Wilson D, Marchant R. 1996. Sheer-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood*. 88:2939-50.

Spector DH, Wade E, Wright DA, Koval V, Clark C, Jaquish D, Spector SA. 1990 Human immunodeficiency virus pseudotypes with expanded cellular and species tropism. *J. Virol.* 64:2298-2308.

Spencer HT, Denning G, Gautney RE, Dropulic B, Roy AJ, Baranyi L, Gangadharan B, Parker ET, Lollar P, Doering CB. 2011. Lentiviral vector platform for production of bioengineered recombinant coagulation factor VIII. *Molecular Therapy*. 302-309.

Stahelin RV, Wang J, Blatner NR, Rafter JD, Murray D, and Cho W. 2005. The origin of C1A-C2 interdomain interactions in protein kinase Cα. *J. Biol. Chem*. 280: 36452-36463.

Steinberg SF. 2008. Structural basis of protein kinase c isoform function. *Physiological Review*. 88:1341-1378.

Strair RK, Schaar D, Goodell L, Aisner J, Chin K, Eid J, Senzon R, Cui XX, Han ZT, Knox B, Rabson A, Chang R, Conney A. 2002. Administration of a phorbol ester to patients with hematological malignancies: preliminiary results from a

phase I clinical trial of 12-O-Tetradecanoylphorbol-13-acetate. *Clinical Cancer Research*. 8: 2512-2518.

Surin W, Barthwal M, Dikshit M. 2008. Platelet collagen receptors, signaling and antagonism: emerging approaches for the prevention of intravascular thrombosis. *Thrombosis Research*. 122:786-803.

Surosky RT, Urabe M, Godwin SG, McQuiston SA, Kurtzman GJ, Ozawa K, Natsoulis G. 1997. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J. Virol.* 71:7951-7959.

Sutherland HG, Newton K, Brownstein DG, Holmes MC, Kress C, Semple CA, Bickmore WA. 2006. Disruption of Ledgf/Psip1 results in perinatal mortality and homeotic skeletal transformations. *Mol. Cell. Bio.* 26:7201-7210.

Sutton RE, Reitsma MJ, Uchida N, Brown PO. 1999. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *J Virol*. 73:3649-60.

Swaroop M, Moussalli M, Pipe S, Kaufman R. 1997. Mutagenesis of a potential immunoglobulin-binding-protein-binding site enhances secretion of coagulation factor VIII. *J Biol Chem*. 272:24121-24124.

Toomey J, Kratzer K, Lasky N, Stnaton J, Broze G. 1996. Targeted disruption of the murine tissue factor gene results in embryonic lethality. *Blood*. 88:1583-1587.

Topalian SL, Solomon D, Rosenberg SA. 1989. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas . *J Immunol*. 142:3714–25.

Tuddenham E, Cooper D, Gritschier J, Higuchim, Hoyer L, Yoshioka A, Peake I, Schwaab R, Olekk, Kazazian H. 1991. Haemophilia A: database of nucleotide

substitutions, deletions, insertions and rearrangements of the factor VIII gene. *Nucleic Acids Res.*19:4821-4833.

Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*. 435:646-51.

Van den Biggelaar M, Bouwens EA, Kootstra NA, Hebbel RP, Voorberg J, Mertens K. 2009. Storage and regulated secretion of factor VIII in blood outgrowth endothelial cells. *Haematologica*. 94:670-8.

Van Damme A, Chuah MK, Collen D, VandenDriessche T. 2004. Onco-retroviral and lentiviral vector-based gene therapy for hemophilia: preclinical studies. *Semin Thromb Hemost*. 30:185-95.

VandenDriessche T, Collen D, Chuah MK. 2001. Viral vector-mediated gene therapy for hemophilia. *Current Gene Therapy*. 1:301-15.

Vargas J Jr, Gusella GL, Najfeld V, Klotman ME, Cara A. 2004. Novel integrase-defective lentiviral episomal vectors for gene transfer. *Human Gene Therapy*. 15:361-372.

Vehar G, Keyt B, Eaton D, Rodriguez H, O'Brien D, Rotblat F, Oppermann H, Keck R, Wood WI, Harkins RN, Tuddenham EG, Lawn RM, Capon DJ. 1984. Structure of human Factor VIII. *Nature*.312:337-342.

Viiala NO, Larsen SR, Rasko JE. 2009. Gene therapy for hemophilia: clinical trials and technical tribulations. *Semin Thromb Hemost*. 35:81-92.

Von Laer D., 1998. $CD34^{+}$ hematopoietic progenitor cells are not a major reservoir of the

human immunodeficiency virus. *Blood*. 76:1281-1286.

Wagenman B, Townsend K, Mathew P, Crookston K. 2009. The laboratory approach to inherited and acquired coagulation factor deficiencies. *Clin Lab Med*. 29:229-252.

Wagner DD, Olmsted JB, and Marder VJ. 1982. Immunolocalization of von Willebrand protein in Wiebel-Palade bodies of human endothelial cells. *J Cell Biol*. 95:355-360.

Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Coller BS, Jordan RE. 1996. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood*. 88:907-914.

Watson S and Nieswandt B. 2003. Platelet-collagen interaction: is GPVI the central receptor? *Blood*. 102:449-461.

Weichold FF. 1998. Neither human immunodeficiency virus-1 (HIV-1) nor HIV-2 infects most primitive human hematopoietic stem cells as assessed in long-term bone marrow cultures. *Blood*. 91:907:915.

Wion L, Kelly D, Summerfield J, Tuddenham E, Lawn M. 1985. Distribution of FVIII mRNA and antigen in human liver and other tissues. *Nature*. 317:726-729.

Wu X, Li Y, Crise B, Burgess SM. 2003. Transcription start regions in the human genome are favored targets for MLV integration. *Science*. 300:1749-51.

Wu Z, Yang H, Colosi P. 2010. Effect of genome size on AAV vector packaging. *Mol Therapy*. 18:80-86.

Wuepper KD, 1973. Prekallikrein deficiency in man. *J Exp Med*. 138:1345-1355.

Xu D, Alipio Z, Fink LM, Adcock DM, Yang J, Ward DC, Ma Y. 2009. Phenotypic

correction of murine hemophilia A using an iPS cell-based therapy. *Proc Natl Acad Sci U S A*. 106:808-13.

Yacoub N, Romanowska M, Haritonova N, and Foerster J. 2007. Optimized production and concentration of lentiviral vectors containing large inserts. *Journal of Gene Medicine*. 9:579-584.

Yang Y, Nunes FA., Berencsi K, Furth EE, Gönczöl E, Wilson JM. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proceedings of the National Academy of Sciences*. 91:4407-4411.

Yarovoi HV, Kufrin D, Eslin DE, Thornton MA, Haberichter SL, Shi Q, Zhu H, Camire R, Fakharzadeh SS, Kowalska MA, Wilcox DA, Sachais S, Montgomery RR, Poncz M. 2003. Factor VIII ectopically expressed in platelets: efficacy in hemophilia A treatment. *Blood*. 102:4006-4013.

Yarovoi HV, Nurden AT, Montgomery RR, Nurden P, Poncz M. 2005. Intracellular interaction of von Willebrand factor and factor VIII depends on cellular context: lessons from platelet-expressed factor VIII. *Blood*. 105:4674-4676.

Yonemura H, Sugawara K, Nakashima K, Nakahara Y, Hamamoto T, Mimaki I, Yokomizo K, Tajima Y, Masuda K, Imaizumi A. 1993. Efficient production of recombinant human factor VIII by co-expression of the heavy and light chains. *Protein Eng*. 6:669-674.

Youjin S, and Jun Y. 2009. The treatment of hemophilia A: from protein replacement to AAV-mediated gene therapy. *Biotechnology Letters*. 31:321-328.

Zavada J. 1982. The pseudotypic paradox. *J. Gen. Virol.* 63:15-24.

Zhang J, Attar E, Cohen K, Crumpacker C, Scadden D. 2005. Silencing

p21(Waf1/Cip1/Sdi1) expression increases gene transduction efficiency in primitive human hematopoietic cells. *Gene Therapy*. 12:1444-1452.

Zhang J, Scadden DT, Crumpacker CS. 2007. Primitive hematopoietic cells resist HIV-1 infection via p21. *J Clin Invest.* 117:473-81.

Zou J, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Beganny S, Chou BK, Chen G, Ye Z, Park IH, Daley GQ, Porteus MH, Joung JK, Cheng L. 2009. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell.* 5:97-110.

Zufferey R, Donello JE, Trono D, Hope TJ. 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virology*. 73:2886-92.

of the five patients admitted in the trial. Following initial expansion, the lymphocytes were modified with a γ-retroviral vector (derived from the Moloney murine leukemia virus) containing a neomycin resistance gene as a marker. No adverse side effects were noted as a result of the gene transfer. In addition, the neomycin resistance gene was detected up to two months following cell infusion (Rosenberg *et al.*, 1990). This study demonstrated successful transfer of a foreign gene to human cells with the use of a retroviral vector. It also alluded to the safety of these vectors as a means to treat other diseases by gene therapy. As a result, preclinical research progressed with the use of similar γ-retroviral vectors for the treatment of monogenic diseases.

In 1990, two children were treated for adenosine deaminase-deficient severe combined immunodeficiency disorder, a congenital disease resulting in frequent opportunistic infections as a result of an impaired immune system. T cells were isolated from each patient, transduced *ex vivo* with a γ-retroviral vector (derived from the Moloney murine leukemia virus) containing the adenosine deaminase gene and readministered back to the patient (Blaese *et al*., 1995). The effectiveness of the trial is debatable due to continued administration of PEGylated bovine adenosine deamainase as a means of protein replacement therapy during the trial. However, ten years later, the modified cells appear to be persisting in the circulation with one of the children exhibiting 20% gene modified lymphocytes (Muul *et al*., 2003). In addition, no adverse effects were experienced as a result of the γ-retroviral vector utilized, once again providing confidence as to the feasibility and safety of gene therapy with the use of these vectors. However, the two aforementioned trials developed a sense of false security in regards to the safety of a γ-retroviral vector derived from the Moloney murine leukemia virus. This was apparent following a clinical trial for X-linked severe combined immunodeficiency. In this trial, $CD34^+$ bone marrow stem cells were modified *ex vivo* to contain a correct copy of the previously unfunctional γc gene (Hacein-Bey-Adenia *et al*., 2002). Although nearly all twenty patients treated have improved immune functioning as quantified by an increase in T and B cells, five patients developed acute lymphoblastic leukemia due to integration near the proto-oncogene LMO2 (Hacein-Bey-Adenia *et al*., 2003). As a result the clinical use of γ-retroviral vectors derived from the Moloney murine leukemia virus has been restricted. Instead, retroviral vectors derived from an HIV-based lentivirus are being utilized for the modification of $CD34^+$ bone marrow stem cells *ex vivo*. Much

clinical progress has been made utilizing this approach for the treatment of X-linked adrenoleukodystrophy, Wiskott-Aldrich Syndrome, β-thalassaemia and AIDS. This has been accomplished without any apparent adverse side effects (Cartier *et al*., 2009; Boztug *et al*., 2010; Cavazzana-Calvo *et al*., 2010; DiGiusto *et al*., 2010).

In regards to hemophilia A, three gene therapy clinical trials to date have been conducted. Each utilized the human cDNA fVIII sequence, but all three differed in the manner whereby the nucleic acid sequence was incorporated into the patients' cells. The first trial by Roth *et al*. (2001) admitted six subjects with severe hemophilia A. Dermal fibroblasts were biopsied from each patient from the upper arm, expanded in tissue culture dishes in a laboratory incubator and electroporated (exposed to a voltage shock in order to perturb the cell membrane layer), resulting in the incorporation of the cDNA sequence into the fibroblast cells. After culturing, stably modified clones were selected and implanted into the omentum of each patient. Transient fVIII expression was observed in three of the patients for up to 6 months, with one patient expressing 4 percent of the normal amount of fVIII twelve weeks after implanting the genetically modified cells. However, the levels of fVIII diminished to less than 0.5% of normal a year later. The second trial included eleven subjects (Powell *et al*., 2003). Four doses (2.8×10^7, 9.2×10^7, 2.2×10^8, and 4.4×10^8 TU/kg) of a γ-retroviral vector based on the Moloney murine leukemia virus were administered via peripheral vein injection over three consecutive days. No adverse effects were observed throughout the 53 week study. Yet once again only a transient expression of fVIII was achieved. Eight of the patients demonstrated greater than 1% of normal fVIII levels on two or more occasions yet these

levels were not sustained throughout the study and could be associated with the administration of exogenous recombinant fVIII. The final trial was performed by GenStar Therapeutics. One patient was injected intravenously with an adenovirus, which contained the full-length human fVIII cDNA. The patient acquired an immune reaction to the virus and the trial was abruptly closed (Berlfein, 2003). Despite promising preclinical data, hemophilia A gene therapy clinical trials have made little advancement in the field due to limited expression of fVIII (High *et al*., 2005).

1.5— Preclinical Gene Therapeutic Applications to Cure hemophilia A

Large transgenes, such as the fVIII transgene, complicate gene therapy applications using viral vectors by (1) limiting the types of vectors available due to encapsidation limitations and (2) reducing the titer of viral vector that can be produced (Kumar *et al*., 2001; Radcliffe *et al*., 2008; Yacoub *et al*., 2007). The different human cDNA transgene lengths chosen for the clinical trials are a reflection of these limitations. The GenStar Therapeutics trial, unlike the other two trials, utilized an adenovirus which has fewer encapsidation constraints than other viral vectors available for gene transfer. As a result, it was the only trial to utilize the full-length cDNA 7-kb sequence. Incorporating a 7-kb transgene into a retrovirus, on the other hand, results in diminished viral titer making it difficult to achieve a high enough number of viral particles to be effective. Reducing the size of the insert, however, can increase viral titer (Yacoub *et al*., 2007). For this reason, many groups using viral vectors have reduced the size of the cDNA to approximately 4.5-

kb. This can be accomplished by removing sequence that encodes the B domain, which has been found to be dispensable for the coagulation activity of fVIII (Toole *et al*., 1986).

As demonstrated above, the complications associated with the large fVIII transgene has led researchers to find innovative ways to apply gene therapy techniques to the transfer of the fVIII transgene. The following sections will discuss the methods that have been proposed for the pre-clinical testing of gene transfer technologies for fVIII. These methods will be outlined in reference to which vector is being used, since each vector is accompanied with its own set of limitations. Both non-viral and viral vectors will be discussed, highlighting the strategies utilized to overcome the limitations experienced due to the use of a large transgene.

Preclinical use of non-viral vectors for hemophilia A

Unlike viral vectors, non-viral vectors themselves do not risk evoking an immunological response, are less expensive to produce, and are less limited by the size of the transgene. As a result as mentioned above, naked DNA gene transfer was among the first methods to be utilized in a clinical trial for hemophilia A (Roth *et al*., 2001). However, this strategy results in transient gene expression because of limited uptake by target cells and further limited integration into the genome of these cells (a process that occurs only randomly through non-homologous recombination) (Essner *et al*., 2005). This issue can be overcome by promoting stable integration into the cell's genome with the use of a transposable element. Transposable elements, although mostly inactive, are found to be littered throughout the human genome (Deininger and Batzer, 2002). In an active form,

transposable elements (transposons) have the ability to jump from one location in the genome to another by a "cut and paste" method through the enzyme transposase, which is encoded in the element. In order for a transposon to be utilized as a gene transfer vehicle, two components must be delivered to the target cell, (1) the transgene flanked by inverted repeat/direct repeat elements that are recognized for integration, and (2) a transposase which can be encoded in the same plasmid or in a second plasmid. These plasmids can be taken up into cells after being complexed to a cationic polymer such as polyethylenimine (PEI). Although transposons can carry an expansive amount of DNA, transposons are still somewhat limited by insert size. Integration efficiency has been shown to decrease with the size of the transgene (Essner *et al*., 2005). This is due to both the difficulties in delivering plasmids containing larger inserts as well as the limitations of the transposase. For example, the transposase enzyme associated with the Sleeping Beauty transposon, the most notable non-viral gene delivery system currently used, is only able to transpose up to 10-kb.

Transposons have been utilized as a non-viral vector for gene therapy of hemophilia A by a number of groups. The Sleeping Beauty transposon system was engineered from an inactive Tc1-like transposable element found in fish (Ivics *et al*., 1997). It has since been used to insert the human B domain deleted fVIII cDNA *in vivo*. fVIII levels remained at ~12% of normal after intravenous plasmid injection through the temporal vein of neonatal hemophilia A mice. However, the presence of inhibitors to fVIII resulted in only partial phenotypic correction (Liu *et al.*, 2006). In order to circumvent the presence of inhibitors, the Largaespada group tolerized neonatal, one day old, hemophilia A mice

to fVIII with a facial vein injection of 0.1U/g of recombinant human fVIII. Eight to twelve weeks later, two high pressure tail vein injections of a Sleeping Beauty transposon were administered. As a result, 16% of normal fVIII levels were seen in these mice at eighty-four days after plasmid injection, which was found to be sustained for 6 months. No inhibitors were detected and an improvement in clotting function was noted (Ohlfest *et al.*, 2005).

A high-pressure method of delivery is accomplished by injecting a high volume into the systemic circulation during a short, < 5 min, time frame (termed hydrodynamic injections). In the mouse, this results in DNA uptake followed by expression from the liver. Designed to increase the delivery of DNA to the nucleus of a cell, high-volume high-pressure injection is not yet applicable for humans (Essner *et al*., 2005). Therefore, other methods of transposon delivery are being explored. One method that is being studied is the cell specific delivery of a transposon by encapsulation in a nanocapsule. A recent report by Kren *et al.* (2009) engineered a nanocapsule to contain the Sleeping Beauty transposon system. The nanocapsule was targeted to liver sinusoidal endothelial cells (LSECs) by coating the capsule with an endogenous ligand for the hyaluronan receptor found on LSECs. Inside the nanocapsule a single plasmid was encapsulated containing both a cis-acting transposase as well as the B domain deleted canine fVIII transgene. Eight-week old hemophilia A mice were injected with 25μg of nanocapsule via tail vein injection. fVIII levels were measurable for a total of 11 months.

Preclinical use of Adeno-associated viral vectors for hemophilia A

Hepatocytes have been modified by a number of adeno-associated viral serotypes. For example, AAV-2, after intraportal administration, transferred a canine B domain deleted version of fVIII to liver cells, which resulted in partial phenotypic correction of hemophilia A mice. Although fVIII activity initially peaked to 8% of normal, expression was not sustained, declining to 2% nine months after injection (Sarkar *et al*., 2003). These findings, although confirmed independently by Scallan *et al*. (2003), are contradictory to similar preclinical and clinical studies for hemophilia B in which therapeutically effective levels of fIX are sustained over time (Manno *et al*., 2005 and Schuettrumpf *et al*., 2005). This may be due to the size constraints associated with the AAV vector because fIX is a significantly smaller transgene than fVIII with a cDNA of 2.8-kb. This smaller sequence allows for the inclusion of larger regulatory elements (such as liver specific promoters and enhancers) in the adeno-associated viral vector that are not able to be incorporated after the inclusion of the fVIII cDNA sequence. In the Scallan (2003) and Sarkar (2003) reports, a minipromoter was all that could be incorporated into the AAV-2 vector to promote fVIII expression. Therefore, it was concluded that regulatory elements, that were unable to be included in the AAV-2 vector (due to insert size restraints associated with adeno-associated viral vectors), were required to enhance fVIII expression. A follow up study evaluated additional adeno-associated viral serotypes (AAV-5, AAV-7, and AAV-8) to determine if other serotypes were more efficient at transducing hepatocytes than AAV-2. For the inefficient transfer of large transgenes, it was hypothesized that by increasing hepatocyte transduction, limited expression could be overcome without the inclusion of regulatory expression-enhancement elements. They found that AAV-8 was superior to other serotypes

regardless of route of administration (intraportally or intravenously) producing near normal physiological levels of fVIII (0.58 + 0.2 IU ml^{-1}) six months post administration at a vector dose of 1 x 10^{11} vector copies / mouse (Sarkar *et al.*, 2004). A similar comparison was performed by Jiang *et al.* (2006), comparing four serotypes (AAV-2, AAV-5, AAV-6, and AAV-8), in both mice and dogs. In mice, transduction efficiency was found to be least with AAV-5 and greatest with AAV-8. However, for dogs no substantial difference was observed among the serotypes. Remarkably though, fVIII expression was sustained in some dogs (2 to 5% of normal) for up to three years, resulting in decreased occurrences of spontaneous bleeds (Jiang *et al.*, 2006). This was a significant contribution to the field being the first multiyear report of therapeutic efficacy and safety in a large animal. Albeit, high vector doses of 6 x 10^{12} and 2.7 x 10^{13} vector genomes/kilogram were required to yield these subtherapeutic levels of fVIII.

Despite the phenotypic improvements noted above, the inclusion of regulatory elements to the vector for enhancing the expression of fVIII would have been the simplest way to address the limited hepatocyte transduction that was seen with AAV-2. Yet since adeno-associated viral vectors are restricted in their genetic carrying capacity, other avenues had to be evaluated to overcome this limitation. Some reports show packaging of genomes greater than 5-kb in adeno-associated vectors (Alloca *et al.*, 2008; Grieger and Samulski, 2005). For example, it is reported that an AAV-8 viral vector was produced containing the B domain deleted human fVIII transgene as well as a full length promoter and enhancer, totaling 5.75-kb (Lu, 2008). However, extensive examination in this report as well as by Wu (2010) clearly showed that inserts ranging from 4.7-kb to 8.7-kb result in

heterogenous virons of varying genome lengths, typically containing truncations at the 5' end. Thus, increasing the size of the adeno-associated vector genome leads to the formation of defective viral particles encapsulating incomplete transgene sequences (Dong *et al*., 1996).

For these reasons, several groups are attempting to overcome the packaging limitation with the use of two different vectors, one for the heavy chain and one for the light chain of fVIII. This strategy resulted from the demonstration of secretable biologically active fVIII following co-transfection in Chinese hamster ovary cells of two plasmids separating the heavy and light chains (Burke *et al*., 1986 and Yonemura *et al*., 1993). In these cells the two polypeptide chains were able to reconstitute a functional fVIII heterodimer that was secreted into the cellular media. This strategy was first performed in C57BL/6 mice by intraportal administration (Burton *et al*., 1999). These mice, able to secrete endogenous fVIII, were chosen since this strain has already been presented with fVIII and potentially would not elicit an immune response to the fVIII transgene. This would allow for expression to be measured without any contraindications. As a result, greater than physiological levels of fVIII were produced. These results were then extended into the hemophilia A mouse model where therapeutic levels of fVIII were achieved in a dose dependent manner. High levels of transduction were noted with twelve percent of hepatocytes being modified with both vectors. Complicating the issue, a chain imbalance was noted with a 25 to 100 fold excess of light chain. This was found to be due to inefficient translational or posttranslational processing that could not be circumvented by changing the administration ratio of heavy to light chain vectors (Scallan *et al*., 2003).

When extended to the hemophilia A dog model, only partial phenotypic correction was achieved, irrespective of adeno-associated serotype used. Although only modest levels of fVIII were observed in the dogs (ranging from 1 to 8% of normal), these levels were found to be sustained for at least two years (Sarkar *et al*., 2006).

Another strategy used to overcome the inability of adeno-associated vectors to deliver large genes is trans-splicing. Trans-splicing attempts to repair the truncated fVIII mRNA *in vivo* by delivering the remaining downstream pre-mRNA. In this strategy, a pre-trans-splicing molecule is delivered by an adeno-associated vector in which complementary mRNA sequences are located at the 5' end of the molecule designed to be spliced with the preexisting truncated fVIII mRNA due to a strong splice site at the 3' end. In this way, a shortened version of the transgene can be delivered *in vivo* to restore the disease phenotype. This method was performed in hemophilia A mice (Chao *et al*., 2003). Hemophilia A mice were created by inserting a neomycin resistance gene into the sixteenth exon of fVIII (Bi *et al*., 1995). As a result truncated fVIII mRNA is still expressed in these mice. By delivering a pre-trans-splicing molecule containing complementary sequence to intron 15, a functional fVIII pre-mRNA was spliced together in vivo resulting in phenotypic correction in eight of the ten injected mice.

Preclinical use of Lentiviral vectors for hemophilia A

Despite these efforts the fact remains that non-integrating adeno-associated viral vectors are unable to stably transduce cells. Therefore, many groups are focusing on the use of integrating vectors. Lentiviral vectors, such as the human immunodeficiency virus (HIV)

and the simian immunodeficiency virus (SIV), have been analyzed for their use in gene therapy since 1996 in which Naldini and colleagues revealed the ability of these vectors to overcome the need for cell division during transduction. Being able to transduce both dividing and non-dividing cells gives lentiviral vectors the same advantage as adeno-associated viral vectors. For this reason, lentiviral vectors are likely to make up a second generation of therapeutic vectors to be tested in clinical trials for the treatment of hemophilia A.

Lentiviral vectors, however, come with a set of limitations (to be discussed in detail in the next section). In particular, lentiviral production is limited. Reduction in titers due to issues associated with transgene size can be overcome by targeting various cell populations *ex vivo* then expanding the genetically modified cells. Therefore, despite diminished titer production, preclinical progress has been made with lentiviral vectors encoding the 4.5-kb B domain deleted fVIII transgene. And, by targeting specific cells, viral transduction can be optimized and enhanced, which further overcomes the reduction in titer due to transgene size. In addition, *ex vivo* modification of cells is considered safer than the *in vivo* delivery of recombinant virus since it eliminates the possible transmission to germline cells, as well as avoiding any systemic toxicity that can result due to direct presentation of the vector particle (Van Damme *et al*., 2004). *Ex vivo* modification also eliminates the issue of modifying antigen-presenting cells, possibly eliminating the development of an immune response to the transgene.

Ex vivo gene therapy for hemophilia A has been analyzed in a broad range of cell types (Viiala *et al*., 2009). Cellular alternatives considered include embryonic stem (ES) cells, bone marrow derived mesenchymal cells, blood outgrowth endothelial cells (BOECs), and hematopoietic stem cells. Stem cells are a reasonable alternative in that they have unlimited replicative potential and contain the ability to differentiate into a wide range of cells. However, the initial use of stem cells in the field of gene therapy resulted in low expression levels. This was thought to either be due to gene inactivation as a result of extensive differentiation (McIvor, 1987) or an inability to effectively transduce stem cells. Regardless, promising results were published utilizing an inducible system for fVIII in ES cells. However, both ethical and safety concerns have inhibited the continuation of these studies (Kasuda *et al*., 2008). This is due to the controversy surrounding the generation of ES cells from human embryos, as well as the formation of teratomas which arose from undifferentiated ES cells used for insulin production (Fujikawa *et al*., 2005). Fortunately, induced pluripotent stem cells (iPS) appear to be comparable to ES cells and have thus been considered to treat monogenic disorders in order to alleviate the ethical concerns surrounding stem cell usage. iPS cells are derived from adult somatic cells which have been reprogrammed to have stem cell characteristics. Significant progress has been made utilizing iPS cell-based therapy for murine hemophilia A (Xu *et al*., 2009). However, these methods are fairly new and still do not address the potential formation of teratomas. Therefore, many obstacles need to be addressed before this therapy can reach the clinics (for further discussion refer to Liras, 2011). Another cell type, bone marrow derived mesenchymal cells, initially yielded therapeutic levels of fVIII from transduced human mesenchymal cells in

immunodeficient mice. Yet fVIII plasma levels deteriorated gradually in spite of the persistence of gene modified cells, suggesting transcriptional repression in this cell type (Van Damme *et al*., 2004).

Human BOECs can be isolated from the peripheral blood of healthy donors and were considered as fVIII gene transfer targets because they express VWF (Jaffe *et al.*, 1973; Wagner *et al.*, 1982). VWF binds with high affinity to fVIII in the circulation and protects fVIII from degradation and uptake by antigen presenting cells (possibly eliminating the development of an immune response to the transgene) (Dasgupta *et al*., 2007). The expression of fVIII in conjunction with VWF has been demonstrated to enhance efficient transport of fVIII through the secretory pathway (Dorner *et al.*, 1989; Kaufman *et al.*, 1989). Therefore, it was hypothesized that modifying these cells to also express fVIII would result in the secretion of fVIII complexed to VWF, which would result in an increased half-life of fVIII, secreted at sites of injury. After transduction, BOECs expressed high levels of fVIII measured at 1.6 pmol/million cells/24hrs, which lasted for over thirty days in culture. fVIII was found to be stored in the same vesicles as VWF in the BOECs but was not found to be released upon agonist stimulation, unlike VWF. Instead, fVIII appeared to be released in a constitutive manner (van den Biggelaar *et al*., 2009). When BOECs were implanted into immunocompetent hemophilic mice, fVIII levels were in the therapeutic range for a total of 27 weeks. Afterwards, the levels declined to baseline due to loss of the implanted BOECs (Matsui *et al*., 2007). Although a benefit for fVIII secretion with VWF was not directly tested, the proof of concept was shown for the expression of fVIII from BOECs.

Similarly, platelets are potentially an ideal source of fVIII expression as specialized secretory cells that endogenously express VWF. In this manner, fVIII expression and stability can be improved by being expressed in conjunction with VWF (Dorner *et al.*, 1989; Kaufman *et al.*, 1989). The following five promoters actively express genes in both megakaryocytes and platetes: GPIIb (αIIb), GPIbα, GPVI, platelet factor 4 and c-mpl (Shi and Montgomery, 2010). However among this list, the GPIIb and the GPIbα promoters have been utilized the most to direct expression of transgenes to platelets. This is due to the high level of expression exhibited by the GPIIb and GPIbα promoters yielding surface densities of approximately 80,000 and 25,000 copies per platelet respectively (Debili *et al.*, 1992; Wagner *et al.*, 1996).

Two separate lines of transgenic mice were produced in which the B-domain deleted human fVIII cDNA was expressed solely in platelets as directed by the GPIIb and GPIbα promoter (Yarovoi *et al.*, 2003; Shi *et al.*, 2006). In both cases, fVIII was found to colocalize with VWF in the α-granules of platelets by immunofluorescent microscopy. The activity of the fVIII in platelets expressed from the GPIIb platelet specific promoter was assessed by a chromogenic assay. Utilizing platelet lysates as well as plasma samples following agonist-induced platelet activation, fVIII activity was measured to be near or slightly above 0.7mU / 10^8 platelets which was able to correct the hemophilia A phenotype as determined by a tail-clip assay (Shi *et al.*, 2006). Similarly, fVIII levels produced as a result of expression directed from the GPIbα promoter were able to correct the phenotype of hemophilia A knockout mice following carotid artery injury. The

occlusion time was equivalent to that achieved with 20% normal fVIII levels (Yarovoi *et al.,* 2003). The results of these reports served as proof-of principle analyses in that it suggested that expression of platelet-derived fVIII by the GPIIb and the GPIbα promoter is efficient for the correction of the hemophilia A phenotype. To address if VWF is necessary for fVIII storage in the α-granules of platelets, the transgenic GPIbα were crossed onto a VWF^{null} background. Despite the lack of VWF, fVIII was localized in the α-granules and was effective at making an improvement in the phenotype of hemophilia A fVIII knockout mice (Yarovoi *et al.*, 2005). In addition, fVIII in the α-granules of platelets was demonstrated to be protected from inhibitory antibodies to fVIII by 6-fold (Gewirtz *et al*., 2008). The restriction of fVIII to platelets has also been observed to serve as a protective measure in regards to the production of inhibitory antibodies to the fVIII transgene. No inhibitory antibodies were observed following a bone marrow transplantation from the transgenic GPIIb mice into fVIII knockout mice that yielded similar levels of fVIII activity and phenotypic correction (Shi *et al.*, 2006). For these reasons, this same group designed a lentiviral vector to transduced bone marrow mononuclear cells as a source of HSCs. The GPIIb promoter was incorporated within the vector as an internal promoter directing the expression of the B-domain deleted human fVIII transgene (Shi *et al.,* 2007). In order to assess the effectiveness of platelet-derived fVIII in the presence of inhibitory antibodies to human fVIII, recipient mice were preimmunized with fVIII prior to transplantation by receiving weekly intravenous injections of recombinant human fVIII (50U/kg). Despite the presence of inhibitors to fVIII, long-term engraftment of the genetically modified HSCs was established following both myeloblative (1100cGy) and nonmyeloblative conditioning regimens (660cGy).

Chromogenic assays revealed active fVIII within platelets that was sustained over time that resulted in phenotypic correction of hemophilia A (Shi *et al.*, 2008). A similar report was published by the same group recently in which minor changes were made to the transduction/transplantation protocol. Instead of utilizing bone marrow mononuclear cells, sca-1^{+} cells were isolated and transplanted into lethally and sublethally irradiated recipient mice. As compared to a parallel non-inhibitor model, fVIII activity was similar and found to be sustained over time at approximately 1.56 mU/10^{8} platelets. Phenotypic correction was found to be achieved at these levels by an electronically induced femoral injury and tail-clip assay. In addition, nonmyeloblative conditioning regimens were used which included busulfan (25-12.5 mg/kg at two and one day prior to transplantation) and anti-(murine)-thymocyte globulin (10mg/kg at two days prior to transplantation) treatment. fVIII activity was undetectable in busulfan alone treated recipients, however the inclusion of anti-(murine)-thymocyte globulin to the conditioning regimen boosted the level of fVIII to that of 1.08 mU/10^{8} platelets (Kuether *et al.*, 2012). Therefore, an HSC gene therapy protocol utilizing a lentiviral vector enconding the GPIIb internal promoter is a viable option for the treatment of individuals with hemophilia A with pre-existing inhibitors to fVIII.

The utilization of platelet-specific promoters for directed expression of fVIII is a plausible tool to be incorporated into an HSC gene therapy application for the treatment of hemophilia A. The GPIIb and the GPIbα platelet-specific promoters have demonstrated efficient expression of fVIII confined in the α-granules of platelets in the absence of vesicular injury. In both cases, curative levels of fVIII were produced as was

evident by phenotypic correction of hemophilia A fVIII knockout mice. However, ectopic expression of fVIII from platelets has been associated with clot instability as a result of temporal and physical maldistribution of fVIII (Neyman *et al.*, 2008). In order for fVIII to be available for the process of secondary hemostasis, platelet degranulation has to occur, a process that begins at the base of the clot and slowly spreads throughout the clot. Thus fVIII is released from platelets in an unconcerted manner. This may be the reason for the increased rate of embolism observed upon phenotype analysis following both a cuticular bleeding model and a cremaster laser arteriole/venule injury model (Greene *et al.*, 2010). Stability of a clot needs to be addressed before these promoters can be utilized in the clinic.

Another cell target, which has been considered, is skeletal muscle cells. Skeletal muscles cells are an ideal target for *in vivo* gene transfer in that skeletal muscle cells are terminally differentiated and provide a consistent source of fVIII persisting throughout the lifetime of an individual. For these reasons, Jeon *et al*. (2010) injected 10^7 lentiviral particles intramuscularly into the thigh of rats and found plasma fVIII levels to increase slightly above that of control mice for up to 4 weeks before deteriorating. Despite the need for follow up studies in order to achieve therapeutic levels of fVIII with this strategy, the results suggested that the *in vivo* administration of a lentivirus targeted at skeletal muscle cells may be an effective strategy for the treatment of hemophilia A.

Most *in vivo* strategies, however, are aimed at targeting hepatocytes, an endogenous producer of fVIII. Lentivirus is administered via either the portal vein or intravenously.

Unfortunately this strategy in the case of fVIII (a protein with extensive immunogenic properties), results in the development of anti-fVIII antibodies due to the possible transduction of antigen-presenting cells. To overcome this, a miRNA sequence was incorporated downstream of the WPRE sequence which would prevent expression in hematopoietic cells (including cells which make up the immune system). This method was found to be very effective in eliminating a fIX immune response (Brown *et al*., 2007), but alone was unable to do so with fVIII. Instead the fVIII-miRNA lentivirus had to be pseudotyped with the baculovirus envelope glycoprotein GP64, which has been shown to restrict transduction away from hematopoietic stem cells (Schauber *et al*., 2004). Combined, the miRNA incorporation and the GP64 pseudotyping were able to restrict fVIII expression to the liver, eliminating the presence of inhibitors, while resulting in about 9% of normal levels of fVIII (0.1U ml^{-1}), which was sustained in mice for a total of 60 weeks (Matsui, 2011). This study was significant in that it modified the current *in vivo* lentiviral gene transfer of fVIII making it safer by restricting expression in the liver.

In contrast to focusing on transferring the missing or malfunctioning gene, constructs that encode proteins that can bypass the missing protein can be used to overcome the difficulties associated with the size of the transgene. In the case of hemophilia A, a smaller gene such as fVII can be used to bypass the need for fVIII. fVII is an extrinsic pathway coagulation factor that along with thromboplastin initiates the blood coagulation proteolytic cleavage cascade (**Figure 1.2**) and has been shown in a recombinant form to be an alternative treatment for hemophilia A (Jurlander *et al*., 2001). For these reasons,

Ohmori *et al.* (2008) transduced HSCs *ex vivo* with a SIV-based lentiviral vector encoding an activated form of fVII expressed from a platelet specific promoter (the GPIbα promoter). As a result, fVII was found to localize to the cell surface following platelet activation in transplanted fVIII-deficient mice. Due to species-specific interactions, the murine TF was unable to interact with the human form of fVII resulting in unimproved hemophilia conditions in the fVIII-deficient mice. However, when a murine fVII was incorporated into the SIV-based lentiviral vector, the clot time and rate of clot formation were significantly reduced, decreasing the mortality rate after tail clipping (Ohmori *et al*., 2008). In addition, fVII was recently incorporated into an adeno-associated vector (AAV-8) being able to overcome the encapsidation limitations of the vector due to the shorter size of fVII. Yet it was shown that in hemophilic dogs, large doses of vector were required to be efficacious (Margaritis *et al*., 2009). Therefore, a bioengineered fVII variant with enhanced intrinsic activity was recently utilized in order to reduce the dose of *in vivo* administered vector. However, adverse thrombotic effects were observed in treated mice which limits enthusiasm for this therapy (Margaritis *et al*., 2011).

In our laboratory, variations of the fVIII transgene have been studied extensively. It was found that expression was increased up to 100 fold utilizing a B domain deleted porcine fVIII transgene (Doering *et al*., 2002). High level fVIII expression was sustained for ten months in hemophilia A mice following transplantation of MSCs modified to contain the porcine fVIII sequence (Gangadharan *et al*., 2006). A subsequent study revealed that high level fVIII expression could be achieved even after low-toxicity pretransplantation

conditioning (Ide *et al*., 2007). Together these studies demonstrated the ability of high-expression porcine sequence elements to function *in vivo* for the correction of fVIII deficiency in the context of *ex vivo* HSC genetic modification. Human/porcine fVIII chimeras were then constructed for the purpose of determining which porcine fVIII domains were responsible for the increased expression. The chimera constructs revealed sequences in the A1 and A3 domains to be responsible for enhanced secretion of porcine fVIII (Doering *et al*., 2004, Dooriss *et al*., 2009). These findings resulted in construction of a high expression chimeric transgene that was utilized to genetically modify HSCs *ex vivo* via lentiviral vectors. The transplantation of these cells resulted in therapeutic levels of fVIII in hemophilia A mice (Doering *et al*., 2009). The chimeric fVIII transgene holds much promise for the gene therapy field in regards to the treatment of hemophilia A.

1.6— Limitations of Gene Therapy

Since the birth of the concept of gene therapy in the early seventies, the field has experienced tremendous highs and lows. A significant number of individuals have experienced clinical benefit as a result of gene therapy. However, these advancements have been overshadowed by highly publicized setbacks. News feeds associated with the death of Jesse Gelsinger, an eighteen year old admitted in a gene therapy trial at the University of Pennsylvania, described the journey of gene therapy as one that has been tainted with a history of hype and unfulfilled promises. This statement was made despite successful reports in the same year from an X-SCID clinical trial in Europe demonstrating improved immune systems in children. It is important to note that this setback and others including the appearance of insertional mutagenesis as a result of the

retrovirus utilized in the X-SCID clinical trial have been treated with sensitivity and resulted in improvements in the current understanding of the technology and methods used during gene replacement. The public perception of gene therapy, in my opinion, has been affected by the disparaging reports unfairly represented by the media. Sadly, the scientific community is not immune to these misconceptions. Upon deciding to join a gene therapy laboratory, a number of my colleagues questioned my involvement in a "dying field." However, these concerns were raised prior to progress made in the field which has resulted in successful gene therapy trials for hemophilia B, AIDS, cancer, X-SCID, adenosine deaminase deficiency, Wiskott-Aldrich syndrome, β-thalassemia and adrenoleukodystrophy (**Table 1.2**).

Table 1.2 – Clinically Beneficial Gene Therapy Trials for Monogenic Diseases

Disease	Vector	Treated	Outcome
X-SCID	γ-retrovirus	20	Full or nearly full correction in 17 patients Normal T-cell subset counts Normal T-cell-mediated immune functions Five developed T-cell leukemia Four in remission following chemotherapy (Cavazzana-Calvo *et al.* 2000; Hacein-Bey-Abina *et al*., 2008)
ADA-SCID	γ-retrovirus	40	Increase of lymphocyte counts Improvement of cellular and humoral responses Enzyme replacement therapy halted in 29 patients No leukemic or oncogenic event (Aiuti *et al*., 2009; Gaspar *et al*., 2011)
WAS	γ-retrovirus	10	Significant clinical benefit Resolution of hemorrhagic diathesis, eczema, autoimmunity and predisposition to recurrent infections One patient developed T-cell leukemia (Boztug *et al.*, 2010)
X-ALD	SIN-lentivirus	2	Cerebral demylenation arrested No clonal dominance or leukemic / oncogenic event (Cartier *et al., 2012)*
β-thalassemia	SIN-lentivirus	1	Stable hemoglobin levels One-third contains vector encoded β-globin Transfusion independent No leukemic / oncogenic event (Cavazzana-Calvo *et al*., 2010)
Hemophilia B	AAV-8	6	FIX levels 2-11% of normal Four discontinued FIX protein replacement therapy Other two had longer spans between prophylaxis At highest vector dose, slight and transient liver-enzyme levels which normalized after glucocorticoid treatment (Nathwani *et al*., 2011)

Abbreviations: X-SCID (X-linked severe combined immunodeficiency), ADA-SCID (adenosine deaminase deficient severe combined immunodeficiency), WAS (Wiskott-Aldrich Syndrome), X-ALD (X-linked adrenoleukodystrophy)

In the field of gene therapy, hemophilia A and B are among the most extensively researched monogenic diseases. Hemophilia A and B are X-linked bleeding disorders attributed to the loss of fVIII and fIX respectively. Hemophilia B is not as prevalent as hemophilia A affecting approximately 1 in 30,000 males. However the size of the fIX transgene is significantly smaller than fVIII with a cDNA of 2.8-kb. The adeno-associated viral (AAV) vector is able to withstand the small size of the fIX transgene which poses as a safer vector existing predominantly as a concatemer of episomes extrachromosomally in cells. Minimal integration with the use of an AAV-based vector reduces the risk of insertional mutagenesis. In addition, long-term expression can be maintained if non-dividing cells are targeted. Since the liver is the main physiological site of fIX synthesis, liver-directed gene transfer of fIX poses an ideal strategy for the treatment of hemophilia B. The first clinical trial for hemophilia B utilized the most prevalent serotype of AAV, AAV-2 which was infused via the hepatic artery. However, this trial presented a number of obstacles to the therapy including the possibility of vertical transmission as a result of transient vector DNA present in the sperm of subjects. This obstacle appeared to be dose-related and could potentially be diminished by the use of lower doses of administered vector. The presentation of humoral and cellular immunity to the vector itself, however, presented itself as the greatest obstacle to be overcome. A number of subjects admitted to the trial prior to admittance contained neutralizing antibodies to AAV, a consequence of utilizing a vector derived from a human pathogen. These antibodies blocked the transduction of target cells and have resulted in the modification of participation requirements for future trials utilizing an AAV-based vector. In addition, hepatocytes that were transduced were eliminated by

memory T cells due to the presentation of AAV capsid-derived antigens resulting in transient fIX expression. Since the first trial, serotypes of AAV have been identified that are capable of transducing hepatocytes more effectively (AAV-8) and thus eliminate the destruction of transduced hepatocytes due to diminished T cell activation. Therefore, a clinical trial was performed utilizing an AAV-8 vector. In addition potential improvements were made to the vector in regards to transduction, the AAV-8 vector was engineered to overcome the limiting transduction step of AAV (the conversion of the single-stranded genome to that of a double-stranded AAV genome) as a self-complementary, double-stranded AAV-8 vector. A total of six subjects with severe hemophilia B were included in the trial and all six subjects experienced levels of fIX above that of a therapeutically relevant increase in fIX (Nathwani *et al*., 2011). The increase in fIX in these subjects drastically changed their lifestyle. Yet more importantly, this was the first trial for hemophilia B that demonstrated sustained levels of fIX representing a significant advancement in the field of gene therapy. Improvements are still needed before this therapy can become widespread, since patients admitted into the trial are still receiving infusions of fIX. Preclinical reports are surfacing in which the specific activity of fIX is increased due to improvements in the transgene. It is likely that the next generation of clinical trials for hemophilia B will include the use of an enhanced fIX transgene.

Gene therapy protocols for the treatment of Hemophilia A, on the other hand, have been more difficult to manage due to the large size of fVIII. The B domain deleted fVIII cDNA is 4.5-kb leaving very little room for the inclusion of regulatory elements to be

packaged in an AAV-based vector. Nonetheless, preclinical attempts have been made to replace the fVIII gene with the use of an AAV-based vector (Burton *et al*., 1999; Chao *et al*., 2003). However when translated to large animal models, therapeutic levels of fVIII can only be achieved upon administration of vector ten-fold higher than the highest dose administered thus far in humans. It is likely that a dose this high will not be tolerated in humans due to the induction of an immune response to the AAV-based vector. For this reason and due to the limited carrying capacity of adeno-associated viral vectors, a lentiviral based vector has been considered for gene therapy applications for the treatment of hemophilia A. The carrying capacity of lentiviral vectors reaches 7-kb, large enough to encompass the B domain deleted fVIII cDNA and relevant promoters. A lentiviral vector is an ideal choice for the genetic modification of HSCs *ex vivo*. However, the utilization of a lentiviral vector does not come without obstacles. First, the production of lentiviral vectors containing an HP-fVIII transgene is limited in HEK-293T cells. For a yet unknown reason, lentiviral titers from vectors containing an HP-fVIII transgene are considerably lower than lentiviral vector titers containing a GFP transgene. Consistently HIV-based lentiviral vectors containing a GFP transgene produced by transient transfection in HEK-293T cells yield titers of approximately 10^8 while vectors containing the HP-fVIII transgene yield titers of 10^7. It has been suggested that the decrease in viral titer correlates to the size of the transgene. The larger the transgene the less viral particles produced (Kumar *et al*., 2001; Yacoub *et al*., 2007). This obstacle may need to be overcome in order to produce a significant amount of vector for a gene therapy protocol aimed at treating a substantial portion of the population affected by hemophilia A. Compounding upon this obstacle is the inefficiency of a lentiviral vector at

transducing HSCs. In theory, only one HSC would need to be modified and engrafted in order to provide a life-long cure to hemophilia A. However the target $CD34^+$ population includes both stem cells and early progenitors of the stem cell. It is possible that the early progenitors present in the $CD34^+$ population are more readily transduced by the lentiviral vector than the pure stem cell, monopolizing the transduction of the lentiviral vector. The sole modification of early progenitors would not attribute to expression of the HP-fVIII transgene over a life-time. Instead, any benefit observed as a result of gene therapy would only be transient due to the shortened persistence of early $CD34^+$ progenitors as compared to the self-renewing stem cell. Therefore, the modification of self-renewing $CD34^+$ stem cells is vital to the success of a gene therapy protocol aimed at modifying HSCs. For this reason, much emphasis in the field has been placed on delineating methods whereby the transduction efficiency of HSCs can be improved. This dissertation has focused on the obstacles presented by the use of a lentiviral vector in a gene therapy protocol designed for the treatment of hemophilia A.

Chapter 2:

Pharmacological Evaluation of Lentiviral Vector Optimized for fVIII Expression

2.1— Introduction

As previously mentioned in chapter 1, hemophilia A is a monogenic disease caused by mutations in the gene encoding fVIII, resulting in the inability to properly form a clot. Hemophilia A is a prime candidate for gene therapy in that only a moderate increase in fVIII (2-5% of normal equating to 2-5 ng ml^{-1}) is required to be therapeutically effective. In addition, the current treatment, consisting of repetitive prophylactic administration of recombinant fVIII as a means of protein replacement, is expensive, invasive and does not always result in patient compliance. Further complicating the current therapy, 30% of individuals with severe and moderately severe hemophilia develop an immunogenic response in the form of neutralizing antibodies against the administered fVIII (Ehrenforth *et al*., 1992). These inhibitors make managing a bleeding episode extremely complicated. Therefore, new therapeutic approaches are needed to treat hemophilia A. fVIII gene therapy attempts to rectify the presence of a mutant F8 gene with either the addition of a functional gene or correction of the original gene. In the case of gene addition, delivery of the fVIII gene is not restricted to a certain cell type because, theoretically, any tissue with exposure to the vasculature is suitable as a cellular target. In addition, the therapeutic window is large, as fVIII levels as high as 150% of normal have not been associated with adverse effects such as thrombosis (VandenDriessche *et al*., 2001). As a result, a number of viral and non-viral delivery strategies have been postulated.

Despite promising preclinical data, hemophilia A gene therapy clinical trials have not progressed past phase 1 trials due to limited expression of fVIII (Roth *et al*., 2001; Powell *et al*., 2003; Berlfein, 2003). Although each trial was unique in regards to the cell

type modified and the viral vector used, all trials yielded less than therapeutic levels of fVIII. To overcome low level transgene expression, we recently compared several high-expression fVIII transgene sequences and demonstrated enhanced expression of a B domain deleted porcine fVIII sequence, both *in vitro* and *in vivo* (Ide *et al*., 2007; Doering *et al*., 2007; Dooriss *et al*., 2009). Upon comparison of a series of hybrid human/porcine cDNAs, the domains responsible for the high expression characteristics of porcine fVIII were identified as the A1 and A3 domains (Doering *et al*., 2004). These findings resulted in the construction of a high expression human/porcine transgene. The bioengineered construct was then introduced via lentiviral vectors into hematopoietic stem cells (HSC) *ex vivo* and used to effectively treat fVIII knockout mice with hemophilia A, yielding therapeutic levels of fVIII (Doering *et al*., 2009). Further optimization resulted in the inclusion of the porcine C1 domain and three alanine substitutions in the A2 domain in order to reduce immunogenicity (Healey *et al*., 2009; Lubin *et al*., 1997). The final high expression B domain deleted fVIII transgene (HP-fVIII) contains human A2 and C2 domains in addition to porcine A1, A3 and C1 domains. This HP-fVIII has been shown to maintain the high expression characteristics of porcine fVIII (Doering *et al*., 2009).

Lentiviral vectors are promising vectors for the delivery of the fVIII transgene because they provide stable integration and are able to transduce both dividing and non-dividing cells (Naldini *et al*., 1996). Lentiviral vectors, unlike adeno-associated viral vectors, are less constrained by the size of the transgene. Vector size constraints are an issue for fVIII gene therapy since the B domain-deleted fVIII cDNA is approximately 4.4-kb (for

review see Johnston *et al*., 2011). For these reasons, lentiviral vectors are reasonable for gene therapy applications aimed at the treatment of hemophilia A utilizing *ex vivo* modification of HSCs. In lentiviral vectors, a woodchuck post-transcriptional regulatory element (WPRE) is routinely added to the 3' end of the transgene. The inclusion of this sequence is for increased transgene expression, as it has been demonstrated that a two to fivefold increase in expression is achieved with a WPRE sequence, which in part is due to an increased export of mRNA and possibly due to facilitating transcript processing (Zuffery *et al*., 1999; Brun *et al.*, 2003; Gonzalea-Murillo *et al.*, 2010). However, a recent report showed that enhanced transgene expression in the presence of a WPRE sequence was dependent on the promoter and cell line used, where in some instances the inclusion yielded no increase or decreased expression (Gonzalea-Murillo *et al*., 2010; Klein *et al*., 2006). In addition, the WPRE codes for the first 60 amino acids of the hepadnavirus X protein, a protein that has been linked to oncogenesis (Kingsman *et al*., 2005). Therefore, the function of the WPRE appears to be more complex than originally assumed and may need to be evaluated in conjunction with individual transgenes, which has not been done for fVIII.

Previous studies have focused on optimizing the fVIII transgene for enhanced fVIII expression (for review see Doering and Spencer, 2009), but the components of the viral vector system have not been as well characterized. The studies presented in this chapter focuses on optimizing the lentiviral vector for virus production, transduction efficiency, and transgene expression with the use of a fVIII transgene that has been bioengineered

for high level fVIII expression (HP-fVIII). The optimized lentiviral vector was utilized in *ex vivo* HSC transduction studies to determine *in vivo* HP-fVIII expression.

2.2— Materials and Methods

Reagents

Dulbecco's Modified Eagle's medium (DMEM)/F-12, Aim V medium and StemPro-34 serum-free medium were purchased from Invitrogen Life Technologies (Carlsbad, CA). Heat-inactivated fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). Penicillin-streptomycin solution was purchased from Mediatech (Manassas, VA). Cell transfections were performed with polyethyleneimine purchased from Fisher Scientific (Pittsburg, PA). Plasmids utilized for viral preparation were isolated from bacterial stocks utilizing QIAGEN Hispeed midiprep plasmid kits (Valencia, CA). Nucleic acid isolation kits were purchased from QIAGEN. Integration events were analyzed using a qPCR SYBR Green Low Rox master mix from Thermo Fisher Scientific (Waltham, MA) an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) and oligonucleotide primers synthesized by Integrated DNA Technologies (Coralville, IA). HP-fVIII RNA was quantifed utilizing fVIII RNA standards generated with an mMessage mMachine kit (Ambion, Austin, TX). Northern blots were performed with the digoxigenin nonradioactive nucleic acid-labeling and detection system (Roche, Indianapolis, IN). Human fVIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KS). Automated activated partial thromboplastin (APTT) reagent was purchased from BioMérieux (Durham, NC). Clotting times were measured using an ST art

Coagulation Instrument (Diagnostica Stago, Asnieres, France). Anti-pfVIII and anti-hfVIII monoclonal antibodies were a kind gift of Dr. Pete Lollar (Aflac Cancer Center and Blood Disorder Services, Emory University, Atlanta, GA). Sca-1^+ cells were isolated using magnetic separation columns purchased from Miltenyi Biotec (Auburn, CA). Exon 16-disrupted hemophilia A mice have been previously described (Bi *et al*., 1995). All antibodies for flow cytometry were purchased from BD Pharmingen (San Diego, CA).

Vector production

Expression plasmids containing the HP-fVIII or eGFP gene along with the necessary packaging plasmids were transiently transfected into HEK-293T cells utilizing polyethylenimine (6 µg PEI/1 µg DNA). A 2:1:1 ratio of expression plasmid to packaging plasmids (expression plasmid:psPAX2:pVSVG) was used to manufacture research-grade HIV-based lentiviral vectors in the LentiMax production system. Research-grade SIV-based lentiviral vectors were manufactured using a 1.3:1:1:1.6 ratio of expression plasmid to packaging plasmids (expression plasmid:pCAG4:pVSVG:pSIV). One day after transfection, the media was replaced with DMEM-F12 containing 10 % fetal bovine serum and 1 % penicillin/streptomycin. Conditioned medium from the HEK-293T viral producing cells was collected for the following three days, passed through 0.45 µmol l^{-1} filter and stored at -80^{o}C until concentration. Virus was concentrated by velocity sedimentation upon centrifugation at 10,000g (4^{o}C) overnight. Viral pellets were resuspended in 1/100th of the original volume of StemPro media, and filtered through a 0.22 µmol l^{-1} filter. Viral titer was

assessed on HEK-293T cells with increasing vector volumes by real-time quantitative PCR (qPCR) seventy-two hours after viral addition. Unconcentrated viral supernatant had titers ranging from $5x10^5$ to $2x10^6$ transducing units ml^{-1} (TU ml^{-1}). Concentrated virus ranged from $5x10^7$ to $1x10^8$ TU ml^{-1}. Virus was stored in 1 ml aliquots at -80^{o}C.

qPCR is a highly sensitive reaction that could overestimate viral titer if residual plasmid remained as a consequence of viral preparation. Therefore, plasmid contamination as a was assessed in order to confirm the viral titers generated by qPCR. Virus was pretreated with 50 units of benzonase per ml of virus for 15 minutes as previously described (Sastry *et al*., 2004). Prior to viral transduction, 100,000 HEK-293T cells were plated in a six well tissue culture treated plate and given 24 hours to adhere to the plate. At the time of viral addition, approximately 200,000 HEK-293T cells were transduced with viral containing media with or with benzonase treatment. DNA was isolated from the cells over time resulting in DNA samples at 24, 48, 72, 240 and 288 hours post transduction. Each time point was performed in triplicate. qPCR analysis was performed on the DNA isolated from each condition. Transgene copies/cell were similar despite benzonase treatment yielding a fold difference near one suggests a lack of plasmid contamination in the viral containing media (**Table 2.1**).

Table 2.1 – The effect of benzonase treatment on copy number analysis

Hours Post Transduction	Fold Difference
24	0.729
48	0.668
72	0.805
240	1.012
288	0.818

Transgene copies/cell were determined at the indicated hours post viral addition by qPCR analysis. The fold difference was determined by dividing the transgene copies/cell from the nontreated wells by the transgene copies/cell of the benzonase treated wells.

Measurement of HP-fVIII transgene copy number

Total genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacture's protocol for cultured cells. DNA was quantified with a spectrophotometer at an absorbance of 260 nm. To determine transgene copy number, 50 ng of each sample was added to a 25 µl real-time quantitative PCR reaction containing 1x SYBR green PCR master mix (Thermo Fisher Scientific, Surrey UK) and 0.01 µM forward and reverse primers. HP-fVIII specific primers annealing to the A1 porcine domain were utilized: forward primer, 5'- CAG GAG CTC TTC CGT TGG -3' at position 164 and reverse primer, 5'- CTG GGC CTG GCA ACG C -3' at position 239. C_t values for each sample were compared to C_t values produced from plasmid standards of known copy quantities. The equivalent copy number was then divided by 8333, the predicted number of genome equivalents in 50 ng of DNA.

To assess the appropriate time following transduction in which to perform the *in vitro* copy number analyses, a time course analysis of copy number was performed in EU1, BHK-M and HEK-293T cells. Approximately 250,000 EU1 (**Figure 2.1 A**) and BHK-M cells (**Figure 2.1 B**) were transduced with SIV-based lentiviral vectors at an MOI of 5. DNA was isolated throughout the analysis as indicated. Each time point was performed in triplicate. Transgene copies/cell were determined by qPCR as previously described. Grey arrows represent when cells were passaged. A gradual decay in copy number is apparent in both EU1 and BHK-M cells that stabilizes 72 hours post transduction (**Figure 2.1 A-B**). The decay observed is consistent with the loss of non-integrated vector over time. A similar protocol was used to assess copy number over time in K562 cells

(performed previously by Kerry Dooriss). A similar decay profile was apparent in K562 cells as was observed in EU1 and BHK-M cells (data not shown).

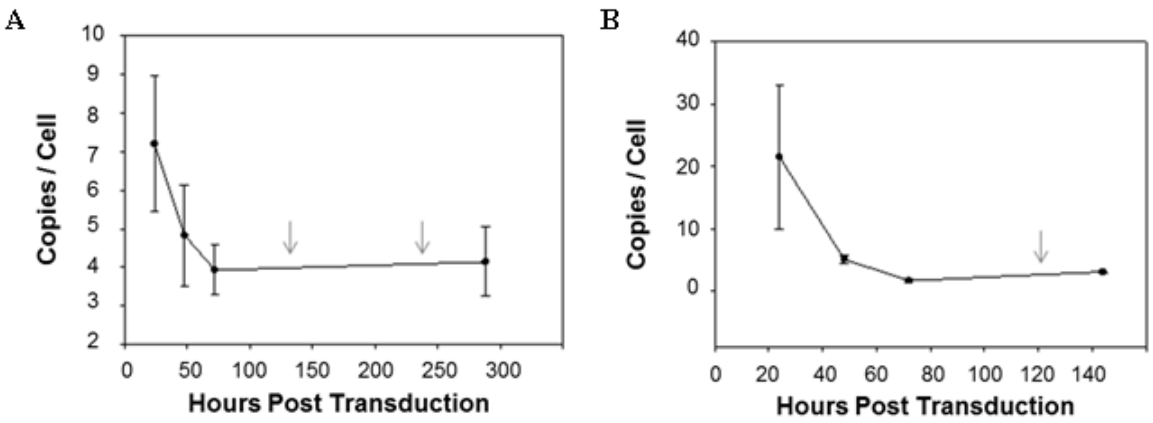


Figure 2.1 Copy number analysis over time reveals loss of non-integrated lentiviral vector by seventy-two hours following viral transduction. (A) EU1 and (B) BHK-M cells were transduced with an SIV-based lentiviral vector at an MOI of 5. DNA was isolated at the indicated time points and assessed for transgene copy number by qPCR. Each time point was performed in triplicate.

Measurement of HP-fVIII transcript expression

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacture's protocol for animal cells 72 hours post transduction. RNA was then quantified spectrophotometrically at an absorbance of 260 nm. HP-fVIII transcripts were measured by quantitative RT-PCR (qRT-PCR) utilizing a porcine fVIII RNA standard as previously described (Doering *et al.*, 2002). qRT-PCR reactions were carried out in 25 µl containing 1x SYBR green PCR master mix, 300 µM forward and reverse primers, 12.5 units MultiScribe, 10 units RNase inhibitor, and 5 ng of sample RNA. Reactions containing the porcine fVIII RNA standard also included 5 ng of yeast tRNA, mimicking the RNA environment of the sample RNA. The oligonucleotide primers utilized for the qRT-PCR reaction annealed in the A2 domain of the the fVIII cDNA sequence at positions 1897-1917 for the forward primer (5'-ATGCACAGCATCAATGGCTAT-3') and at positions 2044-2063 for the reverse primer (5'-GTGAGTGTGTCTTCATAGAC-3'). One-step real-time qRT-PCR was performed by incubation at 48^{o}C for 30 min for reverse transcription followed by one cycle at 95^{o}C for 10 min and 40 amplification cycles of 90^{o}C for 15 sec and then at 60^{o}C for 1 min. Postreaction dissociation was performed to confirm single-product amplification. C_t values for each sample were compared to C_t values produced from the porcine fVIII standards having known transcript quantities.

Measurement of HP-fVIII activity from cell lines

fVIII activity was measured from the supernatant of cells cultured in AIM V media for 24 h before the assay as previously described (Doering *et al.*, 2002). In short, the APTT

reagent-based one stage coagulation assay was performed in duplicate for each supernatant on a ST art Coagulation Instrument in human fVIII-deficient plasma. The clot time for each sample was compared to a standard curve based on dilutions of FACT.

Animals

Exon-16 deleted hemophilia A mice were originally obtained from Dr. Leon Hoyer (Holland Laboratories, American Red Cross, Rockville, MD) by Pete Lollar. The colony has been maintained at Emory University (Atlanta, GA) since 1998. B6.SJL (CD45.1) mice were acquired from Dr. David Archer (Emory University, Atlanta, GA). Both strains were maintained at the animal care facility of Emory University. All procedures were approved by the Institutional Animal Care Committee at Emory University.

Isolation and transduction of murine stem cell antigen-1+ cells

Whole bone marrow was flushed from the femurs and tibias of 8- to 10-week-old CD45.1 mice and then subjected to positive immunomagnetic bead selection. The isolated sca-1^+ cells were cultured at a density of 10^6 cells per ml in StemPro media supplemented with L-glutamine (29 μg ml^{-1}). For 3 days before transduction, the cells were stimulated with murine stem cell factor (100 ng ml^{-1}), murine interleukin-3 (20 ng ml^{-1}), human interleukin-11 (100 ng ml^{-1}), and human Flt-3 ligand (100 ng ml^{-1}). Cells were transduced twice (MOI 15), 8 hours apart, and transplanted via tail-vein injection the following day into lethally irradiated (11*Gy* TBI using a Gammacell 40 Exactor) 8- to 10-week-old recipient hemophilia A mice (CD45.2). Blood was collected from the transplanted mice every two weeks retro-orbitally and fVIII was measured using an

ELISA specific for HP-fVIII. The ELISA only detects properly folded HP-fVIII in that the primary antibody detects the heavy chain (human A2 domain) and the secondary antibody detects the light chain (porcine A3 domain). In addition, HP-fVIII was detected from the plasma using a commercially available chromogenic substrate assay (COATEST SP FVIII) as previously described (Doering *et al.*, 2007).

Statistical analysis

Results are presented as mean ± standard deviation. A student's t-test was used to evaluate a significance of difference between two groups. For comparisons between more than two groups, a one way ANOVA was used. A p value less than 0.05 was considered to be statistically significant.

2.3— Results

2.31— Assessment of the WPRE sequence

Assessment of the requirement for a WPRE element for the production of virus containing an HP-fVIII transgene

A recent report revealed that enhanced transgene expression as a result of a WPRE is dependent on both the promoter and cell line used, and should thus be assessed for individual transgene scenarios (Kingsman *et al.*, 2005). The WPRE was evaluated in the context of an optimized HP-fVIII transgene. Both SIV and HIV expression plasmids were constructed with and without a WPRE sequence (**Figure 2.2 A**) and used to produce recombinant viral vector from HEK-293T cells. Virus, with and without a WPRE, was generated in triplicate under identical conditions. Unconcentrated viral supernatant then

was used to transduce HEK-293T cells to assess viral titer. In this manner, viral production was analyzed from the context of a highly transducible cell line. DNA was extracted from the transduced cells and analyzed for viral copy number by qPCR. For both lentiviral vectors, assuming equal transduction efficiency, the absence of a WPRE did not affect viral production ($P = 0.436$ for SIV and $P = 0.309$ for HIV) (**Figure 2.2 B**).

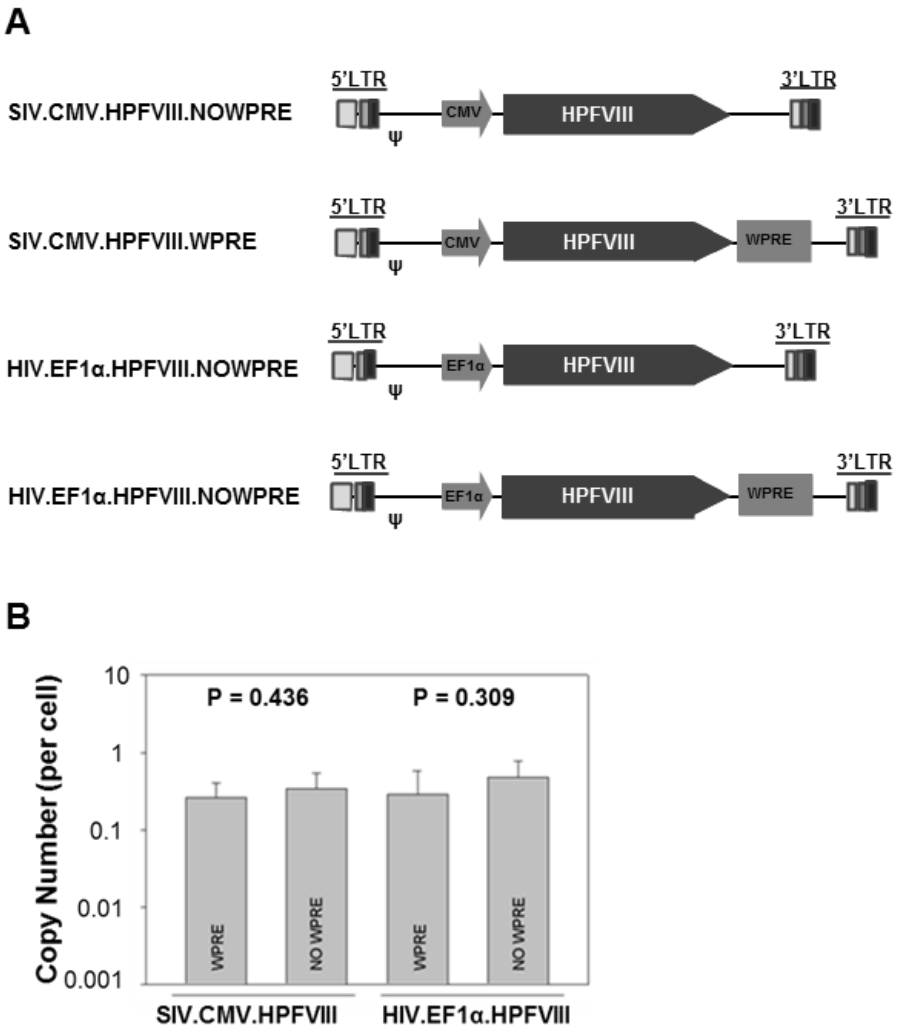


Figure 2.2 Evaluation of the WPRE sequence in SIV and HIV vector systems in regards to viral production. (A) Schematic representation of SIV- and HIV-based lentiviral vectors. (B) Recombinant virus was produced under identical conditions (n = 3). Unconcentrated viral supernatant (0.5 ml) was used to transduce HEK-293T cells. Seventy-two hours later, genomic DNA was isolated from the transduced cells and used to assess viral copy number by qPCR. Each bar represents the mean ± standard deviation of three wells measured in duplicate. *P* values were derived from a student's *t* test.

Evaluation of the expression of the HP-fVIII transgene from lentiviral vectors containing a WPRE element

Two independent internal promoters and vector systems were used to evaluate the effects of a WPRE on expression, a human elongation factor-1 alpha (EF1α) promoter in an HIV lentiviral system and a cytomegalovirus (CMV) promoter in a SIV lentiviral system. The HP-fVIII expression constructs were transiently transfected into HEK-293T cells. Forty-eight hours later, HP-fVIII expression was quantified using an APTT reagent-based one-stage coagulation assay. Comparison of HP-fVIII expression showed that the WPRE did not enhance expression of the transgene driven from either a CMV ($P = 0.757$) internal promoter or an EF1α ($P = 0.405$) (**Figure 2.3 A**). The lack of effect was further analyzed in a hematopoietic context in which two hematopoietic cell lines (EU1 and K562 cells) were transduced with the SIV-based lentiviral vector system. HP-fVIII expression was assessed at both the transcript and protein level. To correct for transduction efficiency, transcript and fVIII activity levels were normalized to copy number for each viral preparation. No enhancement in HP-fVIII transcripts was observed when a WPRE was included in the lentiviral vector (**Figure 2.3 B**). As shown in **Figure 2.3 C**, a similar observation was noted for the activity of expressed HP-fVIII from transduced K562 cells when normalized to copy number. However, significantly more activity was observed from EU1 cells that were transduced with a SIV viral vector devoid of a WPRE sequence than those that were transduced with a SIV virus containing a WPRE sequence ($P = 0.009$) (**Figure 2.3 C**). This relative enhancement was not observed upon normalization of HP-fVIII activity to transcripts (**Figure 2.3 D**). We have previously demonstrated a correlation between fVIII transcript number and fVIII activity (Doering *et al*., 2009).

However, less correlation has been documented between copy number and fVIII expression and would account for the lack of enhancement seen as a result of HP-fVIII activity normalization to transcripts (Spencer *et al*., 2011).

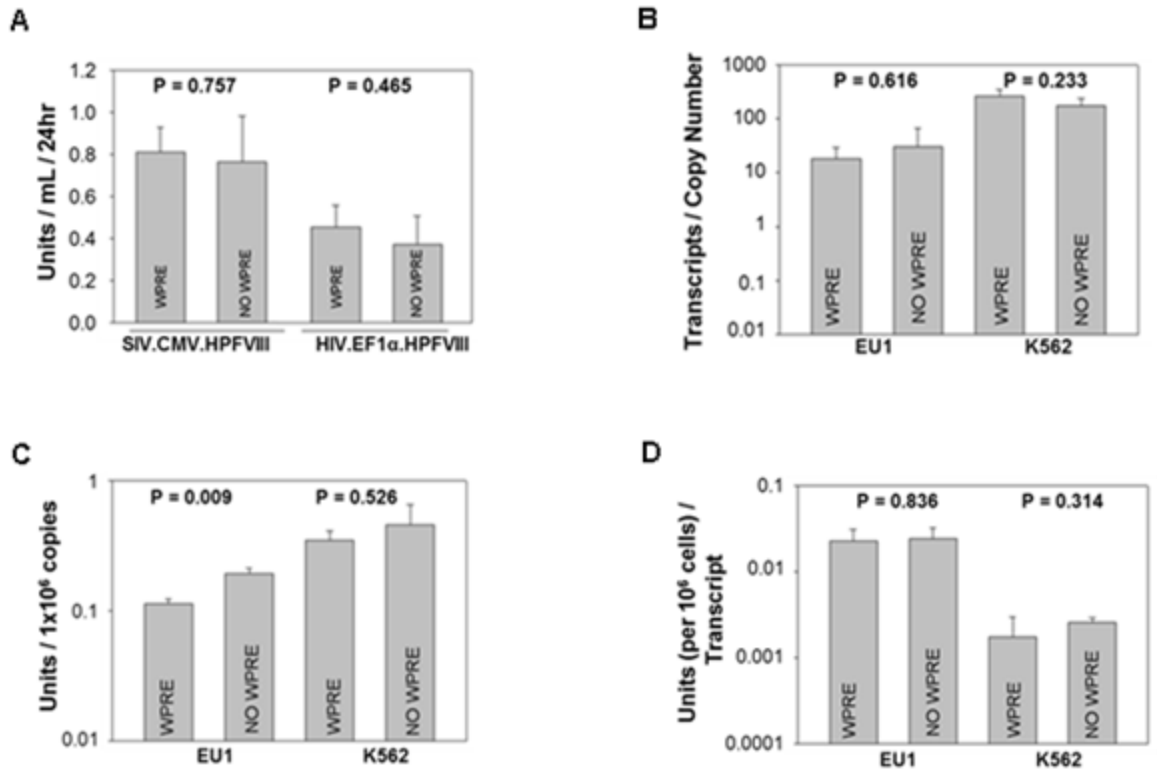


Figure 2.3 Evaluation of the WPRE sequence in regards to HP-fVIII expression. (A) To assess HP-fVIII activity, the expression plasmids were transiently transfected into HEK-293T cells. HP-fVIII activity was detected by an APTT-reagent based one-stage coagulation assay 48 hours post transfection. Twenty-four hours prior to the APTT-reagent based one-stage coagulation assay, conditioned media was replaced with AIM-V (serum-free). Each bar represents the mean ± standard deviation of six wells measured in duplicate. (B) An SIV-based lentivirus also was used to assess HP-fVIII expression in EU1 and K562 cells (MOI 1). Seventy-two hours post-transduction, genomic DNA and total RNA were isolated from the cells. HP-fVIII transcripts and HP-fVIII copy number was assessed by qRT-PCR and qPCR, respectively. (C) HP-fVIII activity was assessed 24 hour after exchange of conditioned media with serum-free AIM-V media by an

APTT-reagent based one-stage coagulation assay. (D) HP-fVIII activity was normalized to transcript levels. Each bar represents the mean ± standard deviation of three wells measured in duplicate. *P* values were derived from a student's *t* test.

Quantification of transcriptional read-through as a result of the presence of a WPRE sequence

It was suggested that the WPRE sequence provides a protective feature in viral vectors by safe guarding against insertional mutagenesis by reducing transcriptional read-through (Higashimoto, 2007). However, none of the constructs tested contained a transgene comparable to the length of HP-fVIII. Therefore, I designed constructs in which an internal ribosomal entry site (IRES) was placed after the 3' viral long terminal repeat (LTR) so that the green fluorescent protein (eGFP) could be expressed only as a result of transcriptional read-through (**Figure 2.4 A**). The IRES eGFP constructs, produced with and without a WPRE, were transiently transfected into HEK-293T cells and assessed for eGFP expression (96 hours after transfection) (**Figure 2.4 B and 2.4 C**). Although minimal transcriptional read-through was apparent, no statistically significant difference was found in the amount of transcriptional read-through when a WPRE sequence was present as compared to when it was removed ($P = 0.905$) (**Figure 2.4 C**). This observation was noted despite similar expression of HP-fVIII ($P = 0.891$) (**Figure 3.4 D**). Taken together, these data demonstrate that the WPRE does not enhance viral titer, transduction or HP-fVIII expression, and led to the removal of the WPRE element from both the HIV-based and SIV-based lentiviral vectors in subsequent studies.

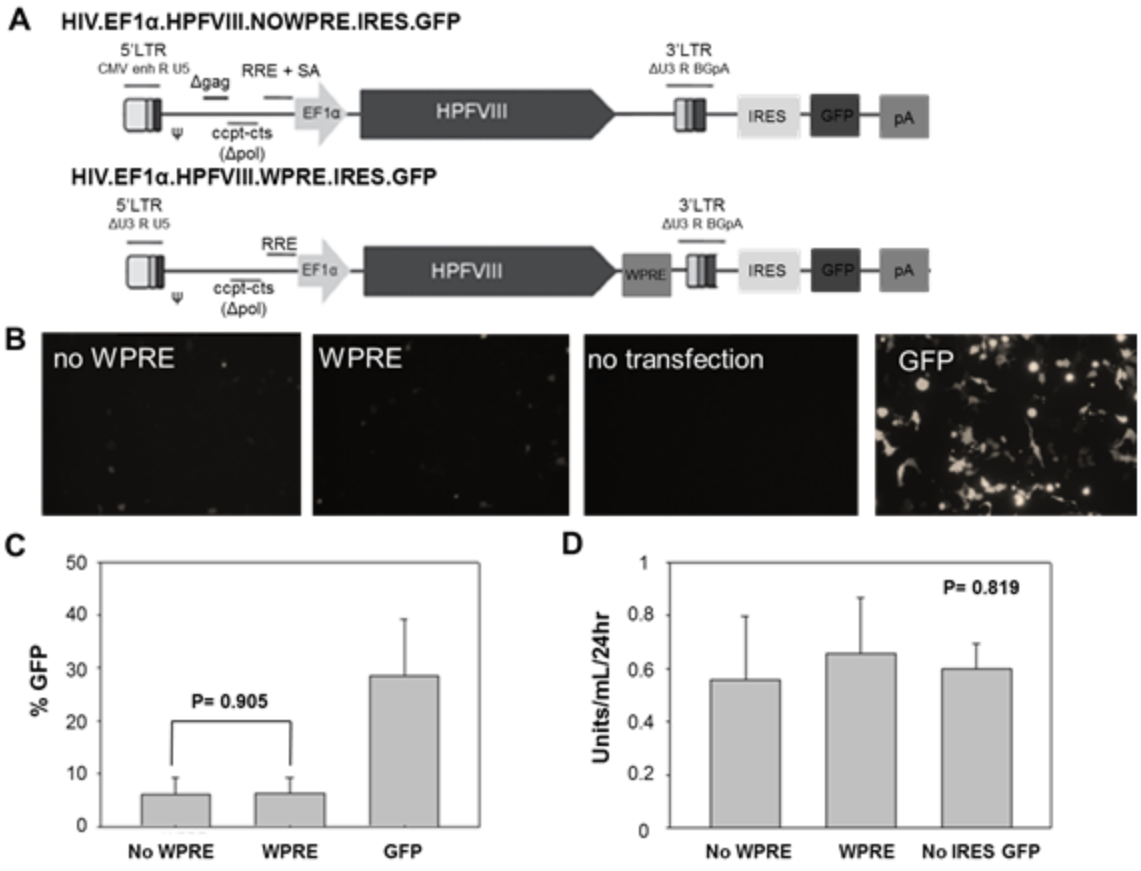


Figure 2.4 Evaluation of transcriptional read-through in the absence of a WPRE sequence. (A) Schematic representation of HIV-based expression plasmids transiently transfected into HEK-293T cells. (B) Fluorescent microscopy images taken 96 hours after transfection. (C)Transcriptional read-through was quantified by flow cytometry. (D) HP-fVIII activity was assessed 24 hours after exchange of conditioned media with serum-free AIM-V media by an APTT-reagent based one-stage coagulation assay. Each bar represents the mean ± the standard deviation of three wells measured in duplicate. *P* values were derived from a one-way ANOVA.

2.32— Comparison of three lentiviral vectors

Comparison of the transduction efficiency and HP-fVIII expression of SIV- and HIV-based lentiviral vectors containing the HP-fVIII transgene

To determine if there are inherent differences in HP-fVIII expression between SIV and HIV gene transfer systems, SIV- and HIV-based expression plasmids were produced in which the only differences were derived from the vector system itself (**Figure 2.5 A**). The HP-fVIII transgene was expressed in both lentiviral vectors from the EF1α internal promoter. Expression plasmids were used to produce virus from HEK-293T cells under identical conditions three separate times. Virus was quantified by assessing viral titer using 1 ml unconcentrated viral supernatant, which was added to the highly transducible HEK-293T cell line as a baseline for comparison. Viral copy number was determined utilizing qPCR with primers specific for the HP-fVIII transgene and compared between SIV- transduced and HIV- transduced cells. Unconcentrated viral vector titers were significantly higher with the SIV vector system ($P = 0.014$) (**Figure 2.5 B**). Recombinant SIV and HIV then was used to transduce BHK-M, K562 and HEK-293T cells at a MOI 5. Seventy-two hours after viral addition, transduction efficiency was measured by analyzing the copy number of HP-fVIII by qPCR. As previously observed in the lab, BHK-M and K562 cells are transduced less efficiently with lentiviral vectors than HEK-293T cells, as noted by lower HP-fVIII copy numbers (**Figure 2.5 C**). Comparison of the two vector systems in cell lines, on the other hand, did not show any difference with respect to the transduction efficiency in both K562 cells ($P = 0.837$) and HEK-293T cells ($P = 0.120$) (**Figure 2.5 C**). Identical copy numbers in HEK-293T cells were observed and expected as these cells were used to calculate viral titer (**Figure 2.5 C**). However, a

slight difference was seen with BHK-M cells. Greater copy numbers were noted when a SIV-based vector was used to transduce BHK-M cells compared with the HIV-based vector ($P = 0.045$).

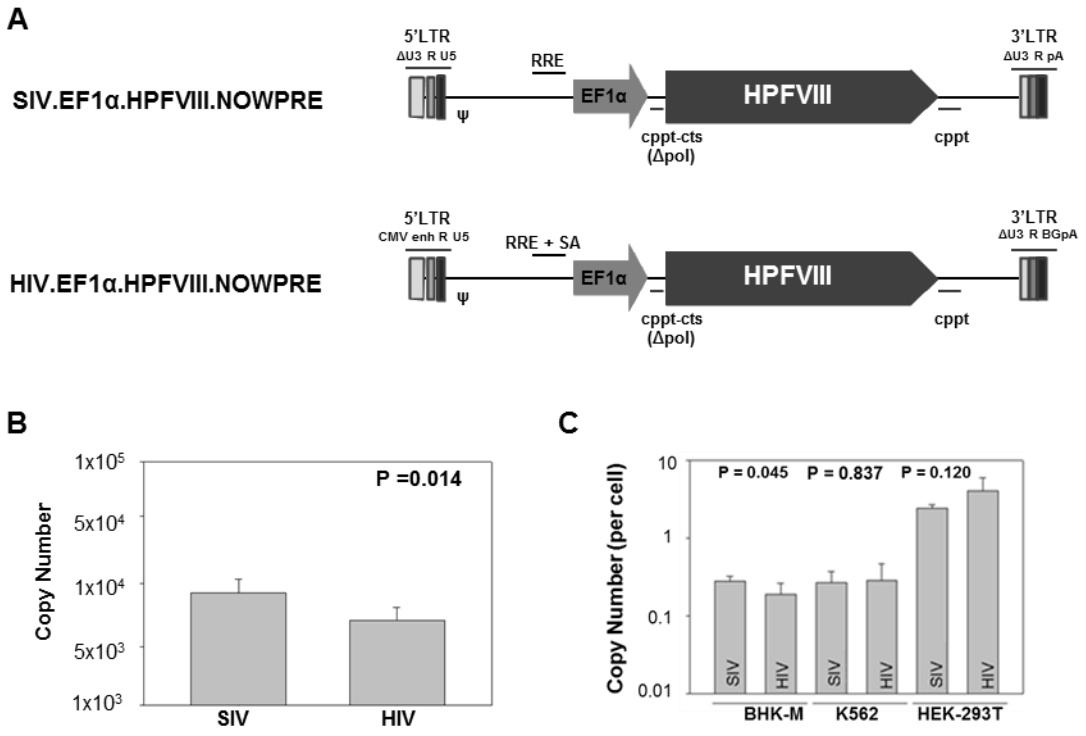


Figure 2.5 Comparison of SIV- and HIV-vector systems encoding HP-fVIII. (A) Schematic representation of the SIV and HIV expression plasmids generated to encode HP-fVIII. (B) Recombinant virus was produced under identical conditions (n = 3) and titered on HEK-293T cells. (C) Each viral preparation was utilized to transduce two wells of BHK-M, K562 and HEK-293T cells at an MOI of 5. Seventy-two hours post-transduction, genomic DNA was isolated and assessed for copy number by qPCR. *P* values were derived from a student's *t* test.

RNA was extracted and fVIII transcripts were quantified using qRT-PCR from the BHK-M, K562 and HEK-293T cells transduced with either SIV- or HIV-based vectors. BHK-M cells were included in this analysis as a high-expressing fVIII cell line. It was expected that the expression of the transgene would be similar between the two viral vectors, as similar MOIs were used and similar copy numbers were determined. However, there was enhanced HP-fVIII RNA expression by the SIV system, which was evident in both BHK-M ($P < 0.001$) and K562 ($P = 0.019$) cell lines (**Figure 2.6 A and 2.6 B**). Enhanced HP-fVIII RNA levels were confirmed by Northern blot analysis (**Figure 2.6 D upper panel**). Supernatants from the transduced cells were used to assess HP-fVIII activity by an APTT reagent-based one-stage coagulation assay. HP-fVIII activity was significantly increased for BHK-M ($P < 0.001$) and K562 ($P = 0.006$) cells transduced with SIV (**Figure 2.6 C**). In BHK-M and K562 cells, greater RNA levels led to greater protein production. This is consistent with previous findings that showed a strong correlation between RNA and fVIII activity (Doering *et al*., 2009). Based on this set of data, the SIV-based vector system was selected for further studies.

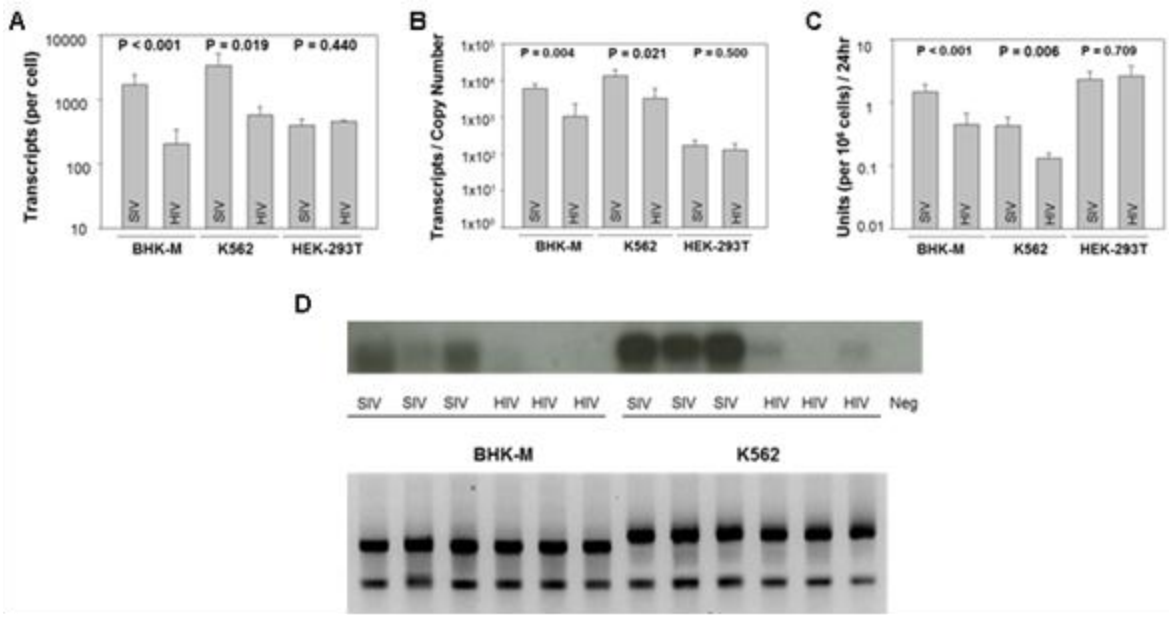


Figure 2.6 Comparison of SIV- and HIV-vector systems encoding HP-fVIII. (A) Seventy-two hours post-transduction, total RNA was isolated and transcript levels were quantified by qRT-PCR. (B) Transcript levels were normalized to copy number. (C) Twenty-four hours prior to isolation, the conditioned media was exchanged for serum-free AIM-V in order to assess the HP-fVIII activity by an APTT reagent-based one-stage coagulation assay. Bars represent the mean ± the standard deviation of three sets of virus each added to two wells, while each well was measured in duplicate. (D) Enhanced transcript levels were confirmed by Northern Blot analysis. Equivalent amounts of ribosomal RNA were apparent among each sample (lower panel). *P* values were derived from a student's *t* test.

Comparison of the transduction efficiency of SIV- and HIV-based lentiviral vectors containing an eGFP transgene

In order to determine if enhanced expression from the SIV-based lentiviral vector was specific to the HP-fVIII transgene, expression plasmids were constructed in which both SIV- and HIV-based lentiviral vectors contained the eGFP transgene (**Figure 2.7 A**). In both gene transfer systems, the eGFP transgene was driven from the EF1α internal promoter. In this manner differences in eGFP expression could be assessed as a consequence of differences derived from the vector system itself. As described earlier, expression plasmids were used to produce virus from HEK-293T cells under identical conditions. Following ultracentrifugation, virus was concentrated and quantified by viral titer using flow cytometry. Titers were comparable among the two viral preparations yielding TU ml^{-1} near 2×10^7.

Transduction efficiency was assessed in BHK-M, K562 and HEK-293T cells. Approximately 200,000 cells were transduced with either the SIV- or HIV-based lentiviral vector at an MOI of 1. The percentage of eGFP positive cells was quantified by flow cytometry. The transduction efficiency of the HIV vector system was greatest with an eGFP transgene in K562 ($P < 0.001$) and HEK-293T cells ($P < 0.001$) (**Figure 2.7 B**). Conversely, the SIV vector system exhibited greater transduction efficiency in BHK-M cells ($P = 0.017$) (**Figure 2.7 B**).

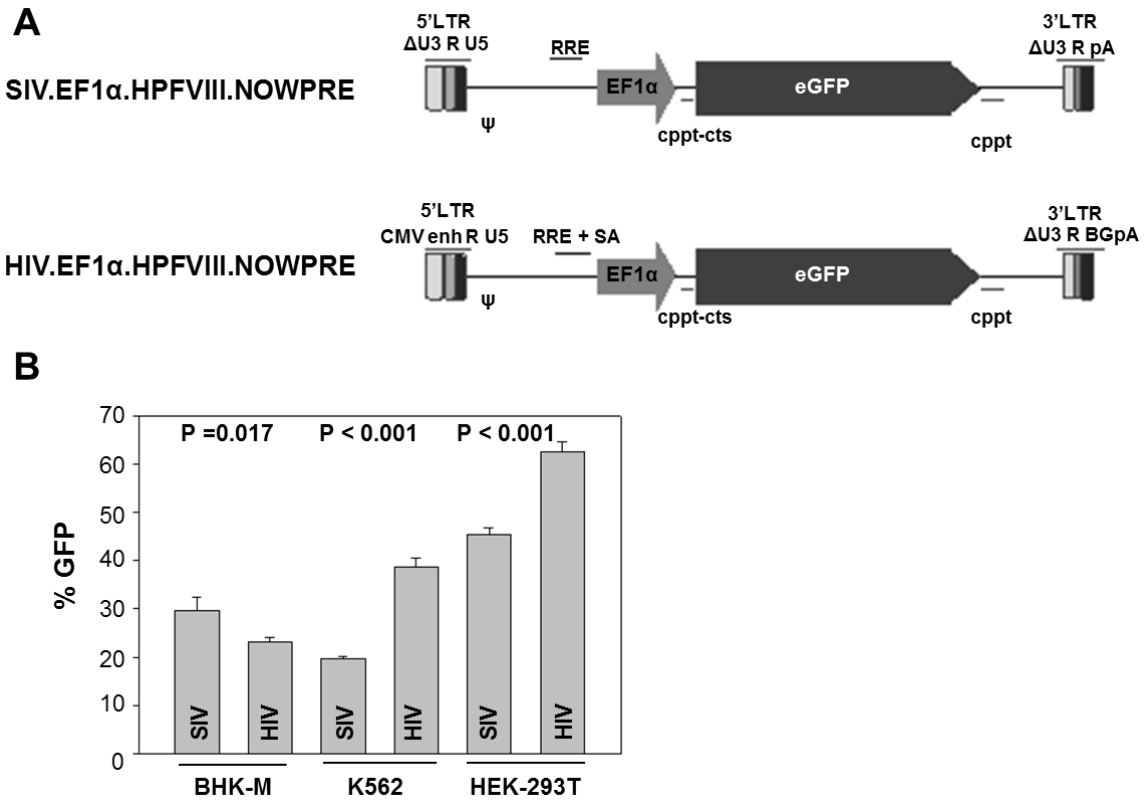


Figure 2.7 Comparison of SIV- and HIV- vector systems encoding eGFP. (A) Schematic representation of the SIV and HIV expression plasmids used to produce recombinant virus containing the eGFP transgene. (B) Single viral preparations were used to transduce three wells of BHK-M, K562 and HEK-293T cells at an MOI of 1. Transduction efficiency was assessed seventy-two hours following transduction by flow cytometry. *P* values were derived from a student's *t* test.

Comparison of the transduction efficiency of an SIV and EIAV-based lentiviral vectors encoding an eGFP transgene

Alternative non-primate lentiviruses have been proposed as vectors for gene transfer. Specifically, a vector has been derived from the equine infectious anemia virus (EIAV) (Olsen, 1998). The EIAV-based vector was previously optimized for eGFP expression (O'Rourke, *et al.* 2005) and obtained as a generous gift from Manji Patel of the University of North Carolina. The optimized EIAV-based vector contained an internal promoter consisting of elements from the CMV and β-actin promoter (Cβ) and included a WPRE sequence (**Figure 2.8 A**).

Transduction efficiencies were compared between the SIV- and EIAV-based lentiviral vectors in sca-1^+ cells. Since expression is not being compared between the two lentiviral vectors, only that of transduction efficiencies, different promoters governing expression of eGFP between the two lentiviral vectors is irrelevant. Sca-1+ cells were isolated from a hemophilia A mouse and stimulated with cytokines for 3 days. Cell counts during stimulation exhibited cell growth indicative of typical sca-1^+ cell functioning (data not shown). Sca-1^+ cells were transduced at an MOI of 20. Seventy-two hours after transduction the sca-1^+ cells were analyzed for eGFP by fluorescent microscopy images (**Figure 2.8 B**) and flow cytometry. Sca-1^+ cells transduced with an SIV-based lentiviral vector yielded 10% GFP positive cells, whereas cells transduced with an EIAV-based lentiviral vector yielded 1.4% GFP positive cells. To confirm the superior transduction efficiency of SIV to EIAV, the titer of the EIAV-based lentiviral vector was reevaluated on HEK-293T cells. Due to the differences in titering protocols,

the EIAV-based lentiviral vector was shown to be tenfold less than originally designated with a titer of $4.6x10^{9}$ TU ml^{-1}. It is predicted that a ten-fold difference would not increase the percentage of GFP positive cells above that of the SIV-based lentiviral vector, suggesting that the EIAV-based lentiviral vector is not superior.

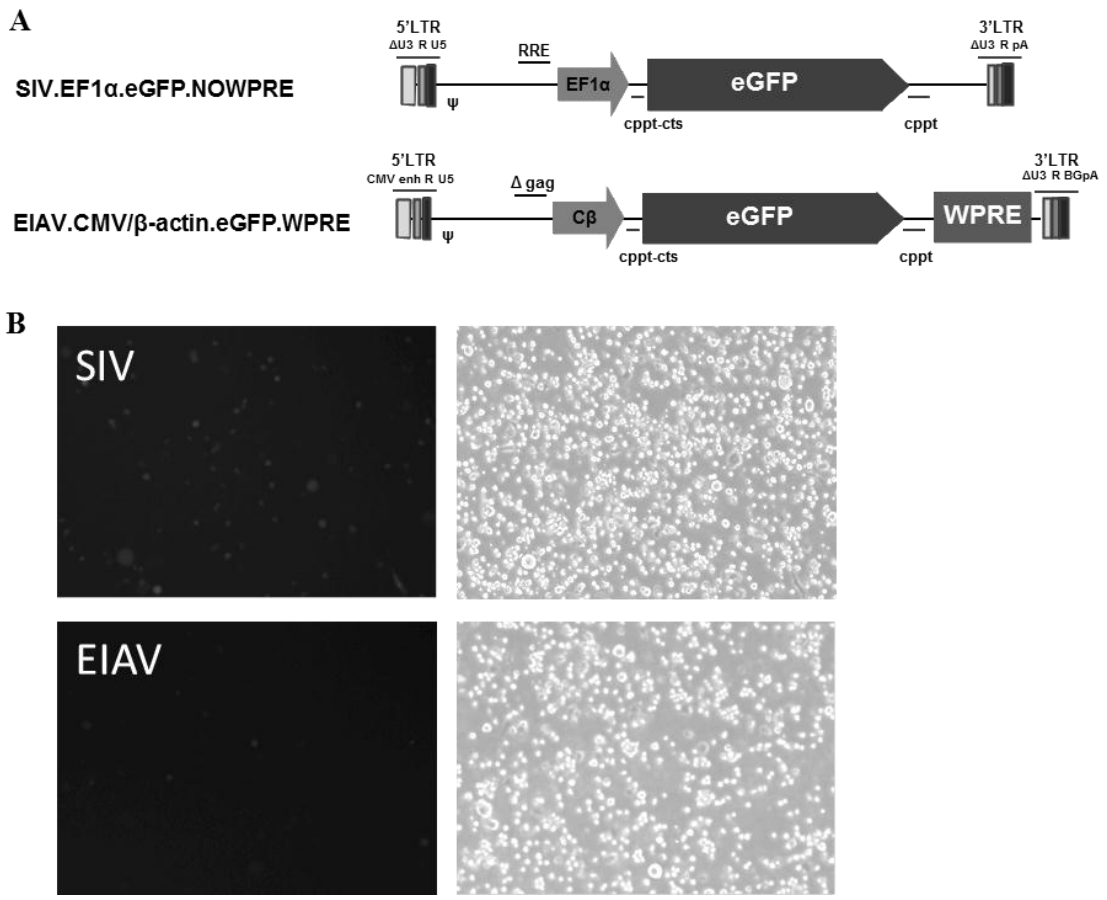


Figure 2.8 Comparison of SIV- and EIAV- based vector systems encoding eGFP. (A) Schematic representation of the SIV and EIAV expression plasmid used for viral preparations in HEK-293T cells. (B) Fluorescence microscopy images were taken prior to flow cytometry. Images depict increased transduction efficiency of an SIV-based vector.

2.33— Analysis of three internal promoters

Self-inactivating (SIN) vectors require internal promoters for transgene expression due to the inactivation of the viral 5' long terminal repeat (LTR) upon transfer of the U3 deletion during integration. A balance between adequate transgene expression and the elimination of transactivation of nearby genes must be maintained by the internal promoter. Three ubiquitous heterologous promoters, varying in enhancer activity, were evaluated in transduced HEK-293T cells. The human EF1α promoter, the CMV promoter, and the yeast phosphoglycerate kinase (PGK) promoter were incorporated into the SIV expression plasmid and used to produce virus under identical conditions (**Figure 2.9 A**). Viral titer was determined for each vector by qPCR. Vector then was added to HEK-293T cells at an MOI of 3. HEK-293T cells, although unable to accurately depict the transcription profile of HP-fVIII in sca-1^+ cells, were chosen due to the inherent superior transduction capabilities as compared to the hematopoietic EU1 and K562 cell lines. Transgene expression was greatest when driven by the CMV promoter and least by the PGK promoter as evaluated by the level of HP-fVIII transcripts from transduced cells (**Figure 2.9 B**). In addition, HP-fVIII activity normalized to copy number showed that HP-fVIII activity is greatest when expression is driven by the CMV promoter and least by the PGK promoter (**Figure 2.9 C**).

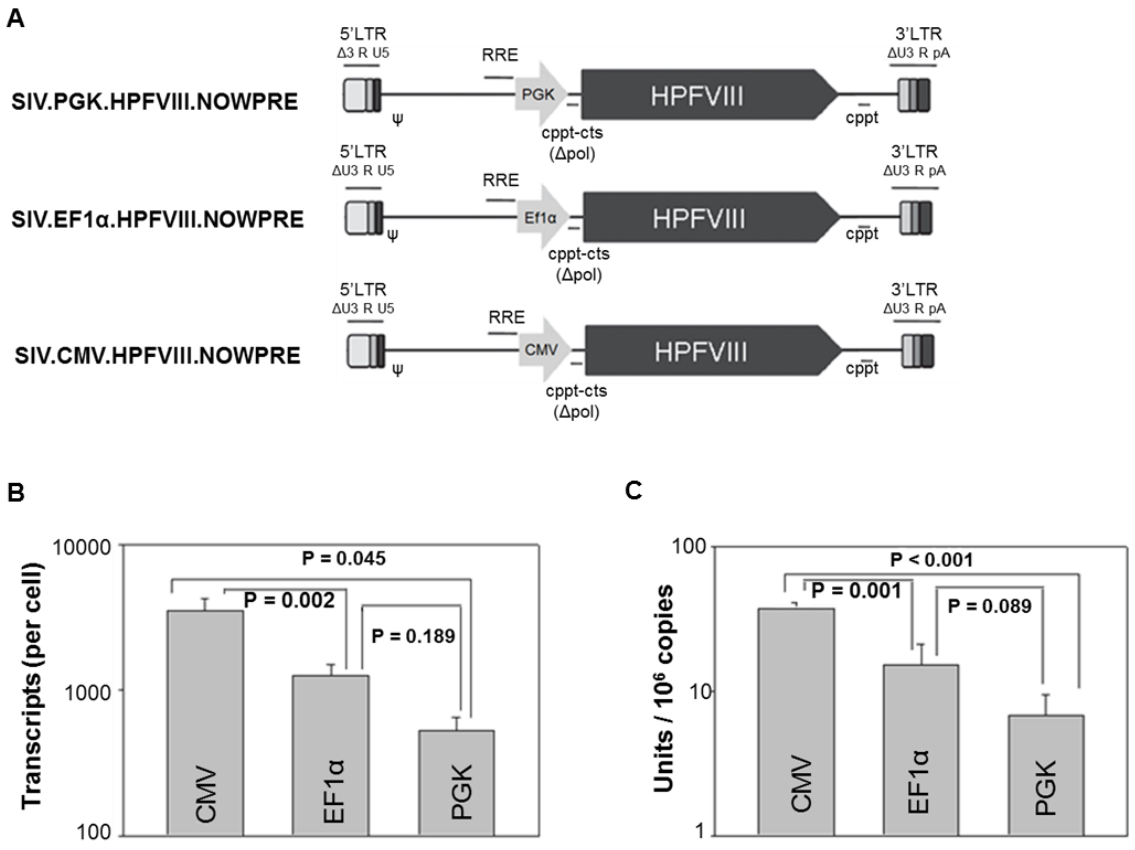


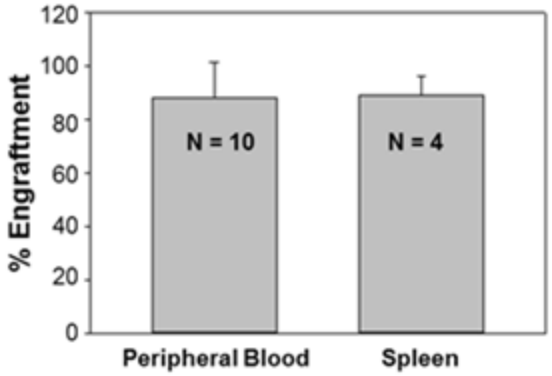
Figure 2.9 Effects of various internal promoters on HP-fVIII activity *in vitro*. (A) Schematic representation of SIN SIV-based expression vectors constructed with the PGK, EF1α or CMV internal promoter to make recombinant lentivirus. (B) HEK-293T cells were transduced at an MOI of 3. Seventy-two hours post-transduction, total RNA and genomic DNA were isolated from the cells and quantified by qPCR for transcript levels. (C) Twenty-four hours before isolation, the conditioned media was exchanged for serum-free AIM-V to assess HP-fVIII activity, which was normalized to copy number to correct for transduction efficiency. Each bar represents the mean ± the standard deviation of three wells. *P* values were determined by a one way ANOVA.

2.34— Hematopoietic stem cell gene therapy for hemophilia A utilizing the optimized lentiviral vector

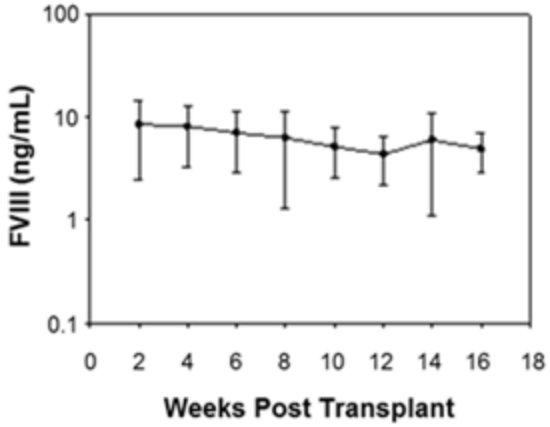
Based on the above analyses, an SIV-based lentiviral vector containing the HP-fVIII transgene expressed from a CMV promoter without the inclusion of a WPRE was predicted to be optimal for HP-fVIII expression *in vitro*. This construct then was evaluated *in vivo* in hemophilia A mice. Sca-1^+ cells were isolated from CD45.1 mice and transduced with the optimized SIV vector encoding HP-fVIII. CD45.1 sca-1^+ cells were transduced twice, 24 h between transductions, at an MOI of 15 each time, which resulted in 30-60% transduction efficiency with similar eGFP encoding vectors. However, with fVIII-containing vectors, only 3-10% is expected as previous findings showed a ten-fold lower transduction efficiency when using a vector encoding fVIII as compared to eGFP (data not shown, Doering *et al*., 2009). Transduction protocols were designed to ensure that transduced cells would only contain, on average, one or fewer copies of the vector. The transduced sca-1^+ cells then were transplanted into lethally irradiated (11 *Gy* split dose total body irradiation) hemophilia A mice. Three months after transplantation, donor cell engraftment was measured by flow cytometry. Average engraftment in the peripheral blood and spleen was shown to be approximately 90% (**Figure 2.10 A**). Every two weeks HP-fVIII levels were quantified by an ELISA assay. HP-fVIII levels persisted for the duration of the study (4 months) with a range between 2 and 20 ng ml^{-1} (**Figure 2.10 B**). A Coatest activity assay showed a similar HP-fVIII expression profile (**Figure 2.10 C**). Copy numbers in six mice remained below the detectable level of five percent gene-modified cells by qPCR with one each having 0.07,

0.10, 0.11 and 0.14 vector copies per genome. No correlation between copy number and HP-fVIII expression was observed in these mice.

A


120
100
80
60
40
20
0
% Engraftment
N = 10
N = 4
Peripheral Blood
Spleen

B


100
10
1
0.1
FVIII (ng/mL)
0 2 4 6 8 10 12 14 16 18
Weeks Post Transplant

C

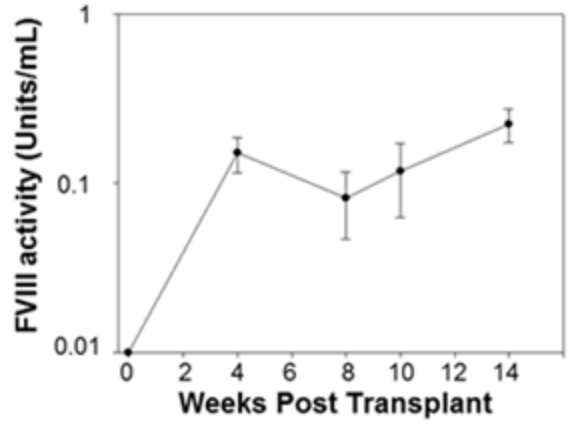

1
0.1
0.01
FVIII activity (Units/mL)
0 2 4 6 8 10 12 14
Weeks Post Transplant

Figure 2.10 *In vivo* expression of the optimized vector in transplanted hemophilia A mice. Sca-1^{+} cells were isolated from CD45.1 mice and transduced with the optimized vector with an MOI of 15 (x 2). Approximately 1 x 10^{6} cells were transplanted into Hemophilia A mice (CD45.2) (n = 14). (A) Engraftment was measured from peripheral blood and splenocytes of three mice and was assessed three months after transplant by flow cytometry. (B) HP-fVIII expression levels were assessed in the mice every two weeks following transplantation by an ELISA. (C) A chromogenic test (Coatest assay) was used to assess HP-fVIII activity in a subset of mice (n = 3).

2.4— Discussion

To date, three clinical trials have been initiated for gene therapy applications to treat hemophilia A (Roth *et al.*, 2001; Powell *et al.*, 2003; Berlfein *et al.*, 2003). However, each trial failed to yield sustained therapeutic levels of human fVIII. To overcome the obstacle of limited expression, a human/porcine fVIII hybrid has been constructed exhibiting expression 19-fold higher than human B domain-deleted fVIII (Doering *et al.*, 2009). Further optimization of the transgene resulted in a theoretically less immunogenic high expression fVIII transgene. With the production of an extensively optimized transgene, efforts can now be directed towards optimizing the viral vector for transgene delivery and expression.

A number of viral vectors have been considered for the modification of cells including both non-integrating and integrating vectors. Non-integrating vectors, such as the adenoviral and adeno-associated viral vectors, exist in cells extrachromosomally and are limited by potential vector genome loss. Yet, adenoviral vectors and adeno-associated viral vectors are appealing in that they efficiently transduce both dividing and non-dividing cells. With respect to the treatment of hemophilia A, adenoviral vectors have been utilized in neonates to produce tolerance (Hu *et al.*, 2011; Hu and Lipshutz, 2011) but currently are less clinically desirable due to toxic side effects experienced in clinical trials (Aruda, 2006). However, adeno-associated viral vectors are being extensively evaluated for use in gene therapy, especially for hemophilia B. Unfortunately, adeno-associated viral vectors are limited by their genetic carrying capacity. The vector cassette associated with the 4.4-kb of the B domain deleted fVIII cDNA, including regulatory

elements, is at and above the carrying capacity of an adeno-associated viral vector for the treatment of hemophilia A. Several groups are attempting to overcome this limitation by separating the genetic payload, that is, the heavy chain and light chain of fVIII, into two different vectors (Hu and Lipshutz, 2011; Scallan *et al*., 2003; Sarkar *et al*., 2006). Other groups are attempting to minimize the regulatory elements by utilizing smaller internal promoters (Scallan *et al*., 2003; Sarkar *et al*., 2004; Jiang *et al*., 2006; Lu *et al*., 2008; Sabatino *et al*., 2011).

Several groups have focused on the use of integrating viral vectors such as lentiviral vectors. Lentiviral vectors are suitable vectors for hemophilia A gene therapy applications in that they (1) stably integrate in the host genome, (2) are able to transduce quiescent cells and (3) can encapsulate large transgenes such as HP-fVIII. Although insertional mutagenesis was observed clinically with the use of integrating γ-retroviral vectors (Wu *et al*., 2003; Hacein-Bey-Abina *et al*., 2003), to date similar issues have not been observed with lentiviral vectors (Cartier *et al*., 2009; Cavazzana-Calvo *et al*., 2010; Biffi et al., 2011). This may be due to integration site preferences between the two vectors. In addition, clinically used lentiviral vectors contain safety measures that are now routinely incorporated, including a 133-bp deletion in the U3 region of the 3' LTR that self-inactivates the vector, creating a replication incompetent or SIN vector (Miyoshi *et al*., 1998; Iwakuma *et al*., 1999), (for review see Pauwels *et al*., 2009). Comparative studies have shown that SIN lentiviral vectors are less oncogenic than γ-retroviruses (Montini *et al*., 2006; Montini *et al*., 2009).

A WPRE often is incorporated into lentiviral vectors since the demonstration of two to fivefold enhanced eGFP and luciferase expression (Zuffery *et al*., 1999; Brun *et al*., 2003; Gonzalea-Murillo *et al*., 2010). However, a recent report showed that the enhanced expression was dependent on the promoter and cell line used, and in some scenarios, the presence of a WPRE resulted in a decrease in transgene expression (Klein *et al*., 2006). It is apparent that the WPRE is more complex than originally assumed and requires individual transgene evaluation. With the HP-fVIII transgene, the WPRE was found to be negligible in regards to viral production, transgene expression and transcriptional read-through. For our high expression construct, there appears to be no benefit to include a WPRE. Thus, based on these results confirmed by Gabriela Denning at Expression Therapeutics, the WPRE was removed from the clinical vector. However, it may be useful for lower expressing fVIII constructs and should be tested in conjunction with each.

The SIV- and HIV-based vector systems were also analyzed in this study in regards to HP-fVIII expression. The production of SIV and HIV lentiviral vectors were optimized separately. Using standard production conditions, higher viral titers routinely were achieved with the SIV system compared with HIV. After each viral preparation was quantified with respect to viral titer, transduction at identical MOIs yielded integration events that were not statistically different in K562 and HEK-293T cells. However, BHK-M and K562 cells transduced with SIV expressed HP-fVIII more efficiently than those cells transduced with identical amounts of HIV. This suggests that although SIV and

HIV integrated at similar levels, the integration events of SIV may be in regions of the genome that promoted greater expression than those regions where HIV integrated. This potential difference requires further study to test this hypothesis. It is interesting that this same phenomenon was not observed with an eGFP transgene and that the enhanced expression of HP-fVIII also was not seen with HEK-293T cells. This suggests a potential transgene and cell type specificity of SIV enhancement. This data also suggests that SIV is, at a minimum, as efficient at gene transfer and expression of HP-fVIII as HIV. In addition, SIV was found to be superior to an EIAV-based lentiviral vector in regards to transduction efficiency. SIV-based vectors may be a safer option for gene therapy applications to combat hemophilia A since a number of adults with hemophilia are HIV-1 positive after receiving plasma-derived fVIII infusion products before HIV testing became routine. The use of HIV vectors can raise safety concerns due to possible recombination events of the vector and wild-type HIV. SIV, on the other hand, is less likely to recombine due to sequence differences between HIV and SIV.

Different internal promoters also were tested as a component of the expression vector for HP-fVIII expression. SIN-lentiviral vectors require internal promoters to direct expression of the transgene due to the inactivation of the 5' LTR during integration. This safety feature removes the enhancer element of the LTRs preventing transactivation of nearby genes. Therefore, the internal promoter must be sufficiently strong to provide adequate expression of the transgene without having the capacity to transactivate genes nearby. The CMV promoter was superior to the EF1α and PGK promoters at expressing HP-fVIII *in vitro* using the SIV-based vector system. The enhanced expression from the

CMV promoter *in vitro* is not unexpected as the CMV promoter contains the strongest enhancer among the three internal promoters. However, with the stronger enhancer activity, the transactivation of nearby genes is a concern with the use of CMV, as well as the possibility of methylation-induced inactivation of the promoter. In hemophilia A mice under limiting transduction protocols using an SIV vector without a WPRE, the CMV promoter directed expression of HP-fVIII at therapeutically relevant levels.

In summary, several fVIII transgenes have been studied extensively for use in gene therapy applications of hemophilia A. HP-fVIII overcomes low-level expression obstacles while theoretically reducing the immunogenicity. Although the transgene has been fairly well described, the expression vector has not been as well characterized. In the studies presented in this chapter, a lentiviral vector was optimized for HP-fVIII expression. Under the conditions tested, the SIV-based lentiviral backbone was found to be more effective for HP-fVIII expression than an HIV-based lentiviral backbone. In the SIV-based lentiviral backbone, the CMV internal promoter was shown to drive HP-fVIII expression efficiently, but it is realized that this promoter may have complications due to possible transactivation and promoter inactivation. The WPRE was found to be unnecessary and was removed from the lentivector. *In vivo* data show that the optimized vector provides sustained HP-fVIII expression in hemophilia A mice, supporting its further development for hemophilia A.

Chapter 3:

Assessment of Alternative Approaches to Lentiviral Production and Transduction

3.1— Introduction

Upon identifying a lentiviral that is optimized for HP-fVIII expression, two limitations associated with the utilization of lentiviral vectors for the modification of HSCs in order to treat hemophilia A were addressed. The first limitation to be confronted is that of the reduced titer apparent with the addition of a large transgene such as fVIII in the lentiviral vector. It is apparent that as the size of the transgene increases the production of vector decreases (Kumar *et al.*, 2001; Yacoub *et al.*, 2007). Thus an alternative approach to lentiviral production was assessed as an attempt to combat this limitation. The second limitation is that of the inherent reduction in infectivity of HSCs to lentiviral vectors. This has been observed clinically in recent gene therapy clinical trials (Cartier *et al.*, 2009; Boztug *et al.*, 2010; Cavazzana-Calvo *et al.*, 2010; DiGiusto *et al.*, 2010). In one particular report as low as 1% gene corrected $CD34^+$ cells were found to be engrafted (DiGiusto *et al.*, 2010). In the case of an HSC gene therapy protocol for hemophilia A, one in which a growth advantage is not expected, greater transduction efficiencies will be needed to achieve therapeutic potential.

Phenotypically mixed viral particles are routinely formed as a result of the infection of a cell with two or more separate viruses (Zavada, 1982). This observation along with the separation of the three retroviral components (gag-pol, viral genome and envelope) onto separate plasmid constructs (refer to **Figure 1.4**) led to the development of pseudotyping methods for lentiviral vectors used in the context of gene therapy. The first alternatively pseudotyped HIV based viral vector contained the amphotropic glycoprotein of the murine leukemia virus (MLV) (Page *et al.*, 1990). This study followed the demonstration

of phenotypic mixing between wild-type HIV and MLV virons (Chesbro *et al*., 1990; Spector *et al*., 1990). The range of host cells transduced by a virus is dependent on the interaction of a viral envelope glycoprotein with specific cell surface receptors. Therefore, other glycoproteins were subsequently used to expand the host range of HIV-based lentiviral vectors (Cronin *et al*., 2005).

The vesicular stomatitis virus glycoprotein (VSVG) gives viral vectors the broadest of host-cell range by interacting with what has historically been thought to be an abundant ubiquitous component of the cellular plasma membrane. The receptor was postulated to be phosphatidyl serine, phosphatidyl inositol or GM3 ganglioside (Schlegel *et al*., 1983, Mastromarino *et al*., 1987; Conti *et al*., 1988). Phosphatidylserine, however, has since been ruled out as the cell surface receptor for VSVG (Coil and Miller, 2004) being attributed solely to a post-binding step of viral entry (Coil and Miller, 2005). Regardless of the exact surface receptor utilized by VSVG, three groups in the same year independently expanded the host range of an HIV-based lentiviral vector by successfully pseudotyping the vector with VSVG (Akkina *et al*., 1996; Naldini *et al*., 1996; Reiser *et al*., 1996). In addition to expanding the host range, another advantage to pseudotyping viral vectors with VSVG is that these vectors can withstand the g-forces of ultracentrifugation in order to yield concentrated vector with high viral titers. Despite the advantageous properties associated with pseudotyping lentiviral vectors with VSVG, VSVG expression is toxic in most cells. Accumulation of VSVG at the surface of HEK-293T cells leads to syncytia formation followed by cell death (Burns *et al*., 1993). As a result, several failed attempts have been made to produce stable packaging cell lines for

the manufacture of VSVG pseudotyped lentivirus. This is mainly due to diminished cell survival. For this reason, the production of lentiviral vectors pseudotyped with VSVG is limited by a transient transfection procedure in HEK-293T cells. By this method, virus can only be produced for a total of three days before the cells become inviable. In addition, vector production in this manner is not ideal being unable to offer a nonvariable source of recombinant virus that can be extensively characterized prior to use in human clinical trials.

Another limitation encountered in the field of gene therapy includes a diminished susceptibility of the hematopoietic stem cell (HSC) to *ex vivo* genetic manipulation. Lentiviral vectors pseudotyped with VSVG, regardless of an increased host range, do not transduce HSCs efficiently. This phenomenon appears to be intrinsic to HSCs being that HSCs are also resistant to wild-type HIV infection regardless of the presence of the CXCR4 cell surface receptors (Von Laer *et al*., 1990; Weichold *et al*., 1998; Lee *et al*., 1999; Shen *et al*., 1999). The exact mechanism responsible for HSC resistance to HIV remains elusive. Therefore, much effort has been directed towards increasing lentiviral transduction of HSCs. One such effort was performed with the use of RNAi technology. p21, a cell cycle checkpoint protein, was transiently silenced in $CD34^+$ hematopoietic stem and progenitor cells. As a result HIV lentiviral transduction was enhanced 2- to 4-fold (Zhang *et al*., 2005). Initially, p21 was chosen as a target being a cyclin-dependent kinase inhibitor necessary for the maintenance of HSC quiescence (Cheng *et al*., 2000). It was stipulated that high efficient gene transduction is cell cycle dependent (Sutton *et al*., 1999). However, the enhancement in HIV lentiviral transduction due to p21 silencing

was independent of a direct effect on cell cycling. Instead, p21 was found to immunoprecipitate with the HIV preintegration complex and increase the abundance of 2-LTR circles indicative of abortive chromosomal HIV integration. In this manner, p21 was suggested to block HIV viral infection by disturbing the endogenous function of HIV integrase (Zhang *et al*., 2007). These reports proposed introducing siRNA to p21 in preclinical transduction protocols. Current methods include HSC pre-stimulation with cytokine cocktails that have been demonstrated to enhance transduction (Santoni *et al*., 2006; Millington *et al*., 2009) but typically result in a loss of gene modified cells following transplantation potentially due to diminished multipotentiality of the HSCs (Zhang *et al*., 2005). RNAi technology, on the other hand, does not disrupt the multipotentiality of HSCs and could potentially be a more advantageous addition to preclinical transduction protocols. In this manner, the multipotentiality of HSCs could remain undisrupted while being more susceptible to transduction.

The studies presented in this chapter depict attempts to overcome some of the previously mentioned pitfalls plaguing the field of gene therapy. VSVG pseudotyped lentivirus production is limited to a transient transfection procedure in which virus is produced for only 3 days. In order to avoid toxicity associated with the overexpression of VSVG in HEK-293T cells, an alternative cell line was assessed for recombinant lentiviral production. In addition, lentiviral transduction enhancement was attempted using two separate methods based off previous reports.

3.2— Materials and Methods

Spinoculation

Spinoculation was performed on K562 and sca-1+ cells in 24 well plates coated with retronectin (1µg µl^{-1}). Cells were plated at a density of 10^5 cells per well and transduced by spinoculation in minimal volume (300 µl) in the presence of polyberene. Spinoculation was performed at 1000g for 2 hours. The effect of spinoculation on lentiviral transduction of K562 cells was assessed with recombinant virus containing either a GFP or HP-fVIII transgene. Transduction with a GFP containing recombinant lentivirus was assessed by flow cytometry, while the effect of spinoculation on the transduction of a recombinant lentivirus containing the HP-fVIII was assessed by the APTT reagent-based one stage coagulation assay as described below.

In vitro fVIII expression

fVIII activity was measured from the supernatant of cells transduced previously with a lentiviral vector containing the high expressing HP-fVIII transgene. The cells were cultured in serum-free media for 24 hours before the assay as previously described in chapter 2. In short, the APTT one stage coagulation assay was performed in duplicate for each supernatant on a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France) in human fVIII-deficient plasma (George King Biomedical, Overland Park, KS). The clot time for each sample was compared to a standard curve based on dilutions of pooled normal citrated human plasma (George King Biomedical, Overland Park, KS).

Isolation of murine stem cell antigen-1^+ (sca-1^+) cells

fVIII knockout mice with hemophilia A due to disruption of exon 16 (as previously described in Bi *et al*, 1995) were treated with 5-FU (150 mg kg^{-1}) intraperitoneally two days prior to bone marrow isolation. The bone marrow was extracted from the femurs and tibias of 9-week old hemophilia A mice upon being flushed with PBS (supplemented with 2% FBS). The flushed bone marrow was then filtered through a 0.7 µmol l^{-1} filter and pelleted at 300g for 5 minutes. Red blood cells were then lysed for 10 minutes with the addition of RBC lysis buffer (Sigma, St. Louis, MO, USA). The remaining cells of the bone marrow were then assessed for purity by flow cytometry utilizing a Ly-6A/E antibody (clone E13-161.7) conjugated to FITC (BD Pharmingen, San Diego, CA). The purity of the population was found to be 85% positive for sca-1^+. Upon confirmation, the sca-1^+ cells were cultured at a density of 10^5 cells per well in a 24 well tissue culture treated plate previously coated with retronectin (1µg $µl^{-1}$). Sca-1^+ cells were cultured in StemPro media supplemented with L-glutamine (29µg mL^{-1}), murine stem cell factor (100ng mL^{-1}), murine interleukin-3 (20ng mL^{-1}), human interleukin-11 (100ng mL^{-1}), and human Flt-3 ligand (100ng mL^{-1}). Transduction began the same day as isolation in the presence of polyberene. Cells were transduced twice, 12 hours apart by spinoculation at a MOI of 15. An *in vivo* assessment of spinoculation was made by transplanting $4x10^5$ sca-1^+ cells via tail-vein injection into lethally irradiated (11*Gy* TBI using a Gammacell 40 Exactor) 8-week-old recipient hemophilia A mice (n = 3). Transplantation occurred 4 days after initial sca-1^+ isolation, 2 days following the second transduction. Blood was collected from the transplanted mice every two weeks beginning one week after transplantation via retro-orbital capillary insertion. HP-fVIII was measured using an

ELISA specific for HP-fVIII. The ELISA only detects properly folded HP-fVIII in that the primary antibody detects the heavy chain (human A2 domain) and the secondary antibody detects the light chain (porcine A3 domain).

Colony-forming Unit Assay

Colony-forming cell assay was performed per manufacturer's protocol (Stemcell Technologies, MethoCult® GF M3434 Optimum with EPO). Briefly, a 10X concentration ($2x10^4$ cells/mL) of sca-1^+ cells was prepared in Iscove's MDM with 2% FBS. A total of 0.3 mL of 10X cell mixture was then added to 3 mL of MethoCult® and mixed by vortexing. MethoCult®/cell mixture was allowed to sit for 5 minutes to allow bubbles to rise followed by dispensing 1.1 mL onto 35 mm dishes in duplicate using a 16 gauge blunt-end needle. The methylcellulose was evenly distributed into the dish by rotation and dishes were placed in a 100 mm culture dish alongside a third dish containing sterile water to maintain proper humidity. Cultures were incubated at 37^oC in 5% CO^2 for 14-16 days for optimal CFC growth. CFU-GM colonies were counted at 5X under an inverted microscope using a 60 mm gridded dish. GFP-positive colonies were scored under fluorescence microscopy.

3.3— Results

3.31— Toxicity of VSVG in BHK-M cells

Lentiviral vector is predominantly produced by transient transfection of the highly transfectable HEK-293T cells. This method of vector production is sufficient to produce vector titers appropriate for laboratory use. However, vector production is limited to 3

days due to toxicity associated with the accumulation of VSVG at the surface of HEK-293T cells (Burns *et al.*, 1993). This drawback has prevented the generation of a stable lentiviral vector-producing HEK-293T cell line in which clinical-grade vectors would ideally be manufactured. BHK-M cells are a suitable alternative for viral production as a highly transfectable cell line. In addition, BHK-M cells are already being used clinically for a portion of the commercial production of recombinant fVIII. BHK-M cells are able to efficiently synthesize recombinant fVIII with all the proper post-translational modifications (**see Figure 1.2**). And, if not susceptible to VSVG toxicity, the production abilities of BHK-M cells could be used to generate substantial amounts of lentiviral vector or even be used to create a packaging cell line. For this reason, the toxicity of VSVG was first assessed in BHK-M cells.

As a transfectable unit, PEI condenses DNA into positively charged particles in order to be brought into the cell via endocytosis upon binding anionic cell surface residues. Once inside the cell, the amines are protonated resulting in osmotic swelling of the vesicle which then bursts releasing the DNA into the cytoplasm. However, PEI is also very cytotoxic and could be the cause of the cell death. Therefore, PEI concentrations were varied to determine the extent of toxicity in BHK-M cells. At a concentration of 3.5 µg ml^{-1}, transfection was not achieved as observed by a lack of expression of GFP (**Figure 3.1 A**). A concentration of 17.67 µg ml^{-1} PEI, however, was tolerable by BHK-M cells (**Figure 3.1 B**). Concentrations higher than 17.67 µg ml^{-1} were followed by a decrease in cell number suggesting cytotoxicity at these higher concentrations (**Figure 3.1 C-E**).

Thus, the optimal concentration of PEI transfection in BHK-M cells appears to be less than 17.67 μg ml^{-1} PEI.

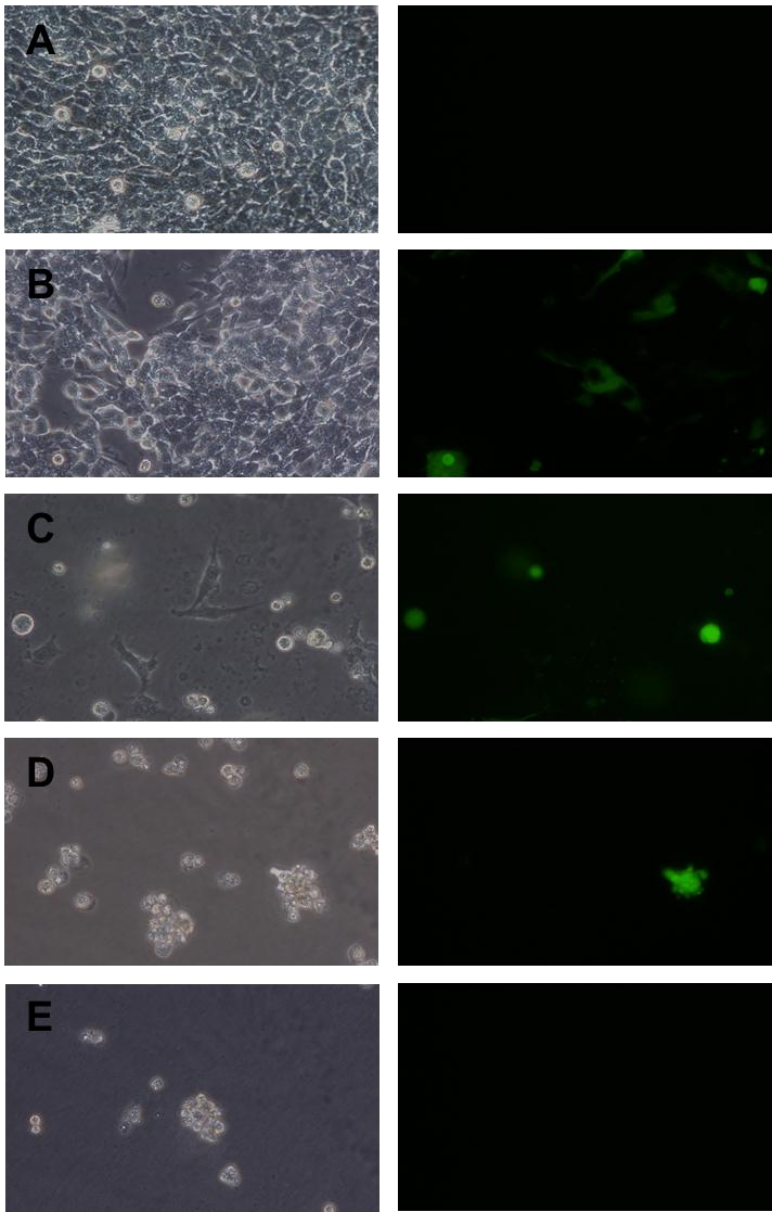


Figure 3.1 PEI toxicity assessment in BHK-M cells. A GFP encoding plasmid was transfected in BHK-M cells in order to assess transfection efficiency by fluorescence microscopy. The following concentrations of PEI were in order to determine the optimal concentration of PEI for BHK-M cellular transfection: (A) 3.5 µg ml^{-1} PEI (B) 17.67 µg ml^{-1} PEI (C) 35.34 µg ml^{-1} PEI (D) 176.7 µg ml^{-1} PEI (E) 353.4 µg ml^{-1} PEI. Images were taken seventy-two hours after transient transfection.

The plasmid containing the VSVG envelope protein was transiently transfected into BHK-M cells using the transfection reagent polyethylenimine (PEI). VSVG plasmid concentrations ranged from 0 to 3200 ng. Fluorescence microscopy images were taken seventy-two hours following transfection in order to assess cell viability (**Figure 3.2 A-F**). Although each well initially contained the same quantity of cells, less cells were adherent at the higher concentrations (**Figure 3.2 D-F**) of VSVG as compared to the well undergoing a mock transfection (**Figure 3.2 A**). In some instances syncytia formation is apparent (**Figure 3.2 F**). This suggests that VSVG is toxic to BHK-M cells. The BHK-M fluorescent images were compared to similar images taken on HEK-293T cells assessed for VSVG toxicity in the same manner. At concentrations as high as 3200 ng, no syncytia formation was observed and cell numbers appeared to be similar to the mock transfection control (**Figure 3.3 A-F**).

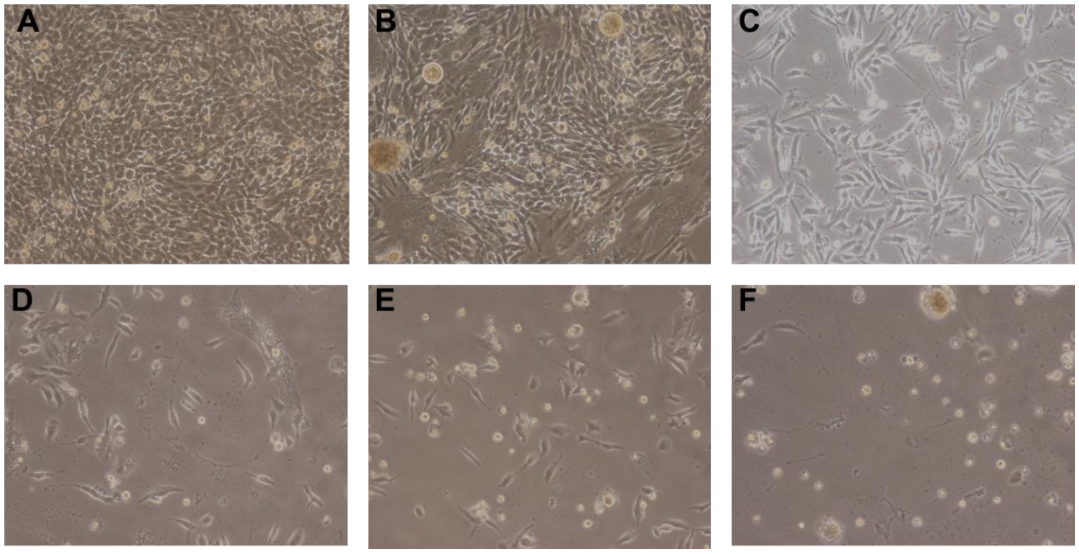


Figure 3.2 VSVG toxicity assessment in BHK-M cells. The VSVG containing lentiviral production plasmid was transiently transfected into BHK-M cells at the following concentrations: (A) no vsvg (B) 200 ng VSVG (C) 400 ng VSVG (D) 800 ng VSVG (E) 1600 ng VSVG (F) 3200 ng VSVG. 6 µg ml^{-1} PEI was used for the transfection to reduce toxicity associated with the transfection agent.

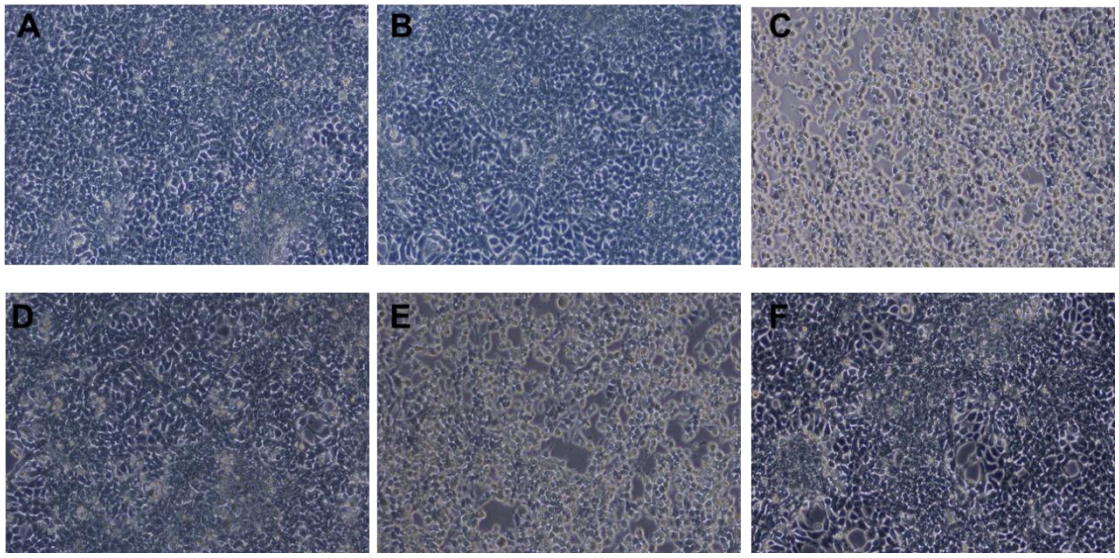


Figure 3.3 VSVG toxicity assessment in HEK-293T cells. VSVG containing plasmid was added to HEK-293T cells at the following concentrations utilizing the transfection reagent PEI: (A) no VSVG (B) 200 ng VSVG (C) 400 ng VSVG (D) 800 ng VSVG (E) 1600 ng VSVG (F) 3200 ng VSVG. Microscopic images were taken seventy-two hours after transfection.

3.32— Effect of alternatively pseudotyping an HIV-based lentiviral vector

Due to the toxicity associated with VSVG in BHK-M cells, three alternative envelopes were assessed for the production of an HIV-based lentiviral vector encoding a GFP transgene in BHK-M cells. Virus was produced by transient transfection in 10cm well plates. Transfection efficiency was assessed seventy-two hours later by fluorescence microscopy. The expression of GFP was similar among all wells despite the concentration or the viral envelope utilized (**Figure 3.4 A-C**). The only exception is that of the BHK-M cells transfected with a VSVG encoding plasmid. As expected, the expression of VSVG diminished BHK-M cell viability as observed by a decrease in BHK-M cells per well in addition to the presence of syncytia formation (**Figure 3.4 D**). Overall, the transfection efficiency appeared to be very low. Conditioned media containing recombinantly produced virus was used to transduce HEK-293T cells to determine viral infectivity. Viral transduction was assessed seventy-two hours after viral addition by examining the presence of GFP positive cells via fluorescence microscopy. GFP expression was not observed in the HEK-293T cells treated with the alternatively pseudotyped viruses (**Figure 3.5 A-D**). This would suggest that the alternatively pseudotyped viruses were not adequately produced, probably due to low transfection efficiency.

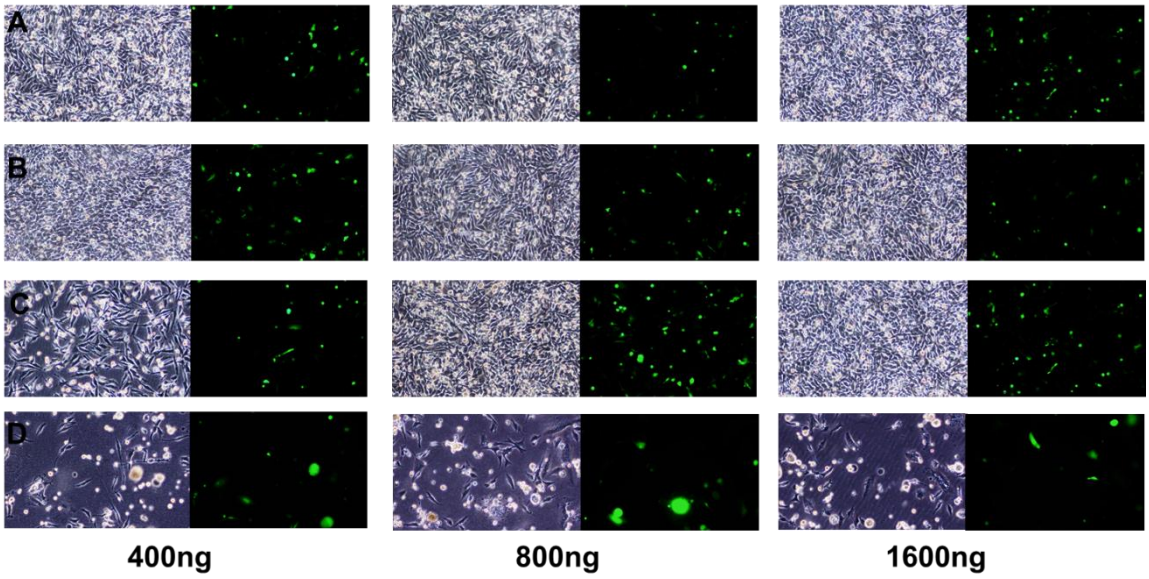


Figure 3.4 Transfection efficiency assessment. BHK-M cells were used to make recombinant HIV-based virus containing a GFP transgene. Four separate envelope plasmids were transfected into the BHK-M cells in order to produce alternatively pseudotyped recombinant virus. Three different concentrations of the (A) Eco (B) pHit (C) 10A1 and (D) VSVG envelope plasmids were transfected into the BHK-M cells using PEI.

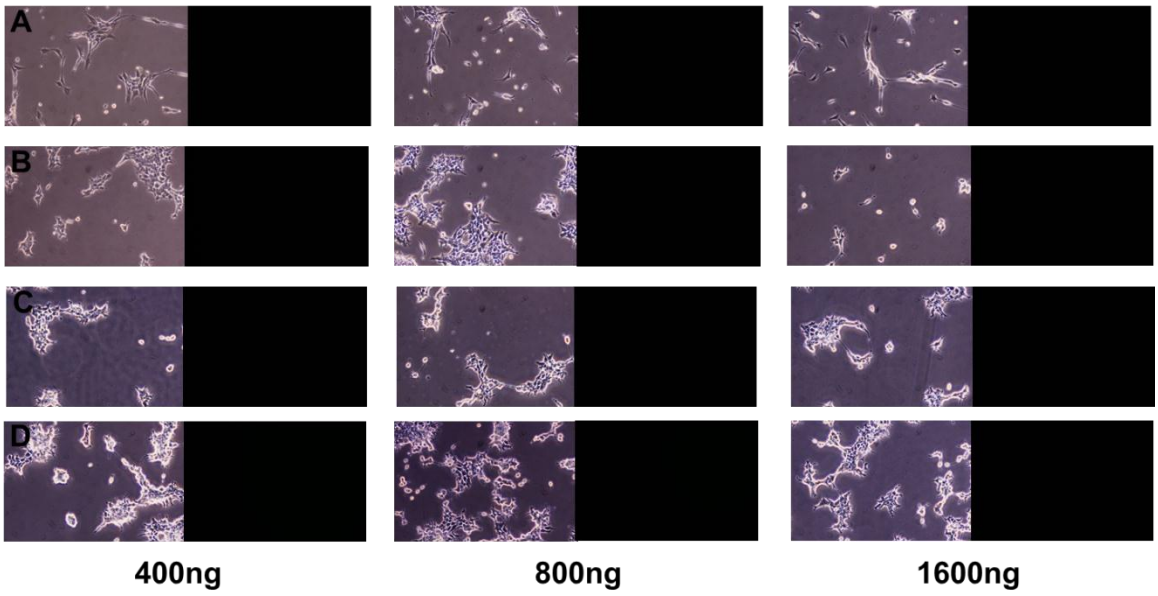


Figure 3.5 Viral infectivity assessment of alternatively pseudotyped HIV-based lentiviral vectors. Conditioned medium containing viral particles was added to HEK-293T cells. Virus was pseudotyped with either (A) Eco (B) pHIT (C) 10A1 or (D) VSVG glycoproteins. In addition, three different concentrations of the envelope plasmid were used to produce the alternatively pseudotyped recombinant virus. Fluorescence microscopy images were taken seventy-two hours after viral addition.

3.33— Knocking down p21

It has been suggested that lentiviral transduction can be enhanced by silencing p21. An HIV-based lentiviral vector was used to transduce human bone marrow-derived $CD34^+$ cells in which p21 had been silenced using RNAi technology. As a result, a 2- to 4-fold increase in GFP positive cells was observed (Zhang *et al*., 2005). An enhancement of that magnitude could be useful in the context of HSC gene therapy for hemophilia A. Therefore, siRNA specific to p21 was obtained and preliminary experimentation was performed.

K562 cells were plated in a 96 well plate at a density of $5x10^4$ cells / well. p21 was silenced by transfecting p21 siRNA into cells using the HiPerfect Transfection Reagent. The K562 cells were transduced forty-eight hours later with an HIV-based lentiviral vector encoding a GFP transgene at an MOI of 1 in the presence of polyberene. Transduction was assessed seventy-two hours after viral addition by flow cytometry. An MOI of 1 transduced approximately 60% of the K562 cells. No significant difference was observed between K562 cells treated with p21 siRNA and nonspecific siRNA (**Figure 3.6 A**). However, a slight increase in mean fluorescent intensity (MFI) was observed in K562 cells treated with p21 siRNA (**Figure 3.6 B**). This suggested a potential to enhance lentiviral transduction of K562 cells, which may not be seen utilizing an MOI of 1. For this reason, the experiment was repeated using lower MOIs. However, no enhancement in transduction efficiency was noted as seen by the percentage of positive GFP cells (**Figure 3.7 A, C, E**). It is unclear why an increase in MFI as seen

with an MOI of 1 (**Figure 3.6 B**) was not observed at the lower MOIs (**Figure 3.7 B, D, F**).

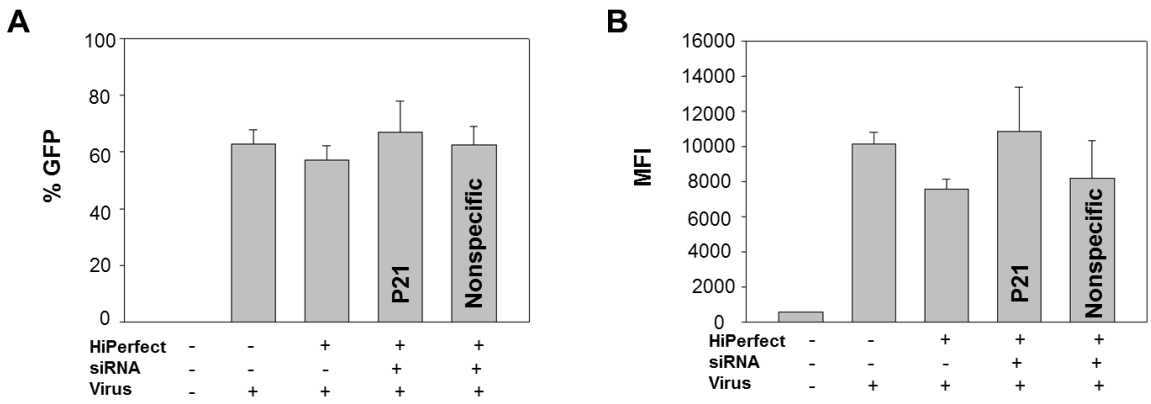


Figure 3.6 Utilizing p21 siRNA to enhance lentiviral transduction. (A) K562 cells were treated with 150nM siRNA (either specific for p21 or nonspecific as a control). An HIV-based lentiviral vector encoding a GFP transgene was used to transduce the cells forty-eight hours after siRNA treatment. Transduction efficiency was assessed seventy-two hours later by flow cytometry. As a result, the percentage of GFP positive cells was quantified and (B) the intensity of fluorescence for each positive cell was recorded as mean fluorescent intensity. A mock transfection control was included (no siRNA just HiPerFect Reagent) as well as an untransduced and an untransfected control.

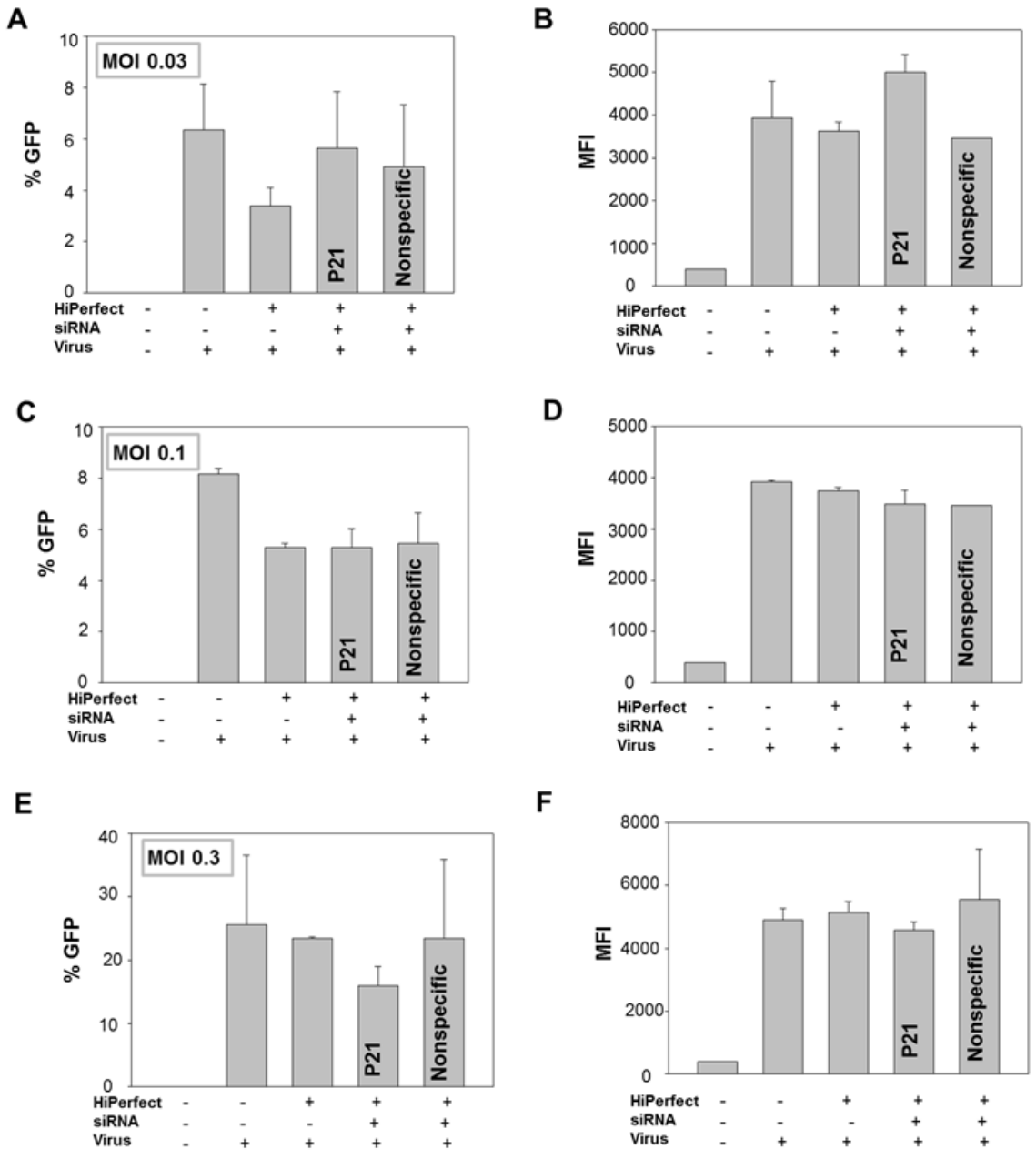


Figure 3.7 The effect of p21 silencing to enhance lentiviral transduction. p21 was silenced forty-eight hours prior to lentiviral transduction. An HIV-based lentiviral vector encoding a GFP transgene was added to K562 cells at three different MOIs as indicated: (A) (B) 0.03. (C) (D) 0.1 and (E) (F) 0.3. Transduction efficiency was quantified by flow cytometry seventy-two hours after viral addition.

3.34— Utilizing spinoculation as a means for enhancing sca-1$^+$ transduction

A recent report demonstrated therapeutic levels of fVIII as high as 225 mU mL^{-1} in hemophilia A mice (Ramezani *et al.*, 2011). Few apparent differences were found between the *in vivo* protocol used in the report and the one used in chapter 2. In both cases, sca-1$^+$ cells were transduced with an SIV-based lentiviral vector containing an enhanced fVIII transgene engineered for high expression. In addition, both protocols included transplanting a limited number of cells in hemophilia A mice after being transduced at a low MOI. Despite the similarities between the protocols, drastically different results were observed. Ramezani *et al*. (2011) achieved average fVIII levels equating to 23% of normal fVIII as compared to an average of 4.5% of normal fVIII observed in chapter 2.

One main difference between the protocols was the addition of a spinoculation step via centrifugation immediately following viral addition to sca-1$^+$ cells. For this reason, the effect of spinoculation was evaluated in regards to the transduction efficiency of the optimized vector outlined in chapter 2. Preliminary analysis was performed *in vitro* using the myeloid erythroid-leukemic K562 cell being that K562 cells are an immortalized cell line as compared to sca-1$^+$ cells obtained upon isolation from the bone marrow. In addition, initial analyses included the use of an SIV-based lentiviral vector containing the GFP transgene in order to assess transduction efficiency rapidly by flow cytometry.

K562 cells were transduced at an MOI of 15 and either immediately spun at 1000g for 2 hours or placed in the incubator overnight (n = 3). In addition, three separate wells were

transduced by spinoculation on two consecutive days at an MOI of 15 each day. Transduction was assessed over time by quantifying the percentage of GFP positive cells in each well. However, an MOI of 15 yielded approximately 100% GFP positive cells in the absence of spinoculation, eliminating the possibility of seeing any enhancement in transduction as a result of spinoculation (**Figure 3.8 A**). However, an enhancement in mean fluorescent intensity (MFI) was noted suggesting some benefit to including a spinoculation step in the transduction protocol ($p = 0.0055$ at 5 days post transduction; $p = 0.0332$ at 7 days post transduction as assessed by the Student's t test between the no spinoculation group and the spinoculation group) (**Figure 3.8 B**). This could be either due to enhanced expression of GFP from the cells transduced by spinoculation or due to an increase in integrated vector copies due to enhanced viral transduction. To determine if the increase in MFI was due to enhanced viral transduction, the analysis was repeated utilizing $1/3^{rd}$ the amount of virus equating to a lower MOI of 5. In the first week, the percentage of GFP positive cells was significantly increased from an average of 68.6% to 89.2% as a result of including a spinoculation step in the transduction protocol ($p = 0.0398$ one week after transduction) (**Figure 3.9 A**). This enhancement was maintained throughout the analysis suggesting an enhancement in lentiviral transduction due to spinoculation. A comparison between the single transduction group and the group transduced twice by spinoculation revealed no significant enhancement in percentage of GFP positive cells over time (**Figure 3.9 A**). However, a significant increase in MFI was observed in the double transduction group as compared to the single transduction group (**Figure 3.9 A**). Once again, this suggests some benefit to transducing K562 cells twice by spinoculation.

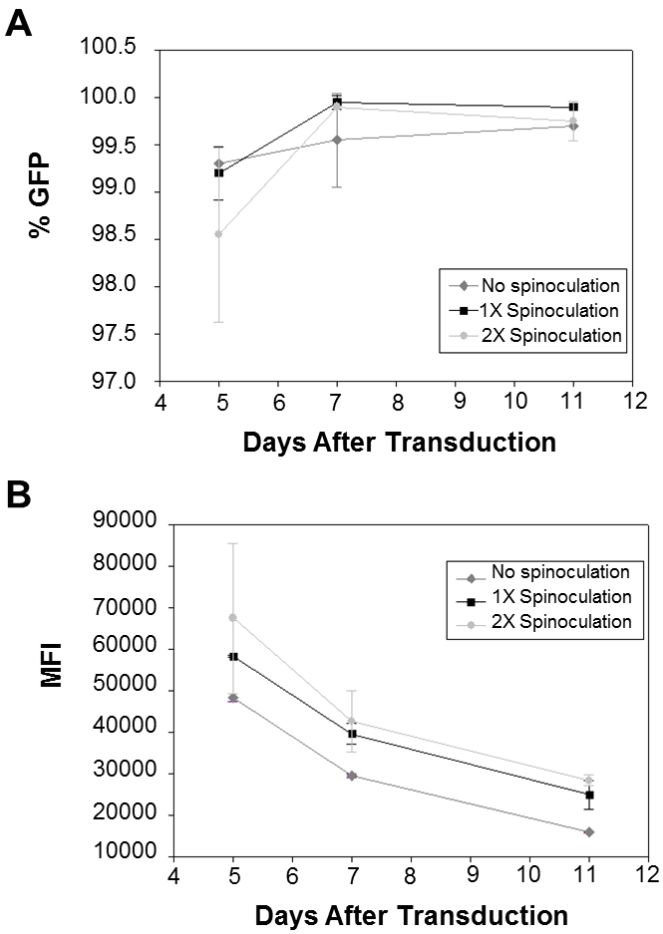


Figure 3.8 The effect of spinoculation on transduced K562 cells with an MOI of 15. (A) An SIV-based lentiviral vector containing a GFP transgene was utilized to transduce K562 cells at an MOI of 15. Transduction was assessed over time by flow cytometry. The percentage of GFP positive cells was compared between K562 cells without spinoculation (dark grey circles), with spinoculation (black squares). K562 cells were transduced twice under spinoculation conditions as a positive control (grey diamonds). (B) The mean fluorescent intensity of the GFP positive cells was quantified by flow cytometry.

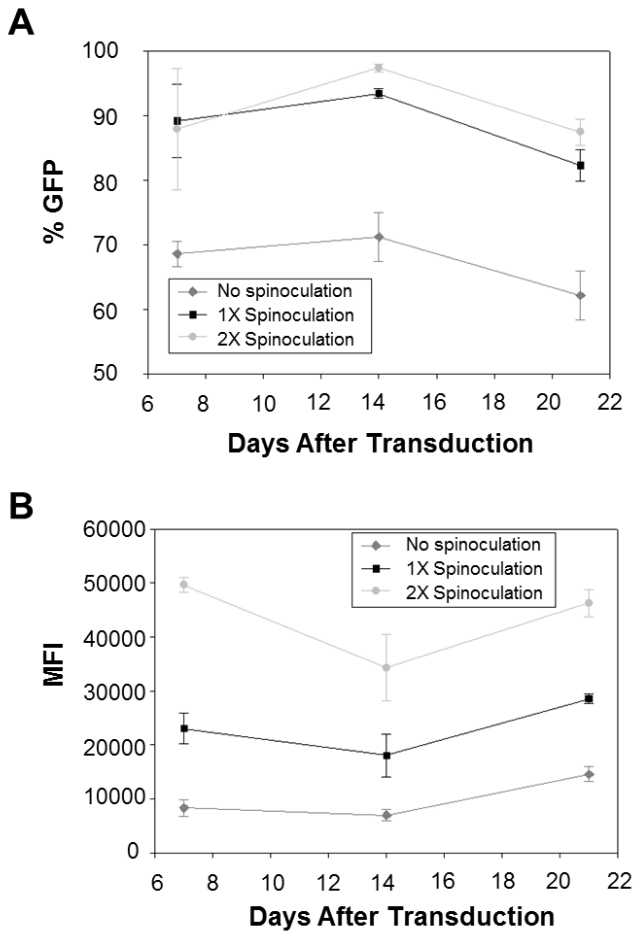


Figure 3.9 The effect of spinoculation on transduced K562 cells with an MOI of 5. (A) Transduction of K562 cells by an SIV-based lentiviral vector containing a GFP transgene (MOI 5) was assessed by flow cytometry. The percentage of GFP positive cells was determined over time and compared among K562 cells without spinoculation (dark grey circles), with spinoculation (black squares) and transduced twice under spinoculation conditions as a positive control (grey diamonds). (B) In the same manner, the mean fluorescent intensity of the GFP positive cells was quantified by flow cytometry.

The enhancement in lentiviral transduction due to spinoculation was then assessed for the optimized lentiviral vector containing the HP-fVIII transgene outlined in chapter 2. K562 cells were transduced with an SIV-based lentiviral vector expressing HP-fVIII from the CMV internal promoter at an MOI of 15. HP-fVIII protein levels were assessed overtime by an APTT reagent-based one stage coagulation assay. K562 cells that underwent spinoculation consistently expressed HP-fVIII at greater levels for a total of eleven days compared to the K562 cells that were not spun after viral addition ($p = 0.003$ three weeks after transduction as determined by a Student's t test) (**Figure 3.10**). This data suggests that SIV-based lentiviral transduction with an HP-fVIII transgene can be enhanced by spinoculation.

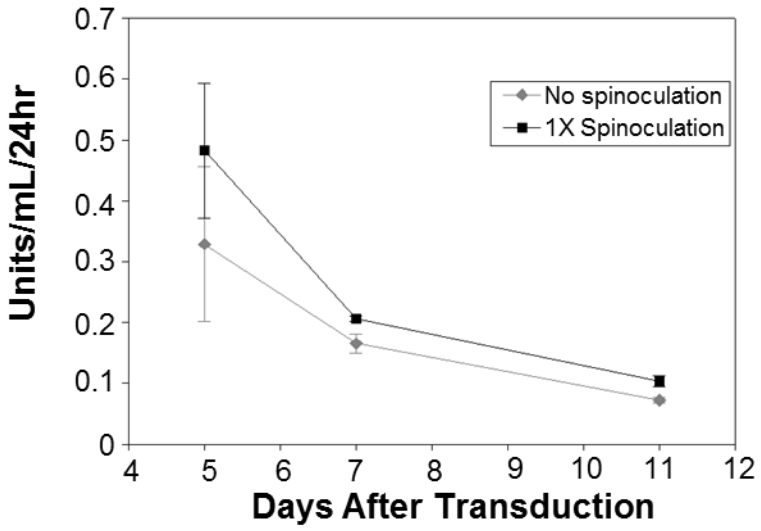


Figure 3.10 HP-fVIII expression overtime in K562 cells. Spinoculation was assessed following transduction of K562 cells with an SIV-based lentiviral vector containing a high-expressing HP-fVIII hybrid transgene expressed from the CMV internal promoter. K562 cells were transduced at an MOI of 15. Immediately following viral addition the cells were either spun for 2 hours at 1000g (black squares) or returned to the incubator (gray circles).

Sca-1^+ cells represent the hematopoietic stem and progenitor population in the mouse. In order to assess the effect of spinoculation on these target cells, sca-1^+ cells were isolated from the bone marrow of the tibias and femurs of 5-FU treated hemophilia A mice. Following isolation, the sca-1^+ cells were transduced with an SIV-based lentiviral vector encoding the GFP transgene. Recombinant virus was added so as to yield an MOI of 15. Transduction was assessed by fluorescent microscopy (**Figure 3.11 A**) and flow cytometry 9 days after transduction. The percentage of GFP positive cells increased from 35% to 68.8% suggesting that SIV-based lentiviral transduction of sca-1^+ can be enhanced by spinoculation (**Figure 3.11 B**). A similar enhancement in transduction was observed in the sca-1^+ cells transduced by spinoculation as was observed in K562 cells.

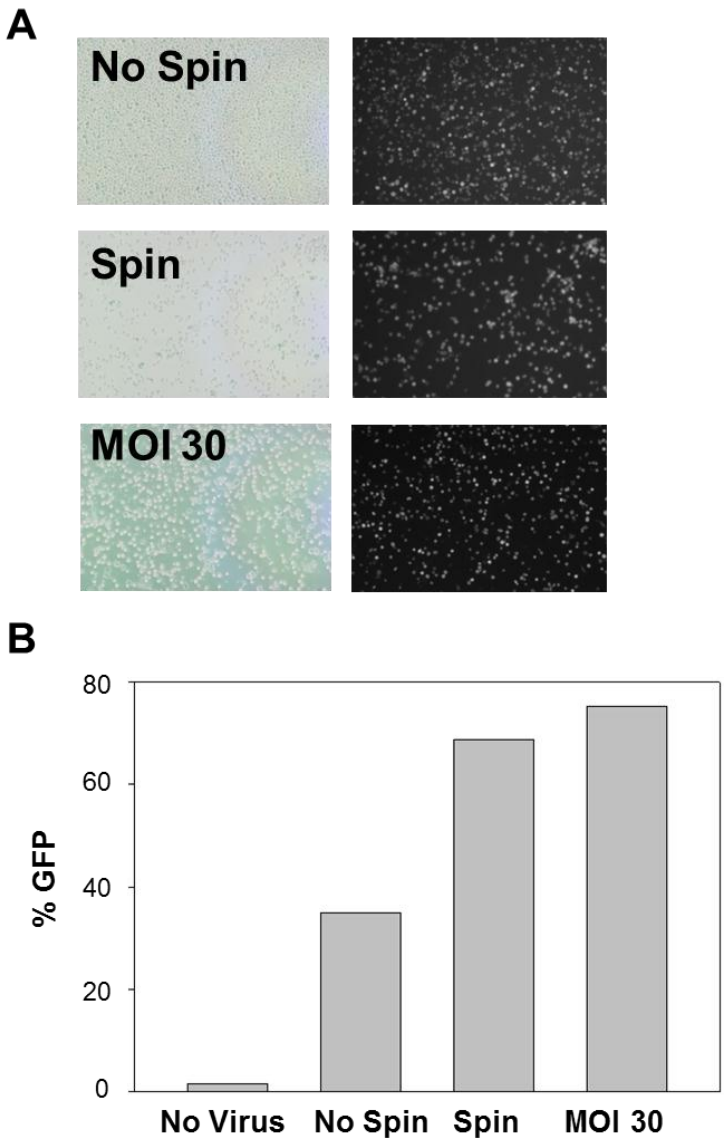


Figure 3.11 The effect of spinoculation on sca-1$^+$ cells. (A) Sca-1$^+$ cells were isolated from hemophilia A mice and transduced with an SIV-based lentiviral vector encoding GFP. Fluorescence microscopy images were taken prior to quantification (B) by flow cytometry.

Due to the results above, an attempt was made to translate these findings *in vivo*. Sca-1^+ cells were isolated and transduced the same day with the optimized lentiviral vector, an SIV-based lentiviral vector containing the HP-fVIII transgene with expression directed from a CMV internal promoter. An identical transduction protocol was utilized as above (**Figure 3.12 A**). Four days after isolation $4x10^5$ cells per mouse were transplanted into lethally irradiated (11*Gy* TBI using a Gammacell 40 Exactor) 8-week-old recipient hemophilia A mice following total body irradiation (n = 3). Despite encouraging preliminary *in vitro* results, minimal HP-fVIII was observed one week after transplantation as assessed by an ELISA assay (**Figure 3.12 B**). In addition, only one mouse remained in the cohort three weeks after transplantation. Although 4×10^5 cells were transplanted per mouse, the fatalities may be due to limited engraftment as a result of transplanting less than optimal sca-1^+ cells. This could also explain the lack of HP-fVIII detected in these mice. A follow-up methyl cell assay confirmed a reduction in sca-1^+ viability following spinoculation (**Table 3.1**).

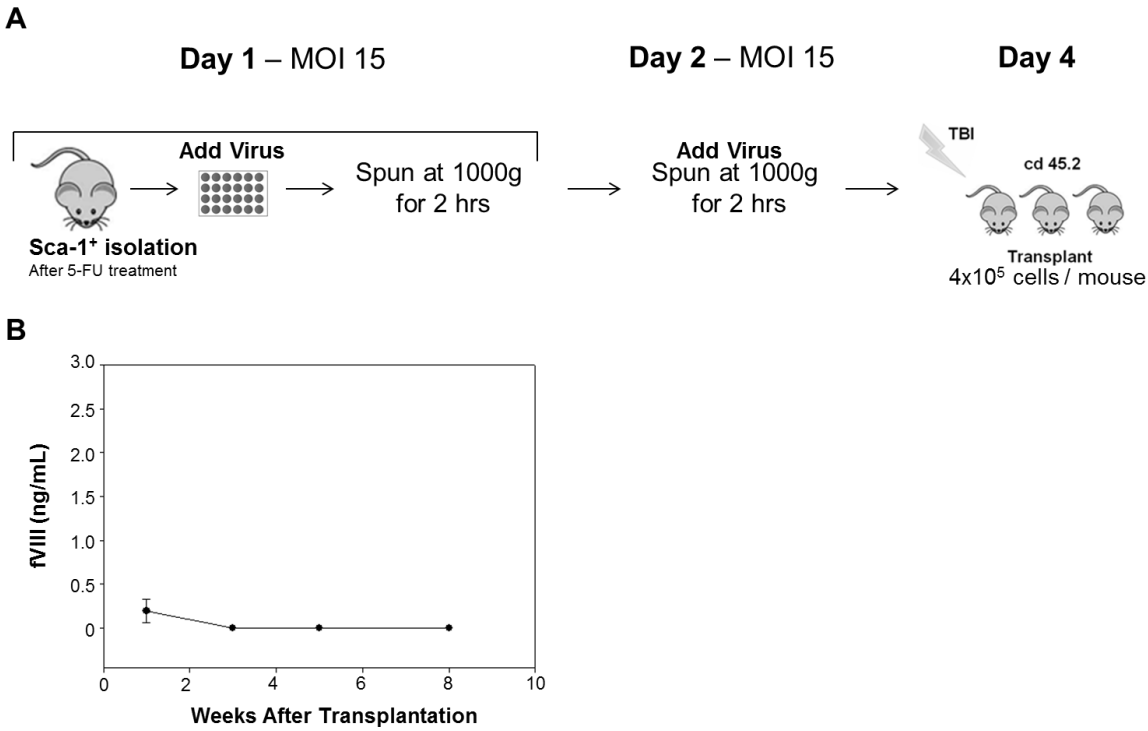


Figure 3.12 The effect of adding spinoculation to the transduction protocol. (A) The transduction protocol was performed as outlined schematically. (B) HP-fVIII was quantified from the peripheral blood of the transplanted mice every two weeks starting one week after transplantation. An ELISA assay was used to quantify HP-fVIII.

Table 3.1 – Sca-1^{+} viability following spinoculation

	Colonies	GFP
Spinoculation	+	
No Spinoculation	+++	++

+ <10 colonies
++ 10-30 colonies
+++ > 30 colonies

3.4— Discussion

In the field of gene therapy, lentiviral vector production is constrained to a transient transfection procedure. HEK-293T cells are predominantly used as a highly transfectable cell line. However, VSVG expression in HEK-293T cells yields syncytia formation followed by a loss in cell viability. Therefore, vector production in this manner is limited. In addition, a transient transfection procedure is not an ideal manufacturing procedure for vector to be utilized in human clinical trials. A stable vector producing cell line would be more appropriate being able to be scaled up and scrutinized as a nonvariable source of vector. For these reasons, BHK-M cells were assessed as an alternative vector producing cell line. Before attempting to create a stable producing cell line, the plasmids required for lentiviral vector production were transfected transiently in order to assess the ability of BHK-M cells to produce a recombinant lentiviral vector. BHK-M cells, however, experienced toxicity associated with VSVG expression. Cell viability was significantly diminished and syncytia formation was evident. In comparison to HEK-293T cells, BHK-M cells appear to be more susceptible to VSVG toxicity. Specifically, HEK-293T cells were found to withstand VSVG expression as a result of transfecting 3200 ng of the envelope plasmid as opposed to the 200 ng threshold observed in BHK-M cells. The increased toxicity in BHK-M cells was not found to be associated with the transfection reagent PEI. Therefore, BHK-M cells are not a viable alternative for VSVG pseudotyped lentiviral production and should not be utilized to generate a stable vector producing cell line.

Many envelopes are currently being evaluated in the field of gene therapy. Since VSVG pseudotyped lentiviral production is not attainable in BHK-M cells, three alternative envelopes to VSVG (Eco, pHit and 10A1), were acquired and assessed for viral production. No apparent toxicity was observed due to the expression of the Eco, pHit or 10A1 glycoproteins. This confirmed a lack of PEI toxicity experienced by BHK-M cells. In addition, cell viability appeared to be unaltered during viral production, potentially overcoming the limitation in days in which lentivirus can be secreted and collected. However, the conditioned medium containing the secreted recombinant virus was unable to sufficiently transduce HEK-293T cells. Recombinant lentivirus virus was not efficiently produced from BHK-M cells. Thus, an improvement was not made to the current HEK-293T transient transfection procedure currently used to produce lentiviral vector. However, if transfection efficiency can be increased vector production may be enhanced in BHK-M cells.

A second limitation in the field of gene therapy is the inability to overcome the inherent resistance of HSCs to lentiviral infectivity. Low transduction efficiencies have been observed clinically and in many cases greater transduction will be required to achieve a therapeutic effect. Therefore, two methods were assessed for the ability to enhance lentiviral transduction. A previous report revealed enhanced expression of bone-marrow derived $CD34^+$ stem and progenitor cells due to the silencing of p21. p21 was suggested to interact with the preintegration complex of HIV inhibiting the integration of the provirus into the genome of the host cell. Silencing p21 would then provide a mechanism in which provirus integration could proceed uninhibited. Therefore, the siRNA to p21

utilized in the report was acquired and assessed on the transduction of K562 cells as an initial indicator of hematopoietic cellular transduction. Three different MOIs were utilized to transduce K562 cells. However, no enhancement in HIV-based lentiviral transduction was observed. This result may be due to cell-type specific pleiotropic effects of p21. For example, depending on cell type, p21 has been attributed to both the inhibition and the progression through the cell cycle. In addition, an opposing role for p21 has been reported in macrophages in regards to HIV infection. p21 levels were found to be upregulated in macrophages as a result of HIV infection and were unable to repress the replication of HIV in this cell type. Therefore, a pleiotropic effect of p21 may account for the lack of enhancement noted in K562 cells following p21 siRNA treatment. However, insufficient knockdown of p21 cannot be eliminated since knockdown was not confirmed.

The use of RNAi technology as a means to enhance lentiviral transduction was ideal in that the effects were transient and were unable to disrupt the multipotentiality of HSCs as is the case with the use of cytokine cocktails. However without an observable enhancement in K562 cells another means of enhancing transduction is required. Spinoculation has been utilized by other groups in the field in the preclinical transduction protocol of sca-1^+ cells. Sca-1^+ cells represent the hematopoietic stem and progenitor population of the mouse. A mechanism providing lentiviral enhancement in this cell could potentially be extrapolated to the $CD34^+$ human bone marrow-derived hematopoietic stem and progenitor cell. Therefore, spinoculation was evaluated for its inclusion into the current transduction protocol utilized in our laboratory. Spinoculation

was found to enhance the lentiviral transduction of K562 cells regardless of the inclusion of a GFP or fVIII transgene. Similarly, the transduction of sca-1^+ cells was enhanced 2-fold with the inclusion of a spinoculation step. However, when included into an *in vivo* transplantation transduction protocol, spinoculation at 1000g for 2 hours diminished the viability of the sca-1^+ cells as evidenced by a lack of hematopoietic reconstitution and gene expression in the lethally irradiated mice. Although spinoculation improved lentiviral transduction, the addition of a spinoculation step did not improve the transduction protocol.

The studies presented in this chapter represent a series of attempts to overcome two of the major hurdles present in the field of gene therapy. Lentiviral production limitations were not able to be overcome by utilizing BHK-M cells as an alternative vector producing cell line. In addition, the transduction protocol was unable to be improved in regards to lentiviral transduction efficiency of the HSC. Silencing p21 did not enhance the transduction efficiency of an HIV-based lentiviral vector. Spinoculation, although able to enhance the percentage of cells infected by the lentiviral vector was unable to disrupt the viability of the stem cell. Therefore, new methods are needed in order to overcome these limitations confronted by the field of gene therapy such as that presented in the next chapter.

Chapter 4:

Pharmacologic Enhancement of Lentiviral Transduction

4.1— Introduction

Hematopoietic stem cells (HSCs) are an attractive cellular target for *ex vivo* gene therapy applications for the correction of several inherited diseases. The reason for their attractiveness is that HSCs have the ability to self-renew as well as differentiate into all lineages of the hematopoietic system. In addition, the use of HSCs is well characterized and these cells are routinely used clinically. However despite their appeal, limited transduction efficiency has been observed in clinical trials (Cartier *et al*., 2009; Boztug *et al*., 2010; Cavazzana-Calvo *et al*., 2010; DiGiusto *et al*., 2010). For example, in a recent phase I clinical trial using HSC gene therapy to treat X-linked adrenoleukodystrophy, $CD34^+$ cells modified with a self-inactivating (SIN) HIV-based lentiviral vector *ex vivo* achieved genetic modification in only 9-14% of blood cells after transplant (Cartier *et al*., 2009). Similarly, in a clinical trial for AIDS, transduction efficiencies were reported to be as low as 1% vector copies per genome in engrafted $CD34^+$ cells (DiGiusto *et al*., 2010). In many scenarios, especially one in which a growth advantage is not expected, greater transduction efficiencies will be needed to achieve therapeutic potential.

Further complicating the issue of limited transduction, high titer viral preparations are not always possible, especially in a scenario in which a large transgene or expression cassette is being utilized. As insert size increases the lentiviral particles recovered by ultracentrifugation have been shown to decrease (Kumar *et al*., 2001; Yacoub *et al*., 2007). A prime example of this can be observed when considering gene therapy applications for the treatment of hemophilia A (for review see Johnston *et al*, 2011). Even after significant reductions in the size of the cDNA encoding fVIII, low viral titer

has been observed (Radcliffe *et al*., 2008). This can limit the amount of virus available to acquire desired multiplicity of infections (MOI) that may be necessary to achieve high transduction efficiencies. Therefore, new strategies would be useful to maximize the transduction efficiency of HSCs.

Multiple attempts have been made towards increasing lentiviral transduction through the use of small molecules and cytokines. In addition, retronectin was shown to enhance the transduction of c-kit^{+} Lin^{-} bone marrow cells 3-fold (Lee *et al.*, 2009) and is thought to act as a bridge for the co-localization of stem cells and viral particles. Small molecules such as ABC transport inhibitors and proteasome inhibitors also have been used with some success (Davis *et al*., 2004; Leuci *et al*., 2011). Both classes of compounds were found to enhance lentiviral transduction of CD34^{+} cells. Similar effects are observed following pre-stimulation with cytokines (Santoni *et al*., 2006). However, a series of comparisons were performed in order to determine an optimal clinically relevant lentiviral transduction protocol using various cytokine combinations and concentrations as well as combining pre-stimulation with the use of retronectin. Overall, low levels of gene-modified cells were observed (Millington *et al*., 2009).

To overcome the transduction barrier, a high-throughput screen was performed to identify compounds that enhance lentiviral transduction. Among the positive compounds identified by the screen were camptothecin and etoposide, which have been shown previously to be enhancers of lentiviral transduction (Groeschel and Bushman, 2005). However, as topoisomerase inhibitors they also are known to cause DNA strand breaks

(Pommier *et al.*, 2010). A potentially safer compound identified was phorbol 12-myristate 13-acetate (PMA). PMA stimulates protein kinase C (PKC), a family of closely related serine/threonine kinases involved in signal transduction pathways regulating an array of cellular processes. The PKC family consists of 10 isoforms classified into three subfamilies according to which secondary messengers are required for PKC activation. Two of the subfamilies, the conventional (PKCα, PKCβ_I, PKCβ_{II}, and PKCγ) and novel PKCs (PKCδ, PKCθ, PKCε, and PKCη) require diacylglycerol (DAG) for activation. PMA, an analog of DAG, activates isoforms in these two subfamilies and associates at the same binding site as DAG in the C1 regulatory domain (Stahelin *et al.*, 2005) (Steinberg, 2008). In the current study, PMA was confirmed as a positive enhancer of lentiviral transduction, enhancing the transduction of hematopoietic cell lines and human $CD34^+$ cells.

4.2— Materials and Methods

Lentiviral Vector Production

SIV-based and HIV-based lentiviral vectors pseudotyped with VSVG were produced from HEK-293T cells upon transient co-transfection utilizing polyethylenimine (6 µg PEI / 1 µg DNA) (Fisher Scientific, Pittsburg, PA). HIV-based lentiviral vectors were manufactured utilizing the LentiMax production system which consists of a 2:1:1 ratio of expression plasmid to packaging plasmids (expression plasmid:psPAX2:pVSVG). A ratio of 1.3:1:1:1.6 of expression plasmid to packaging plasmids (expression plasmid:pCAG4:pVSVG:pSIV) was used to manufacture SIV-based lentiviral vectors. The media was replaced twenty-four hours after transfection with DMEM/F-12

(Invitrogen Life Technologies, Carlsbad, CA) containing 10 % fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1 % penicillin / streptomycin (Mediatech, Manassas, VA). No antibiotics were present during the time of transfection. For the following three days, conditioned medium was collected from the HEK-293T viral producing cells, passed through 0.45 μmol l^{-1} filter and stored at -80^{o}C until concentration by velocity sedimentation upon centrifugation at 10,000 x *g* (4^{o}C) overnight. Viral pellets were resuspended in 1/100th of the original volume of StemPro media (Invitrogen life technologies, Carlsbad, CA), and filtered through a 0.22 μmol l^{-1} filter. Viral titer was assessed on HEK-293T cells with increasing vector volumes by real-time quantitative PCR seventy-two hours after viral addition. Virus was stored in 1mL aliquots at -80^{o}C.

High-throughput Screening

Compounds from the LOPAC^{1280} library (Sigma, St. Louis, MO, USA) were screened in 384 well tissue culture treated clear bottom plates. Each well contained 10^4 K562 cells cultured in phenol-red free DMEM/F-12 media. For each plate, two columns of sixteen wells were used as a baseline for fluorescence containing only K562 cells and virus at an MOI of 0.5. Another set of two columns was used as a positive control containing K562 cells transduced at an MOI of 5. The remaining wells contained K562 cells treated with one compound per well three hours prior to viral addition. Compounds were added at a concentration of 20 μM. Virus was added at an MOI of 0.5. The following day, media in the wells was exchanged for fresh DMEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin / streptomycin. Fluorescence intensity was assessed seventy-

two hours following viral transduction with the use of a fluorescent imager, the Image Xpress. Compounds able to demonstrate a fold increase of 2.5 or higher than the average fluorescence emitted from the baseline columns were identified as positive hits. The positive hits were then confirmed utilizing ten different doses of the compound under the same conditions as the initial screen. Doses ranged from 0.1 μM to 60 μM. Compounds that were able to demonstrate a fold increase in fluorescence intensity a second time were denoted as positive hits.

Culture, treatment and transduction of K562, EU1, BHK-M, HEK-293T, NIH-3T3 and U937 cell lines

K562, EU1, BHK-M, HEK-293T, NIH-3T3 and U937 cell lines were cultured in DMEM/F-12 supplemented with 10% FBS and 1% penicillin / streptomycin. Cells were plated in twenty-four well tissue culture plates and treated with PMA (Fisher Scientific, Pittsburg, PA). Prior to treatment, PMA was dissolved in DMSO to yield an initial concentration of 10 mg mL^{-1}. Subsequent dilutions were performed in DMEM/F-12 supplemented with 10% FBS and 1% penicillin/streptomycin. Following a two hour treatment with PMA, cells were transduced in minimal volume (300 μL) with a lentiviral vector in the presence of polybrene (8 ng μL^{-1}). Transduction efficiency was assessed seventy-two hours by flow cytometry unless otherwise noted. The percentage of GFP positive cells was compared to the percentage of GFP positive cells from cells transduced in the absence of PMA.

Isolation, treatment and transduction of murine stem cell antigen-1^+ cells

The femurs and tibias of 8 to 10 week old exon 16-disrupted hemophilia A mice (as previously described in Bi *et al*, 1995) were flushed with PBS supplemented with 2% FBS. The flushed bone marrow was then subjected to positive immunomagnetic bead selection using magnetic separation columns purchased from Miltenyi Biotec (Auburn, CA) as previously described (Gangadharan *et al.,* 2006). The isolated sca-1^+ cells were then assessed for purity by flow cytometry utilizing a Ly-6A/E antibody (clone E13-161.7) conjugated to FITC (BD Pharmingen, San Diego, CA). The purity of the population was found to be 96% positive for sca-1^+. Upon confirmation, the sca-1^+ cells were cultured at a density of 10^6 cells per mL in StemPro media supplemented with L-glutamine (29µg mL^{-1}) and stimulated for three days with murine stem cell factor (100ng mL^{-1}), murine interleukin-3 (20ng mL^{-1}), human interleukin-11 (100ng mL^{-1}), and human Flt-3 ligand (100ng mL^{-1}). Following stimulation, sca-1^+ cells were plated onto a twenty-four well tissue culture treated plate at a density of $2x10^6$ cells per well and treated with PMA for two hours. Sca-1^+ cells were then transduced in the presence of 8ng μL^{-1} polybrene with a lentivirus containing the GFP transgene. Transduction efficiency was assessed by flow cytometry seventy-two hours following transduction.

Isolation, treatment and transduction of $CD34^+$ cells

$CD34^+$ cells were purchased from AllCells (Emeryville, CA). Prior to arrival, $CD34^+$ cells were mobilized from human bone marrow and isolated from the peripheral blood of 3 individual subjects. Flow cytometry was used to assess the purity of the sample which ranged between 97 and 99 percent of the population found to be positive for CD34. Cells

were shipped over night on dry ice and upon arrival stimulated for 24 hours with recombinant human Flt-3 ligand (300ng mL^{-1}), human recombinant stem cell factor (300ng mL^{-1}), human recombinant thrombopoietin (100ng mL^{-1}), human interleukin-3 (60ng mL^{-1}). $CD34^+$ cells were cultured in GMP serum-free stem cell growth medium (Cell Genix, Portsmouth NH) supplemented with 1% penicillin / streptomycin. After stimulation, the purity of the population was confirmed by flow cytometry. Upon confirmation, $CD34^+$ cells were cultured in a twenty-four well tissue culture treated plate at $2x10^6$ cells per well and treated with PMA for two hours prior to transduction. $CD34^+$ cells were transduced in minimal volume (300 µL) and initially assessed for GFP seventy-two hours following transduction by flow cytometry. The continued purity and transduction efficiency of the $CD34^+$ cells was assessed up to twelve days following transduction.

Colony-forming Unit Assay

Colony-forming cell assay was performed per manufacturer's protocol (Stemcell Technologies, MethoCult® H4035 Optimum without EPO). Briefly, a 10X concentration ($2x10^4$ cells/mL) of $CD34^+$ cells was prepared in Iscove's MDM with 2% FBS. A total of 0.3 mL of 10X cell mixture was then added to 3 mL of MethoCult® and mixed by vortexing. MethoCult®/cell mixture was allowed to sit for 5 minutes to allow bubbles to rise followed by dispensing 1.1 mL onto 35 mm dishes in duplicate using a 16 gauge blunt-end needle. The methylcellulose was evenly distributed into the dish by rotation and dishes were placed in a 100 mm culture dish alongside a third dish containing sterile water to maintain proper humidity. Cultures were incubated at 37°C in

5% CO^2 for 14-16 days for optimal CFC growth. CFU-GM colonies were counted at 5X under an inverted microscope using a 60 mm gridded dish. GFP-positive colonies were scored under fluorescence microscopy.

fVIII Copy Number Analysis

Copy number analysis was performed as outlined in chapter 2. In short, were harvested by centrifugation at 300g for 5 minutes and used to isolate total genomic DNA using the DNeasy® Blood & Tissue Kit (QIAGEN, Valencia, CA). A quantitative PCR reaction was performed utilizing primers specific for the high expressing chimeric fVIII transgene. Ct values for each sample were compared to Ct values produced from plasmid standards of known copy quantities. The equivalent copy number was then divided by 8333, the predicted number of diploid genome equivalents in 50 ng of DNA assuming a haploid genome weight of 3 pg.

fVIII expression

fVIII expression was measured as previously outlined in chapter 2 from the supernatant of cells. In short, the cells were cultured in serum-free media for 24 hours prior to the activated partial thromboplastin reagent-based one stage coagulation assay. The analysis was performed in duplicate for each supernatant on a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France) in human fVIII-deficient plasma (George King Biomedical, Overland Park, KS). The clot time for each sample was compared to a standard curve based on dilutions of pooled normal citrated human plasma (George King Biomedical, Overland Park, KS).

Statistical analysis

All values are reported as mean ± standard deviation. A student's t-test was used for comparisons between two groups. For comparisons between more than two groups, a one way ANOVA was used. Significance was defined as a p value of less than 0.05.

4.3— Results

4.31— High-throughput screen identifies lead compounds

Protocol Optimization of a High-Throughput Screen

In collaboration with Emory's Chemical Biology Discovery Center, a high-throughput screen was performed to identify compounds that could enhance the lentiviral transduction of hematopoietic cells. A library of 1280 pharmacologically active compounds ($LOPAC^{1280}$) was screened. This library was chosen because it covers the major drug target classes and impacts most cellular processes. Although the screen was performed to determine compounds that could enhance the transduction of HSCs, the myeloid erythroid-leukemic K562 cell line was chosen as an initial indicator of enhanced transduction, as K562 cells are less difficult to culture than the cytokine requiring murine sca-1^+ or human $CD34^+$ cells. In addition, a lentivirus containing the GFP transgene was used in order to assess transduction efficiency in a high-throughput manner with the use of a fluorescent imager, the Image Xpress.

The screening protocol was optimized in regards to culturing conditions and endpoint time frame. In order to determine the appropriate culturing condition, a range of K562

cellular densities was tested in a 144 well tissue culture treated clear bottom plate. For each density, cells were transduced with recombinant lentivirus (n = 6) at increasing MOIs. Fluorescent intensity was then assessed seventy-two hours following transduction and compared to nontransduced wells. As shown in **Figure 4.1 A**, the culture of 10^4 cells per well produced the greatest separation in relative fluorescent intensity among wells transduced with increasing MOIs. Therefore the cellular density of 10^4 K562 cells/well yielded an appropriate condition in which to measure transduction enhancement. In order to assess the appropriate endpoint of the screen, 10^4 K562 cells were plated per well and virus was added to each well at increasing amounts (n = 8). Transduction efficiency was assessed at multiple time points after transduction. Well to well variability improved 72 hours following transduction as compared to an assessment at 48 hours (data not shown). In this manner, an appropriate assay window was produced in order to assess enhanced transduction as a fold-increase from the baseline of an MOI of 0.5 (**Figure 4.1 A**). Upon identifying an optimal culturing condition and post transduction read out time for the screen, a total cell image was taken with the use of a Hoechst dye. Minute holes were found in the monolayer of K562 cells that may be the cause for variability among wells (**Figure 4.1 B**). However a distinct difference in fluorescence is clearly noted between an MOI of 0.5 and 4 (**Figure 4.1 C**). This obvious enhancement in fluorescence validates the effectiveness of the screen in being able to determine enhanced transduction as a result of the addition of more virus. It is postulated that a similar increase could be observed as a result of the presence of a molecular compound that enhances transduction.

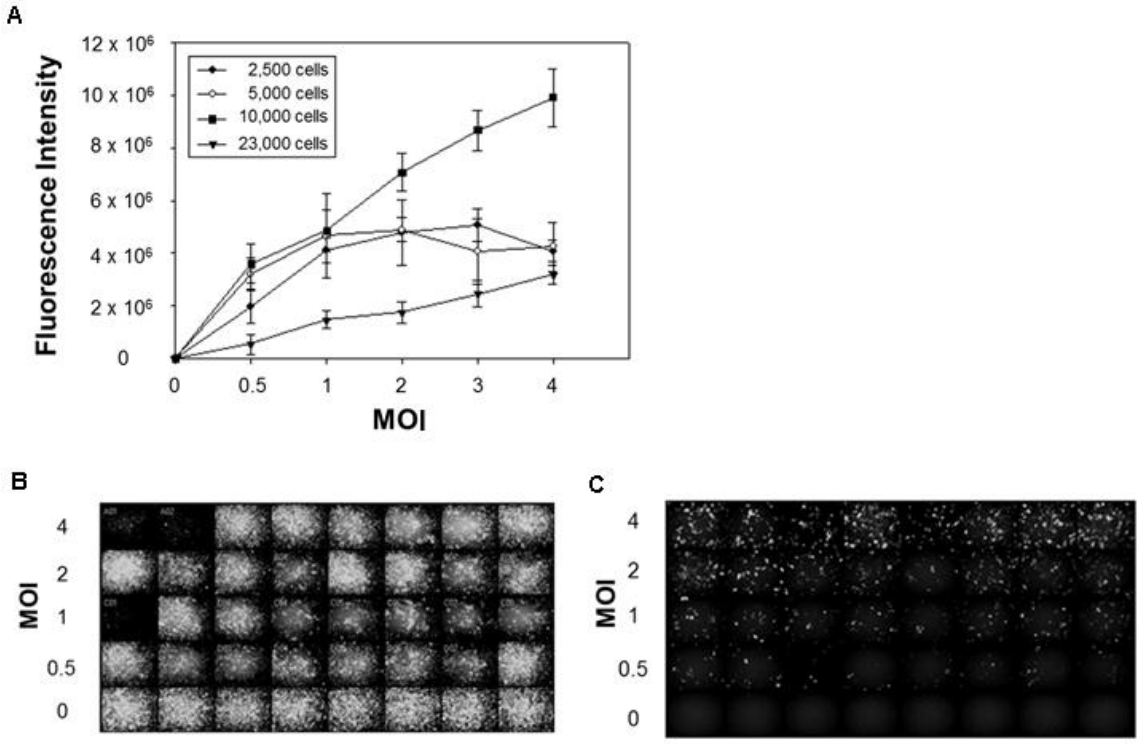


Figure 4.1 Optimization of the high-throughput screening protocol. (A) In order to identify the appropriate cell density at which to yield an appropriate assay window, K562 cells were plated at varying densities (n = 6) and transduced with MOIs increasing from 0 to 4. Fluorescence intensity was quantified seventy-two hours following transduction with the use of a fluorescence imager, the Image Xpress. (B) Cells were stained with a Hoechst dye. (C) Representative fluorescence microscopy image demonstrating observable increases in GFP positive cells as a result of increasing MOI.

High-Throughput Screen

The high-throughput screen was then performed in a tissue culture treated plate in which 10^4 K562 cells were plated per well. The compounds were added at a concentration of 20 μM three hours before viral addition at an MOI of 0.5. Media was exchanged the following day and fluorescence was assessed seventy-two hours following transduction. Fluorescence intensity was compared to baseline fluorescence intensity as determined in the first two columns of the plate, which only contained K562 cells transduced at an MOI of 0.5 (**Figure 4.2 A**). As a positive control, the last two columns of the plate were transduced with 8-fold more virus to confirm increase in fluorescence intensity (**Figure 4.2 A**). Compounds that yielded a fold induction of 2.5 or higher over baseline fluorescence were denoted as positive hits (for example, H22 and O5 of **Figure 4.2 A**) while obvious autofluorescent compounds were not further analyzed (for example, L10 of **Figure 4.2 A**). The screen was performed for both an SIV-based and an HIV-based lentiviral vector. For the SIV-based lentiviral vector, thirty-four compounds were identified that yielded a fold induction in fluorescent intensity of 2.5 or higher. Similarly, thirty-two compounds were identified with the use of an HIV-based lentiviral vector.

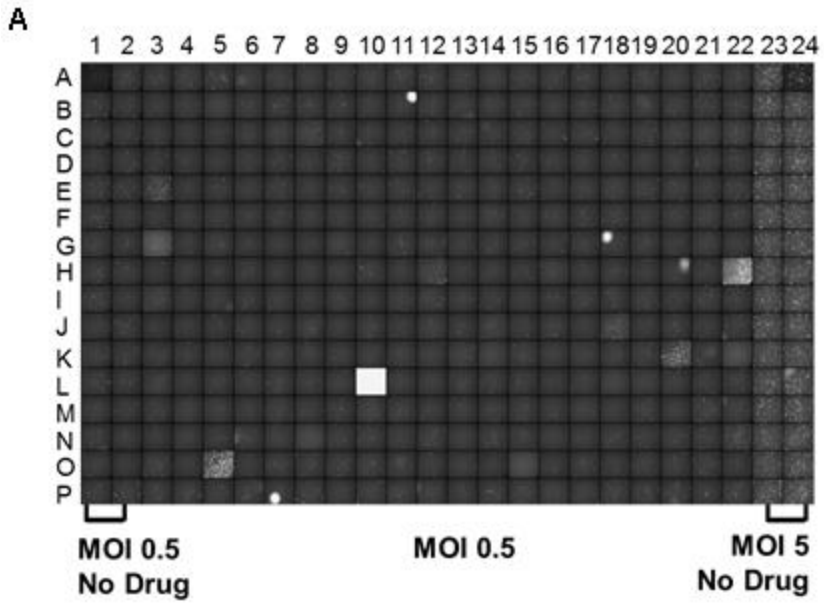


Figure 4.2 Visual representation of the high-throughput screen. (A) Sample fluorescence microscopy image of 1 of 4 plates run during the high-throughput screen. Columns are denoted numerically from 0 to 24. Rows are denoted alphabetically from A to P. The first and last two columns did not contain test compounds. Columns 1 and 2 acted as a baseline from which to assess fold-induction in fluorescent intensity whereas columns 23 and 24 contained K562s transduced with 10 times more virus (positive control). Well L10 represents an autofluorescent compound. Wells H22 and 05 represent compounds identified as positive hits yielding a fold induction greater than 2.5 from that of the first two column means.

4.32— High-throughput screen identifies false positives

Forskolin: False positive

As an FDA approved drug, forskolin was included in the LOPAC^{1280} library. Forskolin was initially identified as a positive hit of the high-throughput screen, which yielded a 6.8 fold induction in fluorescence. A 10-point dose response was then performed utilizing the optimized K562 cellular culturing condition and the optimized endpoint time frame. Forskolin was added at the indicated doses (**Figure 4.3 A**) 3 hours prior to viral transduction at an MOI of 0.5. The results of the 10-point dose assay were promising in that a similar fold induction (7-fold) in fluorescence was observed at 20 μM Forskolin, the concentration used in the initial screen. In addition, a dose response was observed indicating the potential of forskolin to enhance lentiviral transduction (**Figure 4.3 A**). During the validation process, the assay was repeated in a twenty-four well tissue culture treated plate and compared to the initial assessment performed at the screening center in which a three hundred eighty-four well plate was used. K562 cells were plated at a density of 10^5 cells/well and treated with the indicated doses of forskolin in triplicate. An SIV-based lentiviral vector containing a GFP transgene was added to each well at an MOI of 0.5. The percentage of GFP positive K562 cells was quantified by flow cytometry seventy-two hours following viral transduction. However despite the initial observation of forskolin-induced enhanced transduction in a high-throughput format, an increase in GFP positive cells was not able to be reproduced (**Figure 4.3 B**).

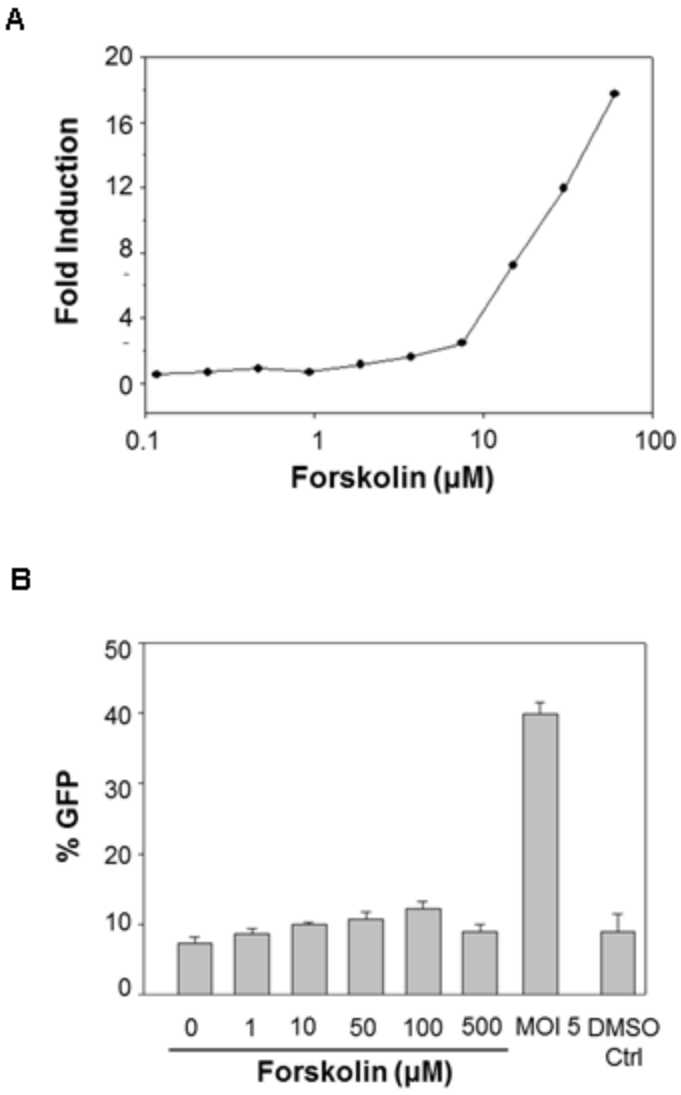


Figure 4.3 Forskolin as an example of a false positive identified from the high-throughput screen. (A) A 10-point dose response curve was performed in the screening center. K562 cells were plated at a cell density of 10^4 cells/well and treated with forskolin at the indicated concentrations for 3 hours prior to viral addition (MOI of 0.5). (B) To validate the 10-point dose response a second dose response analysis was performed utilizing the same concentrations as the screening center with the addition of 500 μM forskolin (n = 3). A twenty-four well plate was seeded with K562 cells at a density of 10^5 cells/well and treated with forskolin at the indicated concentrations and

transduced at an MOI of 0.5. Transduction efficiency was assessed by flow cytometry seventy-hours following viral addition. A DMSO control was added in which the maximum amount of DMSO (used to solubilize forskolin) present in the highest dose of forskolin was added prior to viral addition.

SB 205384: False Positive

The high-throughput screen included the $GABA_A$ partial agonist, denoted as SB 205384. Initially, SB 205384 yielded a fold induction of 7.5 in fluorescence intensity as compared to the control wells transduced in the absence of compound. The 10-point dose response curve performed at the screening center suggested that the enhancement seen in transduction with SB 205384 on K562 cells had yet to be observed at its fullest (**Figure 4.4 A**). Therefore, greater concentrations of SB 205384 were tested on K562 cells plated in a 24 well plate at a density of 10^5 cells/well. K562 cells were treated with SB 205384 at the indicated concentrations (**Figure 4.4 B - C**). Minimal virus was added to each well either in the presence (**Figure 4.4 B**) or absence (**Figure 4.4 C**) of polyberene. Transduction was assessed seventy-two hours later by flow cytometry. Although concentrations greater than 100 μM were used no enhancement in transduction was observed (**Figure 4.4 B - C**). The enhancement in transduction observed at the screening center was unable to be repeated identifying SB 205384 as a false positive.

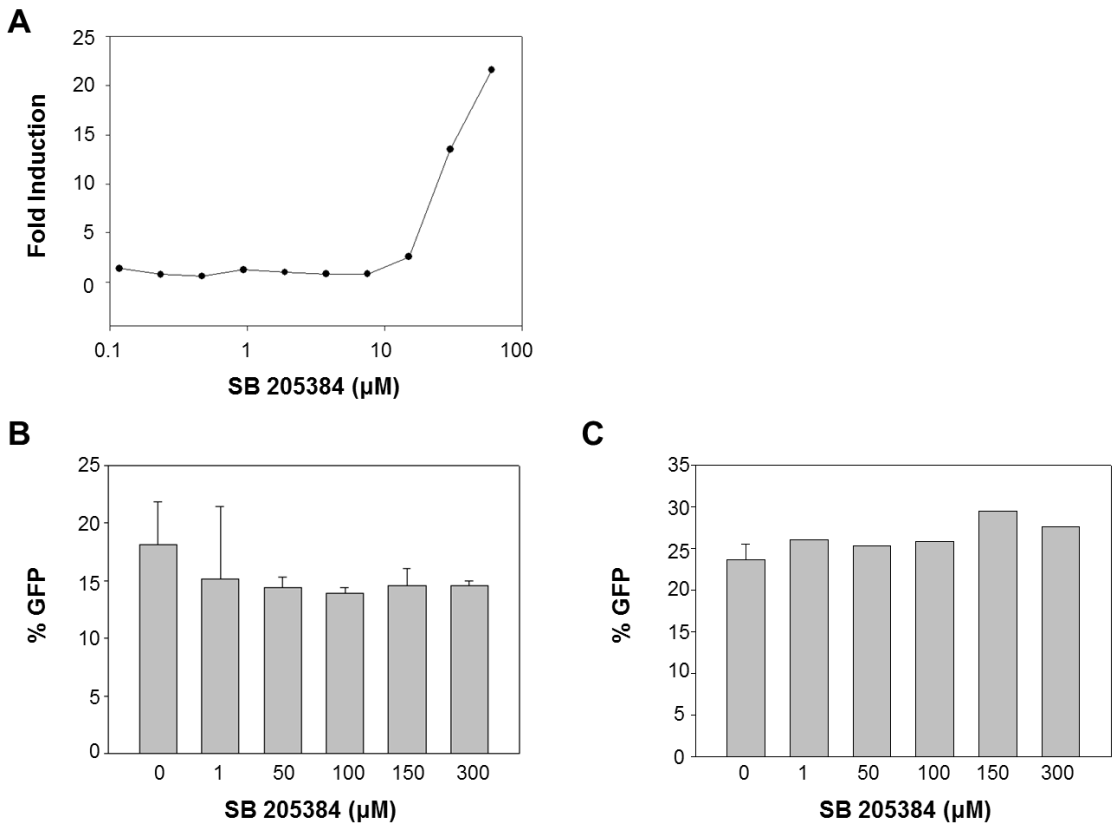


Figure 4.4 SB 205384: a false positive identified from the high-throughput screen. (**A**) In the screening center, a 10-point dose response curve of SB 205384 was performed in order to verify SB 205384 as a positive hit. As previously described above, 10^4 K562 cells were plated per well and treated with SB 205384 at the indicated concentrations for 3 hours prior to viral addition (MOI of 0.5). Fluorescence intensity was measured using the Image Xpress seventy two hours later in which the treated wells were compared to the non-treated wells. (**B**) Similarly, SB 205384 was analyzed in the Spencer laboratory in a dose dependent manner utilizing the same concentrations as the screening center with the addition of 150 and 300 μM SB 205384. Virus was added immediately following compound addition either in the presence or (**C**) absence of polybrene. Transduction efficiency was assessed by flow cytometry seventy-two hours following viral addition.

Cytosine-1-β-D-arabinofuranose hydrochloride: False positive

Cytosine-1-β-D-arabinofuranose hydrochloride was denoted as a positive hit from the screen yielding a 3.1 fold induction in fluorescence intensity from the baseline fluorescence observed from the non-treated wells of the high-throughput screen. The compound was included in the LOPAC screen as an FDA approved drug. Clinically, cytosine-1-β-D-arabinofuranose hydrochloride is used to treat leukemia. As a pyrimidine analog, cytosine-1-β-D-arabinofuranose hydrochloride becomes incorporated into the DNA during synthesis and inhibits cycling through the S phase resulting in apoptosis.

Following the initial high-throughput screen, a 10-point dose response analysis on cytosine-1-β-D-arabinofuranose hydrochloride was performed at the screening center. Fluorescence intensity was increased in a fold dependent manner from the baseline fluorescence of non-treated wells at concentrations up to 15 μM (**Figure 4.5 A**). Concentrations of cytosine-1-β-D-arabinofuranose hydrochloride higher than 15 μM demonstrated a decrease in fluorescence intensity. For this reason, lower concentrations were used to verify cytosine-1-β-D-arabinofuranose hydrochloride as an enhancer of lentiviral transduction in the Spencer laboratory. Cytosine-1-β-D-arabinofuranose hydrochloride was added to K562 cells plated in a 24 well plate at a density of 10^5 cells / well at the indicated concentrations (**Figure 4.5 B - C**). Minimal virus was added the treated and non-treated K562 cells immediately following treatment either in the presence (**Figure 4.5 B**) or absence (**Figure 4.5 C**) of polyberene. The enhancement observed at the lower concentrations of the 10-point dose response analysis performed at the screening center was not able to be repeated.

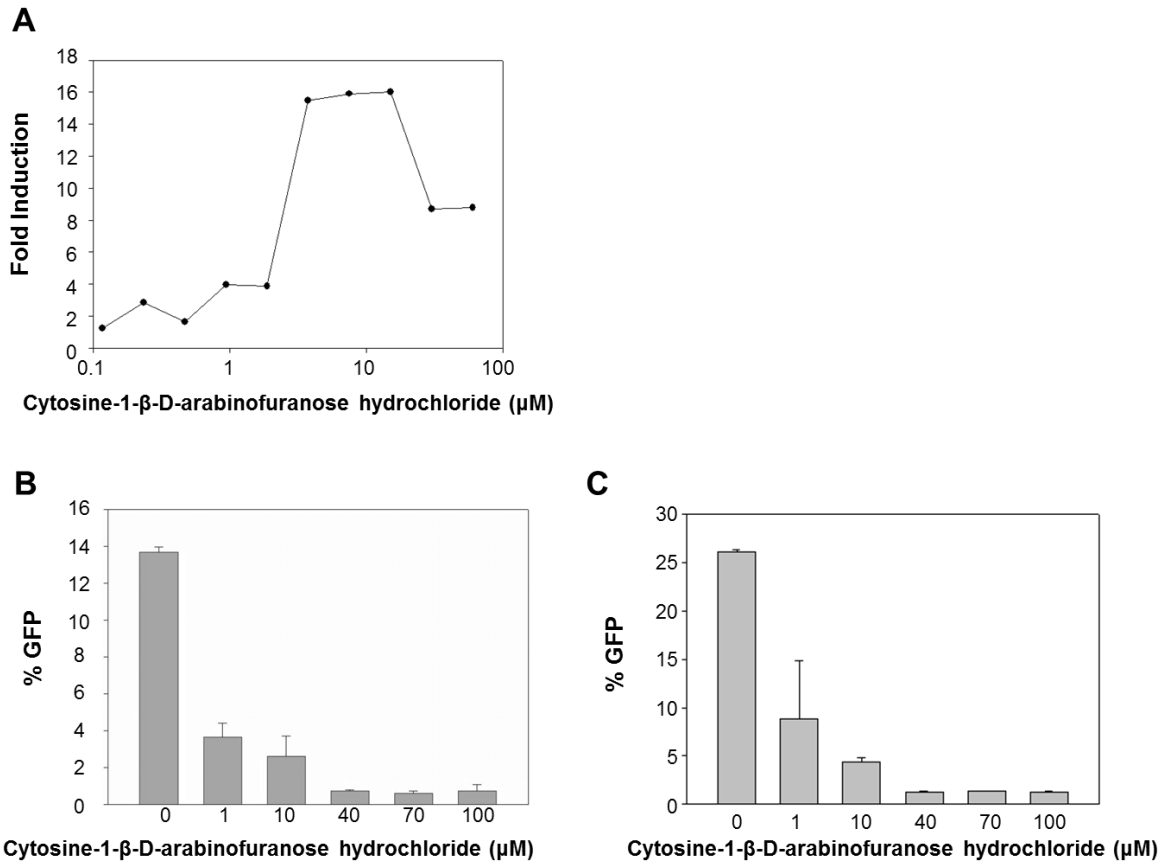


Figure 4.5 Cytosine-1-β-D-arabinofuranose hydrochloride: a false positive of the high-throughput screen. (A) The result of a 10-point dose response assay performed at the screening center for the compound Cytosine-1-β-D-arabinofuranose hydrochloride. The analysis was performed on K562 cells (plated at a density of 10^4 cells/well) treated with the indicated concentrations for 3 hours prior to viral addition (MOI of 0.5). The Image Xpress was used to determine fold induction in fluorescence intensity seventy-two hours after viral addition. (B) Cytosine-1-β-D-arabinofuranose hydrochloride was evaluated in a similar fashion in the Spencer laboratory. K562 cells were plated in 24 well plates at a density of 10^5 cells/well and treated at concentrations similar to those used by the screening center (n = 2). Transduction was assessed by flow cytometry seventy-two hours after viral addition in the presence or (**C**) absence of polybrene.

4.33— High-throughput screen identifies previously confirmed enhancers

Camptothecin: True positive

Camptothecin, etoposide, and taxol were also identified from the screen as positive hits yielding a fold increase in fluorescent intensity of 3.2, 2.6, and 2.7 respectively. Encouragingly, all 3 compounds were previously shown to enhance the transduction of HEK-293T and HeLa cells (Groeschel and Bushman, 2005). In the current study, camptothecin was confirmed as an enhancer of lentiviral transduction of HEK-293T cells. Increasing concentrations of camptothecin were added to HEK-293T cells while simultaneously being transduced with an SIV-based lentiviral vector containing a GFP transgene. Each concentration was performed in triplicate. Transduction efficiency was determined seventy-two hours following transduction by quantifying the percent of GFP positive cells by flow cytometry. As observed by Groeschel and Bushman (2005), transduction efficiency peaked at 0.4 μM camptothecin (**Figure 4.6 A**). Higher concentrations of camptothecin resulted in diminished transduction efficiency, which may be a result of toxicity to the cells. Camptothecin was found to inhibit cell division at concentrations as low as 3 nM (**Figure 4.6 B**), which is likely due to cell cycle arrest in the G2/M phase (Groeschel and Bushman, 2005).

In addition to confirming a previous report identifying camptothecin as a positive enhancer of HEK-293T cell lentiviral transduction, K562 cells were analyzed in order to validate camptothecin as a positive effector of transduction of hematopoietic cells. A similar protocol was performed in which increasing concentrations of camptothecin were added to the cells in triplicate while being transduced with identical amounts of lentiviral

vectors. In this analysis, both HIV and SIV-based lentiviral vectors were used. Similar transduction efficiency curves were noted for both lentiviral vectors. K562 cells yielded a peak in transduction efficiency at 0.08 μM (**Figure 4.6 A**). An inhibition in cellular division as a result of camptothecin was observed with K562 cells as was observed with HEK-293T cells (**Figure 4.6 C**). Because camptothecin and other cytotoxic agents identified in the screen induce DNA damage, they are not considered ideal compounds for clinical use.

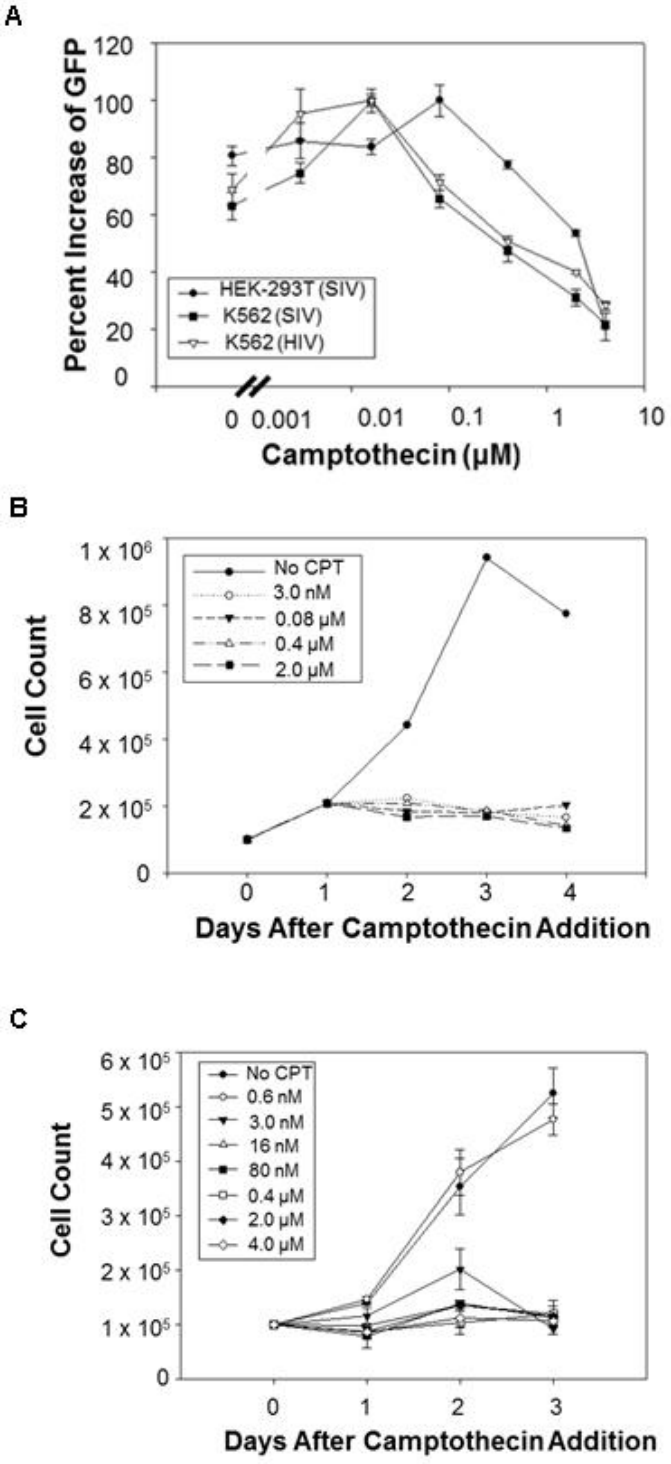


Figure 4.6 Camptothecin enhances lentiviral transduction. (A) In a twenty-four well plate, K562 cells were plated at a density of 10^5 cells/well and treated with the indicated doses of camptothecin (n = 3). K562 cells were transduced with either an SIV-based

(solid squares) or HIV-based (open triangles) lentiviral vector at an MOI of 5. In the same manner, $5x10^4$ HEK-293T cells were plated in a twenty-four well plate twenty-four hours prior to transduction yielding approximately 10^5 cells/well (solid circles). The percentage of GFP positive cells was assessed by flow cytometry seventy-two hours following transduction. (B) HEK-293T cell counts were performed throughout the experiment in which only viable cells were counted after Trypan Blue staining. (**C**) Viable K562 cell counts were obtained up to 3 days after treatment with camptothecin.

4.34— Enhancement of the Positive Hit, PMA is cell dependent

PMA as an enhancer of K562 lentiviral transduction

PMA is an analog of diacylglycerol and is able to bypass the signal transduction pathway leading to PKC activation. PMA was identified from the SIV-based screen as an enhancer of lentiviral transduction and yielded the highest increase in fluorescence intensity observed, which was a 10 fold-increase from the baseline fluorescence. The compound was confirmed as a positive enhancer of lentiviral transduction in a 10-point dose response testing assay. At high nanomolar concentrations, a morphological change is evident (**Figure 4.7 A**), similar to that previously reported (Papayannopoulou *et al*., 1982). Decreasing the concentration of PMA to 2.5 nM diminished this effect (**Figure 4.7 A**). In addition, at the lower doses of PMA a dose response was observed indicating the potential of PMA to enhance lentiviral transduction. This was observed with both the use of an SIV-based (**Figure 4.7 B**) and an HIV-based lentiviral vector (**Figure 4.7 C**). Although no morphological change occurs with PMA treatment at 2.5 nM, a 3.5-fold enhancement in transduction is apparent (**Figure 4.7 B**). Cell proliferation was found to be halted in a dose-dependent manner being completely inhibited at 2.5 nM and higher concentrations (**Figure 4.7 D**). In addition, a time course analysis was performed in order to determine an optimal time at which to add virus after PMA incubation. Virus was added either simultaneously or after a 2, 4, 8, 24 or 48 hour incubation. The analysis was performed under similar culturing conditions in triplicate. Consistently, a 2 hour pre-incubation yielded the greatest enhancement in viral transduction as apparent by the percentage of GFP positive cells determined by flow cytometry seventy-two hours following transduction (**Figure 4.7 E**).

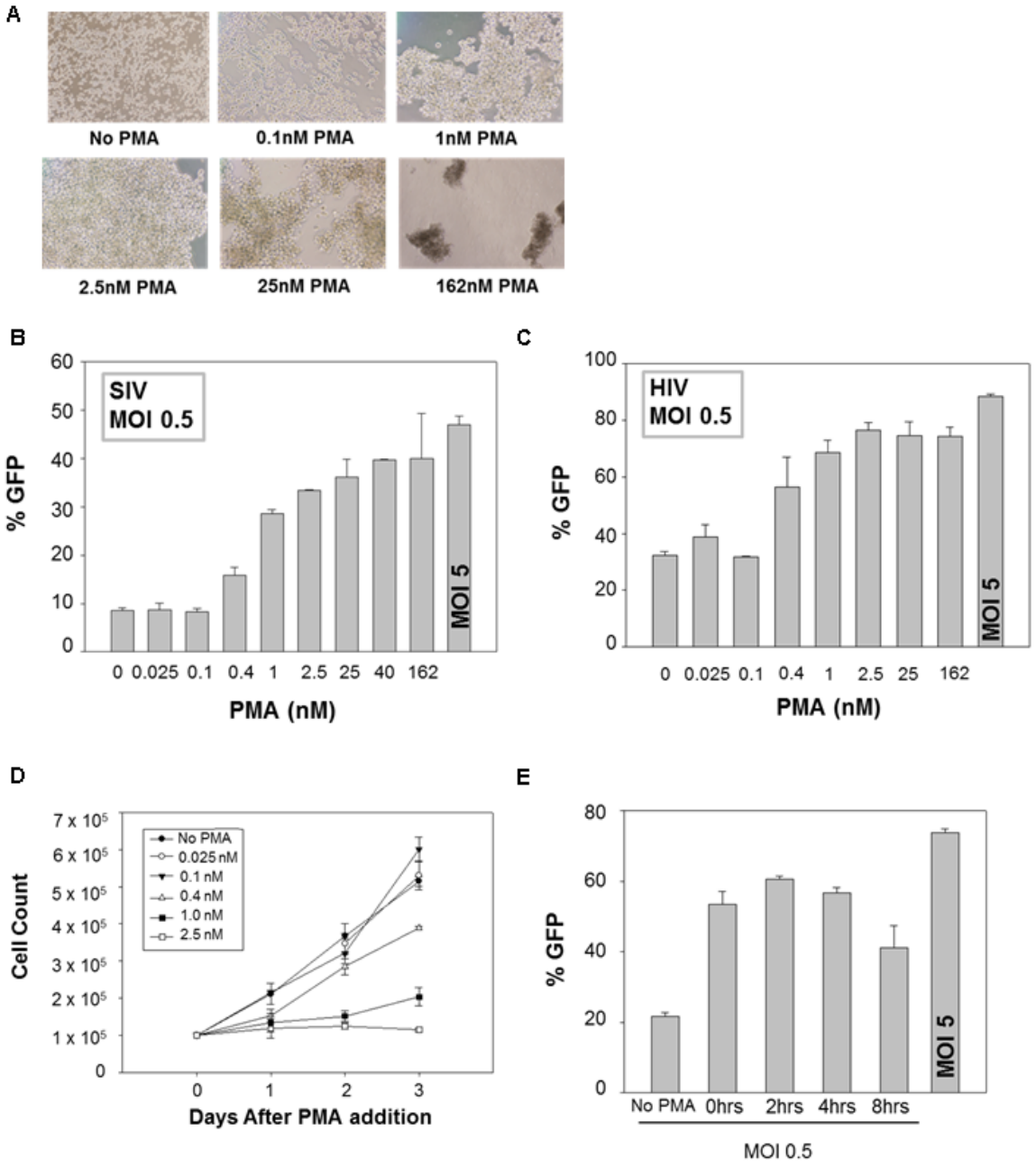


Figure 4.7 PMA enhances lentiviral transduction of K562 cells. (A) Representative microscopy images reveal a morphological change in K562 cells occurring in a dose-dependent manner with PMA treatment. (B) K562 cells were plated at 10^5 cells/well in a twenty-four well plate and treated with PMA at the indicated doses (n = 3) prior to transduction with either an SIV-based or an (C) HIV-based lentiviral vector at an MOI of

0.5. Seventy-two hours post-transduction was assessed by flow cytometry of GFP positive cells. As a positive control, virus was added at an MOI of 5 to the K562 cells in the absence of PMA. (D) Viable cell counts were performed up to 3 days after PMA treatment utilizing a Trypan Blue exclusion assay. (E) In the same manner, a time course analysis was performed in which K562 cells were treated with 162 nM PMA for the indicated hours prior to transduction with an SIV-based lentiviral vector. Transduction efficiency was assessed seventy-two hours later.

PMA enhances lentiviral transduction of EU1 cells

A second human hematopoietic cell line, EU1 cells, which are derived from a B cell acute lymphoblastic leukemia, was analyzed for the effect of PMA. Cells were plated in a twenty-four well tissue culture treated plate at a density of 10^5 cells/well and transduced at an MOI of 5. The analysis was performed with both an SIV-based and an HIV-based lentiviral vector. The cells were treated with PMA at the indicated dose (**Figure 4.8 A**) 2 hours prior to transduction and compared to cells transduced under the same conditions in the absence of PMA. Flow cytometry was performed seventy-two hours following transduction in which the percentage of GFP positive cells was quantified. Non-transduced cells cultured in the presence of PMA were also analyzed as a negative control. Fluorescence microscopy images taken seventy-two hours following transduction demonstrated morphologically unchanged EU1 cells following culture with PMA up to 25 nM and an enhancement in GFP positive EU1 cells was observed by fluorescent microscopy (data not shown). The enhancement in lentiviral transduction of EU1 cells treated with PMA was confirmed by flow cytometry (**Figure 4.8 A**). Using an SIV-based lentiviral vector, transduction of EU1 cells was enhanced almost 3-fold yielding a percentage of GFP positive cells greater than that achieved by adding three times the amount of virus (**Figure 4.8 A**). The percentage of GFP positive cells increased from an average of 43% in the absence of PMA to 75% in the presence of PMA when an HIV-based lentiviral vector was used (**Figure 4.8 B**). As was apparent with K562 cells, EU1 cell proliferation was inhibited by PMA in a dose-dependent manner (**Figure 4.8 C**).

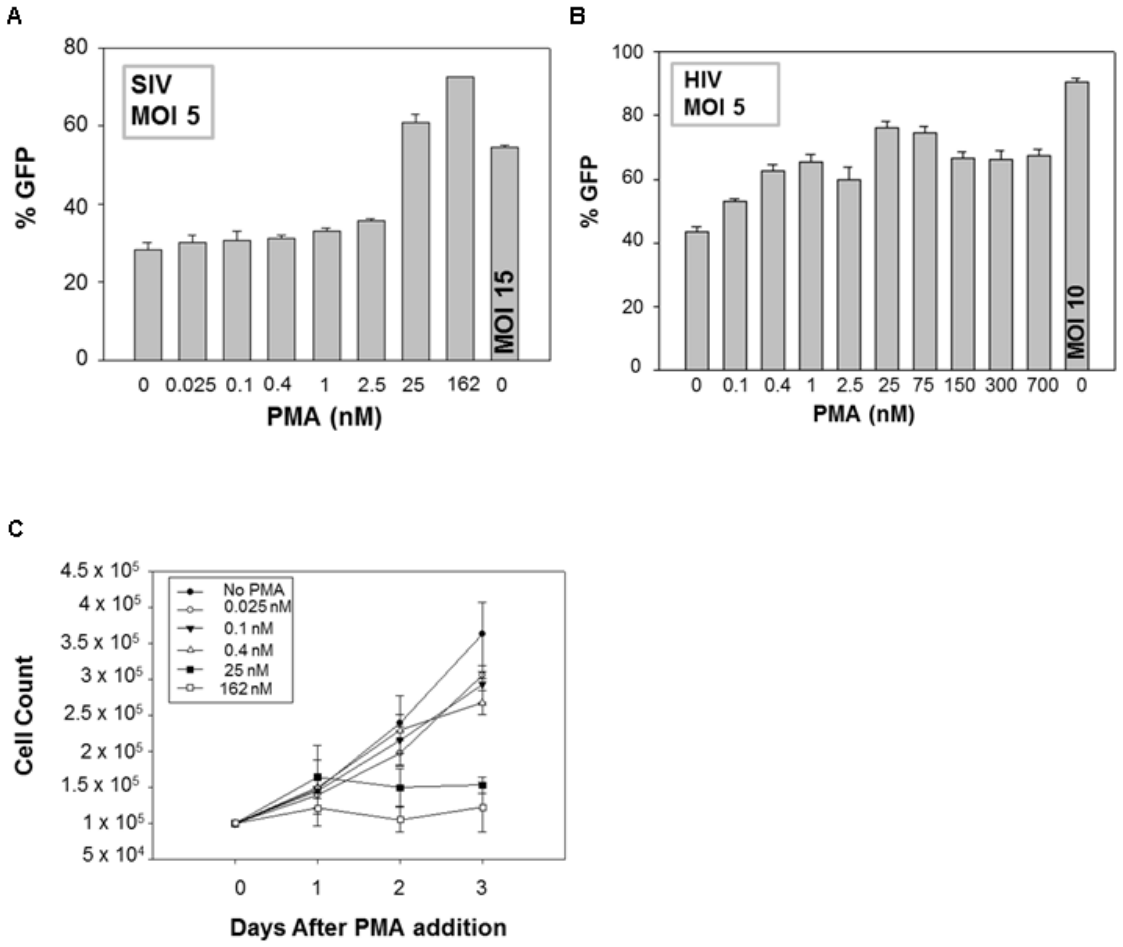


Figure 4.8 PMA enhances the transduction of EU1 cells. (A) EU1 cells were treated with PMA at the indicated doses after being plated at 10^5 cells/well in a twenty-four well plate (n = 3). Transduction was performed 2 hours later at an MOI of 5 with an SIV-based or an (B) HIV-based lentiviral vector. Transduction efficiency was quantified by determining the percentage of GFP positive cells seventy-two hours after transduction by flow cytometry. (C) Viable cell counts were performed throughout the assay up to three days after PMA treatment by a Trypan Blue exclusion assay.

PMA enhances the transduction of a lentiviral vector containing a high-expressing fVIII transgene

A high-expression B domain deleted human/porcine fVIII hybrid was previously used to effectively cure a murine model of hemophilia A using HSC transplantation gene therapy (Doering, 2009). This high-expressing fVIII transgene was incorporated into an HIV-based lentiviral vector (**Figure 4.9 A**) and used in this study to assess the enhancement properties of PMA with the use of a therapeutic transgene. K562 cells were plated at a density of $5x10^5$ cells per well of a twenty-four well plate and transduced at an MOI of 5 (n = 3). The K562 cells were transduced following a 2 hour PMA incubation and compared to K562 cells not transduced in the presence of PMA. Media was exchanged twenty-four hours prior to a one-stage coagulation assay which was performed 12 days following transduction. FVIII activity was significantly greater from K562 cells treated with 10 nM PMA prior to transduction ($p = 0.002$) (one way ANOVA)(**Figure 4.9 B**). DNA was isolated from the transduced cells and assessed for vector copies per genome by qPCR. Enhanced transduction was noted for K562 cells treated with 10 nM PMA prior to transduction as shown by an increase in vector copies per genome ($p = 0.003$) (one way ANOVA)(**Figure 4.9 C**).

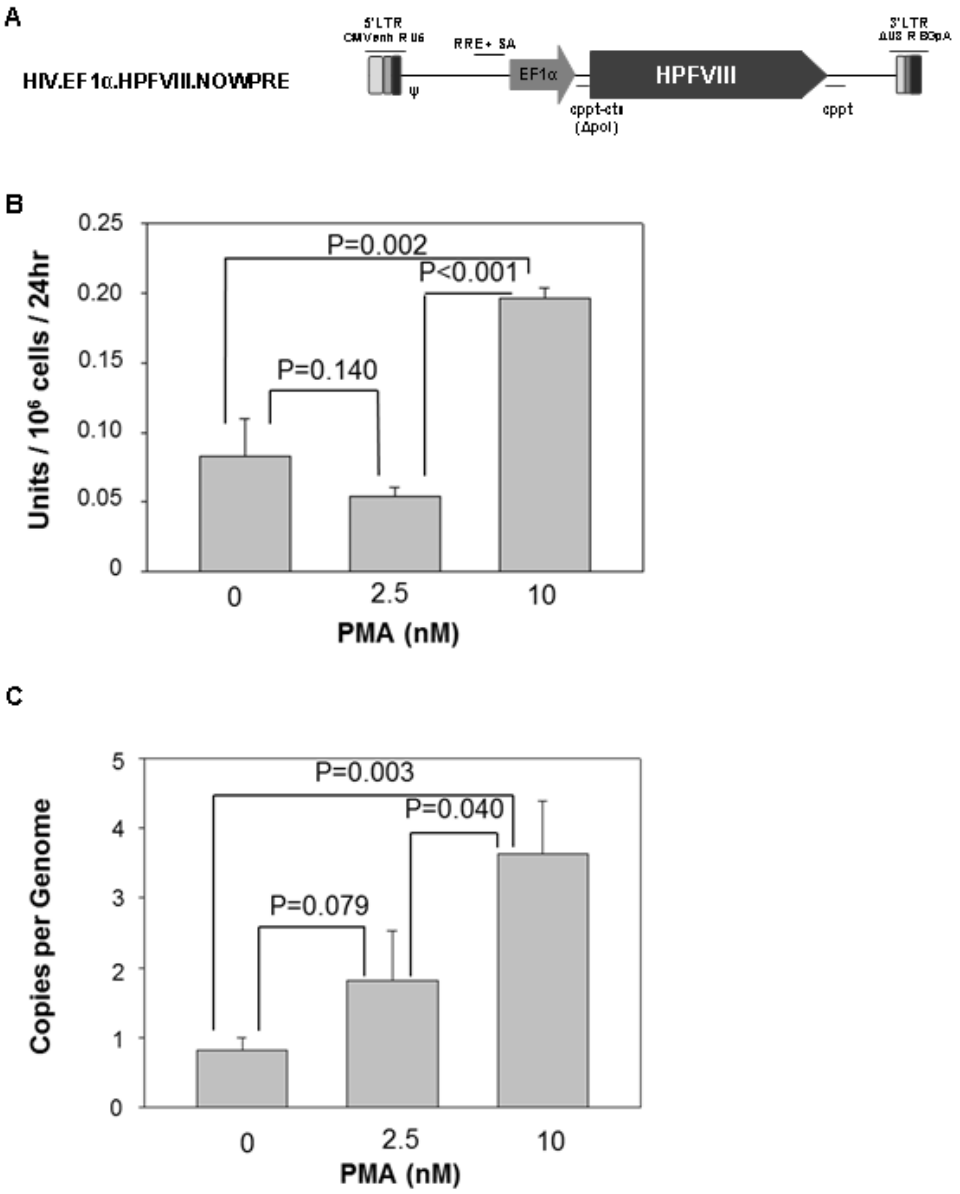


Figure 4.9 PMA enhances the transduction of a lentiviral vector encoding a high-expressing fVIII transgene. (A) Schematic representation of the HIV-based lentiviral vector utilized to transduce K562 cells. (B) fVIII expression was quantified by an aPTT reagent-based one-stage coagulation assay. Twenty-four hours prior to isolation, the conditioned media was exchanged for serum-free AIM-V in order to assess the fVIII activity. Bars represent the mean ± the standard deviation of three transduced wells with each well measured in duplicate. (C) DNA was isolated twelve days following

transduction and assessed for integrated vector copies per genome by qPCR. Bars represent the mean ± the standard deviation of 3 transduced wells measured in duplicate. A one way ANOVA was utilized to determine the significance between groups treated with and without PMA.

Abbreviations: HPFVIII, high expressing porcine/human chimeric fVIII; EF1α, elongation factor 1α.

The effect of PMA on U937, HEK-293T, NIH-3T3 and BHK cell lentiviral transduction

A panel of cell lines was assessed for the effect of PMA on lentiviral transduction. Included in the panel was a third hematopoietic cell type, the U937 cell line originating from a monocytic lymphoma. As with the K562 and EU1 cell lines, 10^5 cells were transduced per well in a twenty-four well tissue culture treated plate. The analysis was performed with both an SIV-based (MOI of 5) and an HIV-based lentiviral vector (MOI of 1). The cells were treated with PMA at the indicated dose (**Figure 4.10 A**) 2 hours prior to transduction and compared to cells transduced under the same conditions in the absence of PMA. Flow cytometry was performed seventy-two hours following transduction in which the percentage of GFP positive cells was quantified. Non-transduced control cells cultured in the presence of PMA were also analyzed as a negative control. No enhancement was noted even upon increasing the concentration of PMA to 700 nM. This observation was noted with both SIV and HIV-based lentiviral vectors (**Figure 4.10 A**). Despite the lack of enhancement, cell proliferation was inhibited over time in a dose-dependent manner (data not shown).

Three adherent cell lines were also included in the panel to be analyzed. HEK-293T (human embryonic kidney cells), NIH-3T3 (mouse fibroblasts) cells, and BHK (baby hamster kidney cells) were plated at a density of $2.5x10^4$ cells per well of a twenty-four well tissue culture treated plate a day prior to transduction. Cells approached $5x10^4$ the following day and were transduced at an MOI of 0.5 with either SIV or HIV-based lentiviral vectors. Cells were treated with the indicated doses of PMA two hours prior to transduction. Transduction was assessed by fluorescence microscopy and quantified by

flow cytometry seventy-two hours after transduction. The percentage of GFP positive cells as compared to those cells transduced with a lentivirus in the absence of PMA. No enhancement was observed upon addition of PMA in HEK-293T cells transduced with an SIV-based or an HIV-based lentiviral vector (**Figure 4.10 B**). A similar observation was apparent for NIH-3T3 cells (**Figure 4.10 C**). As expected, the transduction efficiency of BHK cells was less than that observed with HEK-293T and NIH-3T3 cells at the same MOI. The percentage of GFP positive cells following PMA treatment was not enhanced in BHK cells with either SIV-based and HIV-based lentiviral vectors (**Figure 4.10 D**). In addition, proliferation of HEK-293T, NIH-3T3 or BHK cells was not inhibited in the presence of PMA (data not shown).

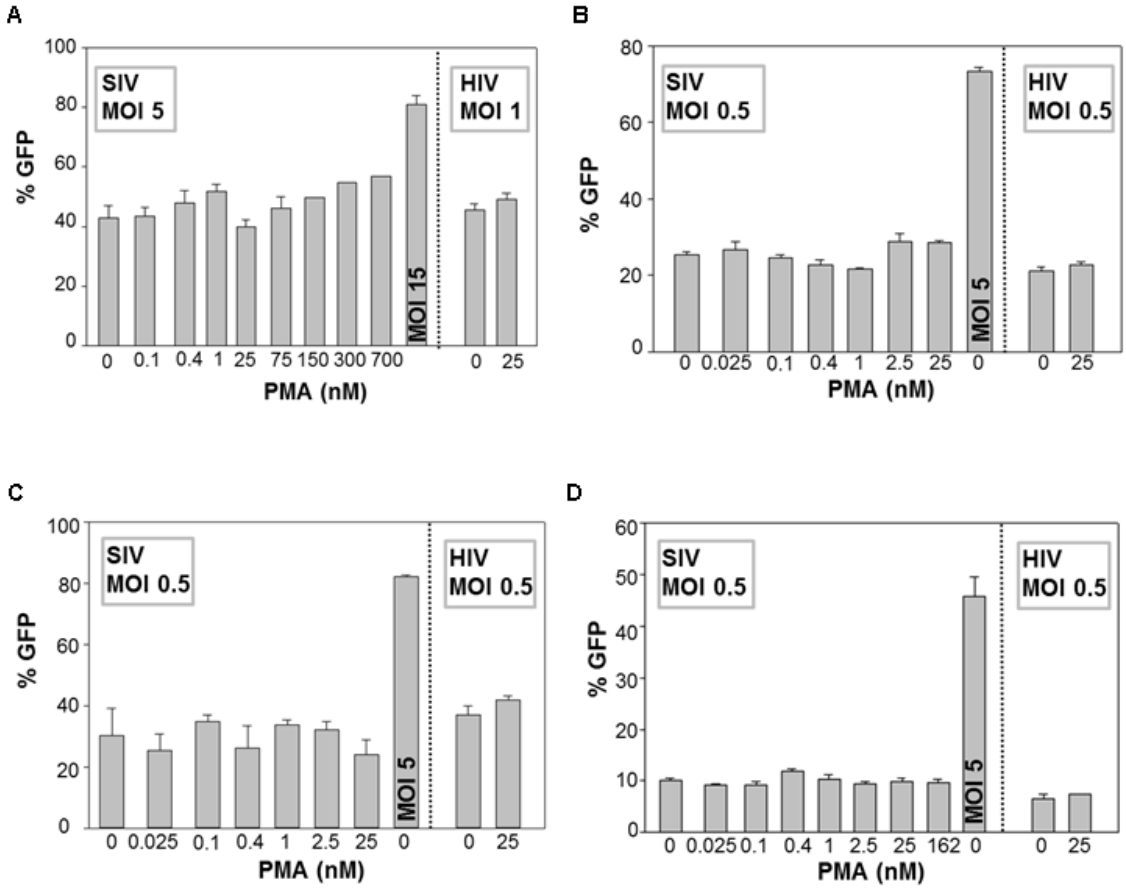


Figure 4.10 Assessment of the effect of PMA on a panel of cell lines. (A) U937 (B) HEK-293T (C) NIH-3T3 and (D) BHK-M cells were treated with the indicated doses of PMA. Two hours later, the cells were transduced with either an SIV-based or an HIV-based lentiviral vector (n = 3). Transduction efficiency was assessed by flow cytometry seventy-two hours after transduction.

PMA effect on murine stem cell antigen-1^+ cells

Murine preclinical models of genetic disease are common. Sca-1^+ cells represent the HSC early and late progenitor population. PMA was tested on sca-1^+ cells isolated from the tibia and femurs of C57BL/6 mice. Sca-1^+ cells were stimulated with cytokines for 3 days. Cell counts during stimulation showed cell growth indicative of typical sca-1^+ cell functioning (data not shown). Following stimulation, $2x10^5$ cells were plated per well of a twenty-four well tissue culture treated plate and treated with PMA at the indicated doses 2 hours prior to transduction. The percentage of GFP positive cells was quantified by flow cytometry seventy-two hours later. Despite increasing the concentration of PMA to 1 μM, no enhancement was observed with sca-1^+ cells transduced with an SIV-based lentiviral vector in the presence of PMA as compared to those cells transduced in the absence of PMA (**Figure 4.11 A**). A similar observation was noted with the use of an HIV-based lentiviral vector (data not shown). In addition, no inhibition of cell proliferation was observed at concentrations up to 100 nM (**Figure 4.11 B**).

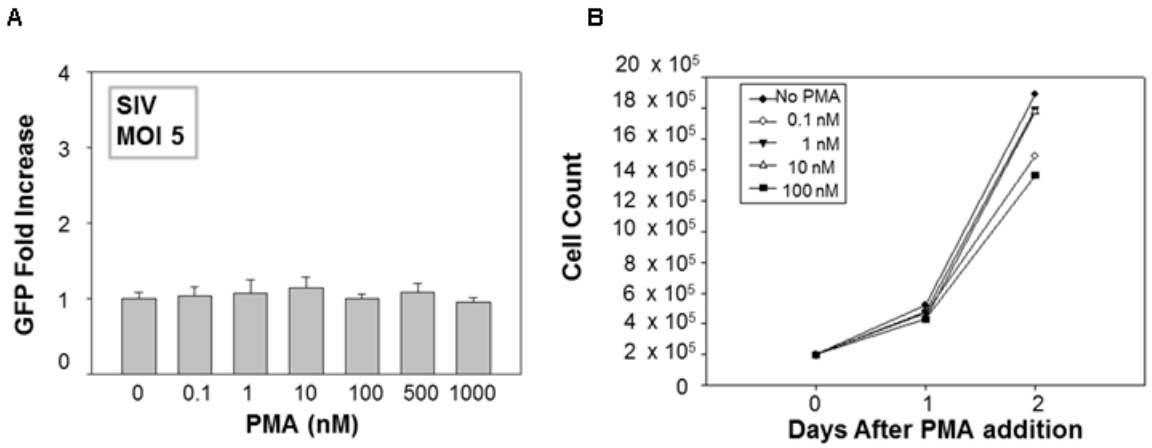


Figure 4.11 Lack of PMA effect on murine hematopoietic stem and progenitor cells. (A) Sca-1^+ cells isolated from the femurs and tibias of C57BL/6 mice werestimulated with cytokines for 3 days prior to PMA treatment. Sca-1^+ cells were treated with PMA at the indicated doses for a total of 2 hours prior to viral transduction at an MOI of 5 with an SIV-based lentiviral vector encoding GFP (n = 3). Transduction efficiency was quantified seventy-two hours following transduction by flow cytometry. (B) Viable cell counts were obtained up to 2 days following PMA treatment utilizing a Trypan Blue exclusion assay.

PMA effect on bone marrow-derived $CD34^+$ cells

The most important clinical target to be evaluated in the presence of PMA is the $CD34^+$ cell population which includes hematopoietic stem and early progenitor cells. Similar protocols as above were used to assess the effect of PMA on $CD34^+$ cells mobilized and isolated from human peripheral blood. $CD34^+$ cells were plated in twenty-four well tissue culture treated plate at a density of $2x10^5$ cells per well. Following a 2 hour incubation with PMA at the indicated doses, cells were transduced with either an SIV-based or an HIV-based lentiviral vector at an MOI of 20. Transduction efficiency was initially assessed seventy-two hours following transduction and quantified by flow cytometry. With both an SIV (**Figure 4.12 A**) and HIV-based lentiviral vector (**Figure 4.12 B**), the percentage of GFP positive cells was increased from 5% to greater than 20% in the presence of PMA. A significant enhancement in lentiviral transduction was observed at as low as 1 nM PMA (**Figure 4.12 A-B**). Initial assessments were performed at seventy-two hours, however prolonged culture revealed similar percentages of GFP positive cells 5 days after transduction (**Figure 4.12 C**). In order to assess longer term transgene expression, the $CD34^+$ cells were cultured for a total of 2 weeks. Over time, the percentage of $CD34^+$ cells positive for GFP was found to have diminished. Yet the fold-induction as a result of PMA treatment was preserved (**Figure 4.12 D**). In addition, $CD34^+$ cells were found to retain the CD34 and CD45 markers after culture with PMA. Flow cytometry was performed seventy-two hours after the cells were transduced with the HIV-based lentiviral vector. Cells not treated with PMA were 97.2% positive for CD34 and CD45 while cells treated with PMA were found to be 95.6% (0.1 nM PMA) and 93.0% (1 nM PMA) positive for both markers (**Figure 4.12 E**). In regards to cellular

proliferation, PMA was found to inhibit cellular proliferation of $CD34^+$ cells over the expanse of 4 days (**Figure 4.12 F**). In addition, $CD34^+$ cells were transduced with a clinical grade HIV-based lentiviral vector following a 2 hour incubation with 1nM PMA. $CD34^+$ cells treated with PMA were found to exhibit colony forming units (CFU) 2-fold greater than those cells not treated with PMA however this is in the range of standard error as outlined in the manufacturer's protocol (**Figure 4.12 G**). Total DNA was extracted from the colonies and analyzed for copy number by real-time quantitative PCR utilizing primers that bind to the rev responsive element of HIV. In the absence of PMA, the copies/cell averaged 0.06 whereas the number of copies/cell for the PMA treated colonies averaged 0.29.

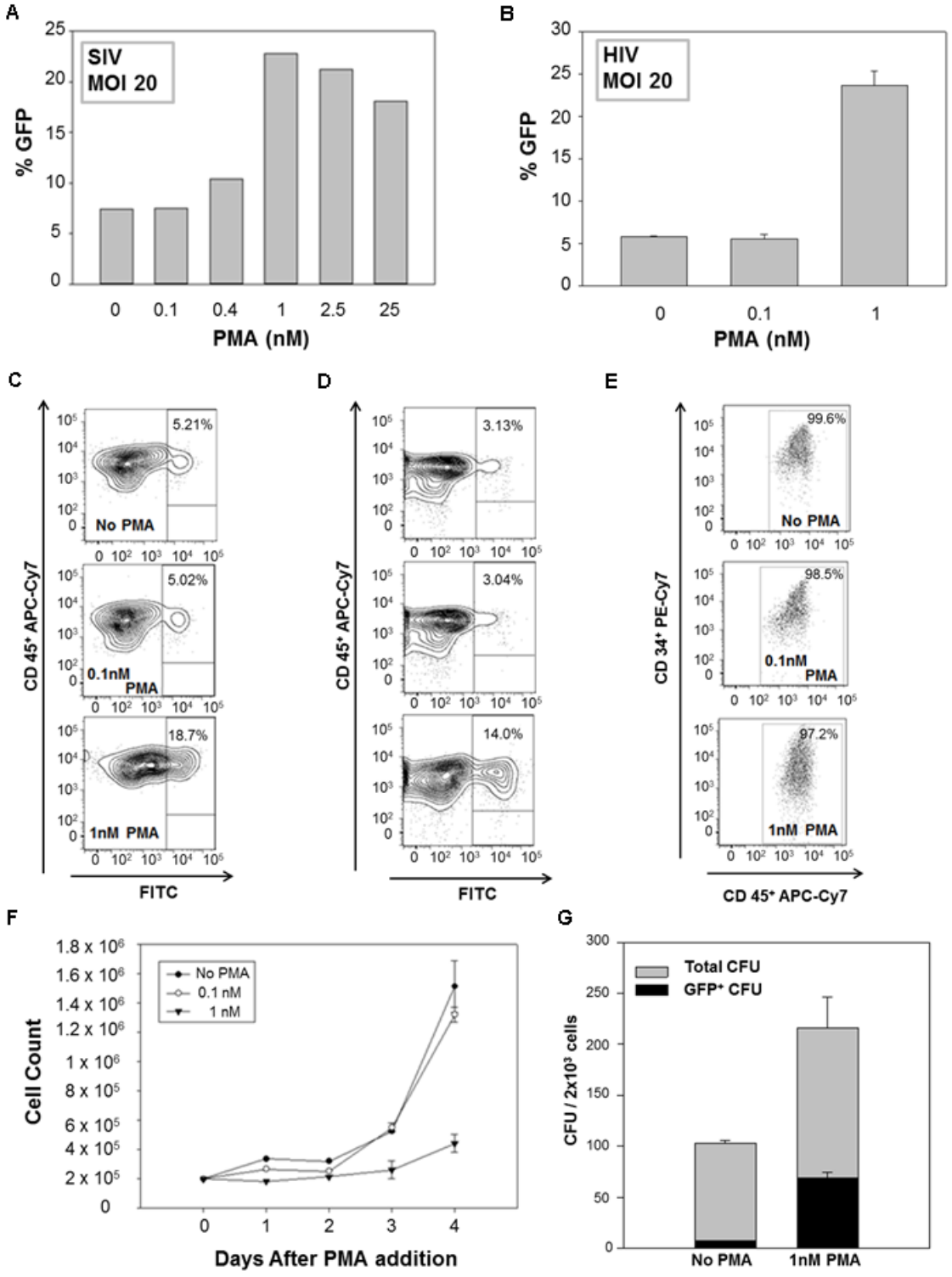


Figure 4.12 PMA enhances lentiviral transduction of CD34$^+$ cells. (A) Mobilized CD34$^+$ cells isolated from human peripheral blood were treated with PMA at the

indicated doses for 2 hours prior to viral addition at an MOI of 20 with an SIV-based and (B) an HIV-based lentiviral vector (n = 3). The percentage of GFP positive cells was quantified seventy-two hours after transduction assessing transduction efficiency in the presence of PMA. The persistence of gene modification was assessed by flow cytometry 5 (C) and 13 days (D) following PMA treatment. Anti-CD45 antibody conjugated to APC-CY^7 was used to identify the population. (E) The persistence of CD34 and CD45 markers was assessed by flow cytometry seventy-hours following PMA treatment. Anti-CD34 antibody conjugated to PE-Cy7 was used. (F) Viable cell counts were performed up to 4 days following PMA treatment by a Trypan Blue Exclusion assay. (G) In addition, the appearance of CFUs was quantified from $CD34^+$ cells transduced with a clinical grade HIV-based lentiviral vector encoding a codon-optimized GFP transgene. Fluorescence microscopy was used to determine genetically-modified colonies.

4.35— Mechanism for PMA effect on K562 cells

As a phorbol ester, PMA binds to the C1 domain of PKC resulting in PKC translocation and activation (Stahelin *et al.*, 2005; Steinberg, 2008). Eight PKC isoforms are activated by PMA and have been implicated in an array of cellular processes. The lack of enhanced lentiviral transduction in HEK-293T, NIH-3T3, BHK, U937 and sca-1+ cells may be due to the different PKC isoform found in these cells as compared to those found in K562, EU1 and $CD34^+$ cells. However, PMA may be acting on more than just PKC. Approximately 50 amino acids located in the C1 domain are responsible for PMA/DAG binding (Ono *et al.*, 1989; Kazanietz *et al.*, 1994, 1995; Quest *et al.*, 1994). These amino acids have been found to constitute similar C1 domains on nonkinase phorbol ester receptors which include chimaerins (a family of Rac GTPase activating protins), RasGRPs (exchange factors for Ras/Rap1), and Munc13 isoforms (scaffolding proteins involved in exocytosis) (Ron and Kazanietz, 1999; Kazanietz, 2000). Further analysis is needed to assess the mechanism by which PMA is enhancing lentiviral transduction.

Enhanced Transduction as a result of DNA damage

To access DNA damage as a result of PMA treatment, DNA fragmentation was measured by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. By this method, a free 3'-hydroxyl residue generated during DNA fragmentation is labeled by a biotinylated nucleotide via the enzyme terminal deoxynucleotidyl transferase. The biotinylated nucleotides are then quantified with the use of a streptavidin-FITC conjugate by flow cytometry.

In this manner, K562 cells were treated with PMA for 6, 4, 2 or zero hours prior to enzyme and biotinylated nucleotide addition. As a positive control, K562 cells were treated with nuclease for thirty minutes to generate DNA breaks in every cell. Immediately following the labeling reaction, flow cytometry was performed in order to quantify the amount of incorporated biotinylated nucleotides at the site of a DNA nick with the use of a streptavidin-FITC conjugated antibody. Extensive DNA fragmentation was observed in the nuclease treated K562 cells, in that seventy-six percent were positive for FITC (**Figure 4.13**). However, less than 1% of the K562 cells treated with PMA were found to be positive for FITC. In addition, no significant difference was observed between the PMA treated and non-treated K562 cells (**Figure 4.13**). Therefore, breakage in the DNA does not appear to be increased as a result of PMA treatment.

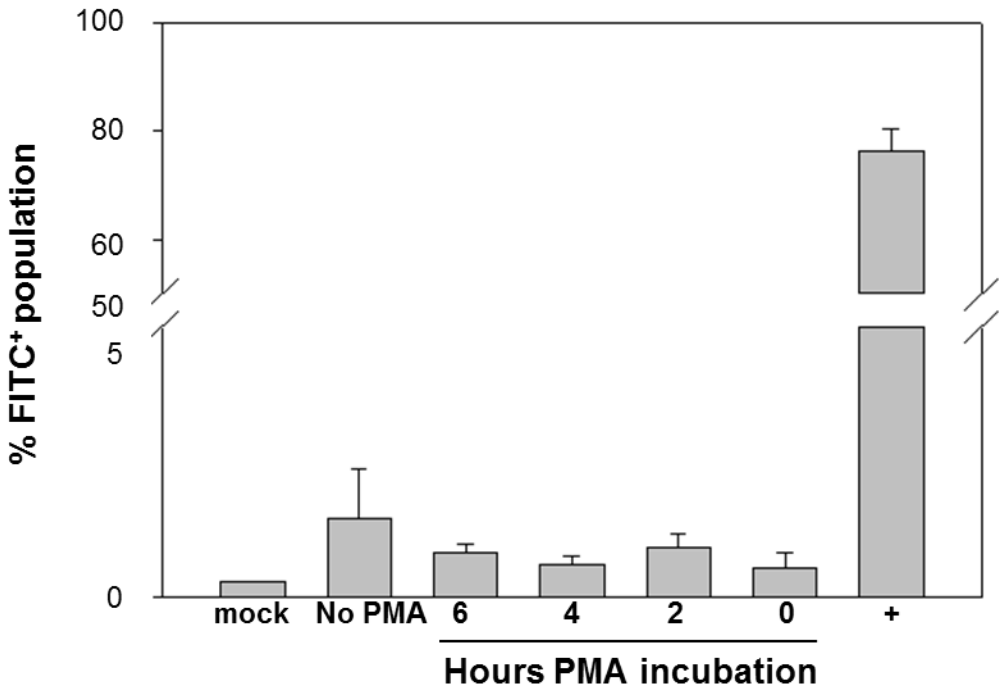


Figure 4.13 Assess of DNA fragmentation as a result of PMA treatment. A TUNEL assay was performed in order to quantify the amount of DNA nicks present as a result of PMA treatment. K562 cells were plated at a density of 10^6 cells / well in a 6 well plate and treated with 10nM PMA at the indicated times (n = 4). Cellular pellets were acquired and fixed with a 3.7% formaldehyde solution. DNA nicks were labeled with biotinylated nucleotides by the enzymatic activity of terminal deoxynucleotidyl transferase. As a mock control, the terminal deoxynucleotidyl transferase enzyme was omitted. Quantification of the incorporated nucleotides was performed with the addition of a streptavidin-FITC antibody measured by flow cytometry. In addition to the mock control, a subset of K562 cells was treated with nuclease prior to the labeling reaction as a positive control in order to fragment the DNA. Non PMA-treated K562 cells were utilized as a baseline for comparison.

Enhanced Transduction as a result of PKC activation

In order to assess the involvement of PKC in the transduction enhancing process of PMA on K562 cells, a PKC inhibitor was utilized. Calphostin C targets the regulatory domain of PKC inhibiting translocation and activation. With an IC_{50} of 50nM, K562 cells were treated with calphostin C (50nM) for 4 hours prior to PMA addition (10 nM). Two hours following PMA addition, an SIV-based lentivirus encoding the GFP transgene was used to transduce the treated K562 cells at an MOI of 0.5. Flow cytometry was performed seventy-two hours later to assess transduction efficiency. The average percentage of K562 cells positive for GFP as a result of PMA treatment averaged 76.9 percent while K562 cells pretreated with 50 nM calphostin C prior to PMA treatment averaged 45.4 percent. A significant reduction in the enhancement achieved by the addition of PMA was inhibited following calphostin C treatment at 50 nM (**Figure 4.14 A**). This suggests that PKC is involved in the mechanism whereby which PMA enhances lentiviral transduction in K562 cells. To determine which isoform of PKC was involved, inhibitors selective for PKC isoforms were utilized in the same manner as calphostin C. K562 cells were treated with the inhibitors for 4 hours prior to PMA treatment for 2 hours. After treatment, cells were transduced with a GFP containing lentivirus at an MOI of 0.5. Transduction was determined seventy-two hours after viral addition by flow cytometry. (**Figure 4.14 B**)

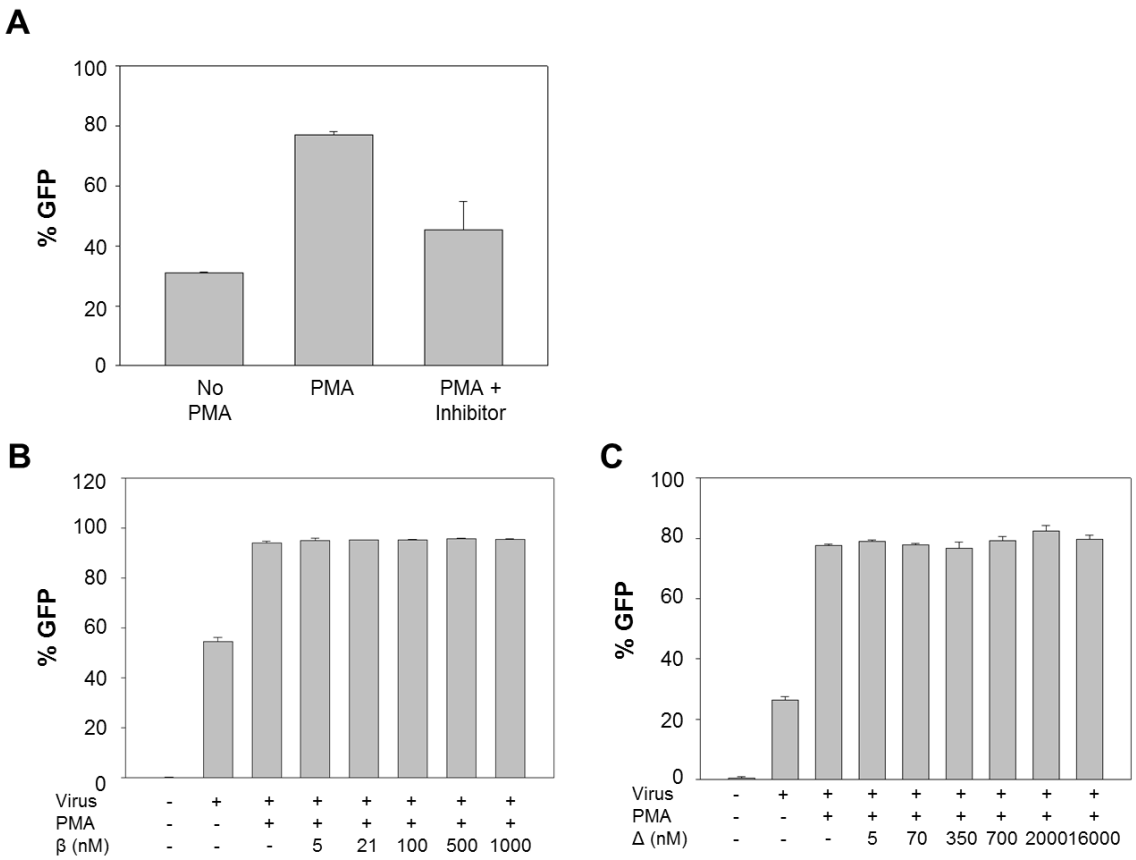


Figure 4.14 Use of a PKC inhibitor as a means to determine the mechanism whereby which PMA enhances the lentiviral transduction of K562 cells. (A) K562 cells were treated with the 50 nM calphostin C for 4 hours prior to PMA treatment at 10 nM. An SIV-based lentiviral vector encoding a GFP transgene was used to transduce the K562 cells at an MOI of 0.5 two hours following PMA treatment. Transduction efficiency was assessed by flow cytometry seventy-two hours after viral addition. In the same manner, K562 cells were treated with selective inhibitors specific for the (B) β_2 (N-(2-ethyl-2H-1,2,3-triazol-4-yl)-2-[4-(4-methoxypyrimidin-2-yl)-1H-pyrazol-1-yl]acetamide) isoform and (C) the Δ (1-(3-methylphenyl)-5-{[(2-methylphenyl)amino]methylene}-2-thioxodihydro-4,6(1H,5H)-pyrimidinedione) isoform of PKC for 4 hours prior to PMA treatment at 10 nM.

Assessment of LEDGF expression as a result of PMA treatment

HIV infection has been found to be expedited by the transcriptional co-activator identified as the lens epithelium-derived growth factor (LEDGF). LEDGF interacts with the lentiviral integrase facilitating integration of the transgene into the genome of the target cell. LEDGF contains a zinc-finger in the N-terminal region of the protein which has been determined to bind to cellular DNA. Despite as the name infers, LEDGF is ubiquitously expressed at all stages of life (Nishizawa *et al.*, 2001). Yet its role in development is significant, being that mice born with an inactivated LEDGF gene die perinatally upon experiencing severe skeletal abnormalities (Sutherland *et al.*, 2006). However, the complete biological function of LEDGF remains unknown.

LEDGF expression was evaluated in the context of PMA due to a report demonstrating LEDGF interaction with HIV integrase. In this manner, LEDGF directs viral integration into the genome more efficiently (Ciuffi, 2008; Shun *et al.*, 2008). Thus K562 cells were treated with PMA and assessed for the levels of LEDGF protein by Western blot analysis. LEDGF protein levels were not increased in K562 cells as a result of PMA treatment (**Figure 4.15 A-B**). This suggests that an enhancement in lentiviral transduction as a result of PMA treatment is not due to increased levels of LEDGF.

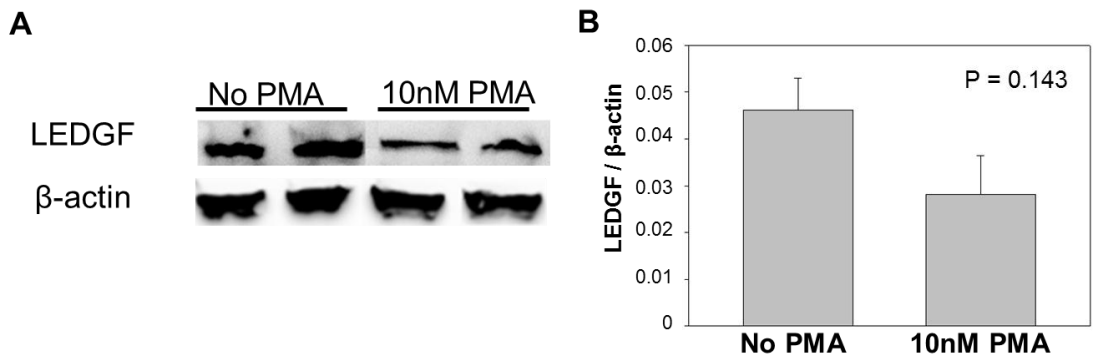


Figure 4.15 Western Blot detecting LEDGF levels following PMA treatment. (A) K562 cells were treated with PMA (10 nM) and harvested 12 hours later for protein. Protein concentration was analyzed by a BCA assay. Equivalent amounts of protein (75 µg) were added to each lane of an SDS-PAGE gel. Following transfer to a 0.2 micron membrane, LEDGF and β-actin levels were detected by a chemiluminescent Western Blot. (B) The p75 LEDGF and β-actin bands were quantified using ImageJ. The p75 LEDGF band was normalized to β-actin as graphically. A t-test was utilized to determine significance.

4.4— Discussion

Limiting transduction of hematopoietic stem cells has been observed clinically with the use of lentiviral vectors (Cartier *et al*., 2009; Boztug *et al*., 2010; Cavazzana-Calvo *et al*., 2010; DiGiusto *et al*., 2010). This obstacle is compounded among other things by the loss of modified cells after transplantation. This was observed in a previous HSC gene therapy phase I clinical trial designed to treat X-linked adrenoleukodystrophy. Prior to transplantation, 50% of the $CD34^+$ population was genetically modified, but only 9-14% of blood cells were found to be positive after transplantation (Cavazzana-Calvo *et al*., 2010). This discrepancy could either be a result of culturing parameters *ex vivo* that may significantly reduce the repopulation potential of $CD34^+$ cells (Kustikova *et al*., 2009) or due to the difficulty surrounding the transduction of pure HSCs as opposed to differentiated progenitor cells in the $CD34^+$ population. Enhancing the transduction of HSCs would prove beneficial for many gene therapy applications that utilize HSCs for gene delivery.

A library of 1280 pharmacologically active compounds was screened for the purpose of identifying enhancers of SIV and HIV-based lentiviral transduction. One apparent shortcoming of the performed high-throughput screen would be that the $LOPAC^{1280}$ library was not entirely inclusive. The proteasome inhibitor, MG132, was not a part of the library and thus not included in the screen despite the previous demonstration of MG132 to enhance lentiviral transduction of $CD34^+$ cells and T lymphocytes (Leuci, *et al*., 2011). Camptothecin, etoposide and taxol, on the other hand, were included in the $LOPAC^{1280}$ library. All three compounds have been previously identified to enhance the

transduction of an HIV-based lentiviral vector (Groeschel and Bushman, 2005). In the high-throughput screen, these 3 compounds were noted as positive hits. However, validation of camptothecin as a positive hit independent of the high-throughput screen showed an enhancement in the transduction of K562 cells with both a SIV and an HIV-based lentiviral vector. Thus camptothecin represents a false negative of the HIV-based lentiviral vector screen. Therefore, false negatives are an issue for identifying compounds with the use of a high-throughput screen as well as false positives, such as described for forskolin, SB 205384 and cytosine-1-β-D arabinofuranose hydrochloride.

Despite the ability of camptothecin or other topoisomerase inhibitors to enhance lentiviral transduction, these compounds are not ideal for clinical use because they induce DNA damage (Pommier *et al*., 2010). Therefore, our focus was on non-DNA damaging agents such as PMA. PMA, another positive hit from the high-throughput screen, represents a potentially safer compound than topoisomerase inhibitors. Initial assessment was performed with K562 cells in which a 4-fold increase in modified cells was apparent when culturing with PMA prior to transduction. This increase was achieved at a concentration as low as 2.5 nM PMA, where no morphological change was evident (Papayannopoulou *et al*., 1983). On the other hand, inhibition of cellular proliferation, on the other hand, was observed at 2.5 nM PMA.

In addition to enhancing the transduction efficiency of K562 cells, PMA also enhanced the transduction of the human hematopoietic cell line, EU1. EU1 cells are derived from the lymphoid lineage as opposed to K562 cells which are of the myeloid lineage. Once

again, an inhibition of cellular proliferation was apparent following PMA treatment, suggesting that inhibition of the cell cycle might be a method whereby PMA is exhibiting its effects. This is consistent with the observation that G_2/M arrest was responsible for the increased efficiency of HIV infection in HEK-293T, HeLa and IMR90 cells treated with camptothecin (Groschel and Bushman, 2005). The exact method whereby arresting cellular proliferation results in a favorable cellular condition for lentiviral gene transfer is unknown. Transduction could be enhanced at one or possibly multiple steps during lentiviral infection. If transduction is enhanced at the entry phase, a simple explanation would be to conclude that G_2/M arrest provides a greater surface area for which a lentivirus can attach being that the surface area of a cell stalled prior to division is expected to be 2-fold (Mitchison, 2003). However, this scenario is unlikely in that transduction was not enhanced with U937 cells or cytokine deprived sca-1^+ cells in which proliferation was inhibited (data not shown). A more likely explanation would be that a molecular event is occurring in the cell as a result of PMA treatment resulting in a cellular change more conducive to lentiviral infection.

As a phorbol ester, PMA binds to the C1 domain of PKC resulting in PKC translocation and activation (Stahelin *et al.*, 2005; Steinberg, 2008). Eight PKC isoforms are activated by PMA and have been implicated in an array of cellular processes. The lack of enhanced lentiviral transduction in HEK-293T, NIH-3T3, BHK, U937 and sca-1+ cells may be due to the different PKC isoform found in these cells as compared to those found in K562, EU1 and $CD34^+$ cells. However, PMA may be acting on more than just PKC. Approximately 50 amino acids located in the C1 domain are responsible for PMA/DAG

binding (Ono *et al.*, 1989; Kazanietz *et al*., 1994, 1995; Quest *et al*., 1994). These amino acids have been found to constitute similar C1 domains on nonkinase phorbol ester receptors which include chimaerins (a family of Rac GTPase activating protins), RasGRPs (exchange factors for Ras/Rap1), and Munc13 isoforms (scaffolding proteins involved in exocytosis) (Ron and Kazanietz, 1999; Kazanietz, 2000). Further analysis is needed to assess the mechanism by which PMA is enhancing lentiviral transduction.

A novel finding of this study is the ability of PMA to enhance the lentiviral transduction of $CD34^+$ cells, a clinical target of gene therapy applications. Transduction was enhanced 3-fold with both an SIV-based and an HIV-based lentiviral vector, where at a concentration as low as 1 nM PMA increased the percentage of modified cells from 7 to 22 percent. This 3-fold difference remained apparent two weeks after transduction. Cells treated with PMA retained the CD34 and CD45 markers in a similar fashion to cells not treated with PMA. In addition, these cells were able to give rise to a subset of lineage committed cells as evidenced by the ability of $CD34^+$ cells treated with PMA to produce colonies in methylcellulose. Similar to the enhancement in transgene copy number using a fVIII transgene and K562 cells, the increase in percentage of GFP positive cells observed with PMA treatment of $CD34^+$ cells is due to an enhancement in lentiviral transduction as opposed to simply an enhancement in GFP expression. Additional studies are needed to determine the engraftment potential of $CD34^+$ cells following treatment with PMA.

Despite recent preclinical and some clinical progress with the use of HSC gene therapy, the transduction efficiency of HSCs remains a critical hurdle. Limited transduction often results in limiting expression of the transgene, which could result in little to no therapeutic benefit. In the current study, a high-throughput screen identified PMA as an enhancer of lentiviral transduction. Furthermore, PMA was shown to enhance the transduction of hematopoietic cell lines from both the myeloid and lymphoid lineages. PMA enhanced the transduction of a clinically relevant population of hematopoietic stem and progenitor cells. PMA has been approved for use in a phase I clinical trial to treat hematological malignancies in which the doses used (ie systemic µM concentrations) were tolerable without any apparent renal, hepatic or hematological toxicity establishing the feasibility of utilizing phorbol esters in humans (Strair *et al.*, 2002). Therefore, the use of PMA clinically to enhance the transduction of $CD34^+$ cells *ex vivo* is a viable option.

Chapter 5:

Conclusions and Future Perspectives

5.1— Conclusions

Optimization of the Lentiviral Vector

To date, three hemophilia A gene therapy clinical trials have been initiated. However the results of all three trials resulted in limited transient fVIII levels unable to circumvent the bleeding episodes experienced in these patients. Limited fVIII expression demonstrated in these trials could potentially be due to the inefficient transport of the human fVIII cDNA from the endoplasmic reticulum to the Golgi apparatus. fVIII is retained in the endoplasmic reticulum via a resident protein chaperone, BiP, which binds to a hydrophobic cluster in the A1 domain. In addition, efficient secretion of fVIII has been associated with carbohydrate-facilitated transport via the protein, lectin mannose binding-1 (LMAN1) which binds mannose residues attached to the B domain posttranslationally. With this in mind, several high-expression fVIII transgene sequences have been postulated in order to overcome the low level transgene expression. We recently compared these sequences to that of a B domain deleted porcine fVIII sequence. Enhanced expression of the B domain deleted porcine fVIII sequence was demonstrated both *in vitro* and *in vivo* (Ide *et al*., 2007; Doering *et al.*, 2007; Dooriss *et al*., 2009). The responsible domains for the high expression characteristics of porcine fVIII were found to be attributed to the A1 and A3 domains (Doering *et al.*, 2004). In addition, the immunogenicity of fVIII was found to be reduced by the addition of the porcine C1 domain and three alanine substitutions in the A2 domain (Healey *et al*., 2009; Lubin *et al*., 1997). Thus a hybrid human/porcine B domain deleted fVIII transgene containing the human A2 and C2 domains and the porcine A1, A3 and C1 domains was constructed (HP-fVIII). The bioengineered construct contains 91% human amino acid sequence and

has been shown to maintain the high expression characteristics of porcine fVIII (Doering *et al*., 2009).

As outlined above, much effort has been focused towards the optimization of the fVIII transgene. However, little attention has been directed towards the lentiviral vector itself in regards to fVIII expression. For this reason, the components of the lentiviral vector were evaluated and optimized for enhanced expression of fVIII. Three components were specifically assessed as a part of the lentiviral vector (1) the WPRE sequence (2) the lentiviral backbone and (3) the internal promoter.

The WPRE, although originally observed to increase the expression of GFP, was not found to exert any effect on the expression of HP-fVIII. A panel of cell lines was assessed for lentiviral vectors differing only in the presence or absence of a WPRE sequence. For all cell lines considered, no increase in HP-fVIII was observed at both the transcript and protein level. In addition, the WPRE was not found to be beneficial in regards to the amount of transcriptional read-through observed. Thus without any benefit to HP-fVIII expression or safety, the WPRE was eliminated from the lentiviral vector. Of note prior to these studies, the inclusion of a WPRE sequence in a gene therapy viral vector was considered a standard operation. Direct evidence confirming enhanced transgene expression as a result of a WPRE sequence was not required by the Recombinant DNA Advisory Committee prior to clinical application. However, due to this report as well as that of Klein *et al.* (2006) and Kingsman *et al.* (2005) it has been

advised that the utility of the WPRE sequence be scrutinized more closely in regards to enhanced transgene expression and safety

The second component of the lentiviral vector to be evaluated was the lentiviral backbone itself. A total of three different lentiviral backbones were evaluated. As a result, the SIV-based lentiviral vector was found to be superior in regards to HP-fVIII expression. Interestingly, this was not the case with a GFP transgene. The reason for this discrepancy is unknown. It is possible that the integrase associated with the SIV-based lentiviral vector has an integration profile that favors the expression of a large transgene such as HP-fVIII. However an integration analysis will need to be performed in order to affirm such a hypothesis. If this is the case, this information could be useful for future gene therapy applications for hemophilia A utilizing non-viral vectors able to integrate the transgene at specific sites in the genome such as zinc finger nucleases and tal effector nucleases. These non-viral vectors could be designed to incorporate the fVIII transgene at safe harbors or locations in the genome conducive to efficient fVIII expression. However, the technology associated with these non-viral vectors is not advanced enough for human application. Therefore the use of an SIV-based lentiviral vector for the treatment of hemophilia A by gene therapy is a viable first-generation clinical vector. However before this can become a reality, clinical-grade SIV-based lentiviral vectors will need to be produced in large quantities as is currently available for HIV-based lentiviral vectors. Until this is accomplished, an HIV based lentiviral vector is a suitable choice to begin treating individuals with hemophilia A via gene therapy and could serve as a substitute first-generation clinical vector. The SIV-based lentiviral vector may then serve

as a second-generation vector aimed at treating the subpopulation of individuals with hemophilia A living with AIDS (estimated to be between 6000 to 10,000 in the United States alone) (Meier, 1996). A large portion of adults with hemophilia A are HIV-1 positive due to receiving plasma derived fVIII as a means of protein replacement therapy prior to routine testing of blood-borne pathogens from donated blood products. Therefore, an SIV-based lentiviral vector may be a safer lentiviral vector since utilizing an HIV-based lentiviral vector in these individuals could potentially result in unsafe recombination events between the vector and wild-type HIV in the genome. Regardless of the limitations associated with the production of a clinical-grade SIV-based lentiviral vector, theSIV-based lentiviral backbone was used to analyze the third component of the lentiviral vector, the internal promoter.

Three ubiquitous promoters were assessed. In terms of HP-fVIII expression, the CMV internal promoter was found to be most efficient in HEK-293T cells. This result was not unexpected due to the strong enhancer found in the CMV promoter. However, the use of a CMV internal promoter clinically is constrained by the potential transactivation of nearby genes as a result of the strong enhancer found in the CMV promoter as compared to the enhancers associated with the EF1α and PGK internal promoters. This raises safety concerns surrounding the use of a CMV promoter in humans. In addition, the CMV promoter has been associated with methylation-induced inactivation which could result in transient expression of HP-fVIII. Therefore, further analysis may need to be performed in order to determine a more suitable internal promoter for clinical gene therapy applications designed for the treatment of hemophilia A. This may include the

clinically applicable EF1α internal promoter, demonstrating levels of HP-fVIII in HEK-293T cells greater than that of the PGK internal promoter. However, a larger panel of internal promoters including cell-specific promoters needs to be evaluated. Regardless of the drawbacks to utilizing a CMV internal promoter, as a result of these studies, a lentiviral vector was optimized for HP-fVIII expression and demonstrated therapeutic levels of HP-fVIII in a fVIII deficient mouse model. The results of this study was used to help delineate a lentiviral vector that received approval by the Recombinant DNA Advisory Committee (**Figure 5.1**).

Gene Therapy for the Treatment of Hemophilia A

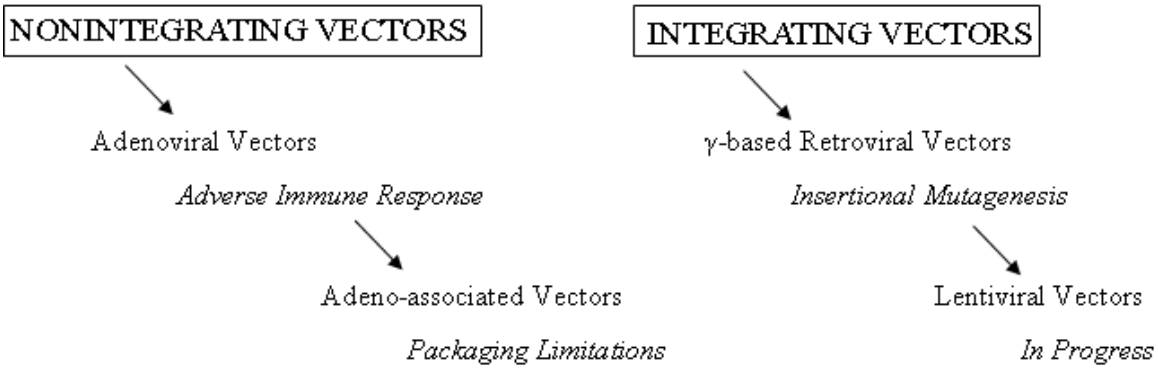


Figure 5.1 Viral vectors proposed for gene therapy protocols for the purpose of treating hemophilia A. Two types of viral vectors have been proposed for gene transfer, the nonintegrating and integrating vectors. Adenoviral vectors were one of the first vectors to be used clinical to treat hemophilia A. However, the trial was abruptly halted due to an adverse immune response to the vector. Thus adeno-associated vectors have been utilized preclinically to transfer the fVIII gene to the liver of mice. Yet encapsadiation limitations are the major setback associated with the use of adeno-associated vectors for hemophilia A. Integrating vectors, on the other hand, provide a means for stable integration. The γ-based retroviral vectors were the first retroviral vectors to be utilized in the field. However, their use in the context of hemophilia A has been hindered by the presentation of insertional mutagenesis as seen in the clinical trial for X-SCID. The lentiviral vector, able to package a large transgene such as fVIII, has a safer integration profile than the γ-based retroviral vectors. In addition, the transfer of fVIII has been achieved preclinically in a hemophilia A mouse in which therapeutic levels were observed. Thus, a lentiviral vector is an appropriate vector to use for the treatment of hemophilia A.

Alternate Lentiviral Production Protocol

The delineation of an optimized lentiviral vector for HP-fVIII expression was demonstrated above. However, the production limitations associated with such a lentiviral vector remain a concern in the field. Lentiviral production is currently confined to a transient transfection procedure in HEK-293T cells due to the toxicity associated with the VSVG viral envelope. Lentiviral vectors are pseudotyped with the VSVG envelope protein in order to increase the range of host cells capable of being genetically modified. However, the VSVG protein accumulates at the cell surface of HEK-293T cells forming syncytia and ultimately leading to diminished cell viability. HEK-293T cells are viable for approximately three days following the onset of VSVG expression. Thus the generation of a stable packaging cell line for the mass production of VSVG-pseudotyped lentiviral vectors has yet to be delineated. Production of lentiviral vectors in this manner would be ideal for lentiviral vectors utilized in human clinical trials, in that the lentiviral vector could not only be produced in mass quantities but could also come from an extensively characterized nonvariable source. In order to establish a stable lentiviral packaging cell line an alternative cell line was analyzed for VSVG pseudotyped lentiviral production. BHK-M cells were chosen as a highly efficient clinically applicable protein producing cell line. The production of lentivirus was assessed in BHK-M cells following transient transfection. However, it was apparent that VSVG was more toxic in BHK-M cells than in HEK-293T cells. This observation is probably due to an increase in VSVG expression in BHK-M cells as compared to HEK-293T cells. Thus BHK-M cells are not a viable option for the production of lentiviral vectors pseudotyped

with VSVG. Therefore, other avenues will need to be explored in order to overcome the production limitation of lentiviral vectors, especially those encompassing large transgenes such as fVIII. This includes establishing a stable producing cell line in which VSVG expression is induced. This would diminish toxic side effects associated with the expression of VSVG until lentiviral production is required. In addition, alternative nontoxic envelopes may be utilized.

Enhancement of HSC transduction by a lentiviral vector

Another obstacle encountered in the field of gene therapy with the use of a lentiviral vector is the inefficient transduction of HSCs by lentiviral vectors. HSCs are innately resistant to lentiviral transduction by a yet unknown mechanism. However, the transduction of a self-renewing and fully capable differentiating $CD34^+$ cell is pertinent to the success of *ex vivo* gene therapy applications for the treatment of hemophilia A. Therefore, several attempts were made to enhance the transduction efficiency of lentiviral vectors.

The most progress was made as a result of a high-throughput screen. 1280 compounds were screened as a part of the LOPAC library. Three previously identified enhancers of lentiviral transduction were fortuitously included in the LOPAC library. The high-throughput screen confirmed all three compounds as enhancers of HIV-based lentiviral transduction as well as identifying the compounds as enhancers of SIV-based lentiviral transduction. However, these compounds induce DNA damage and are not ideal for clinical use. PMA, on the other hand, was also identified as an enhancer of lentiviral

transduction as a positive hit of the high-throughput screen yet not found to induce DNA damage. Initially, PMA was found to enhance the transduction of K562 cells. However, further analysis revealed that PMA was also able to enhance the transduction of the hematopoietic EU1 and most importantly the clinically therapeutic target, the $CD34^+$ population of hematopoietic stem and early progenitor cells. PMA was not able to enhance all cell lines assessed. The adherent HEK-293T and BHK-M cells, as well as the hematopoietic U937 and $sca\text{-}1^+$ cell line were resistant to the positive effects of PMA on lentiviral transduction. The discrepancy associated with the positive and negative effects of PMA is postulated to be due to the specific isoform of PKC prevalent in each individual cell type. However, at this time it is unclear which isoform PMA is acting on.

The ability of a universal PKC inhibitor (Calphostin C) to prevent the effect of PMA does suggest that PKC is the route in which PMA enhances transduction. Although the involvement of PKC in the mechanism whereby PMA enhances transduction has only been experimentally portrayed in K562 cells, potentially this same pathway is being utilized in EU1 and $CD34^+$ cells. PKC activation depending on the cell type can result in a multitude of downstream effects. The identification of this pathway will aid in the incorporation of PMA into a clinical transduction protocol.

The incorporation of a molecule that behaves in the same manner as PMA into a transduction protocol would ensure adequate genetic manipulation. Since PMA was revealed to enhance the transduction of the clinically relevant $CD34^+$ population of hematopoietic stem and progenitor cells, it is likely that transduction would be efficient

enough to result in adequate expression of a transgene such as fVIII. In regards to safety, reports following a phase I clinical trial designed for the treatment of hematological malignancies revealed that PMA was well tolerated following administration of PMA at μM concentrations without the appearance of any renal, hepatic or hematological toxicity (Strair *et al*., 2002). Therefore, PMA can act as a lead compound for the determination of a chemical with improved potency and/or selectivity for enhanced lentiviral transduction of the clinically relevant $CD34^+$ cells. This compound can then be utilized in a gene therapy clinical trial (**Figure 5.2**). However, it is likely that the transduction protocol will include such a compound during a second-generation clinical trial.

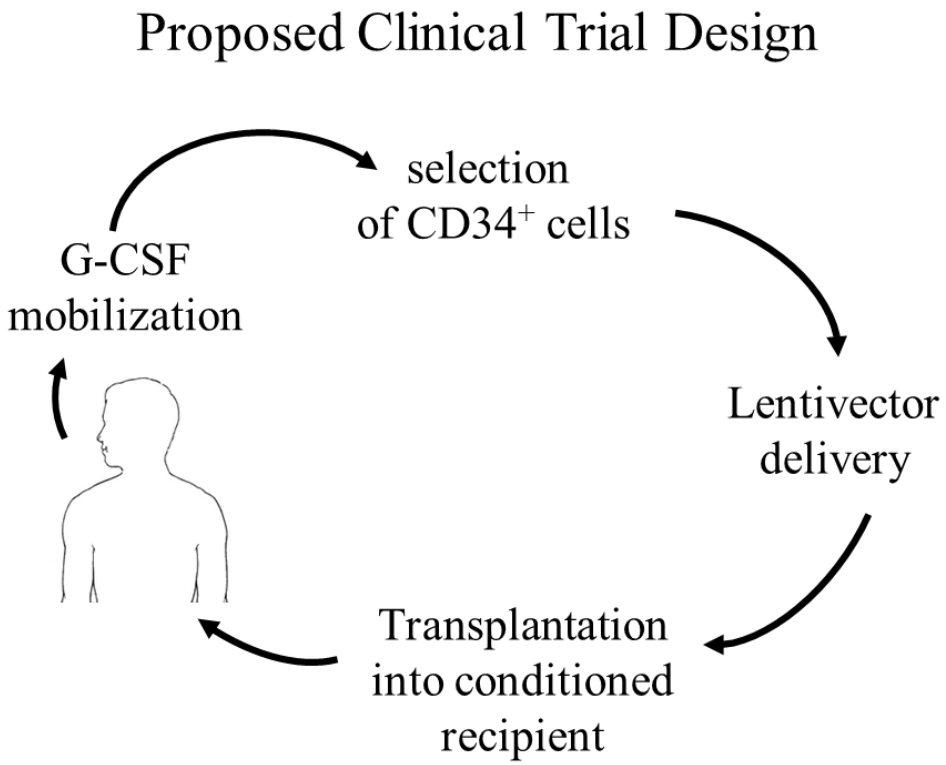


Figure 5.2 Schematic of gene therapy protocol for the treatment of hemophilia A. A clinical trial to be conducted at Emory University has been reviewed and approved by the Recombinant DNA Advisory committee. In addition, the protocol which includes the components involved have been deemed as a viable candidate for further development following a preinvestigational new drug and biosafety review. Following submission of and approval from the institutional review board and investigational new drug application, subjects with hemophilia A will be admitted into a gene therapy clinical trial in which $CD34^+$ cells will be mobilized from the hematopoietic stem cell compartment by G-CSF administration. G-CSF stimulates the bone marrow to produce and release granulocytes and stem cells into the bloodstream. Upon collection, the $CD34^+$ cells will be genetically modified *ex vivo* with a first-generation HIV-based lentiviral vector

lacking a WPRE sequence while encoding the high expressing human/porcine fVIII transgene expressed from the clinically approved EF1α internal promoter. The $CD34^+$ cells will then be removed from the viral containing medium prepping the cells for transplantation back into the patient in order to deliver a renewable supply of fVIII.

Abbreviation: G-CSF (Granulocyte colony-stimulating factor)

5.2— Future Perspectives

In my opinion, the field of gene therapy is migrating away from utilizing integrating viral based vectors as a means of gene replacement. This is mainly due to the risk of insertional mutagenesis. Although insertional mutagenesis has not been observed clinically with the use of lentiviral vectors (Cartier *et al*., 2009; Cavazzana-Calvo *et al*., 2010; Biffi *et al*., 2011), the potential of the integrated lentiviral cDNA to activate a protooncogene or disrupt a tumor suppressor gene is still a major concern in the field. One avenue explored as a means to eliminate insertional mutagenesis has capitalized on the fact that lentiviruses and lentiviral vectors, in addition to integrating in the genome, produce circular extrachromosomal forms of DNA. Two types of circular episomes can be formed. The first consists of 2 adjacent long terminal repeats (LTR) formed as a result of nonhomologous end joining of the linear reverse transcribed viral cDNA. The second type of circular episome contains one LTR following homologous recombination. As with AAV-based vectors, gene transfer of this nature is lost overtime as cells divide due to a lack of origin of replication in the 1- and 2-LTR episomal circles. However, these circular viral episomes may persist in terminally differentiated cells such as hepatocytes. For this reason, integrase-defective lentiviral vectors have been established in order to eliminate the potential risk of insertional mutagenesis associated with integration.

The lentiviral integrase protein consists of three domains, two zinc-finger binding domains (one in the N-terminal region which binds the viral DNA and another in the C-terminal region of the protein which binds the host DNA) and a catalytic core domain. Three amino acids of the core domain have been found to be highly conserved among the

retroviral integrase proteins and have thus been denoted as the catalytic triad. Therefore, the majority of the missense mutations introduced to produce an integrase-defective lentiviral vector occur at one of these three amino acids. These mutations prevent the integration of the transgene and if utilized to modify non-dividing cells can potentially persist for the lifetime of the host.

Integrase-defective lentiviral vectors could be an ideal route of transferring the fVIII gene into hepatocytes, the endogenous producer of fVIII. The route of administration would be similar to that of an AAV-based vector containing fIX for hemophilia B recently performed clinically. Likewise, insertional mutagenesis would not be a concern since the genome would not be disrupted by an integrase-defective lentiviral vector. But unlike AAV-based vectors, an integrase-defective lentiviral vector would not be constrained by the size of fVIII. Thus, integrase-defective lentiviral vectors encompass all of the advantages that AAV-based vectors offer while providing a means to overcome their shortcomings. However, as is a major concern in most gene therapy applications, the level of fVIII expression driven from the 1- and 2-LTR episomal circles is questionable. fVIII will need to be equivalent if not superior to those applications utilizing a lentiviral vector. Unfortunately, the integrase-defective lentiviral vector is not able to overcome the production limitations associated with lentiviral vectors. Up to this point, integrase-defective lentiviral vector titers for the most part have been reported to be similar to those of integrating lentiviral vectors (Saenz *et al.*, 2004; Nightingale *et al.*, 2006; Negri *et al.*, 2007; Coutant *et al.*, 2008). However, some reports have indicated lower titers than those

of integrating lentiviral vectors (Vargas *et al*., 2004; Philippe *et al*., 2006; Apolonia *et al*., 2007).

Integrase-defective lentiviral vectors are promising tools for providing a safer method of achieving gene transfer of fVIII. However, another method in which safer integration can be achieved is by utilizing a vector that provides a means for site-directed integration. Integrating viruses, such as the HIV and SIV lentiviruses, integrate into the host genome in a somewhat nonspecific manner, showing preference for active transcriptional units (Mitchell *et al*., 2004). Although this is a safer integration profile than that of the γ-retroviral vector utilized in the X-SCID clinical trial which favored integration near transcription start sites, an ideal vector would integrate at a specific location in the genome recognized as a safe harbor. This can be achieved with the use of either zinc finger nucleases or tal effector nucleases.

Zinc finger nucleases (ZFN) are fusion proteins comprised of a nonspecific FokI endonuclease domain and a zinc finger DNA-binding domain. Integration specificity comes from the ZFN DNA-binding domain which can be engineered to recognize a specific sequence. With 3-6 zinc finger DNA-binding domains arranged in tandem, a 9-18 base-pair target site can be achieved since each domain recognizes three base pairs. When two FokI endonuclease domains dimerize, a double strand break is formed. This double strand break can then be repaired naturally by the cell via nonhomologous end joining or homologous recombination. In the case of homologous recombination, the cell uses the undamaged sister chromatid to repair the break by copying the sequence across

the break. However, an exogenous donor DNA template consisting of a transgene flanked by homologous regions could also be used to repair the double strand break while at the same time transferring the gene encoding fVIII into the cell (Porteus, 2011).

Tal effector nucleases (TALENs) perform site-directed integration of a transgene in a similar manner as ZFNs. Like ZFNs, TALENS contain a FokI endonuclease domain fused with a DNA-binding domain. To achieve integration at a specific location in the genome of the target cell, the DNA-binding domain takes advantage of the ability of TAL effector proteins to specifically recognize a DNA sequence. TAL effector proteins contain a series of repeating subunits each thirty-four amino acids in length. The amino acid residues at position twelve and thirteen determine the recognition of a single nucleic acid. These residues can be modified in each repeat to engineer a TAL effector that is able to recognize a specific piece of DNA (Moscou *et al*.., 2009).

Both ZFNs and TALENs have been utilized to target endogenous disease-related mammalian genes (Urnov *et al*., 2005; Zou *et al*., 2009; Connelly *et al*., 2010; Mussolino *et al*., 2011). However, the frequency of gene targeting with the use of these nucleases is not yet substantial enough in primary patient-derived cells. In addition, toxic side effects have been observed with the use of ZFNs (Porteus and Baltimore, 2003). This is probably due to the production of off-target double-strand breaks which is not only concerning in regards to cytotoxicity but would also pose questions as to the safety profile of a ZFN. TALENs, on the other hand, have been shown to be less toxic and may

be a more ideal platform to accomplish site-directed gene targeting (Mussolino *et al*., 2011).

Until these technologies are improved, the utilization of a lentiviral vector for the treatment of hemophilia A is appropriate (**Figure 5.2**). One of the major limitations with the utilization of lentiviral vectors for *ex vivo* HSC modification has potentially been overcome by the results of this dissertation. Lentiviral transduction of $CD34^+$ cells has been enhanced by treatment prior to viral addition with the phorbol ester, PMA. The mechanism whereby PMA enhanced transduction has been delineated to be a result of PKC. Before PMA can be clinically applicable as a part of the gene therapy protocol for hemophilia A, the effects on integration will need to be adequately explored in regards to safety. Enhancing the transduction of $CD34^+$ cells with PMA is unfavorable if the likelihood of an insertional mutagenic event is increased as a consequence of an integration preference towards transcription start sites. This has yet to be analyzed. In addition, the effect of PMA on the multipotentiality of $CD34^+$ cells will need to be addressed. As demonstrated in chapter 4, $CD34^+$ cells treated with PMA produced colonies in methycellulose indicative of having the potential to give rise to lineage committed cells. However, the colonies were not specifically analyzed for the exact lineage that the $CD34^+$ cells differentiated into. Without this assessment, it is unclear whether the PMA treated $CD34^+$ cells have maintained the ability to differentiate into all hematopoietic lineages necessary for reconstitution and not just a subset. However, transplantation of PMA treated $CD34^+$ cells into irradiated NOD-SCID mice could answer this question as well as confirming that PMA does not affect the transplantation

efficiency of $CD34^+$ cells. Transducing the $CD34^+$ cells with a GFP containing virus prior to transplantation would allow one to identify lineage committed transplanted $CD34^+$ cells by identifying GFP positive cells that also contain lineage specific markers by flow cytometry. In this manner, the multipotentiality of the PMA treated $CD34^+$ cells could be assessed as well as confirming their persistence overtime indicative of a lack of effect of PMA on transplantation efficiency.

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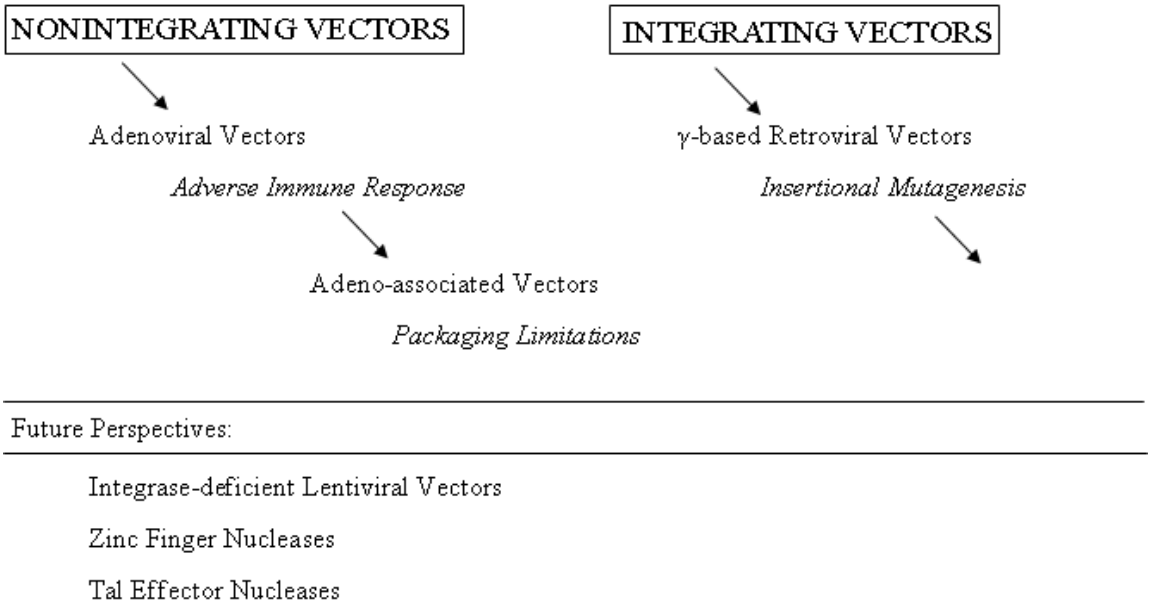

Figure 5.3 Modes of gene transfer proposed for the treatment of hemophilia A. Future perspectives include the use of an integrase-deficient lentiviral vector in which the transgene would remain episomal in the target cell. In addition, site-directed integration by zinc finger nucleases and tal effector nucleases will be utilized in the future providing a safer integration profile. However, until these technologies are improved the use of a lentiviral vector in a gene therapy protocol is an appropriate mode to transfer fVIII for the treatment of hemophilia A.

Limited expression of fVIII as apparent in all previous clinical attempts to treat hemophilia A with gene therapy has been potentially overcome by the construction of a high-expression chimeric fVIII transgene. HP-fVIII was used to genetically modify hematopoietic stem cells and effectively treat hemophilia A mice in order to overcome the low expression barrier. Prior to advancing to the clinic, the gene transfer system was optimized by analyzing the various components of the self-inactivating lentiviral vectors in terms of viral production, transduction efficiency and transgene expression. The data outlined in this dissertation was used to help develop a clinical vector that was recently approved by the Recombinant DNA Advisory Committee. In addition to optimizing the lentiviral vector, the obstacle associated with the resistance of HSCs to lentiviral infection was overcome by PMA as identified from a high-throughput screen. Notably, the viral transduction of $CD34^+$ cells was enhanced from 7% to >20%. PMA enhancement seems to correlate with an inhibition in cell division. Further analysis will be needed before PMA can be incorporated into a gene therapy protocol. Otherwise, an efficient lentiviral vector and protocol for the treatment of hemophilia A has been delineated and is currently being evaluated by the FDA for a phase I clinical trial to treat and possibly cure hemophilia A.

REFERENCES

Aiuti A, Cattaneo F, Galimberti S, Benninghoff U, Cassani B, Callegaro L, Scaramuzza S, Andolfi G, Mirolo M, Brigida I, Tabucchi A, Carlucci F, Eibl M, Aker M, Slavin S, Al-Mousa H, Ghonaium A, Ferster A, Duppenthaler A, Notarangelo L, Wintergerst U, Buckley RH, Bregni M, Marktel S, Valsecchi MG, Rossi P, Ciceri F, Miniero R, Bordignon C, Roncarolo MG. 2009. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*. 360:447-58.

Akkina RK, Walton RM, Chen M.L, Li QX, Planelles, V, Chen IS. 1996. High-efficiency gene transfer into $CD34^{+}$ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J. Virology*. 70:2581-2585.

Allocca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D, Kim SR, Maguire A, Rex TS, Di Vicino U, Cutillo L, Sparrow JR, Williams DS, Bennett J, Auricchio A. 2008. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invst.* 118:1955-1964.

Apolonia L, Waddington SN, Fernandes C, Ward NJ, Bouma G, Blundell MP, Thrasher AJ, Collins MK, Philpott NJ. 2007. Stable gene transfer to muscle using non-integrating lentiviral vectors. *Molecular Therapy*. 15:1947-1954.

Aruda V. 2006. Toward gene therapy for hemophilia A with novel adenoviral vectors: success and limitations in canine models. *J. Thromb. Haemost*. 4:1215-7.

Bagnall RD, Waseem N, Green PM, Giannelli F. 2002. Recurrent inversion breaking

intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood*. 99:168-174.

Berlfein J. 2003. Clinical trial update. *Hemaware*. 8:52-3.

Bevers E, Comfurius P, Zwall R. 1982. Changes in membrane phospholipid distribution platelet activation. *Biochim Biophs Acta.*736:57-66.

Bhopale G, Nanda R. 2003. Blood coagulation factor VIII: An overview. *J. Biosci.* 28: 783-789.

Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr. 1995. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nature Genetics*. 10:119-21.

Biffi A, Bartolomae CC, Cesana D, Cartier N, Aubourg P, Ranzani M, Cesani M, Benedicenti F, Plati T, Rubagotti E, Merella S, Capotondo A, Sgualdino J, Zanetti G, von Kalle C, Schmidt M, Naldini L, Montini E. 2011. Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood*. 117:5332-9.

Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, Greenblatt JJ, Rosenberg SA, Klein H, Berger M, Mullen CA, Ramsey WJ, Muul L, Morgan RA, Anderson WF. 1995. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science*. 270:475-480.

Boztug K, Schnmidt M, Schwarzer A, Banerjee PP, Díez IA, Dewey RA, Böhm M,

Nowrouzi A, Ball CR, Glimm H, Naundorf S, Kühlcke K, Blasczyk R, Kondratenko I, Maródi L, Orange JS, von Kalle C, Klein C. 2010. Stem-cell therapy for Wiskott-Aldrich syndrome. *N.Engl. J. Med.* 363: 1918-27.

Bowen DJ. 2002. Haemophilia A and haemophilia B: molecular insights. *Molecular Pathology*. 55:1-18.

Brinke A, Tagliavacca L, Naylor J, Green P, Giangrande P, Giannelli F. 1996. Two chiameric transcription units result from an inversion breaking intron 1 of the factor VIII gene and a region reportedly affected by reciprocal translocations in T-cell leukaemia. *Hum Mol Genet.* 5:1945-1951.

Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A, Baccarini A, Lazzari G, Galli C, Naldini L. 2007. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nature Technology*. 25:1457-1467.

Brun S, Faucon-Biguet N, Mallet J. 2003. Optimization of transgene expression at the posttranscriptional level in neural cells: implications for gene therapy. *Molecular Therapy*. 7:782-9.

Brunetti-Pierri N, Palmer DJ, Beaudet AL, Carey KD, Finegold M, Ng P. 2004. Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Human Gene Therapy*. 15:35-46.

Burns JC, Friedmann T, Driever W, Burrascano M. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *PNAS*. 90:8033-8037.

Butenas S, DiLorenzo ME, Mann KG. 1997. Ultrasensitive fluorogenic substrates for serine proteases. *Journal of Thrombosis and Haemostasis*. 78:1193-1201.

Burton M, Nakai H, Colosi P, Cunningham J, Mitchell R, Couto L. 1999. Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. *Proc Natl Acad Sci U S A*. 96:12725-30.

Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, Vidaud M, Abel U, Dal-Cortivo L, Caccavelli L, Mahlaoui N, Kiermer V, Mittelstaedt D, Bellesme C, Lahlou N, Lefrére F, Blanche S, Audit M, Payen E, Leboulch P, l'Homme B, Bougnéres P, von Kalle C, Fischer A, Cavazzana-Calvo M, Aubourg P. 2009. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science. 326:818-23.

Cartier N, Hacein-Bey-Abina S, Von Kalle C, Bougnères P, Fischer A, Cavazzana-Calvo M, Aubourg P. 2010. Gene therapy of x-linked adrenoleukodystrophy using hematopoietic stem cells and a lentiviral vector. *Bull Acad Natl Med*. 194:255-64.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*. 288:669-672.

Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F, Down J, Denaro M, Brady T, Westerman K, Cavallesco R, Gillet-Legrand B, Caccavelli L, Sgarra R, Maouche-Chrétien L, Bernaudin F, Girot R, Dorazio R, Mudler GJ, Polack A, Bank A, Soulier J, Larghero J, Kabbara N, Dalle B, Gourmel B, Socie G,

Chrétien, S, Cartier N, Aubourg P, Fischer A, Cornetta K., Galacteros F, Beuzard Y, Gluckman E, Bushman F, Hacein-Bey-Abina S, Leboulch P. 2010. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature*. 467:318-22.

Chao H, Mansfield SG, Bartel RC, Hiriyanna S, Mitchell LG, Garcia-Blanco MA, Walsh CE. 2003. Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing. *Nat Med*. 9:1015-1019.

Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden DT. 2000. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science*. 287:1804-1808.

Chesebro B, Wehrly K, Maury W. 1990. Differential expression in human and mouse cells of human immunodeficiency virus pseudotyped by murine retroviruses. *J. Virology*. 64:4553-4557.

Ciuffi A. 2008. Mechanisms governing lentivirus integration site selection. *Current Gene Therapy*. 8:419-429.

Coil DA, and Miller AD. 2004. Phosphatidylserine is not the cell surface receptor for vesticular stomatitis virus. *J. Virol.* 78:10920-10926.

Coil DA, and Miller AD. 2005. Enhancement of enveloped virus entry by phosphatidylserine. *J. Virol.* 79:11496-11500.

Colman RW, Bagdasarian A, Talamo RC, Scott CF, Seavey M, Guimaraes JA, Pierce JV, Kaplan AP. 1975. Williams trait. Human kiniongen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with

abnormalities of the Hageman factor-dependent pathways. *J. Clin. Invest.* 56:1650-1662.

Connelly JP, Barker JC, Pruett-Miller S, Porteus MH. 2010. Gene correction by homologous recombination with zinc finger nucleases in primary cells from a mouse model of a generic recessive genetic disease. *Molecular Therapy*. 18:1103-1110.

Conti C, Mastromarino P, Ciuffarella MG, Orsi N. 1988. haracterization of rat brain cellular membrane components acting as receptors for vesicular stomatitis virus. *Arch. Virol.* 99:261-269.

Coutant F, Frenkiel MP, Despres P, Charneau P. 2008. Protective antiviral immunity conferred by a nonintegrative lentiviral vector-based vaccine. *Plos One.* 3:3973.

Cronin J, Zhang X, Reiser J. 2005. Altering the tropism of lentiviral vectors through pseudotyping. Curr. Gene Ther. 5:387-398.

Dasgupta S, Repessé Y, Bayry J, Navarrete AM, Wootla B, Delignat S, Irinopoulou T, Kamaté C, Saint-Remy JM, Jacquemin M, Lenting PJ, Borel-Derlon A, Kaveri SV, Lacroix-Desmazes S. 2007. VWF protects FVIII from endocytosis by dendritic cells and subsequent presentation to immune effectors. *Blood.* 109:610-620.

Davi G, and Patrono C. 2007. Platelet activation and atherothrombosis. *New England Journal of Medicine*. 357:2482-2494.

Davis BM, Humeau L, Slepushkin V, Binder G, Korshalla L, Ni Y, Ogunjimi EO,

Chang LF, Lu X, and Dropulic B. 2004. ABC transporter inhibitors that are substrates enhance lentiviral vector transduction into primitive hematopoietic progenitor cells. *Blood*. 104:364-373.

Debili N, Issaad C, Masse JM, Guichard J, Katz A, Breton-Gorius J, Vainchenker W. 1992. Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation. *Blood*. 80:3022-3035.

Deininger P. and Batzer M. 2002. Mammalian Retroelements. *Genome Research*. 12: 1455-1465.

DiGiusto DL, Krishnan A, Li L, Li H, Li S, Rao A, Mi S, Yam P, Stinson S, Kalos M, Alvarnas J, Lacey SF, Yee JK, Li M, Couture L, Hsu D, Forman SJ, Rossi JJ, Zaia JA. 2010. RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. *Sci. Transl. Med*. 2:36-43

Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. 2002. High level expression of recombinant porcine coagulation factor VIII. *J Biol Chem*. 277:38345-9.

Doering C, Parker E, Healey J, Craddock H, Barrow R, Lollar P. 2002. Expression and characterization of recombinant murine FVIII. *Thromb Haemost.* 88:450-458.

Doering C, Healey JF, Parker ET, Barrow RT, Lollar P. 2004. Identification of porcine coagulation factor VIII domains responsible for high level expression via enhanced secretion. *J Biol Chem*. 279:6546-6552.

Doering CB, Gangadharan B, Dukart HZ, Spencer HT. 2007. Hematopoietic stem cells encoding porcine factor VIII induce pro-coagulant activity in hemophilia A mice with pre-existing factor VIII immunity. *Molecular Therapy*. 15:1093-9.

Doering CB, Denning G, Dooriss K, Gangadharan B, Johnston JM, Kerstann KW, McCarty DA, Spencer HT. 2009. Directed engineering of a high-expression chimeric transgene as a strategy for gene therapy of hemophilia A. *Molecular Therapy*. 17:1145-54.

Doering CB, and Spencer HT. 2009. Advancements in gene transfer-based therapy for hemophilia A. *Expert Rev Hematol*. 2:673-83.

Dooriss KL, Denning G, Gangadharan B, Javazon EH, McCarty DA, Spencer HT. Doering CB. 2009. Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A. *Human Gene Therapy*. 20:465-78.

Dorner AJ, Wasley LC, Kaufman RJ. 1989. Increased synthesis of secreted proteins induces Expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol Chem*. 264(34):2062-2067.

Eaton D, Rodriguez H, Vehar G. 1986. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulation activity. *Biochemistry*. 25:505-512.

Ehrenforth S, Kreuz W, Scharrer I, Kornhuber B. 1992. Factor VIII inhibitors in haemophiliacs. *Lancet* 340:253.

Eigenbrot C. 2002. Structure, function, and activation of coagulation factor VII. *Curr Protein Pept Sci*. 3:287-299.

Emi N, Friedmann T, Yee JK. 1991. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *Journal of Virology*. 65:1202-1207.

Essner JJ, McIvor RS, Hackett PB. 2005. Awakening gene therapy with Sleeping Beauty transposons. *Current Opinion in Pharmacology*. 5:513-519.

Fay PJ. 1988. Subunit structure of thrombin-activated human factor VIIIa. *Biochim Biophys Acta*. 952:181-190.

Fischer A. and Cavazzana-Calvo M. 2008. Gene therapy of inherited diseases. *Lancet.* 371:2044-2047.

Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. 2005. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol*. 166:1781-91.

Gangadharan B, Parker E, Ide L, Spencer HT, Doering C. 2006. High level expression of porcine FVIII from genetically modified bone marrow-derived stem cells. *Blood.* 34:461-475.

Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Zhang F, Adams S, Bjorkegren E, Bayford J, Brown L, Davies EG, Veys P, Fairbanks L, Bordon V, Petropoulou T, Kinnon C, Thrasher AJ. 2011. Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Sci Transl Med*. 3:97ra80.

Gewirtz J, Thornton MA, Rauova L, Poncz M. 2008. Platelet-delivered factor VIII provides limited resistance to anti-factor VIII in hibitors. *J. Thromb. Haemost.* 6:1160-1166.

Gitschier J, Wood W, Goralka T, Wion K, Chen E, Eaton D, Vehar G, Capon D, Lawn R. 1984. Characterization of the human factor VII gene. *Nature*. 312:326-330.

Gonzalez-Murillo A, Lozano ML, Alvarez L, Jacome A, Almarza E, Navarro S, Segovia JC, Hanenberg H, Guenechea G, Bueren JA, Rio P. 2010. Development of lentiviral vectors with optimized transcriptional activity for the gene therapy of patients with Fanconi anemia. *Human Gene Therapy*. 21:623-30.

Gorman L, Suter D, Emerick V, Schumperli D, Kole R. 1998. Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs. *Proceedings of the National Academy of Sciences.* 95:4929-4934.

Greene TK, Wang C, Hirsch JD, Ahai L, Gewirtz J, Thornton MA, Miao HZ, Pipe SW, Kaufman RJ, Camire RM, Arruda VR, Kowalska MA, Poncz M. 2010. In vivo efficacy of platelet-delivered, high specific activity factor VIII variants. *Blood.* 116:6114-6122.

Groeschel B. and Bushman F. 2005. Cell Cycle Arrest in G2/M Promotes Early Steps of Infection by Human Immunodeficiency Virus. *Journal of Virology*. 79:5695–5704.

Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M. 2002. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N. Engl. J. Med*. 18:1185-93.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P. 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 302:415-9.

Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier

E, Caccavelli L, Delabesse E, Beldjord K, Asnafi V, MacIntyre E, Dal Cortivo L, Radford I, Brousse N, Sigaux F, Moshous D, Hauer J, Borkhardt A, Belohradsky BH, Wintergerst U, Velez MC, Leiva L, Sorensen R, Wulffraat N, Blanche S, Bushman FD, Fischer A, Cavazzana-Calvo M. 2008. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest.* 118:3132-3142.

Hallek M, Girod A, Braun-Falco M, Wendtner CM, Bogedain C, Hörer M. 1998. Recombinant adeno-associated virus vectors. *IDrugs*. 1:561-573.

Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. 2009. The comparative immunogenicity of human and porcine factor VIII in haemophilia A mice. *Thrombosis and Haemostasis*. 102:35-41.

Hermonat PL. and Muzyczka N. 1984. Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue cultured cells. *Proceedings of the National Academy of Sciences*. 81:6466-6470.

Higashimoto T, Urbinati F, Perumbeti A, Jiang G, Zarzuela A, Chang LJ, Kohn DB, Malik P. 2007. The woodchuck hepatitis virus post-transcriptional regulatory element reduces readthrough transcription from retroviral vectors. *Gene Therapy*. 14:1298-1304.

High K. 2005. Gene transfer for hemophilia: can therapeutic efficacy in large animals be safely translated to patients? *J Thromb Haemost.* 3:1682-1691.

Hockin MF, Jones KC, Everse SJ, Mann KG. 2002. A model for the stoichiometric regulation of blood coagulation. *Journal of Biological Chemistry*. 277:18322-33.

Hu C, Cela RG, Suzuki M, Lee B, Lipshutz GS. 2011. Neonatal helper-dependent

adenoviral vector gene therapy mediates correction of hemophilia A and tolerance to human factor VIII. *Proceedings of National Academy of the Sciences*. 108:2082-7.

Hu C. and Lipshutz GS. 2012. AAV-based neonatal gene therapy for hemophilia A: long-term correction and avoidance of immune responses in mice. *Gene Therapy*. 2012; e-pub ahead of print 12 January 2012; doi:10.1038/gt.2011.200

Ide LM, Gangadharan B, Chiang KY, Doering CB, Spencer HT. 2007. Hematopoietic stem-cell gene therapy of hemophilia A incorporating a porcine factor VIII transgene and nonmyeloablative conditioning regimens. *Blood*. 110:2855-63.

Iwakuma T, Cui Y, Chang LJ. 1999. Self-inactivating lentiviral vectors with U3 and U5 modifications. Virology. 261:120-32.

Jaffe EA, Hoyer LW, Nachman RL. 1973. Synthesis of antihemophnilic factor antigen by cultured human endothelial cells. *J. Clin, Invest.* 52:2757-2764.

Jaffe EA, Hoyer LW, Nachman, R.L. 1974. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci.* 71:1906-1909.

Jeon HJ, Oh TK, Kim OH, Kim ST. 2010. Delivery of factor VIII gene into skeletal muscle cells using lentiviral vector. *Yonsei Med J.* 51:52-7.

Jiang H, Lillicrap D, Patarroyo-White S, Liu T, Qian X, Scallan CD, Powell S, Keller T, McMurray M, Labelle A, Nagy D, Vargas JA, Zhou S, Couto LB, Pierce GF. 2006. Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood*. 108:107-15.

Johnston J, Doering, CB, Spencer HT. 2011. Gene Therapy Strategies Incorporating

Large Transgenes. In: Chunsheng Kang (ed). Gene Therapy - Developments and Future Perspectives. InTech: Croatia, pp 121-142.

Jurlander B, Thim L, Klausen NK, Persson E, Kjalke M, Rexen P, Jørgensen TB, Østergaard PB, Erhardtsen E, Bjørn SE. 2001. Recombinant activated factor VII (rFVIIa): characterization, manufacturing, and clinical development. *Semin Thromb Hemost*. 27:373-84.

Kasuda S, Kubo A, Sakurai Y, Irion S, Ohashi K, Tatsumi K, Nakajima Y, Saito Y, Hatake K, Pipe SW, Shima M, Yoshioka A. 2008. Establishment of embryonic stem cells secreting human factor VIII for cell-based treatment of hemophilia A. *J Thromb Haemost*. 6:1352-9.

Kaufman RJ, Wasley LC, Davies MV, Wise RJ, Israel DI, Dorner AJ. 1989. Effect of von Willebrand factor coexpression on the synthesis and secretion of factor VIII in Chinese hamster ovary cells. *Mol Cell Biol*. 9(3):1233-42.

Kaufman RJ. and Pipe SW. 1999. Regulation of factor VIII expression and activity by von Willebrand factor. *Thromb Haemost*. 82:201-208.

Kazanietz MG, Bustelo XR, Barbacid M, Kolch W, Mischak H, Wong G, Pettit BR, Bruns JD, Blumberg PM. 1994. Zinc finger domains and phorbol ester pharmacophore. Analysis of binding to mutated form of protein kinase C zeta and the vav and c-raf proto-oncogene products. *J. Biol Chem*. 269:11590-11594.

Kazanietz MG, Barchi JJ Jr., Omichinski JG, Blumberg PM. 1995. Low affinity binding of phorbol esters to PKC and its cysteine-rich region in the absence of phospholipids. *J. Biol. Chem*. 270, 14679-14684.

Kazanietz, MG. 2000. Eyes wide shut: protein kinase C isozymes are not the only

receptors for the phorbol ester tumor promoters. *Mol Carcinog*. 28, 5-11.

Kingdon HS, and Hassell TM. 1981. Hemophilic dog model for evaluating therapeutic effectiveness of plasma protein fractions. *Blood.* 58:868-872.

Kingsman SM, Mitrophanous K, Olsen JC. 2005. Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE). *Gene Therapy*. 12:3-4.

Klein R, Ruttkowski B, Knapp E, Salmons B, Gunzburg WH, Hohenadl C. 2006. WPRE-mediated enhancement of gene expression is promoter and cell line specific. *Gene*. 372:153-61.

Kuether EL, Schroeder JA, Fahs SA, Cooley BC, Chen Y, Montgomery R, Wilcox DA, Shi Q. 2012. Lentivirus-mediated platelet gene therapy of murine hemophilia A with pre-existing anti-factor VIII immunity. *J Thrombosis and Haemostasis*. 10:1570-1580.

Kulkarni S, Dopheide S, Yap C, Ravanat C, Freund M, Mangin P, Heel KA, Street A, Harper IS, Lanza F, Jackson SP. 2000. A revised model of platelet aggregation. *J Clin Invest.* 105:783-791.

Kumar M, Keller B, Makalou N, Sutton RE. 2001. Systemic determination of the packaging limit of lentiviral vectors. *Human Gene Therapy*. 12:1893-905.

Kustikova OS, Schiedlmeier B, Brugman MH, Stahlhut M, Bartels S, Li Z, Baum C. 2009. Cell-intrinsic and vector-related properties cooperate to determine the incidence and consequences of insertional mutagenesis. *Mol. Ther*. 17:1537-47.

Lakich D, Kazazian HH Jr., Antonarakis SE, Gitschier J. 1993. Inversions disrupting

the factor VIII gene are a common cause of severe haemophilia A. *Nat Genet*. 10:236-241.

Laughlin CA, Tratschin JD, Coon H, Carter BJ. 1983. Cloning of infectious adenoassociated virus genomes in bacterial plasmids. *Gene*. 23:65-73.

Lee B, Ratajczak J, Doms RW, Gewirtz AM, Ratajczak MZ. 1999. Coreceptor/chemokine receptor expression on human hematopoietic cells:biological implications for human immunodeficiency virus-type 1 infection. *Blood*. 93:1145-1156.

Lee HJ, Lee YS, Kim HS, Kim YK, Kim JH, Jeon SH, Lee HW, Kim S, Miyoshi H, Chung HM, Kim DK. 2009. Retronectin enhances lentivirus-mediated gene delivery into hematopoietic progenitor cells. *Biologicals*. 37:203-209.

Lenting PJ, van Mourik JA, Mertens K. 1998. The life cycle of coagulation factor VIII in view of its structure and function. *Blood*. 92:3983-3996.

Leuci V, Mesiano G, Gammaitoni L, Cammarata C, Capellero S, Todorovic M, Jordaney N, Circosta P, Elia A, Lesnikova M, Georges G, Piacibello W, Fagioli F, Cignetti A, Aglietta M, Sangiolo D. 2011. Transient proteasome inhibition as a strategy to enhance lentiviral transduction of hematopoietic $CD34^{+}$ cells and T lymphocytes: Implications for the use of low viral doses and large-size vectors. *J. Biotechnology*. 156:218-226.

Leyte A, van Schijndel H, Niehrs C, Hunger W, Verbeet M, Mertens K, van Mourik J. 1991. Sulphation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. J Biol Chem. 266:740-746.

Lippi G, Favaloro E, Franchini M, Guidi G. 2009. Milestones and perspectives in coagulation and hemostasis. *Semin Thromb Hemost.* 35:9-22.

Lu H, Chen L, Wang J, Huack B, Sarkar R, Zhou S, 2008. Complete correction of hemophilia A with adeno-associated viral vectors containing a full-size expression cassette. *Human Gene Therapy*. 19:648-54.

Lubin IM, Healey JF, Barrow RT, Scandella D, Lollar P. 1997. Analysis of the human factor VIII A2 inhibitor epitope by alanine scanning mutagenesis. *J Biol Chem*. 272:30191-5.

Mackman N, Tilley R, Key N. 2007. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arterioscler Throm. Vasc Biol.* 27:1687-1693.

Mann KG. and Kalafatis M. 2003. Factor V: a combination of Dr Jekyll and Mr Hyde. *Blood*. 101:20-30.

Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, Ozelo MC, Hoots K, Blatt P, Konkle B, Dake M, Kaye R, Razavi M, Zajko A, Zehnder J, Rustagi PK, Nakai H, Chew A, Leonard D, Wright JF, Lessard RR, Sommer JM, Tigges M, Sabatino D, Luk A, Jiang H, Mingozzi F, Couto L, Ertl HC, High KA, Kay MA. 2005. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med.* 12:42-247.

Margaritis P, Roy E, Aljamali MN, Downey HD, Giger U, Zhou S, Merricks E, Dillow A, Ezban M, Nichols TC, High KA. 2009. Successful treatment of canine hemophilia by continuous expression of canine FVIIa. *Blood.* 113:3682-3689.

Margaritis P, Roy E, Faella A, Downey HD, Ivanciu L, Pavani G, Zhou S, Bunte RM,

High KA. 2011. Catalytic domain modification and viral gene delivery of activated factor VII confers hemostasis at reduced expression levels and vector doses in vivo. *Blood.* 117:3974-3982.

Marquette K, Pittman D, Kaufman R. 1995. A 110 amino acid region within the A1-domain of coagulation factor VIII inhibits secretion from mammalian cells. *Thromb Haemost.* 72:155-158.

Mastromarino P, Conti C, Goldoni P, Hauttecoeur B, Orsi N. 1987. Characterization of membrane components of the erythrocyte involved in vesicular stomatitis virus attachment and fusion at acidic pH. *J. Gen. Virol.* 68:2359-2369.

Matsui H, Shibata M, Brown B, Labelle A, Hegadorn C, Andrews C, Hebbel RP, Galipeau J, Hough C, Lillicrap D. 2007. Ex vivo gene therapy for hemophilia A that enhances safe delivery and sustained in vivo factor VIII expression from lentivirally engineered endothelial progenitors. *Stem Cells.* 25:2660-2669.

Matsui H, Hegadorn C, Ozelo M, Burnett E, Tuttle A, Labelle A, McCray PB Jr, Naldini L, Brown B, Hough C, Lillicrap D. 2011. A microRNA-regulated and GP64-pseudotyped lentiviral vector mediates stable expression of FVIII in a murine model of Hemophilia A. *Mol Therapy*. 19:723-730.

McIvor RS, Johnson MJ, Miller AD, Pitts S, Williams SR, Valerio D, Martin DW Jr, Verma IM. 1987. Human purine nucleoside phosphorylase and adenosine deaminase: gene transfer into cultured cells and murine hematopoietic stem cells by using recombinant amphotropic retroviruses. *Mol Cell Biol.* 7:838-46.

Meier B. 1996. "Blood, Money and AIDS: Hemophiliacs Are Split; Liability Cases Bogged Down in Disputes". *The New York Times*. 6-11-1996.

Millington M, Arndt A, Boyd M, Applegate T, Shen S. 2009. Towards a clinically relevant transduction protocol for primary human $CD34^{+}$ hematopoietic stem/progenitor cells. *Plos One*. 4:1-10.

Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR, Bushman FD. 2004. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *Plos One*. 2:1127-1137.

Mitchison JM. 2003. Growth during the cell cycle. Int. Rev. Cytol. 226:165-258.

Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. 1998. Development of a self-inactivating lentivirus vector. *Journal of Virology*. 72(10):8150-7.

Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C. 2006. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol*. 24:687-96.

Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, Benedicent F, Sergi LS, Ambrosi A, Ponzoni M, Doglioni C, Di Serio C., von Kalle C, Naldini L. 2009. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *Journal of Clinical Investigation*. 119:964-75.

Moscou MJ. and Bogdanove AJ. 2009. A simple cipher governs DNA recognition by TAL effectors. *Science*. 326:1501.

Muller MJ. and Baer HP. 1983. Relaxant effects of forskolin in smooth muscle: Role of cyclic AMP. *Naunyn- Schmiedeberg's Archives of Pharmacology*. 322:78-82.

Muruve DA, Barnes MJ, Stillman IE, Libermann TA. 1999. Adenoviral gene

therapy leads to rapid induction of multiple chemokines and acute neutrophil dependent hepatic injury in vivo. *Human Gene Therapy*. 10:965-976.

Mussolino C, Morbitzer R, Lütge F, Dannemann N, Lahaye T, Cathomen T. 2011. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Research.* 39:9283-9293.

Muul LM, Spiess PJ, Director EP, Rosenberg SA. 1987. Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma . *J Immunol*. 138:989–95.

Muul LM, Tuschong LM, Soenen SL, Jagadeesh GJ, Ramsey WJ, Long Z, Carter CS, Garabedian EK, Alleyne M, Brown M, Bernstein W, Schurman SH, Fleisher TA, Leitman SF, Dunbar CE, Blaese RM, Candotti F. 2003. Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. *Blood*. 101:2563-2569.

Nachman R, Levine R, Jaffe EA. 1977. Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *J. Clin. Invest.* 60:914-921.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 272:263-7.

Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, Chowdary P, Riddell A, Pie AJ, Harrington C, O'Beirne J, Smith K, Pasi J, Glader B, Rustagi P, Ng CY, Kay MA, Zhou J, Spence Y, Morton CL, Allay J, Coleman J, Sleep S, Cunningham JM, Srivastava D, Basner-Tschakarjan E, Mingozzi F,

High KA, Gray JT, Reiss UM, Nienhuis AW, Davidoff AM. 2011. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med.* 365:2357-2365.

Negri DR, Michelini Z, Baroncelli S, Spada M, Vendetti S, Buffa V, Bona R, Leone P, Klotman ME, Cara A. 2007. Successful immunization with a single injection of non-integrating lentiviral vector. *Molecular Therapy.* 15:1716-1723.

Neto M, Lunardi C, Rodrigues G, Bendhack L. 2011. Vasodilatation induced by forskolin involves cyclic GMP production. *J. Biopysical Chem.* 2:373-379.

Neyman M, Gewirtz J, Poncz M. 2008. Analysis of the spatial and temporal characteristics of platelet-delivered factor VIII-based clots. *Blood.* 112:1101-8.

Ni H, Denis C, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. 2000. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest.* 106:385-392.

Nightingale SJ, Hollis RP, Pepper KA, Petersen D, Yu XJ, Yang C, Bahner I, Kohn DB. 2006. Transient gene expression by nonintegrating lentiviral vectors. *Molecular Therapy.* 13:1121-1132.

Nishizawa Y, Usukura J, Singh DP, Chylack LT Jr, Shinohara T. 2001. Spatial and temporal dynamics of two alternatively spliced regulatory factors, lens epithelium-derived growth factor (ledgf/p75) and p52, in the nucleus. *Cell Tissue Res.* 305:107-14.

Novotny WF, Brown SG, Miletich JP, Rader DJ, Broze GJ Jr. 1991. Plasma antigen levels of the lipoprotein-associated coagulation inhibitor in patient samples. *Blood.* 78:387-393.

Ohmori T, Ishiwata A, Kashiwakura Y, Madoiwa S, Mitomo K, Suzuki H, Hasegawa M, Mimuro J, Sakata Y. 2008. Phenotypic correction of hemophilia A by ectopic expression of activated factor VII in platelets. *Molecular Therapy*. 16:1359-1365.

Olsen JC. 1998. Gene transfer vectors derived from equine infectious anemia virus. *Gene Therapy*. 5:1481-1487.

Olson ST, Bjork I, Shore JD. 1993. Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. *Methods Enzymol*. 222:525-559.

Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U, Nishizuka Y. 1989. Phorbol ester binding to protein kinase C requires a cysteine-rich zing-finger-like sequence. *Proc Natl Acad Sci USA*. 86, 4868-4871.

O'Rourke JP, Olsen JC, Bunnell BA. 2005. Optimization of equine infectious anemia derived vectors for hematopoietic cell lineage gene transfer. *Gene Therapy*. 12:22-29.

Osterud B. and Rapaport, S.I. 1977. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. *Proceedings of the National Academy of Sciences*. 74:5260-5264.

Page KA, Landau NR, Littman DR. 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J. Virol*. 64:5270-5276.

Papayannopoulou T, Nakamoto B, Yokochi T, Chait A, and Kannagi R. 1983. Human

erythroleukemia cell line (HEL) undergoes a drastic macrophage-like shift with TPA. *Blood*. 62:832-845.

Pauwels K, Gijsbers R, Toelen J, Schambach A, Willard-Gallo K, Verheust C, Debyser Z, Herman P. 2009. State-of-the-art lentiviral vectors for research use: risk assessment and biosafety recommendations. *Current Gene Therapy*. 9:459-74.

Pelletier R, Caron SO, Puymirat J. 2006 RNA based gene therapy for dominantly inherited diseases. *Current Gene Therapy*. 6:131-146.

Pezeshkpoor B, Rost S, Oldenburg J, El-Maarri O. 2012. Identification of a third rearrangement at Xq28 that causes severe hemophilia A as a result of homologous recombination between inverted repeats. *J Thromb Haemost.* 10:1600-1608.

Philippe S, Sarkis C, Barkats M, Mammeri H, Ladroue C, Petit C, Mallet J, Serguera C. 2006. Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. *Proc Natl Acad Sci.* 103:17684-17689.

Pipe S, Morris J, Shah J, Kaufman R. 1998. Differential interaction of coagulation factor VIII and Factor V with protein chaperones calnexin and calreticulin. *J Biol Chem*. 273:8537-8544.

Pittman D, and Kaufman R. 1992. Identification and functional importance of tyrosine sulfate residues within recombinant factor VIII. *Biochemistry*.31:3315-3325.

Pommier Y, Leo E, Zhang H, and Marchand C. 2010. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chem. and Biol*. 17:421-433.

Porada CD, Sanada C, Long CR, Wood JA, Desai J, Frederick N, Millsap L, Bormann C, Menges SL, Hanna C, Flores-Foxworth G, Shin T, Westhusin ME, Liu W, Glimp H, Zanjani ED, Lozier JN, Pliska V, Stranzinger G, Joerg H, Kraemer DC,

Almeida-Porada G. 2010. Clinical and molecular characterization of a re-established line of sheep exhibiting hemophilia A. *J Thromb Haemost*. 8:276-285.

Porteus MH and Baltimore D. 2003. Chimeric nucleases stimulate gene targeting in human cells. *Science*. 300:763.

Powell JS, Ragni MV, White GC, 2nd, Lusher JM, Hillman-Wiseman C, Moon TE, Cole V, Ramanathan-Girish S, Roehl H, Sajjadi N, Jolly DJ, Hurst D. 2003. Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood*. 102:2038-45.

Quest AF, Bardes ES, Bell RM. 1994. A phorbol ester binding domain of protein kinase C gamma. Deletion analysis of the Cys2 domain defines a minimal 43-amino acid peptide. *J. Biol. Chem*. 269:2961-2970.

Quinonez R. and Sutton R. 2002. Lentiviral vectors for gene delivery into cells. *DNA and Cell Biology*. 21:937-951.

Radcliffe P, Sion CJM., Wilkes EJ, Custard EJ, Beard GL, Kingsman SM, Mitrophanous K.A. 2008. Analysis of factor VIII mediated suppression of lentiviral vector titres. *Gene Therapy*. 15:289-297.

Ramezani A, Zweier-Renn LA, Hawley RG. 2011. Factor VIII delivered by haematopoietic stem cell-derived B cells corrects the phenotype of haemophilia A mice. *Thromb Haemost*. 105:676-87.

Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML. 2003 Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol. Genet. Metab*. 80:148-158.

Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. 1996 Transduction of nondividing cells using pseudotyped defective high-titer HIV type I particles. *PNAS*. 93:15266-15271.

Rivera J, Lozano M, Navarro-Nunez L, Vincente V. 2009. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*. 94:700-711.

Ron D, and Kazanietz MG. 1999. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J*. 13:1658-1676.

Rosen E, Chan J, Idusogie E, Clotman F, Vlasuk G, Luther T, Jalbert L, Albrecht S, Zhong L, Lissens A. 1997. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature*. 390:290-294.

Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA. (1988) Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: a preliminary report . *N. Engl. J. Med*. 319:1676–80.

Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, Merino M, Culver K, Miller AD, Blaese RM, Anderson WF. (1990) Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med*. 323:570-8.

Roth DA, Tawa NE, Jr., O'Brien JM, Treco DA, Selden RF. 2001. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *New England Journal of Medicine* 344:1735-42.

Sabatino DE, Lange AM, Altynova ES, Sarkar R, Zhou S, Merricks EP, Franck HG,

Nichols TC, Arruda VR, Kazaian HH Jr. 2011. Efficacy and safety of long-term prophylaxis in severe hemophilia A dogs following liver gene therapy using AAV vectors. *Molecular Therapy*. 19:442-9.

Saenz DT, Loewen N, Peretz M, Whitwam T, Barraza R, Howell KG, Holmes JM, Good M, Poeschla EM. 2004. Unintegrated lentivirus DNA persistence and accessibility to expression in nondividing cells: analysis with class I integrase mutants. *Journal of Virology*. 78:2906-2920.

Samulski RJ, Berns KI, Tan M, Muzyczka N 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proceedings of the National Academy of Sciences*. 79:2077-2081.

Santoni de Sio FR., Cascio P, Zingale A, Gasparini M, Naldini L. 2006. Proteasome activity restricts lentiviral gene transfer into hematopoietic stem cells and is down-regulated by cytokines that enhance transduction. *Blood*. 107:4257-4265.

Sarkar R, Xiao W, Kazazian HH Jr. 2003. A single adeno-associated virus (AAV)-murine factor VIII vector partially corrects the hemophilia A phenotype. *J Thromb Haemost*. 1:220-6.

Sarkar R, Tetreault R, Gao G, Wang L, Bell P, Chandler R, *et al.* 2004. Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood*. 103:1253-60.

Sarkar R, Mucci M, Addya S, Tetreault R, Bellinger DA, Nichols TC, Kazazian HH Jr. 2006. Long-term efficacy of adeno-associated virus serotypes 8 and 9 in hemophilia a dogs and mice. *Human Gene Therapy*. 17:427-39.

Sastry L, Xu Y, Cooper R, Pollok K, Cornetta K. 2004. Evaluation of plasmid DNA removal from lentiviral vectors by benzonase treatment. *Human Gene Therapy*. 15:221-226.

Savage B, Saldivar E, Ruggeri Z. 1996. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 84:289-297.

Scallan CD, Liu T, Parker AE, Patarroyo-White SL, Chen H, Jiang H, Vargas J, Nagy D, Powell SK, Wright JF, Sarkar R, Kazazian HH, McClelland A, Couto LB. 2003. Phenotypic correction of a mouse model of hemophilia A using AAV2 vectors encoding the heavy and light chains of FVIII. *Blood*. 102:3919-26.

Scallan CD, Lillicrap D, Jiang H, Qian X, Patarroyo-White SL, Parker AE, Liu T, Vargas J, Nagy D, Powell SK, Wright JF, Turner PV, Tinlin SJ, Webster SE, McClelland A, Couto LB. 2003. Sustained phenotypic correction of canine hemophilia A using an adeno-associated viral vector. *Blood*. 102:2031-7.

Schauber CA, Tuerk MJ, Pacheco CD, Escarpe PA, Veres G. 2004. Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. *Gene Therapy*. 11:266-275.

Schecter A, Spirn B, Rossikhina M, Giesen P, Bogdanov V, Fallon J, Fisher E, Schnapp L, Nemerson Y, Taubman M 2000. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res.* 87:126-132.

Schlegel R, Tralka TS, Willingham MC, Pastan I. 1983. Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site? *Cell.* 32:639-646.

Schneider S, Nuschele S, Wixforth A, Gorzelanny C, Alexander-Katz A, Netx R. 2007. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proceedings of National Academy of Sciences*. 104:7899-903.

Schnell MA, Zhang Y, Tazelaar J, Gao GP, Yu QC, Qian R, Chen SJ, Varnavski AN, LeClair C, Raper SE, and Wilson JM. 2001. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Molecular Therapy*. 5:708-22.

Schuettrumpf J, Herzog RW, Schlachterman A, Kaufhold A, Stafford DW, Arruda VR. 2005. Factor IX variants improve gene therapy efficacy for hemophilia B. *Blood*. 105:2316-2323.

Shen H, 1999. Intrinsic human immunodeficiency virus type 1 resistance of hematopoietic stem cells despite coreceptor expression. *Journal of Virology*. 73:728-737.

Shi Q, Wilcox DA, Fahs SA, Weiler H, Wells CW, Cooley BC, Desai D, Morateck P, Gorski J,Montgomery R. 2006. Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J. Clin. Invest*. 116:1974-1982.

Shi Q, Fahs SA, Wilcox DA, Kuether E, Morateck P, Mareno N, Weiler H, Montgomery R. 2008. Syngenic transplantation of hematopoietic stem cells that are genetically modified to express factor VIII in platelets restores hemostasis to hemophilia A mice with preexisting FVIII immunity. *Blood*. 112:2713-2720.

Shi Q, and Montgomery R. 2010. Platelets as delivery systems for disease treatments. *Advanced Drug Delivery Reviews*. 62:1196-1203.

Shun MC, Botbol Y, Li X, Di Nunzio F, Daigle JE, Yan N, Lieberman J, Lavigne M, Engelman A. 2008. Identification and characterization of PWWP domain residues critical for LEDGF/p75 chromatin binding and human immunodeficiency virus type 1 infectivity. *Journal of Virology*. 82:11555-11567.

Siedlecki CA, Lestini B, Kottke-Marchant K, Eppell S, Wilson D, Marchant R. 1996. Sheer-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood*. 88:2939-50.

Spector DH, Wade E, Wright DA, Koval V, Clark C, Jaquish D, Spector SA. 1990 Human immunodeficiency virus pseudotypes with expanded cellular and species tropism. *J. Virol.* 64:2298-2308.

Spencer HT, Denning G, Gautney RE, Dropulic B, Roy AJ, Baranyi L, Gangadharan B, Parker ET, Lollar P, Doering CB. 2011. Lentiviral vector platform for production of bioengineered recombinant coagulation factor VIII. *Molecular Therapy*. 302-309.

Stahelin RV, Wang J, Blatner NR, Rafter JD, Murray D, and Cho W. 2005. The origin of C1A-C2 interdomain interactions in protein kinase Cα. *J. Biol. Chem*. 280: 36452-36463.

Steinberg SF. 2008. Structural basis of protein kinase c isoform function. *Physiological Review*. 88:1341-1378.

Strair RK, Schaar D, Goodell L, Aisner J, Chin K, Eid J, Senzon R, Cui XX, Han ZT, Knox B, Rabson A, Chang R, Conney A. 2002. Administration of a phorbol ester to patients with hematological malignancies: preliminiary results from a

phase I clinical trial of 12-O-Tetradecanoylphorbol-13-acetate. *Clinical Cancer Research*. 8: 2512-2518.

Surin W, Barthwal M, Dikshit M. 2008. Platelet collagen receptors, signaling and antagonism: emerging approaches for the prevention of intravascular thrombosis. *Thrombosis Research*. 122:786-803.

Surosky RT, Urabe M, Godwin SG, McQuiston SA, Kurtzman GJ, Ozawa K, Natsoulis G. 1997. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J. Virol.* 71:7951-7959.

Sutherland HG, Newton K, Brownstein DG, Holmes MC, Kress C, Semple CA, Bickmore WA. 2006. Disruption of Ledgf/Psip1 results in perinatal mortality and homeotic skeletal transformations. *Mol. Cell. Bio.* 26:7201-7210.

Sutton RE, Reitsma MJ, Uchida N, Brown PO. 1999. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *J Virol*. 73:3649-60.

Swaroop M, Moussalli M, Pipe S, Kaufman R. 1997. Mutagenesis of a potential immunoglobulin-binding-protein-binding site enhances secretion of coagulation factor VIII. *J Biol Chem*. 272:24121-24124.

Toomey J, Kratzer K, Lasky N, Stnaton J, Broze G. 1996. Targeted disruption of the murine tissue factor gene results in embryonic lethality. *Blood*. 88:1583-1587.

Topalian SL, Solomon D, Rosenberg SA. 1989. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas . *J Immunol*. 142:3714–25.

Tuddenham E, Cooper D, Gritschier J, Higuchim, Hoyer L, Yoshioka A, Peake I, Schwaab R, Olekk, Kazazian H. 1991. Haemophilia A: database of nucleotide

substitutions, deletions, insertions and rearrangements of the factor VIII gene. *Nucleic Acids Res.*19:4821-4833.

Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*. 435:646-51.

Van den Biggelaar M, Bouwens EA, Kootstra NA, Hebbel RP, Voorberg J, Mertens K. 2009. Storage and regulated secretion of factor VIII in blood outgrowth endothelial cells. *Haematologica*. 94:670-8.

Van Damme A, Chuah MK, Collen D, VandenDriessche T. 2004. Onco-retroviral and lentiviral vector-based gene therapy for hemophilia: preclinical studies. *Semin Thromb Hemost*. 30:185-95.

VandenDriessche T, Collen D, Chuah MK. 2001. Viral vector-mediated gene therapy for hemophilia. *Current Gene Therapy*. 1:301-15.

Vargas J Jr, Gusella GL, Najfeld V, Klotman ME, Cara A. 2004. Novel integrase-defective lentiviral episomal vectors for gene transfer. *Human Gene Therapy*. 15:361-372.

Vehar G, Keyt B, Eaton D, Rodriguez H, O'Brien D, Rotblat F, Oppermann H, Keck R, Wood WI, Harkins RN, Tuddenham EG, Lawn RM, Capon DJ. 1984. Structure of human Factor VIII. *Nature.*312:337-342.

Viiala NO, Larsen SR, Rasko JE. 2009. Gene therapy for hemophilia: clinical trials and technical tribulations. *Semin Thromb Hemost*. 35:81-92.

Von Laer D., 1998. $CD34^{+}$ hematopoietic progenitor cells are not a major reservoir of the

human immunodeficiency virus. *Blood*. 76:1281-1286.

Wagenman B, Townsend K, Mathew P, Crookston K. 2009. The laboratory approach to inherited and acquired coagulation factor deficiencies. *Clin Lab Med*. 29:229-252.

Wagner DD, Olmsted JB, and Marder VJ. 1982. Immunolocalization of von Willebrand protein in Wiebel-Palade bodies of human endothelial cells. *J Cell Biol*. 95:355-360.

Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Coller BS, Jordan RE. 1996. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood*. 88:907-914.

Watson S and Nieswandt B. 2003. Platelet-collagen interaction: is GPVI the central receptor? *Blood*. 102:449-461.

Weichold FF. 1998. Neither human immunodeficiency virus-1 (HIV-1) nor HIV-2 infects most primitive human hematopoietic stem cells as assessed in long-term bone marrow cultures. *Blood*. 91:907:915.

Wion L, Kelly D, Summerfield J, Tuddenham E, Lawn M. 1985. Distribution of FVIII mRNA and antigen in human liver and other tissues. *Nature*. 317:726-729.

Wu X, Li Y, Crise B, Burgess SM. 2003. Transcription start regions in the human genome are favored targets for MLV integration. *Science*. 300:1749-51.

Wu Z, Yang H, Colosi P. 2010. Effect of genome size on AAV vector packaging. *Mol Therapy*. 18:80-86.

Wuepper KD, 1973. Prekallikrein deficiency in man. *J Exp Med*. 138:1345-1355.

Xu D, Alipio Z, Fink LM, Adcock DM, Yang J, Ward DC, Ma Y. 2009. Phenotypic

correction of murine hemophilia A using an iPS cell-based therapy. *Proc Natl Acad Sci U S A*. 106:808-13.

Yacoub N, Romanowska M, Haritonova N, and Foerster J. 2007. Optimized production and concentration of lentiviral vectors containing large inserts. *Journal of Gene Medicine*. 9:579-584.

Yang Y, Nunes FA., Berencsi K, Furth EE, Gönczöl E, Wilson JM. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proceedings of the National Academy of Sciences*. 91:4407-4411.

Yarovoi HV, Kufrin D, Eslin DE, Thornton MA, Haberichter SL, Shi Q, Zhu H, Camire R, Fakharzadeh SS, Kowalska MA, Wilcox DA, Sachais S, Montgomery RR, Poncz M. 2003. Factor VIII ectopically expressed in platelets: efficacy in hemophilia A treatment. *Blood*. 102:4006-4013.

Yarovoi HV, Nurden AT, Montgomery RR, Nurden P, Poncz M. 2005. Intracellular interaction of von Willebrand factor and factor VIII depends on cellular context: lessons from platelet-expressed factor VIII. *Blood*. 105:4674-4676.

Yonemura H, Sugawara K, Nakashima K, Nakahara Y, Hamamoto T, Mimaki I, Yokomizo K, Tajima Y, Masuda K, Imaizumi A. 1993. Efficient production of recombinant human factor VIII by co-expression of the heavy and light chains. *Protein Eng*. 6:669-674.

Youjin S, and Jun Y. 2009. The treatment of hemophilia A: from protein replacement to AAV-mediated gene therapy. *Biotechnology Letters*. 31:321-328.

Zavada J. 1982. The pseudotypic paradox. *J. Gen. Virol.* 63:15-24.

Zhang J, Attar E, Cohen K, Crumpacker C, Scadden D. 2005. Silencing

p21(Waf1/Cip1/Sdi1) expression increases gene transduction efficiency in primitive human hematopoietic cells. *Gene Therapy*. 12:1444-1452.

Zhang J, Scadden DT, Crumpacker CS. 2007. Primitive hematopoietic cells resist HIV-1 infection via p21. *J Clin Invest.* 117:473-81.

Zou J, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Beganny S, Chou BK, Chen G, Ye Z, Park IH, Daley GQ, Porteus MH, Joung JK, Cheng L. 2009. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell.* 5:97-110.

Zufferey R, Donello JE, Trono D, Hope TJ. 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virology*. 73:2886-92.